INTERACTION OF VANADATE WITH HUMAN THROMBIN, SOME AMINO ACIDS AND ASCORBIC ACID AT PHYSIOLOGICAL pH

BY

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CERTIFICATION

This thesis entitled "Vanadate Interaction with Human Thrombin, some Amino Acids and Ascorbic Acid at the Physiological pH" by Ismaila Yahaya AbdulMumin meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to scientific and literary presentations.

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DEDICATION

This Project is Dedicated to the memory of my late friend Sheikh ABD AL QAWIYY UMAR ABD ALL'AH LL.B (ISLAMIC LAW), BL, for the love we shared while we were together. May the Almighty Allah have Mercy on his soul and forgive his sins, Amin, Amin.

DECLARATION

I hereby declare that this thesis is the report of my research work carried out by me and has not been accepted on my previous application for a higher degree. All quotations are distinguished by quotation marks and all information from other sources have been acknowledged by means of references.

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In the name of Allah, the Beneficent, the Merciful, the Provider, the Forgiver. May Allah ta'ala's Blessings, Salutation and Benediction be upon our beloved Prophet, Messenger and Servant Mohammad, his progeny and his followers. Praise be to Allah ta'ala the Lord of the worlds.

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ABSTRACTS

The interaction of vanadate with human thrombin some L-oc amino acids and L-ascorbic acid were studied at the physiological pH (7.40) using ultraviolet difference spectroscopy. It was concluded that thrombin specifically and reversibly binds 2 equivalents of vanadate at the metal-binding sites of the protein. The vanadium (V) - thrombin complex was formed, by addition of vanadate to thrombin. Reversibility experiments carried out using Fe(III) and Ga(III) showed displacement of bound vanadium following addition of these ions which exhibited greater interaction with thrombin compared to vanadium (V) as reflected by their higher values of molar absorptivities. The binding constant for the second equivalent of vanadate is 10^{48} in 0.02M phosphate buffer, pH 7.40 at 29.5°C. From these studies, vanadate binds on the metal binding sites of the thrombin as a cation analogue.

The interaction of vanadate with some L-cc amino acids and L-ascorbic acid were studied at the physiological pH using pHmetric titration and ultraviolet charge transfer spectra. The most stable stoichiometric ratio of complexes formed as observed in the pH metric titrations were selected and their charge transfer spectra studied for possible binding modes.

At the physiological pH, L-ascorbic acid formed a stable ML type complex with vanadate; L-histidine, L-proline and model ligands ethylamine and histamine all formed stable ML2 type complexes with the Vanadate system; L-alanine, L-aspartic

acid and L-tyrosine and model ligands acetic acid and 2,3-diamino propaionic acid all formed stable ML3 type complexes with the Vanadate system; and L-threonine formed stable ML4 type complex with the vanadate system in 0.02M phosphate buffer, 0.02M ionic strength, at 29.5°C.

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ABBREVIATIONS

Acet	Acetate
2,3-DAPA	2,3 diamino propaionic acid
Ethy	ethylamine
Hist	Histamine
µg	microgram
ng	nanogram
ppb	parts per billion
UV	Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION

SECTION A

1.1.1 Vanadium: Occurrence and its Importance.

Vanadium, third in the first transition series is ubiquitous element. It is common in many foods, cereals and vegetables. Vanadium has natural affinity for fats and oils; food oils have high concentrations. Municipal water supplies may contain on the average about 1 to 6 ppb. Urban air contains some vanadium, perhaps due to the use of petroleum products (Cotton, 1987).

There has been some suggestions that vanadium is useful, if not essential in various biological systems. A haematopoietic effect has been postulated. Iron deficient rats respond more rapidly to added iron in the diet when vanadium is also present. Vanadium also decreases cholesterol and phospholipid content in liver of experimental animals (Casarett, 1975).

1.1.2 Vanadium: Toxicity and Excretion from the Body.

The average body burden of vanadium has been estimated at about 30mg (0.03g); the largest single compartment is in the fat. Bones and teeth stores contribute to the body burden. It has been postulated that some homeostatic mechanism controls the normal level of vanadium in the face of excessive intake, since the element in most forms, is moderately absorbed. The principal route of excretion is urine. The normal serum level is 35 to 48μg/100mL. When excess amounts of vanadium are in the diet, the concentration in the red cells tend to increase (Quist and Hokin, 1978; Nielson, 1979, 1980). The toxic action of vanadium is largely confined to the respiratory tracts. Bronchitis and

Bronchopneumonia are more frequent in workers exposed to vanadium compounds. Gastrointestinal distress, nausea, vomiting, abdominal pain, cardiac palpitation, tremor, nervous depression and kidney damage too, have been linked with industrial exposure (Casarett, 1975). Medicinal uses of vanadium compounds (V_2O_5) produced gastrointestinal disturbance, slight abnormalities of clinical chemistry related to renal functions and nervous systems effects (Myron et al. 1978). However, it is possible that these diseases may involve metabolic defects that lead to an enhanced susceptibility of certain individuals to chronic vanadium-toxicity (Nechay and Saunders, 1978).

1.1.3 Biological Functions of Vanadium.

Evidence from the nutritional essentiality of vanadium is not conclusive. Strasia (1971) found that rats fed less than 100ng of vanadium/g diet exhibited lower growth and had higher plasma and bone iron and higher hematocrits than rats fed 0.5æg of vanadium/g of diet. Hopkins and Mohr (1974) reported that the only effects of vanadium deprivation on rats was an apparent impaired reproductive performance that became apparent only in the fourth generation.

Recent findings that suggest vanadium does have a physiological role, have not come from nutritional, but from In Vitro studies with (Na, K)-ATPase and ATP phosphorylase. Rifkin(1965) was first to report that vanadium potently inhibits (Na,K)-ATPase, Cantley et al. (1978) found that vanadate was transported to the red blood cells where it inhibited the sodium pump by binding to the (Na, K) - ATPase from the cytoplasmic side(the site of ATP hydrolysis). The ability of vanadium to inhibit cholesterol biosynthesis has also been reported (Curan et al. 1968). The site of the inhibition was the microsmal enzyme system referred to as

squalene synthetase. Unfortunately vanadium did not lower blood cholesterol in older animals, or humans where elevated cholesterol levels is a risk factor for coronary artery diseases(Hopkins and Mohr, 1974).

1.1.4 Vanadium (V) Hydrolysis.

Vanadium(V) has an electron configuration of argon ($1s^2 2s^2\ 2p^6\ 3s^2 3p^6$). The 4s and 3d orbitals are completely empty. A large number of hydrolysis products are known, most of which are definitely established as a result of many different studies. The only cation at ordinary concentration is VO_2^+ (Dyrssen and Sekine, 1964) and at slightly alkaline pH, the V(V) species and some of the equilibria of the systems are:

Dilute solutions where only mononuclear species occur at concentrations of vanadium(V) below $2x10^{-5}$ M, namely VO_2^+, $VO(OH)_3$ (aq), $VO_2(OH)$, $VO_3(OH)^{2-}$, and VO^{3-}.

The decavanadate region in which the predominant species are:

$V_{10}O_{26}(OH)^{4-}$, $V_{10}O_{27}(OH)^{5-}$, $V_{16}O_{28}^{6-}$ at concentrations in excess of 0.01M between pH 1 and 6. The metavanadate region where the species of the general formula $(VO_3)_x^{x-}$ predominates at $6 \leq pH \leq 9$. The pyrovanadate region where the species $V_2O_7^{4-}$ predominates at $9 < pH < 12$. Vanadium has a rich and varied chemistry especially in the (IV) and (V) oxidation states. The form of vanadium, usually an oxyanion or oxycation i.e (VO_3^-, VO_2^+), depends upon its concentration and pH of the medium (Pope, 1968; Baes and Mesmer, 1976).

SECTION B

1.2.1 Proteins

The term protein is used to describe a polypeptide containing more than 40 amino acid residues. Proteins are the instruments by which genetic information is expressed. The molecular weights of proteins are in the range of 104 to 106 g. The determination of the chemical structure and the spatial configuration of proteins is a very important matter, as this is bound up with biological function of the proteins. At the present time an increasing number of protein structures have been determined to a high degree of resolution by X-ray diffraction techniques. This is one of the best methods for determining the complete structure. The body handles metals carefully and usually keeps them tightly bound within proteins. Since metals are important constituents of numerous enzymes, they may possibly appear in a more loosely bound form as a result of the increased protein degradation that has been shown to occur during certain activities i.e exercise Scenario (Jenkins, 1993). The role of proteins may be divided into work proteins e.g transport, digestion, catalysis etc. This work is controlled by nucleic acid systems which have the power to pass on information; and catalytic proteins (enzymes). The enzymes do not work themselves but persuade other systems to work faster. They have molecular weights from 5000 up to many millions. Since they are only catalysts, they must have a means of regenerating themselves i.e enzymes must not finish up or be destroyed at the end of the reaction. This power of regeneration applies to many of the other types achieved by a finesse in the molecular geometry of the protein that requires four levels of structure:

Primary structure: This is the unique sequence of amino

acid residues that varies from protein to protein.

Secondary structure: This is the pattern into which the chains are bent.

Ternary structure: This is the way in which chains mutually interweave around and through other chains, each stabilising the other. Together, two, three or four of these chains combine to give the overall shape of the protein such as the long thin strands of fibrous proteins and the compact pellets of globular proteins such as haemoglobin. Only the amino acid residues around the outer edges of a protein and possibly those lining a cleft that can chemically react. Nevertheless, most of the side-groups help in setting up the correct electrostatic environment for enzyme activity.

Quaternary structure: This is the way in which one protein attaches itself to another, forming shapes familiar and visible to us, e.g a combination of keratins gives hair (Hughes, 1972; Hardy, 1979).

1.2.2 Thrombin

Thrombin is the enzyme of blood that causes clotting by forming fibrin. Fibrin is an elastic thread-like network of insoluble protein formed from fibrinogen by the action of thrombin in the clotting of blood. Thrombin has 36,000g molecular weight on an average, and is a serine proteinase that catalyses the cleavage of certain arginyl and lysyl bonds(Doolittle, 1965, Hardy, 1979).

1.2.3 Structure and Properties of Thrombin

Thrombin, prothrombin and fibrinogen have been found in the blood plasma in all classes of vertebrates (Doolittle, 1962). The essential features of the mammalian coagulation of prothrombin to thrombin, limited thrombic proteolysis of fibrinogen with the release of fibrinopeptides (Doolittle, 1965),and self assembly

of the resulting fibrin monomer to form an insoluble polymeric fibrin gel have all been thoroughly studied(Robins, 1944; Doolittle, 1965). Thrombin has been proposed to be a complex molecule consisting of a total number of 309 amino acid residues arranged in two chains, with the first chain A, containing 49 residues and chain B, 260 amino acid residues(Appendix 1).

The amino acids of thrombin have been reported to be of high specific activity (Magnusson, 1971). Table 1.1 shows the 20 normal amino-acid protein components with symbols (according to the instruction to authors in the Biochemical Journal, 1978).

Table 1.1. 20 normal amino acid protein components

Amino acid	Symbols
Alanine	Ala
Arginine	Arg
Aspartic acid	Asp
Cysteine	Cys
Glutamic acid	Glu
Glutamine	Gln or Glx
Asparagine	Asn or Asx
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
proline	Pro
Serine	Ser
Threonine	Thr
Tyrosine	Tyr
Valine	Val

It can be seen from the appendix therefore that the thrombin molecule is extremely gigantic. But since the molecule consists of amino acid residues linked together, at least some properties of thrombin are imminent due to the presence of several amino, carbonyl, terminal amino and carboxyl groups. From the common

behaviour of these groups in their reactions, the complexing behaviour of thrombin can be predicted. Two specific sites have been reported to be involved in the thrombin system: The "acceptor" sites which are the γ-carboxyamide groups of two glutamine residues in the γ-chain, and the "donor" sites which are the amino groups two lysine residues in the α and γ chains(Chen, 1969; Lorand, 1969).

1.2.4 Trypsin: Occurrence, Composition and Properties.

Trypsin like thrombin, is a serine proteinase that catalyzes the cleavage of certain arginyl and lysyl bonds. It has a molecular weight of 23,200 (Murray et al, 1990). In the pancreatic cells of vertebrates, the inactive precursor of trypsin, trypsinogen, is synthesized and subsequently converted to the active enzyme (Desauelle, 1959). This protein-splitting enzyme, derived from trypsinogen, in the pancreatic juice has the same amino acid composition as those found in the thrombin. A consideration of the analogy between trypsin and chymotrypsin leads to the assumption that the specific site of trypsin to which positively charged groups of substrates or inhibitors bind electrostatically, contains the negatively charged aspartic acid or glutamine residues (Smith and Shaw, 1969). This residue at such position has been found so far only in trypsin, thrombin and trypsin-like proteinase from streptomyces griseus (Hardy, 1979). Kinetic evidence was presented for an auxiliary site on trypsin to which at least some neutral molecules preferentially bind. Confirmation of the existence of ternary complex formation was sought using the technique of equilibrium dialysis. Interesting conclusions emerged from observations that trypsin possesses two binding sites close to each other, one to which charged molecules are bound preferentially and the other for preferential binding of neutral molecules (Sanborn and Byran, 1968).

SECTION C

1.3.1 Amino Acids

Amino acids are simple organic compounds with the general formula (R - CH -(NH_2) - COOH), R may be an acidic, neutral or basic group which makes the naturally occurring acids differ in nature. Amino acids are the fundamental building blocks of proteins which take part in all living processes. The amino acid distribution pattern in the blood plasma and urine are of great diagnostic importance in medicine. Some specific metabolic roles are the biosynthesis of phosphatide, phosphorins and nucleotides(Hughes, 1972; Murray et al, 1990). The sequence of amino acid residues will determine the physical and chemical properties of the protein in terms of the chemical and physical interactions occurring between the side chains R in NH CH(R)COOH. The important side chains in this connection are those involving aromatic groups, sulphur-containing groups, and - NH, -OH and-COOH groups. The nature of these residues may generate hydrophobic or hydrophylic environments in certain regions of the protein chain. Each amino acid has at least two ionizable groups, the amino and carboxyl groups. The NH group is less acidic than the carboxyl group and so, between pH 4-9, the aminoacid exists as a zwitterion, H N^+ CH(R)COO^-. The α-carboxyl and amino functions are involved in the formation of the peptide link and so each peptide has terminal -NH and -COOH groups, together with peptide links and side chains. These are all possible metal binding sites (Perutz, 1969).

1.3.2 Ascorbic Acid

Ascorbic acid ($C_6H_8O_6$, pK_a 4.19) is an α-keto-lactone. The electron-transfer property is exhibited by the reaction of ascorbic acid with nicotinamide

solution resulting in an instantaneous production of a yellow colour due to complex formation (Lewin, 1976). The isolated product was an ascorbate nicotinamide salt with some association between the acid group and a secondary hydroxyl group of the acid. The various reactions of the ascorbate anion in forming complexes with metal ions and its other activities have been studied by many workers(Schubert, 1952; Barr and King, 1956). The optical absorbance spectrum of the ascorbate ion shows a peak at λ_{max} between 265nm and 266nm (Hewitt, 1961). Ascorbate anion can exercise significant influence on biological activities in various ways. Some of them are as follows:

Directly, that is via its characteristics - physico chemical properties of ionized state, Oxidation/Reduction, its capacity to lower interfacial tension and H.....bonding participation.

Indirectly, by, (I) influencing the concentration levels of the cyclic nucleotides; (II) affecting the level of enzymatic activity; (III) involvement in hormone production, such as those of adrenaline and serotonin etc; (IV) influencing enzyme biosynthesis; (V) detoxicating activity of endogenous toxic substances i.e the reducing action of ascorbate on adrenochrome(which interferes with many enzymatic reactions), and exogenous toxic substances i.e the increased resistance by rats to lethal doses of barbiturates when given vitamin C and similarly by humans to the toxic effects of Pb^{2+} and Hg^{2+} (Lewin, 1976).

SECTION D

1.4.0 Literature Review

1.4.1 Concept of Metalloproteins and Metalprotein Complexes.

Most naturally occurring metal ions are bound by proteins. The question of the nature and extent of metal ion-protein interaction is therefore a most important one. In metalloproteins(including metalloenzymes), the metal ion and protein are firmly linked together so that the metal ion can be regarded as an integral part of the protein structure, from which it cannot be separated except by extreme chemical attack. The activity of the metalloprotein is usually lost if the metal is replaced by another. In metal activated proteins or metal-protein complexes-(including metal activated enzymes), the metal ion is combined reversibly with the protein. Systems such as these are much less amenable to study, than the metalloproteins for which it is usually possible to say something about the site symmetry of the metal and the nature of the binding groups that hold it to the protein. The metal ion-binding group interaction for this second class will of course be much less than that involved in the first class. This will be an important factor in the mechanisms of the reactions catalysed by these metal activated enzymes. An example is the role of Mg^{2+} in the hydrolysis of ATP by the appropriate enzyme. The binding of metal ions to free ATP is through the phosphate groups. The hydrolysis of ATP does not change considerably from metal to metal in model systems. However, the state of affairs is very different in enzymatic reactions. Cd(II), Ni(II) and Co(II) have an inhibitory effect while Mg(II)and Mn(II) have a catalytic effect. This phenomenon demonstrates the inhibitory effect by certain site on the enzyme which is necessary for catalysis,

while the weaker binding of the metals magnesium and manganese has resulted in this site being free (Perutz, 1968; Hughes, 1972; Murray et al. 1990).

1.4.2 Back Ground Knowledge on Vanadate Interaction with some Proteins.

In the recent years much interest has been shown on the interaction of vanadate with proteins/enzymes (Jaswinder and Tracey, 1993; Thermistoklis et al. 1994). Vanadium (V) forms a complex series of monomeric and polymeric vanadate species (Baes and Mesmer, 1976; Cotton and Wilkinson, 1987). At concentrations less than 10^{-4}M , the monomeric vanadate ion VO_4^{3-} undergoes a series of three protonations with pk_a's of 13.0, 8.2 and 3.8 (Smith et al. 1976). Most vanadium toxicology has been concentrated on vanadium(V) compounds (Waters, 1977). Thus at micromolar levels in blood, vanadium(V) exists as a mixture of HVO_4^{2-} and $H_2VO_3^-$ (O'Donnel et al. 1976). Reports on the interaction of vanadium(V) and desferroxamine B in strongly acid medium have been extensive. Desferroxamine B, $NH_2(CH_2)_5$-{N(0H)C(O)$(CH_2)_2$C(O)N(H) $(CH_2)_5\}_2$ N(OH)C(O) CH_3 = H_3DFB is a linear trihydroxamate-based siderophore produced by certain microorganism. Iron (III) also very strongly chelates vanadium ions. This siderophore is currently used as a drug in the treatment of patients suffering form Cooley's Anaemia or chronic dialysis (Luterotti, 1986; Keller, 1991; Bartinic-Haberle, 1991). The discovery of vanadium in biomolecules such as marine algal bromoperoxidase (Morningstar et al. 1987; Everette and Butler,1980), and nitrogenase from Azobacter Vinelandii (De Boer et al. 1988) as well as by its ability to produce significant physiological effects (Butler and Carrano, 1991) stimulated a lot of interests. It has been shown that vanadium(V) interact with acid

groups and gives complexes with a vanadium-O_{amide} mode of coordination, which though air sensitive, are definitely stable in the solid state and quite stable in solution under an inert atmosphere. The chemical significance lies in the fact that the x-ray structures provide first solid - state evidence that the vanadium-O_{amide} binding is a possible mode of action in proteins(Thermistoklis et al. 1994). The ultraviolet absorbance spectroscopy has been used to investigate the interaction of vanadate with methyl -β-D-ribofuranoside, adenosine and inosine(Tracey, 1991). The interaction of vanadate(V) anion with transferrin has also been studied potentiometrically and spectrophotometrically (Wills, 1988). Vanadate complexes so formed have been established by ultraviolet spectrokinetics (Ray and Post, 1990) in which studies of pH effect and charge transfer spectra were used to determine the stoichiometry of complexes formed. The interaction of vanadate with human erythrocytes have also been studied extensively (Cantley, 1978;1979; Macara et al. 1980).

1.4.3 Metal Ion Interaction with Amino Acids.

Each of the twenty naturally occurring L - α amino acids may form a stable five membered chelate ring with a metal ion. In the absence of complicating donor side chains, usually the donor groups are the amino and carboxylate groups. Under other pH conditions this may not be the case; thus at lower pHs the amino acid may coordinate as a neutral ligand. Potentially polydentate ligands such as the amino acids may serve as a bidentate or tridentate ligand using different combinations of their donor atoms. Much works have been carried out on amino acid-copper complexes (Wilson et al. 1970). As the ligand field of the four in-plane donor toms increased, so the axial interactions become weaker. The

Pauling's Electroneutrality principle states that a complex is most stable when the charge on each atom lies between-1 and +1. In the present case, as the charge transferred to the metal in the plane increases, so there is less need for transfer of charge from axial ligands. Five coordination could thus be favoured by the presence of four strongly bound donor atoms e.g three peptide donors and a water molecule. The fifth position would probably be filled by a side chain carboxyl group or a water molecule (Hardy, 1979; Murray 1990). The UV spectroscopic studies and pH metric titrations have been used extensively to study metal ion-amino acid interaction (Djordjevic, 1989; Xiao et al. 1990).

1.4.4 Ascorbic Acid Physiologically Reviewed

Ascorbates are the most efficient inhibitors of lipid peroxidation when vitamin E is present (Mcay, 1985; Beudich et al 1986; Levin, 1986). It is essential to keep ascorbate level high since the compound may actually become a prooxidant in low concentrations (Burkit and Gilbert, 1989). Exercises may increase the requirement for ascorbate (Belko, 1987). Deficiency leads to poor athletic performance (Howald and Segasser, 1975). At high physiological levels of ascorbate, there is enhancement in the cellular uptake of the copper and iron, they also interfere with binding of copper to superoxide dismutase protein, presumably the interference with protein binding would leave Cu available to catalyze lipid peroxidation (Dasgeyta and Tudunek,1992).

SECTION E

1.5.0 Relevance of Study and Research Objectives

Vanadium is essential to human body. It may cause imbalance in certain biological functions of the human body if the required concentration is not present.

Due to its immense importance, many studies have been carried out on the complexing behaviour of vanadate with polydentate ligands in crystalline salts. The reactivity of vanadate remains a challenging area of investigation. There has been increasing interest in the interaction of vanadate with proteins. Vanadium has been reported to interact with proteins as a phosphate analogue(Cantley et al. 1978; Chasteen, 1983) and as iron-analogue(Johnson et al, 1974; Sabbioni et al. 1978; Carrano et al. 1984).

Thrombin is an important protein, composed of eighteen amino acids, and has many physiological functions; it is capable of binding metal ions as a protein especially transition metal ions. Amino acids are potentially polydentate ligands and are reported to form complexes with different metal ions. Some of the amino acids are responsible for the peak function of the brain (Perutz, 1969).

The importance of ascorbic acid cannot be over emphasized. It is an important constituent in the absorption of iron and copper in the body; it also functions as a detoxicating agent on harmful metal ions. There is no report on the complexing behaviour of vanadate with thrombin, the amino acids selected and ascorbic acid. Therefore, this project was undertaken with a view to studying the interaction of Vanadate with Thrombin, selecteda mino acids and ascorbic acid at the physiological pH. The objectives of this work can be outlined as follows:

1. To study the interaction of vanadate with thrombin. All experiments will be carried out at physiological pH (7.40) using 0.02M phosphate buffer. pH dependent UV spectra of vanadate will be run. The difference UV spectra of the interaction of vanadate with thrombin will be run with the aim of finding the stoichiometry of vanadium(V)-thrombin complex formed in solution. Reversibility

experiments using Ga(III) and Fe(III) will be carried out in order to find the probable binding sites of the protein i.e whether the vanadate would behave as an iron(III)-analogue or as a phosphate-analogue.

The complexing behaviour of vanadate with model ligand-trypsin will be compared with thrombin.

2. To study the interaction of vanadate with some L-amino acids and L-Ascorbic acid. The interaction will be studied in solution using pH metric titration and UV spectroscopy with aim of establishing the binding sites of the ligands and the stoichiometry of the complexes formed at physiological pH. The interaction of the model ligands employed, with vanadate will be compared with the nature of the amino acids interaction. Ascorbate enhance the cellular uptake of Fe and Cu, its effect on vanadium absorption has not been reported. This will probe the nature of vanadate - ascorbate interaction at physiological level.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Equipment and Apparatus

Spectrophotometers: PYE Unicam Model SP 8-100 and Milton Roy Spectronic 1001 Plus model were used during these studies.

pH Meter: Model 79 Griffith pH meter was used throughout these studies.

Weighing Balance: Mettler weighing balance model H8-25 was used for the weighings.

Oven: Gallenkamp Hotbox oven, size one was used for drying.

Chemicals: All chemicals used were AnalaR grade of British DrugHouse (BDH) unless otherwise stated:

Ammonium Vanadate

Ammonium Iron(III) sulphate

Gallium(III) oxide

Thrombin (Human) (sigma chemicals)

Trypsin (porcine)(sigma chemicals)

L - Alanine

L - Ascorbic acid

L - Aspartic acid

L - Histidine

L - Proline

L - Threonine

L - Tyrosine

Histamine

Acetic acid

Elthylamine

2,3 - Diaminopropionic acid (2,3 - DAPA)

Disodium salt of EDTA

Sodium dihydrogen phosphate

Disodium hydrogen phosphate

Sodium perchlorate

Perchloric acid

Hydrochloric acid

Sodium hydroxide

2.2 Preparation of Standard Solutions and Reagents

2.2.1 Metal Ions

Standard solutions of vanadium(V), Iron(III) and Ga(III)ions were prepared as reported in vogel's quantitative inorganic analysis (Vogel, 1989).

A stock solution containing vanadium(V) ions was prepared by dissolving 2.5250g (0.02moles) oven dried ammonium vanadate in 50mL of distilled water and then quantitatively transferred to 1000mL standard volumetric flask and making the volume up to mark with distilled water. This was standardized by titrating with 0.05M EDTA solution and using Eriochrome Black T indicator, the concentration of the stock solution was 2.1580×10^{-2}M. By appropriate dilutions, working standards solutions of vanadium(V) were prepared as 1.08×10^{-4}M and 2.75×10^{-4}M.

A stock solution of iron(III) was prepared by dissolving 6.0000g (0.023 moles) of ammonium iron(III) sulphate in 50mL of distilled water containing 1mL

of concentrated sulphuric acid. The solution was then transferred to a 250mL standard volumetric flask and the volume made up to the mark with distilled water. This solution was standardized by titrating with 0.05M EDTA solution. The concentration was 5.6390×10^{-3}M. By appropriate dilutions, working standard of iron(III) was prepared from the stock solutions as 6.5×10^{-4}M.

Standard solutions of gallium(III) ions, 6.0000g (0.032moles)in 250mL flask, was prepared using the procedure in the preparation of iron(III) solution, but without adding sulphuric acid. Standardization was done by titration with 0.05M EDTA solution using Variamine Blue B indicator. The concentration was 8.0030×10^{-3}M. The working standard was prepared by appropriate dilutions as: 7.4×10^{-4}M.

2.2.2 Thrombin

0.0400g of dried thrombin was weighed into a volumetric flask containing 1.0mL of 0.1M perchloric acid. The mixture was swirled to dissolve the thrombin. The resulting solution was then transferred to a 75mL standard volumetric flask. 5mL of 0.02M phosphate buffer, pH 7.40, was added and the solution was made up to the mark with distilled water. Appropriate dilutions were made to working concentration of 1.45×10^{-5}M.

2.2.3. Trypsin

0.0500g of porcine trypsin was dissolved in a volumetric flask containing 1.0mL of 0.1M perchloric acid and quantitatively transferred to 100mL standard volumetric flask. 5mL 0.02M phosphate buffer, pH 7.40 was added and the

solution made up to the mark with distilled water (Schewert and Takenaka, 1955). Appropriate dilution was made to prepare working concentration of 5.00×10^{-5}M.

2.2.4 Amino Acids and Ascorbic Acid

L - Alanine was dried at 29.5± 0.5°C. Stock concentration of L-Alanine was prepared by weighing 0.4450g (0.0050 moles) in 50mL of distilled water containing 1mL of sodium perchlorate and then transferring quantitatively into a 250mL standard volumetric flask and made up to the mark. The concentration of approximately 0.02M was confirmed by titration thus: A portion(5mL) was added to glacial acetic acid(25mL)at 29.5 ± 0.5°C. The solution was titrated against a standard iodine solution with starch as indicator until a pink colour appeared. On addition of water (30mL) the colour changed to purple, and the excess iodine was back titrated with sodium thiosulphate (Perrin, 1968).

Using the above procedure, standard solutions of L-Ascorbic acid (1.0300g); L-Aspartic acid (0.6655g); L-Histidine (0.7750g);L-Proline(0.5757g); L-Threonine (0.6455g); L-Tyrosine (0.9060g),were prepared in 250mL flasks.

The following model ligands were prepared as above but were not standardized by titration: 2,3 - Diaminopropionic acid(0.7050g, 0.0050 moles); Histamine (0.5557g w/v, 0.0050 moles);Ethylamine (0.3222g, 0.0050 moles) and Acetic acid (0.2888g w/v,0.0050 moles) in 250mL flasks.

2.2.5 EDTA

0.10M disodium hydrogen ethylenediaminetetracetate dihydrate was prepared by dissolving 37.2250g of the salt in 100mL of deionized water. The solution was then transferred quantitatively to 1000mL standard volumetric flask and the volume made up to mark with deionized water. Working standards of

0.05M and 0.01M concentrations in 250mL flasks respectively were prepared by diluting appropriate volumes of the 0.01M EDTA stock solution.

2.2.6 Indicators

Eriochrome Black T indicator was prepared by dissolving 0.02000g of the dyestuff in 15mL of - triethanolamine and 5mL of absolute ethanol making a 1.0% solution.

Variamine Blue B indicator of 1.0% aqueous solution was similarly prepared.

2.2.7 Buffers

Phosphate buffer stocks were prepared by dissolving: 71.6400g, 0.19 moles, of $Na_2 HPO_4 .12H_2 0$ in 1000mL distilled water and swirled to dissolve after which the 1000mL mark was made up to the mark with distilled water; 31.2100g, 0.20 moles, of $NaH_2 PO_4.2H_2 O$ was dissolved in a 1000mL volumetric flask and containing 100mL distilled water, swirled to dissolve and made up to the 1000mL mark with distilled water (Gomori, 1955). The mixture for the appropriate working standards were then carried out(Christian, 1977).

2.3 Theory of Ultraviolet Spectroscopic Determination

The electronic spectra of molecules are found in the wavelength range 100-800nm of the electromagnetic spectrum. The visible region to which the human eye is sensitive corresponds to the range of wavelengths between 400 and 800nm. The range between 200 and 400nm is referred to as the near ultraviolet region. The region below 200nm is called far or vacuum ultraviolet region. There are two classes of spectra, namely emission spectrum and absorption spectrum. An emission spectrum is obtained by analysing the light emitted by a luminous source.

An absorption spectrum is obtained by the spectroscopic analysis of the light transmitted by an absorbing medium which is placed between the light source and the spectroscope. It is on this latter principle the studies in this work are based upon. When a molecule absorbs radiation, its energy changes - may be electronic, vibrational or rotational. These three processes by which a molecule can absorb radiation; all involve raising the molecule to a higher internal energy level, the increase in energy being equal to the energy of the absorbed radiation (h). Since each of these internal energy transitions is quantized, they will occur only at definite wavelengths corresponding to an energy h equal to the quantized jump in the internal energy.

Beer-Lambert's law of light absorption states that the functioning of the incident light absorbed is proportional to the number of molecules in the path and length of the path. That is if a substance is dissolved in a solvent, the absorption by the solution will be proportional to its molecular concentration provided the solvent does not have any absorption in that region. The law is expressed as:

$$\text{Absorbance (A)} = \epsilon CL \quad \ldots\ldots\ldots\ldots \quad 2.1$$

Where is the molar absorptivity in M^{-1} cm^{-1} , C is the concentration, mole L^{-1} , and L is the cell path length usually 1cm. The electronic transitions that take place in spectrum all due to the absorption of radiation by specific types of groups within the molecule (Rao, 1975).

2.3.1 Difference Methods

A difference method is a technique in which an absorbing solution is used in the reference beam instead of the normal blank. The resulting absorbance reading is then the difference between the absorbance of sample and of reference (Harris,

1990; Tracey, 1991). The effect of a known compound on the spectrum of a mixture can be balanced out by using a solution of the known compound in the reference beam, preferably in a variable cell. The resulting spectrum is that of the rest of the mixture.

2.4 Theory of pH Metric Titrations

Since the pH of solution changes during an acid-base titration, a convenient way to follow the titration is by potentiometry, with a glass electrode - reference electrode pair. The cell potentials are measured by a pH meter. Plotting of these data, which are read in pH units versus mL of titrant yields the titration curve for the acid - base system. The end-point in the titration curve can be determined by selecting the point of greatest inflexion. From pH measurements, it is possible to calculate the dissociation constant, buffer ratios, hydrolysis effects and to obtain additional useful information about the system. In biological systems, pH of the arterial blood is remarkably constant ranging from 7.38 to 7.42. From a physiological point of view, a change of about 0.05 pH units is very significant. In general, the two physiologically important buffer systems are phosphate and carbonate. Medically any change of as little as ±0.05 pH units is an indication of metabolic acidosis or alkalosis and can be the result of one of several different diseases (Christian, 1977; Pietryzyk, 1979).

2.5 Complex Formation

The formation of complexes in aqueous solution may be recognised by a number of different methods, of which the classical test of modification of chemical properties is only one, and somewhat unreliable one at that: all reactions have equilibrium constants, and chemical tests are often only investigations of relative

values of equilibrium constants. Physical methods (such as investigations of colligative properties, electronic or vibrational spectra, solubility, conductivity, or electrode potentials) provide more reliable evidence as well as, in favourable circumstances, leading to values of equilibrium constants for complex formation. Let us consider a metal ion M and a ligand L forming a series of complexes ML, ML ....ML_n in aqueous solutions. Although each stage in complex formation really represents successive replacement of water from the hydration shell of the metal ion, on the convention that the activity of water in such a solution is unity it can be disregarded as a reactant or product. For the formation of successive complexes, then, provided equilibrium is attained.

$$M + L \rightleftharpoons ML \qquad K = \frac{[ML]}{[M][L]} \qquad 2.2$$

$$ML + L \rightleftharpoons ML_2 \qquad K = \frac{[ML_2]}{[ML][L]} \qquad 2.3$$

$$ML_{n-1} + L \rightleftharpoons MLn \qquad Kn = \frac{[MLn]}{[ML_{n-1}][L]} \qquad 2.4$$

The equilibrium constants K , K .....Kn are called stepwise formation (or stability) constants (Sharpe, 1987).

2.6 Ultraviolet Titrations

All the parameters were adjusted as recommended for the instrument in the manufacturer's handbook. The instrument was allowed to warm up to 30 minutes before use.

2.6.1 Vanadate pH Dependent UV Titration.

1.08×10^{-4}M solution of vanadate prepared by the method described in 2.2.1, was adjusted over a p range of 5.60 to 10.55 in 0.02M phosphate buffer using 0.01M sodium hydroxide and/or dilute hydrochloric acid (0.01M) at the appropriate working pH. Distilled water was used in the reference cuvette, and vanadate in the sample cuvette. The pH dependence UV spectra were recorded with spectrophotometer using 2mL of sample and distilled water in the respective cuvettes.

2.2.6 Vanadate-Thrombin Difference Spectra

1.45×10^{-5}M thrombin solution prepared from stock as described in 2.2.2, was buffered at pH 7.40 using the appropriate 0.02M phosphate buffer mixture on adjustment with 0.01M sodiumhydroxide solution. 2mL of the prepared thrombin solution were placed in both the sample and reference cuvettes. Aliquots of 2.75×10^{-4}M ammonium vanadate solution at pH 7.40 (prepared as in 2.6.1) were added, using 1mL capacity syringe, to the sample cuvette, and the corresponding volume of distilled water added to the reference cuvette. After allowing adequate time following each addition (about five minutes), a difference spectra was recorded. Aliquots added were 10, 30, 50, 70, 90, 110, 130,150, 170, 195, 260, 360μL.

2.6.3 Vanadate-Trypsin Difference Spectra

5.00×10^{-5}M trypsin solution prepared from stock solution as described in 2.2.3, was buffered at pH 7.40 using the 0.02M phosphate, pH 7.40, buffer mixture. The procedure for vanadate addition, equilibration and spectral

determination as described in 2.6.2 were repeated in this study. Aliquots added were equally the same.

2.6.4 Vanadate-Thrombin Complex Difference Spectra Following the Addition of Gallium (III).

7.40×10^{-4}M solution of gallium(III) was prepared by adjustment of 10mL of stock volume (2.2.1) to pH 7.40 using 0.01M sodium hydroxide and buffered with 0.02M phosphate, pH 7.40 buffer mixture. 2mL of 1:1 vanadate-thrombin complex (formed by allowing 30 minutes equilibration time after mixing) were added to both sample and reference cuvettes. Aliquots of gallium(III)were then added to the sample cuvette and equivalent amount of distilled water to the reference cuvette using 1mL syringes respectively (allowing about 5 minutes for equilibration) and then the difference spectra were recorded. Aliquots added were 1, 5, 10, 15, 20, 30, 40, 50, 60μL.

2.6.5 Vanadate-Thrombin Complex Difference Spectra Following the Addition of Iron(III)

The procedure is the same as in 2.6.4 except that 6.50×10^{-4}M iron(III) solution, pH 7.40 was used in place of gallium(III)solution. Aliquots added were 10, 15, 20, 30, 40, 50μL.

2.6.6 Vanadate-Thrombin Complex (1:1) Difference Spectrum as a Function of pH

The procedure for the various pH studied for the 1:1 (equal volume) mixing of vanadate and thrombin was done in accordance with the procedures in 2.6.1 and 2.6.2. The mixing at each pH was made 1:1 (equal volumes; 5mL of each solution at the required pH mixed to form a total of 10mL solution) and allowed to equilibrate for 30 minutes. The spectra were taken with a 1:1 ratio of

thrombin distilled water in the reference cuvette. The pH studied were 6.20, 6.75, 7.00, 7.40, 8.60, 9.20.

2.7 pH Titrations of Vanadate with some L-Amino Acids, L-Ascorbic Acid and Model Ligands

Vanadate solution, $1.08X10^{-4}M$, pH 7.40 was prepared as described in 2.6.1. All measurements were carried out at 29.5±0.5°C, in the presence of 0.01M sodium perchlorate. The amino acid and ascorbic acid were prepared as described in 2.2.4. They were used on adjustment of the pH to 7.40 using either 0.01M hydroxide and buffered with 0.02M phosphate, pH 7.40, buffered mixture. The procedure for the titrations of all the amino acids, ascorbic acid and the model ligands were the same and it as thus:

10mL vanadate solution ($1.08x10^{-4}M$) pH 7.40 was placed in a 100mL beaker, and then at intervals aliquots of 5mL of the ligand 0.02M i.e L-Alanine, was added, allowing about 5 minutes equilibration time after each addition, the pH changes were recorded. The pH changes were monitored for aliquot additions at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mL.

2.8 Ultraviolet Charge Transfer Spectra of Vanadate-Ligand Complexes

From the pH titrations, the most stable vanadate: ligand ratio for complexes formed were selected and their charge transfer spectra were studied. The equivalence point(s) in the pH titration curves gives possible stoichiometry of the vanadate-Ligand complex so formed. Thus, from the studies in 2.7, the most

stable vanadate: ligand ratio of complex formed at 1:1, or 1:2, 1:3, or at 1:4 were studied spectrophotometrically thus:

For a 1:1 stable complex, 10mL of vanadate solution, 1.08×10^{-4}M, pH 7.40 were added each to two beaker. Equal volumes of ligand and distilled water were added respectively and allowed to equilibrate for 30 minutes.

2mL of each beaker content were then added to a sample and reference cuvettes respectively and the spectra taken. The same procedures were followed for a stable 1:2, 1:3 and 1:4, vanadate-Ligand complexes except that the volume of ligand and distilled water additions were respectively doubled, tripled and quadrupled at each stable ratio studies.

CHAPTER THREE

3.0 RESULTS

3.1 Preparation and Characterization

3.1.1 Ammonium Vanadate pH Dependent Solution

The ammonium vanadate solution was prepared by dissolving stoichiometric amount in perchlorate medium and buffered with 0.02M phosphate buffer over the pH range studied. Table 3.1 shows the relationship between the pH increments and molar absorptivities at vanadate concentration 1.08×10^{-4}M.

3.1.2 Ammonium Vanadate-Thrombin Interaction at pH 7.40

Aliquot addition of ammonium vanadate 2.75×10^{-4}M at pH 7.40 to the solution of thrombin, 1.45×10^{-5}M at pH 7.40, resulted instantly in the complex formation as the light yellow colouration of the vanadate changes gradually to colourless solution. This was allowed to equilibrate at each aliquot addition (about five minutes) and the spectra taken.

3.2 Ultraviolet Spectral Data

3.2.1 Vanadate pH Dependent Spectra

The vanadate ion has its own pH dependent UV spectra as shown in figure 3.1. The HVO_4^{2-} species has a λ_{max} 255nm. A protonation constant for HVO_4^{2-} was obtained as $10^{8.12}$ at 29.5°C which is in agreement with the literature value of $10^{8.5}$ obtained at 25°C (Smith, 1976). The profanation to form $H_2VO_4^-$ affects the vanadate spectra.

3.2.2 Vanadate-Thrombin Interaction Difference Spectra

The addition of aliquots of ammonium vanadate to the solution of thrombin generates the family of spectra shown in figure 3.2. The λ_{max} 256nm is close to that of the free vanadate ion, λ_{max} 266nm, but the molar absorptivity of the vanadium(V) -protein λ_{max} complex is more than 9000 M^{-1} cm^{-1} per metal ion compound with only about 2000M^{-1} cm^{-1} for the free vanadate ion.

The absorbance maximum at 256nm reflects the perturbations in the binding site which is characteristic of binding at the high affinity binding site of the thrombin (Fenton, 1988). To normalize the results from run to run, the absorbance at 256nm was divided by the analytical thrombin concentration to give an apparent absorptivity, for each titration point (Harris, 1990).

Table 3.2 shows the relationship between $\Delta\epsilon$ and r where r is the [vanadate]: [Thrombin] ratio obtained as equation 3.1 at each titration point.

$$r = \frac{[\text{Vanadate}]}{[\text{Thrombin}]} \qquad \ldots\ldots\ldots\ldots 3.1$$

Values of $\Delta\epsilon$ were plotted versus r, to give the titration curve shown in figure 3.3. The titration of thrombin with vanadate shows linear plots from r = 0 to r = 1.86 with a slope of 11,005 M^{-1} cm^{-1}, which then curves into a second linear segment from r = 1.86 to r = 3.41 that has a slope of about 4500 M^{-1} cm^{-1}.

TABLE 3.1. CHARACTERISTIC MOLAR ABSORPTIVITIES OF pH DEPENDENT [VANADIUM(V)] = 1.08 X 10^{-4}M, UV SPECTRA

pH	Absorbance	ϵ (M^{-1} cm^{-1})
10.55	0.368	3407
9.60	0.355	3287
9.20	0.325	3009
8.84	0.310	2870
8.65	0.290	2685
8.30	0.260	2407
8.00	0.250	2315
7.75	0.235	2176
7.40	0.210	1944
7.00	0.185	1713
6.50	0.175	1620
5.60	0.160	1481

TABLE 3.2. RELATIONSHIP BETWEEN υ, r, $\in_{VO_3^-}$, $\Delta\in_{obs}$, $\Delta\in_{V\text{-}Thr}$

$\Delta\in_{obs}$ $M^{-1}cm^{-1}$	r	$\in_{VO_3^-}$ $M^{-1}cm^{-1}$	$\Delta\in_{V\text{-}Thr}$ $M^{-1}cm^{-1}$	υ
833	0.09	1916	10082	0.08
2465	0.28	1916	10082	0.24
4060	0.48	1916	10082	0.38
6119	0.66	1916	10082	0.59
8203	0.86	1916	10082	0.80
10082	1.05	1916	10082	0.98
12241	1.24	1916	10082	1.20
14818	1.42	1916	10082	1.48
17476	1.62	1916	10082	1.76
20312	1.86	1916	10082	2.05
23522	2.48	1916	10082	2.30
27250	3.41	1916	10082	2.53

TABLE 3.3. BINDING CONSTANT OF SECOND EQUIVALENT OF VANADIUM (V) - THROMBIN COMPLEX.
$K = [V_2 - THROMBIN]/[V\ THROMBIN][VO_3^-]$

TITRATION	Log K
	± 0.28
1	5.60
2	5.20
3	5.00
4	4.90
5	4.80
6	4.80
7	4.70
8	4.70
9	4.70
10	4.70
11	4.70
12	4.60

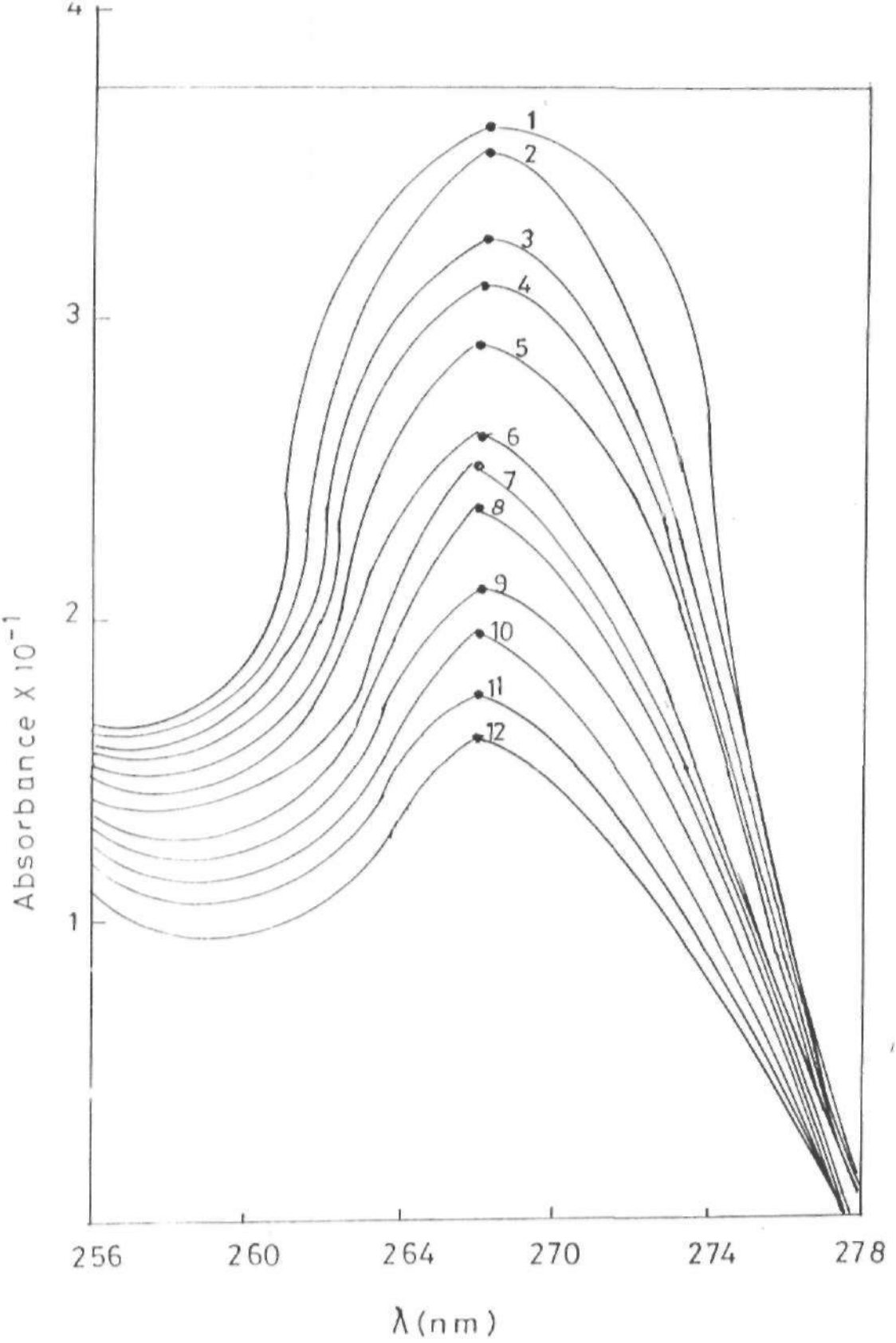


Figure 3.1. UV spectra of ammonium vanadate as a function of pH [Vanadate] = 1.08×10^{-4} M. curve 1, pH = 10.55; 2, 9.60; 3, 9.20; 4, 8.84; 5, 8.65; 6, 8.30; 7, 8.00; 8, 7.75; 9, 7.40; 10, 7.00; 11, 6.50; 12, 5.60; 0.02M Phosphate buffer, temperature = 29.0 ± 0.5°C

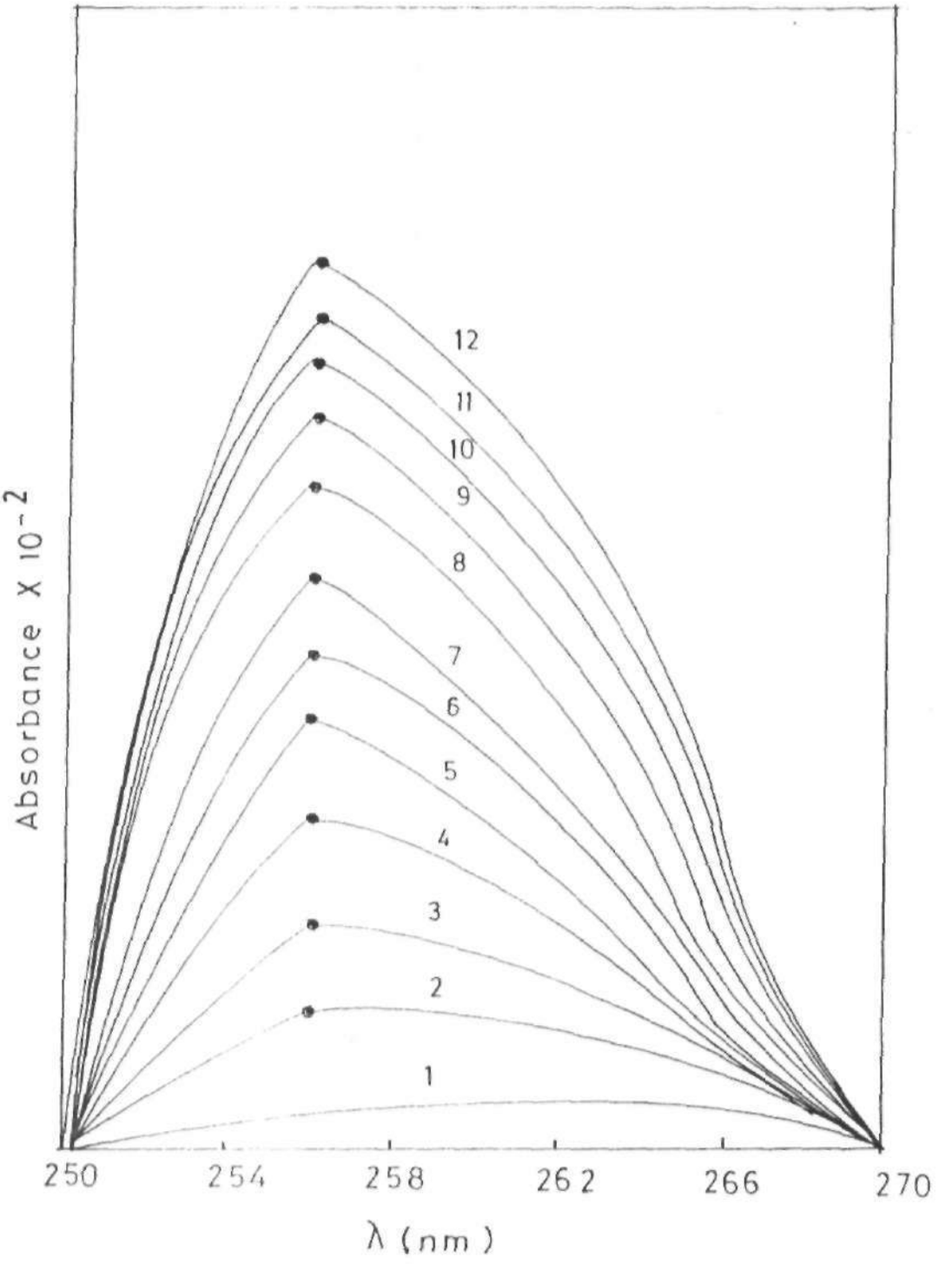


Figure 3·2. Difference UV spectra from titrations of thrombin with ammonium vanadate in 0·02 M phosphate buffer, at pH 7·40, t = 29·0 ± 0·5°C [Thrombin] = $1·45 \times 10^{-5}$ M, [VO_3^-] $2·75 \times 10^{-4}$ M, Curve 1, 10 µl. VO_3^-; 2, 30; 3, 50; 4, 70; 5, 90; 6, 110, 7, 130; 3, 150; 9, 170; 10, 195; 11, 260; 12, 360.

It is easier to visualize the extent of vanadium(V) binding to thrombin by mathematically removing the absorbance due to free vanadate. This can be achieved by plotting υ versus r (Table3.2), where υ is the average number of vanadium(V) ions bound per thrombin molecule and is defined as

$$\upsilon = \frac{(\Delta\epsilon_{obs} - (r x \epsilon_{vo_3}))}{(\Delta\epsilon_{v\text{-}Thr} - \epsilon_{vo_3})} \qquad \text{.....3.2}$$

where $\Delta\epsilon_{obs}$ is the observed absorptivity following each addition of vanadate, $\Delta\epsilon_{v\text{-}Thrombin}$ is the absorptivity of vanadate-Thrombin at 1:1 complex, ϵ_{vo_3} is the molar absorptivity of the free vanadate at pH 7.40, and r is as obtained in equation 3.1. As long as there is essentially complete binding at each aliquot addition of vanadate to the thrombin, plots of υ versus r will always be a slope equal to 1.

3.3 Determination of Binding Constants for the Vanadate- Thrombin Complex

At pH 7.40 the binding of the first equivalent as indicated by the linear plots of υ versus r from r = 0 to r = 1.86, figure 3.4, no equilibrium constant for the binding of the first equivalent of vanadium(V) can be calculated from these data. The vanadate titration plots begin to curve downward in the area between r = 1.86 and 3.00 indicative of a weaker binding after the second equivalent. These quantitative observations are confirmed by calculation of vanadate-thrombin binding constants (Table 3.3). The binding constants for the second equivalent of vanadium(V)was calculated as shown in equation 3.3.

$$K = \frac{[V_2 \text{ - Thrombin}]}{[V \text{ - Thrombin})(VO_3^-]}$$

where V = vanadate species binding.

3.4 Reversibility Studies

There is the possibility that the vanadate might produce a difference UV spectrum by irreversibly oxidizing the donor group(s) at the thrombin metal binding site. Such behaviour has been reported for the periodate anion with transferrin (Geoghegan et al. 1980). The reversibility of vanadium(V) to thrombin was therefore evaluated by forming the vanadate- thrombin complex, 1:1, allowing about 30 minutes for complete reaction, then adding the more strongly binding iron (III) and gallium(III) ions to displace the thrombin bound vanadium(V) figures 3.5 and 3.6. The vanadium-thrombin complex displays essentially no absorbance in the visible region of the spectrum. The displacement of vanadium(V) by gallium(III) was much faster as higher absorbance was recorded for the gallium(III) spectra figure 3.6, compared to the spectra of iron (III) displacement of the vanadate, figure 3.5. This suggests that the donor group(s) to which the vanadate is bounded is not irreversibly oxidized and that the binding site in the protein for vanadate, gallium(III) and iron(III) is the same and most probably a cation binding site.

3.5 Trypsin Model Studies

Thrombin shows the same site specificity as trypsin and plasmin in its preference for arginine and lysine ester substrates studied (Sherry, 1954; Elmore and Curragh, 1963). The Kinetics of thrombin, trypsin and plasmin have been extensively characterized (Martin et al. 1959; Chase and Shaw, 1969). A solution of trypsin 5.00×10^{-5}M was titrated spectrophotometrically with ammonium vanadate, difference UV spectra was recorded, figures 3.7 and 3.8.

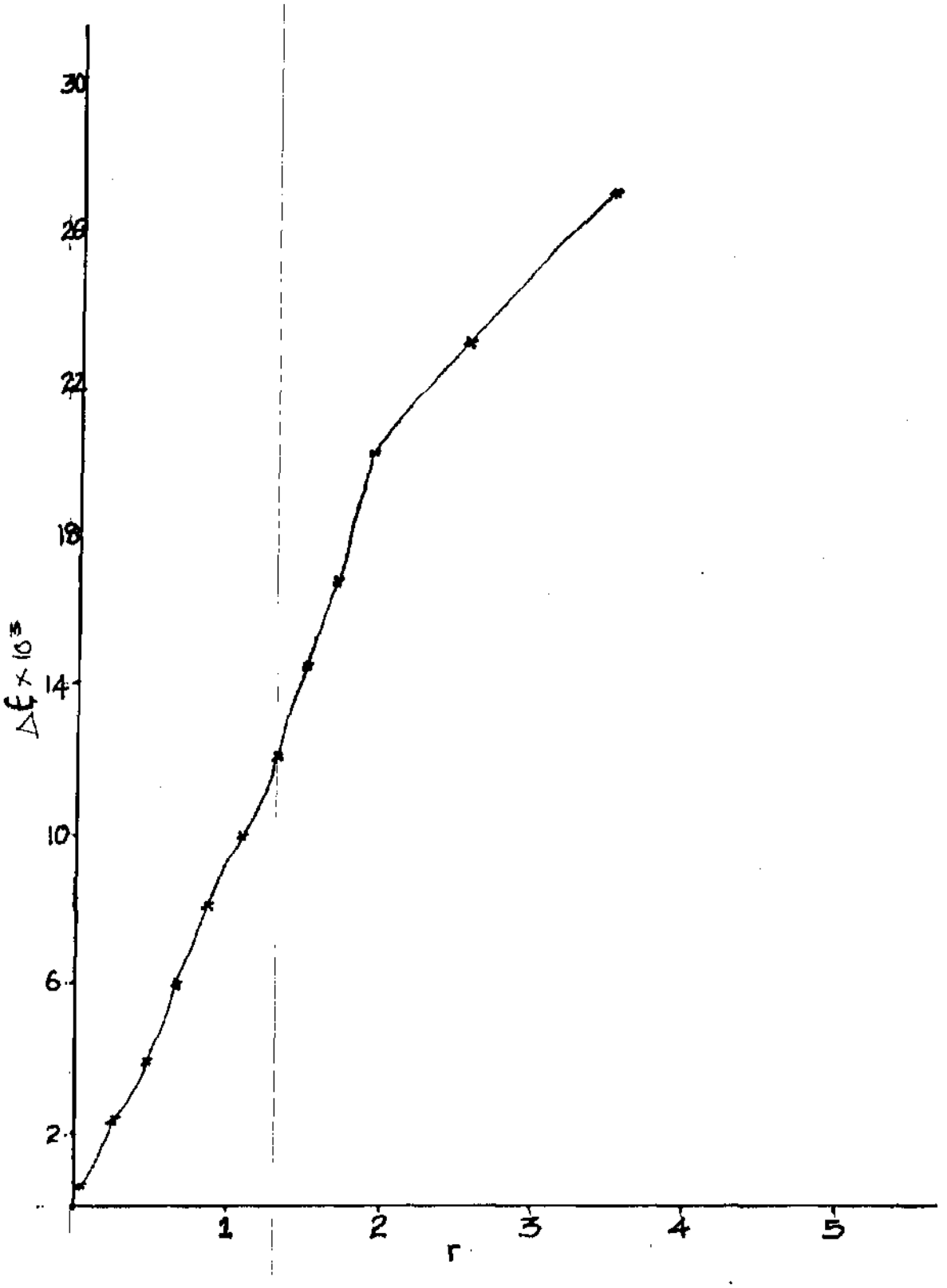


FIGURE 3.3 PLOTS OF $\Delta\epsilon$ VS r FOR THE TITRATION OF THROMBIN WITH VANADATE.

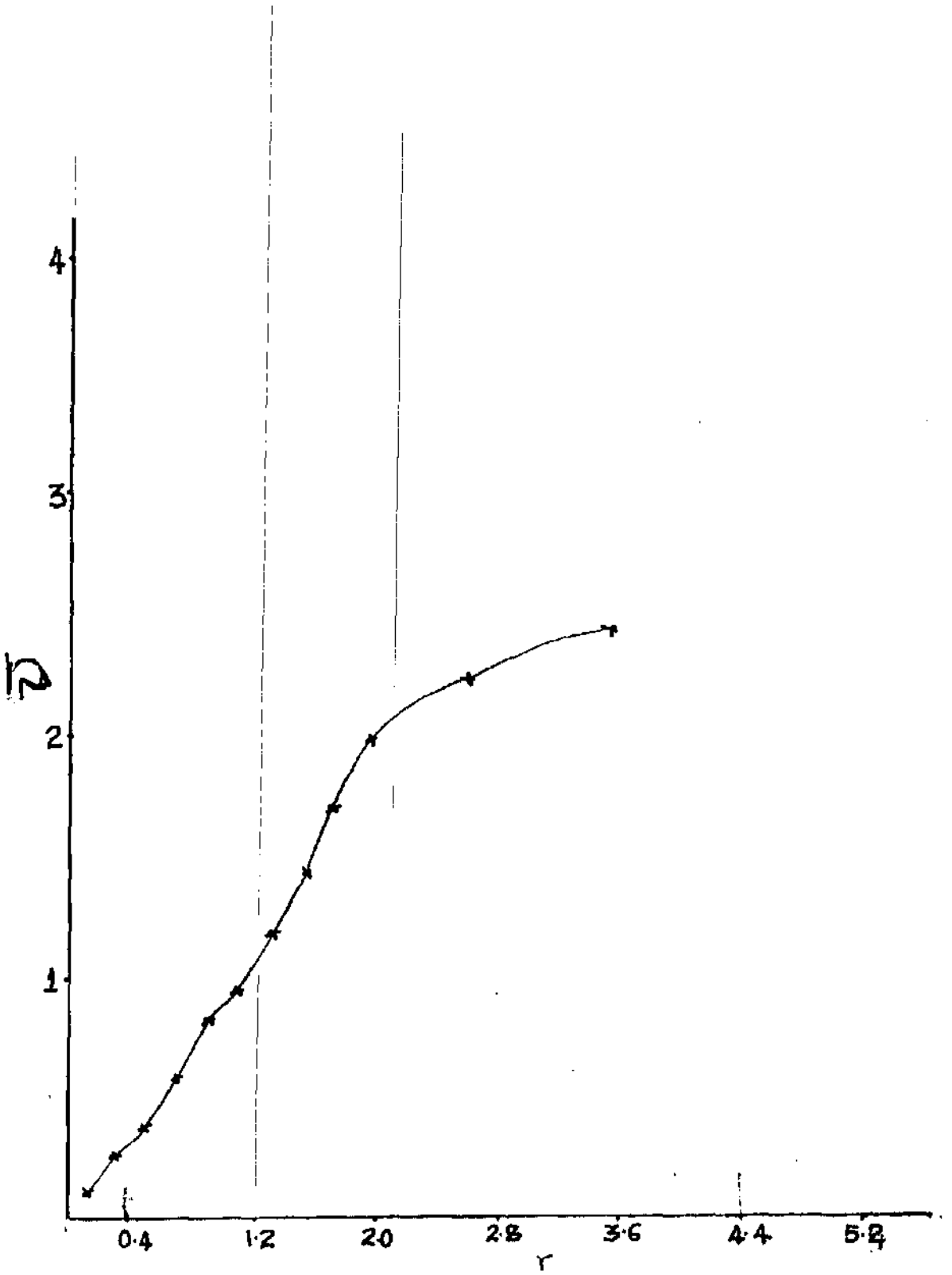


FIGURE 3.4 PLOTS OF $\bar{\nu}$ VS r FOR THE TITRATIONS OF THROMBIN WITH VANADATE.

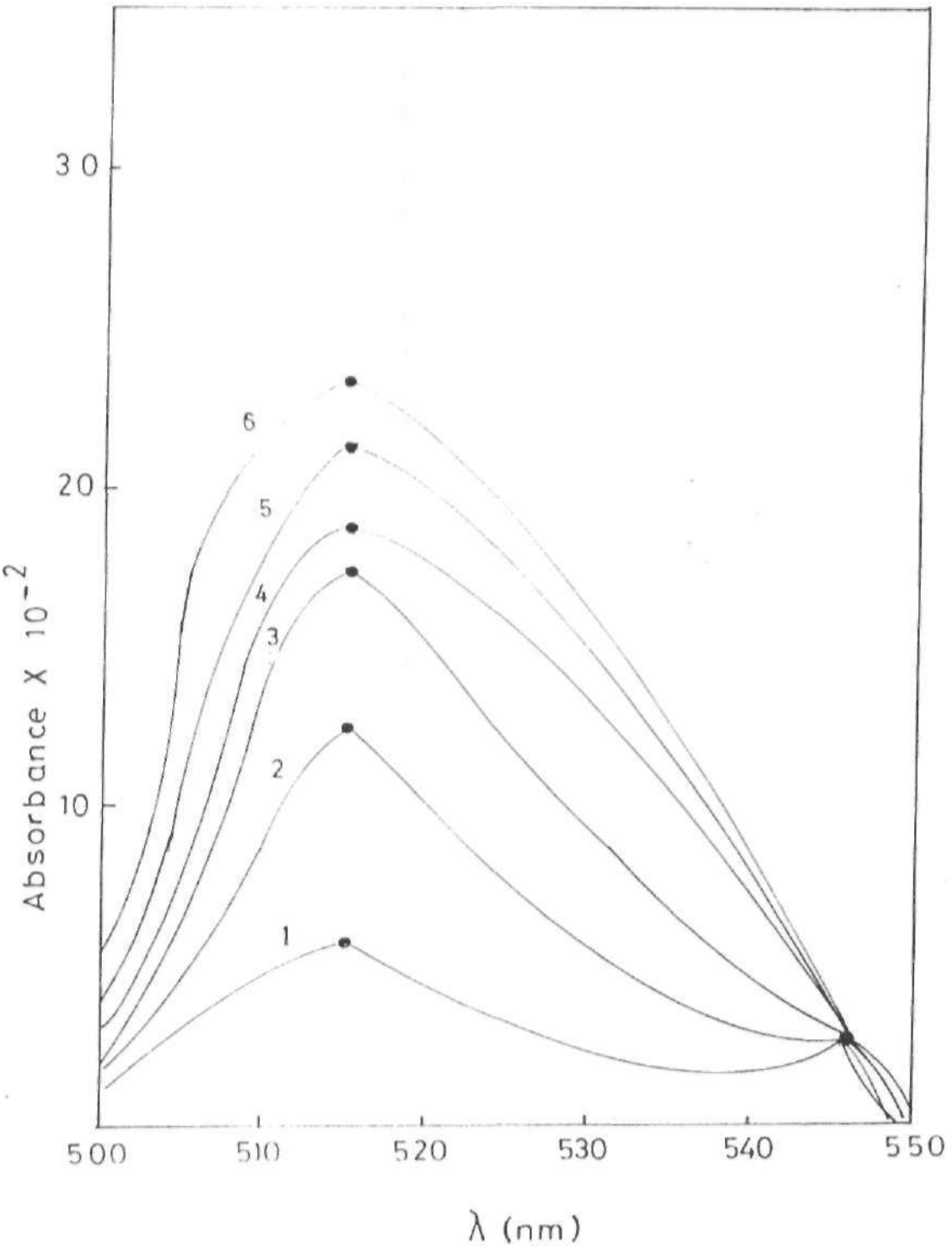


Figure 3·5. Difference UV spectra of 1:1 Vanadium (V) – thrombin complex following additon of aliquots of Fe (III), [Thrombin] = 1.5×10^{-5} M, [Fe] = 6.5×10^{-4} M, pH 7.40, 0·02M Phosphate buffer, curve 1, 10 µL Fe, 2, 15; 3, 20; 4, 30; 5, 40; 6, 50. temperature 29.0 ± 0.5°C.

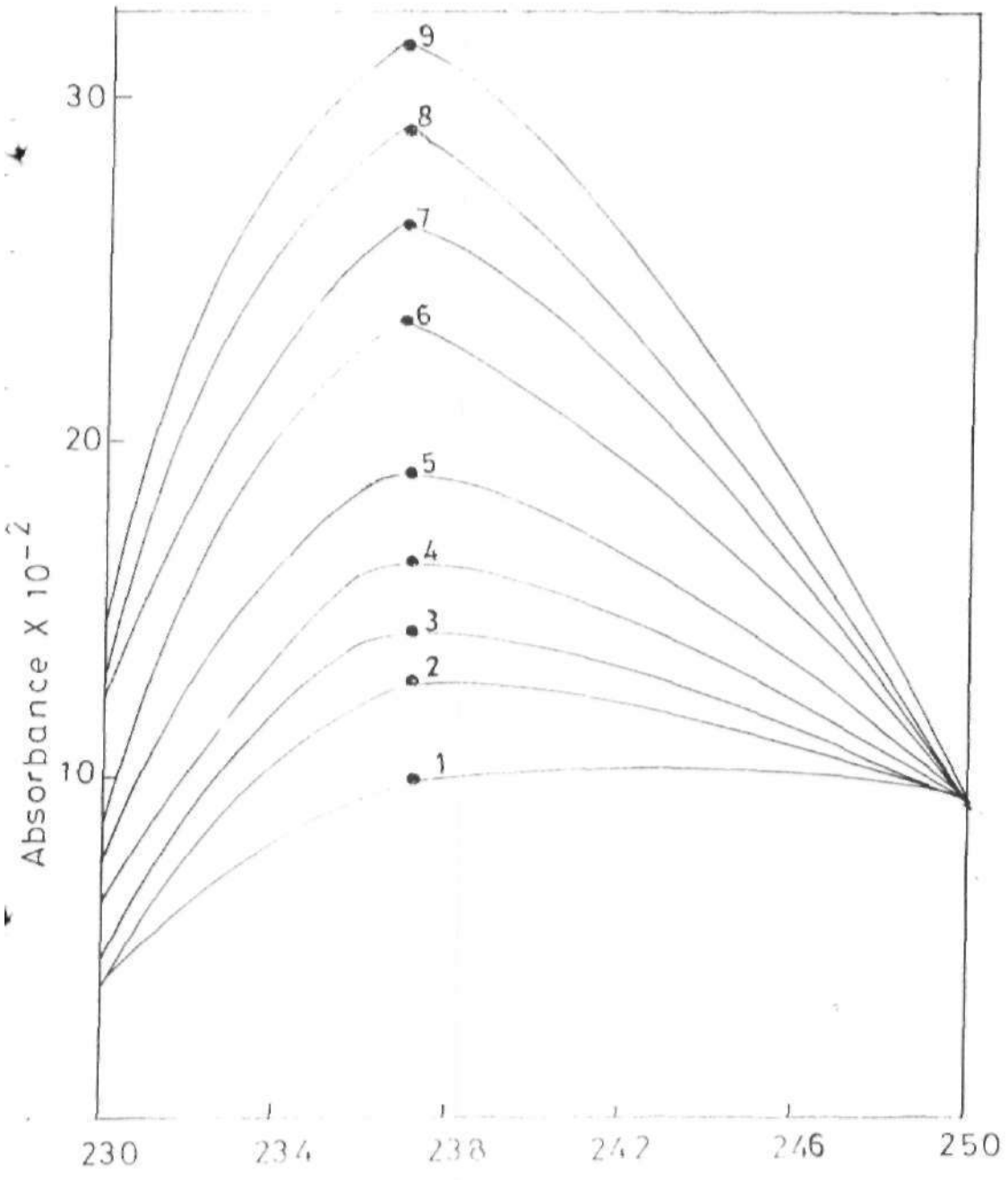


Figure 3·6. Difference UV spectra of 1:1 Vanadium (V) - Thrombin complex following addition of Aliquots of gallium (III). [Thrombin] [1.5×10^{-5}], [Ga] = 7.4×10^{-4} M, pH 7.40, 0.02 M phosphate buffer curve 1, 1μL Ga; 2, 5; 3, 10; 4, 15; 5, 20; 6, 30; 7, 40; 8, 50; 9, 60.

3.6 Vanadate-Thrombin, 1:1, Complex: pH Dependence Spectra

The unique pH dependence of the UV spectra of vanadate -thrombin, 1:1 complex, figure 3.25, indicates that metal -centered protonation is occurring over the pH range 6.20-9.20. This could involve either protonation of an oxo group to form ahydroxo ligand, equation 3.4, or further protonation of a hydroxo group to form a bound water molecule, equation 3.5.

$$VO_2^{+} + H^{+} \quad VO(OH)^{2+} \quad \ldots\ldots 3.4$$

$$VO(OH)^{2+} + H^{+} \quad VO(H_2O)^{3+} \ldots\ldots 3.5$$

proton displacement studies which would help establish the stoichiometry of the thrombin complex, are essentially precluded by the tendency of vanadate to polymerize at the higher concentrations needed for such studies (Baes and Mesmer, 1976).

3.7 Potentiometric Titrations of the Vanadate-Ligand Complexes at pH 7.40

Vanadate complexes of the ligands - representative pH metrictitration curves for the ligands and vanadate ion complexation can be seen from figures 3.9 to 3.16. The complex formation in the vanadate-L-Alanine, vanadate-L-Aspartic acid,vanadate-L-Histidine, vanadate-L-proline and vandate-L-Threonine systems are limited to pH 7.40 with differing stoichiometries. While the vanadate-L-Ascorbic acid is formed at the acidic pH of 6.40, and the vanadate-L-Tyrosine is formed in the approximate pH range of 7.20 and 7.40. Table 3.5 shows complexes of moderate stability formed at the indicated pH with respective stoichiometries. From the potentiometric measurements Log β values (section 2.5) were determined. The calculated values areas shown in table 3.5

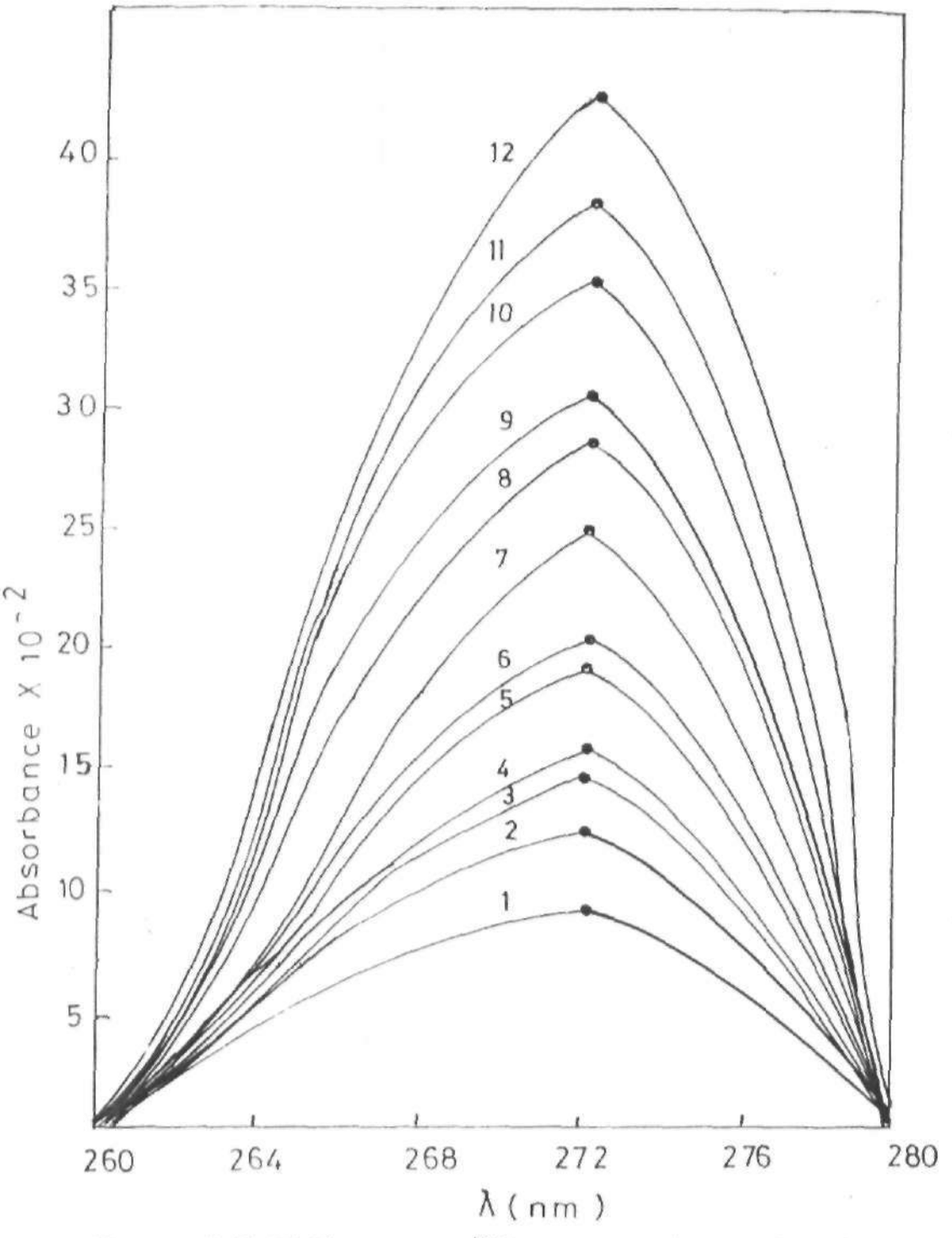


Figure 3·7. Difference UV spectra from titrations of trypsin with ammonium vanadate in 0·02M phosphate buffer at pH 7·40, t = 29·0 ± 0·5°C. [Trypsin] = 5.00×10^{-5} M, $[VO_3^-] = 2.75 \times 10^{-4}$, curve 1, 10 µL VO_3; 2, 30; 3, 50; 4, 70; 5, 90; 6, 110; 7, 130; 8, 150; 9, 170; 10, 195; 11, 260; 12, 360.

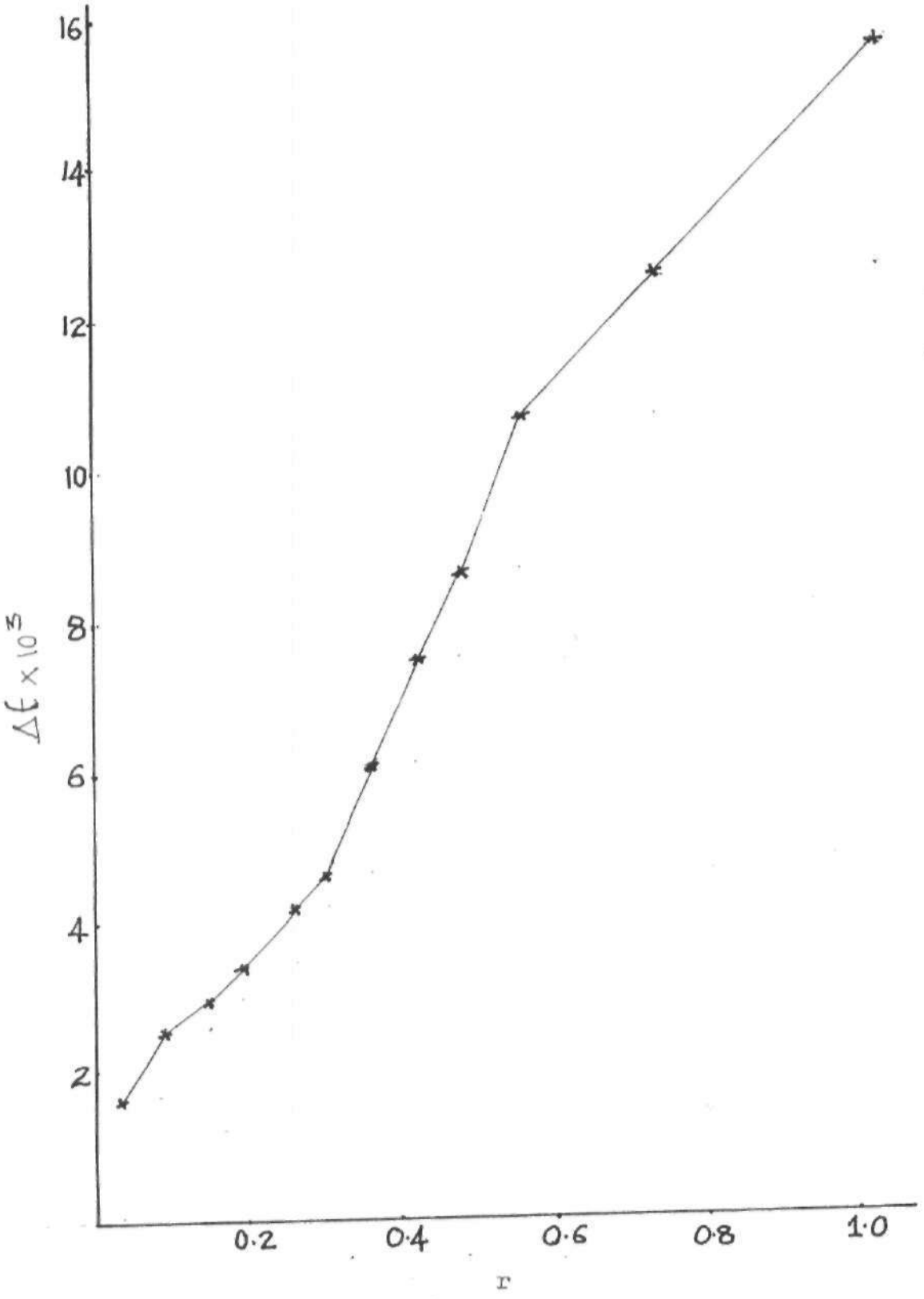


FIGURE 3.8 PLOTS OF $\Delta\epsilon$ OBSERVED VERSUS r FOR V.H.ADATE-TRYPSIN COMPLEX AT pH 7.40

3.8 Charge-Transfer Spectra of the Vanadate-Ligand Complexes at the Stable Metal-Ligand Stoichiometry.

Table 3.6 is a summary of the change-transfer spectra of the vanadate-ligand complexes at various equivalence ratios. Amino acids do not absorb visible light (they are colourless) and with the exception of aromatic amino acids - Tryptophan, Tyrosine, phenylalanine and, Histidine, they do not absorb above 240nm.

TABLE 3.5. STABILITY DATA FOR COMPLEXES PRESENT IN THE VANADATE - LIGAND, ML_n SYSTEMS

COMPLEX	RATIO	Log β ±0.20	pH	λ_{max} (nm)
[M-Ala_3]	1:3	4.49	7.40	204
[M-Asc]	1:1	1.39	6.40	200
[M-Asp_3]	1:3	4.49	7.40	202
[M-His_2]	1:2	2.92	7.40	216
[M-Pro_2]	1:2	2.92	7.40	208
[M-Thr_4]	1:4	6.09	7.40	196
[M-Tyr_3]	1:3	4.49	7.20	198
[M-$Acet_3$]	1:3	4.49	7.20	209
[M-$Hist_2$]	1:2	2.92	7.60	215
[M-$Ethy_2$]	1:2	2.92	7.75	205
[M-$DAPA_3$]	1:3	4.49	7.30	201

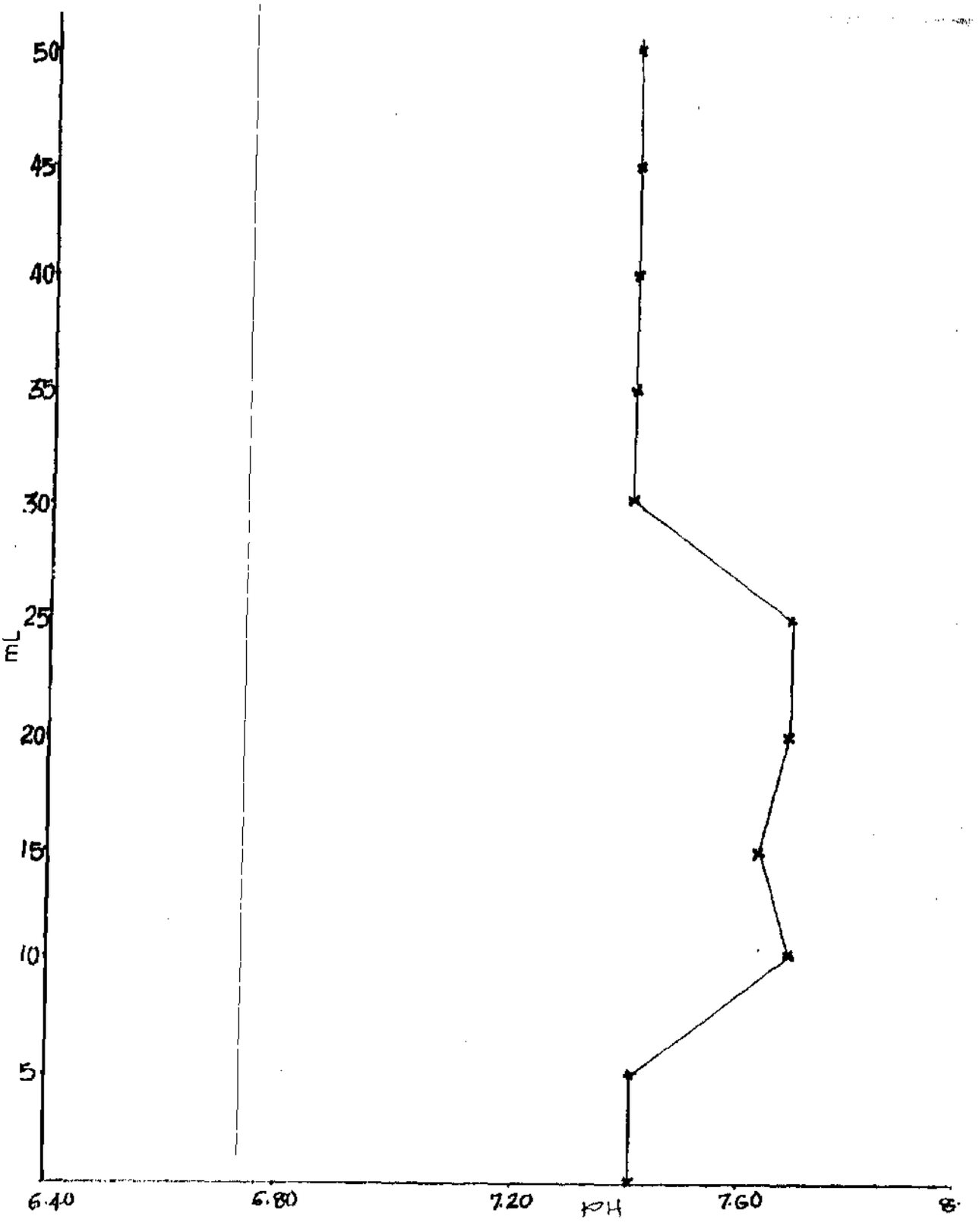


FIGURE 3·9 Curve for pH titrations of [Vanadate] = 1.08×10^{-4} M, and [L-Alanine] = 0.02M; 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M

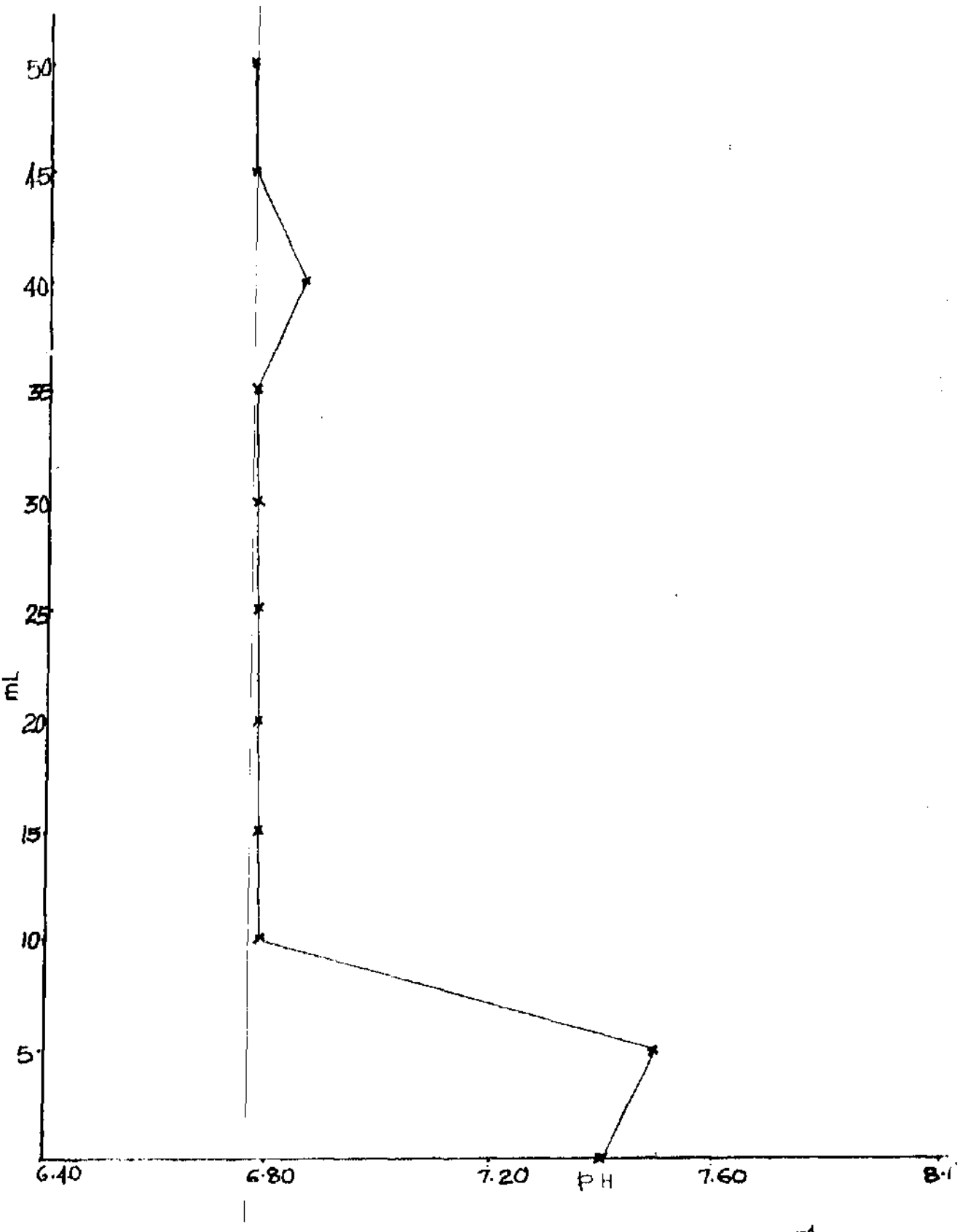


FIGURE 3.10 Curve for pH titrations of [Vanadate] = 1.08×10^{-4} M, and [L-Ascorbic Acid] = 0.02M; 0.02M Phosphate buffer, t = 29.5 ± 0.50°C, I = 0.02M

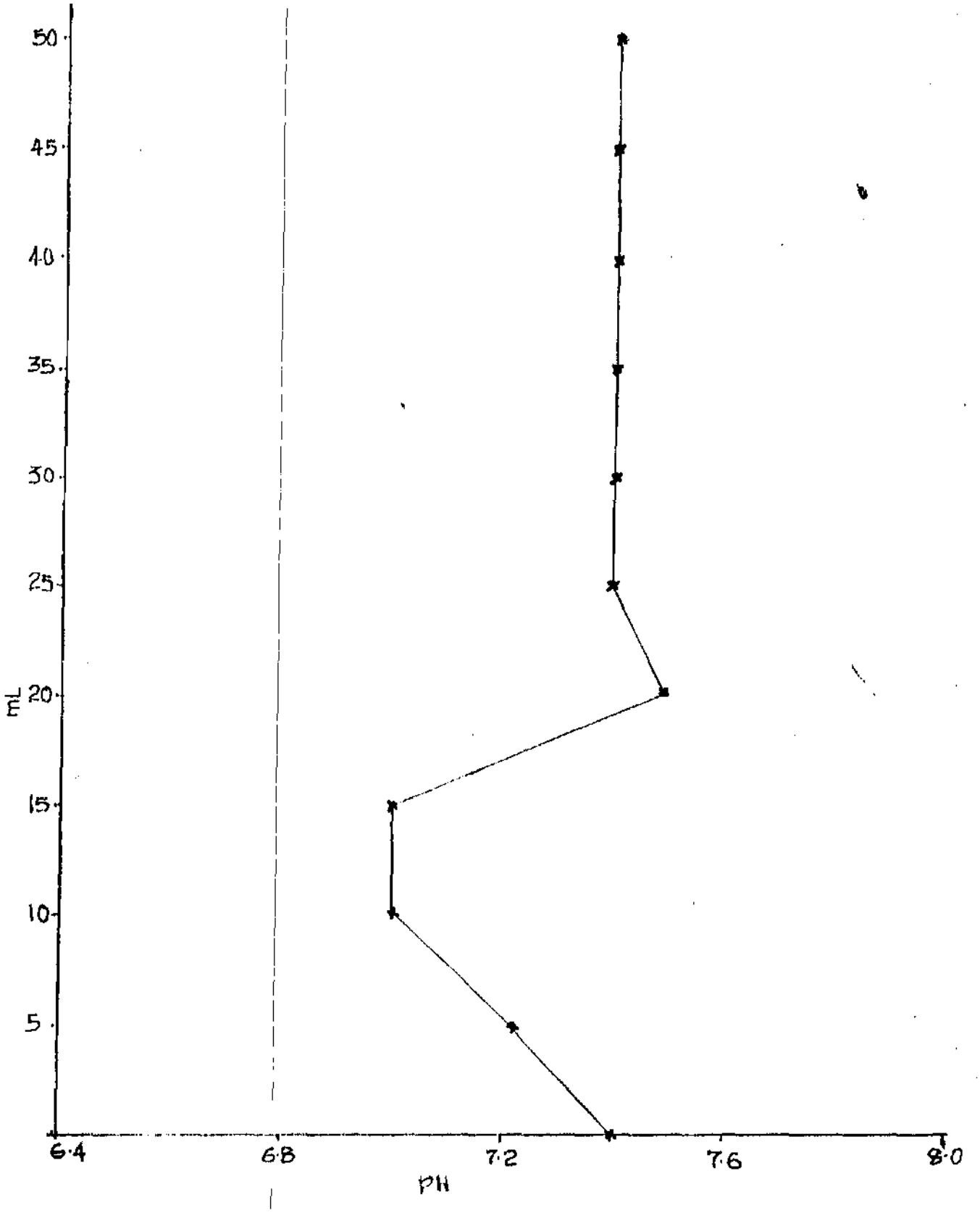


FIGURE 3.11 Curve for pH titrations of [Vanadate] = 1.08×10^{-4}M and [L-Aspartic Acid] = 0.02M, 0.02M Phosphate buffer, t = 29.50 ± 0.50°C

I = 0.02M

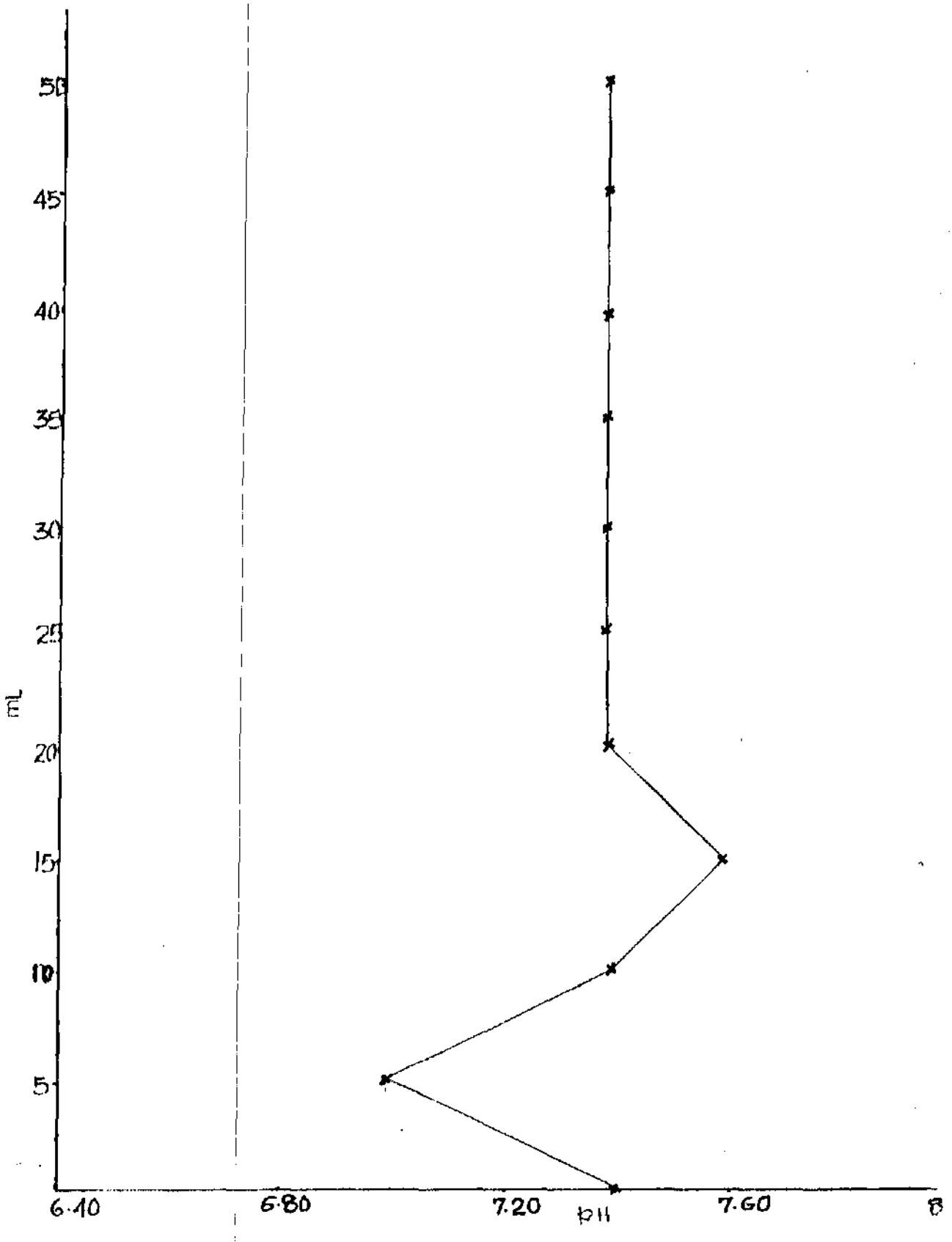


FIGURE 3-12 Curve for pH titrations of [Vanadate] = 1.08×10^{-4}M and [L-Histidine] = 0.02M; 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M

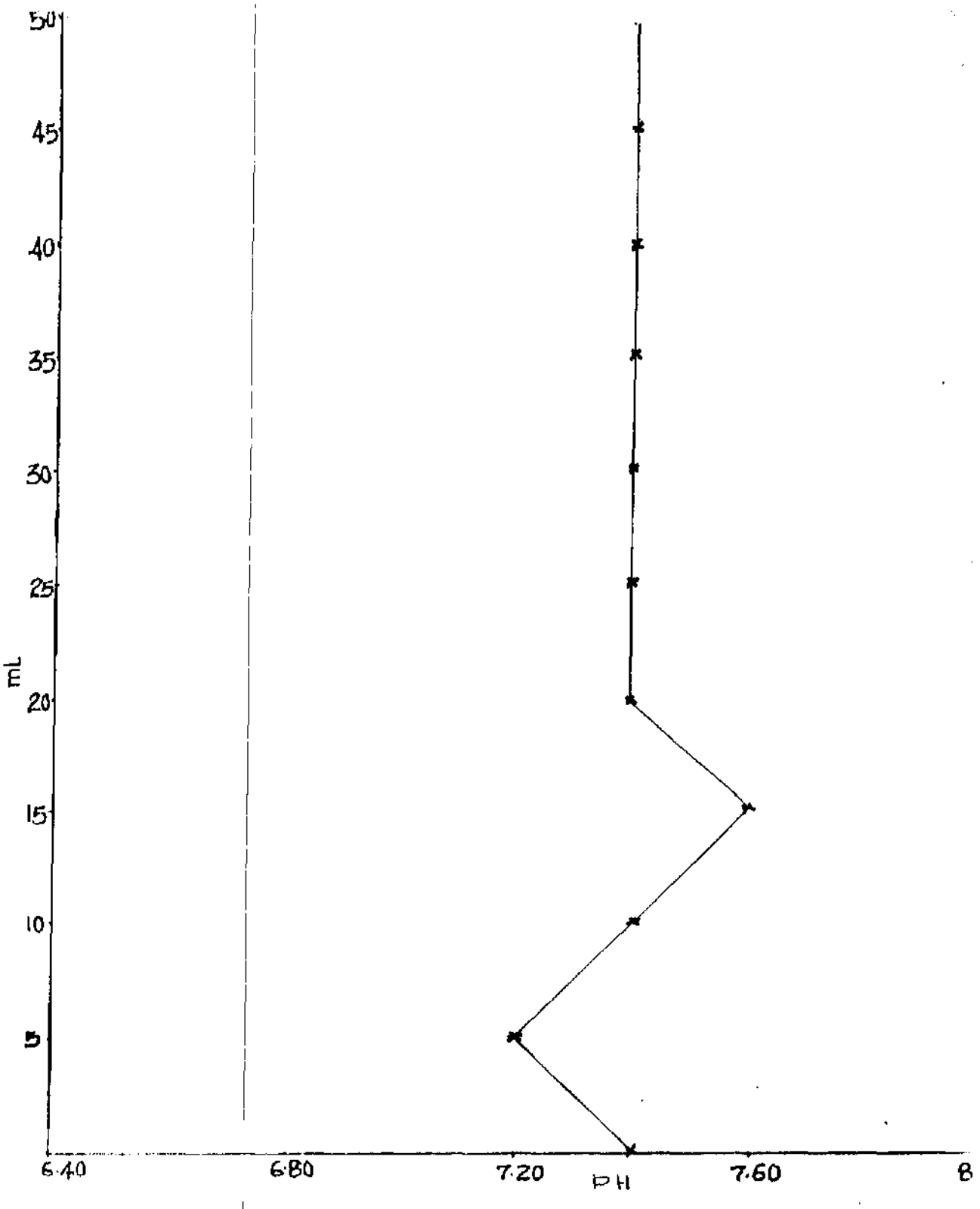


FIGURE 3.13 Curve for pH titrations of [Vanadate] = 1.08×10^{-1} M, and [l -Proline] = 0.02M; 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M

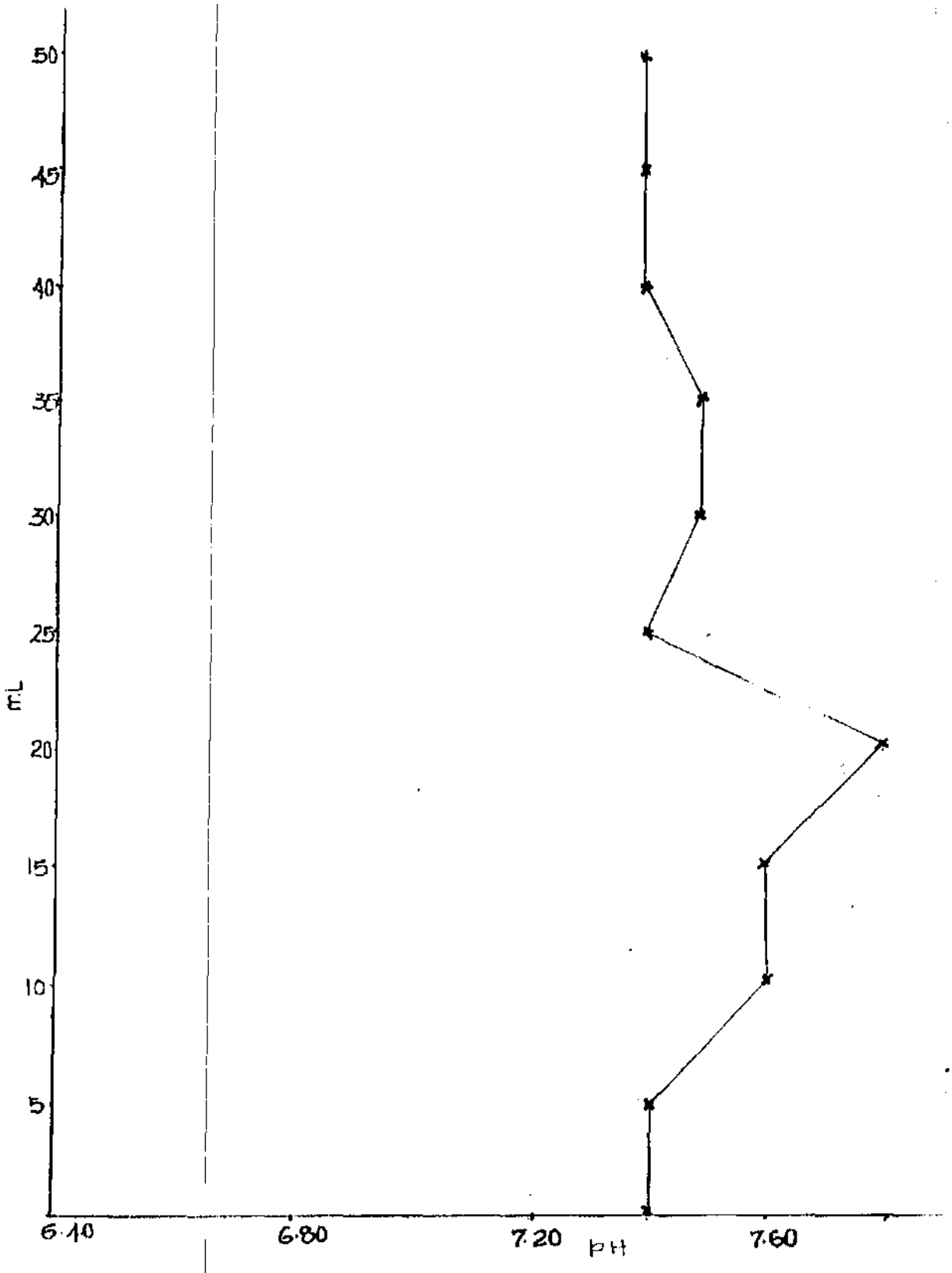


FIGURE 3·14 Curve for pH titrations of [Vanadate] = 1.08×10^{-4}M and [L-Threonine] = 0.02M: 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M

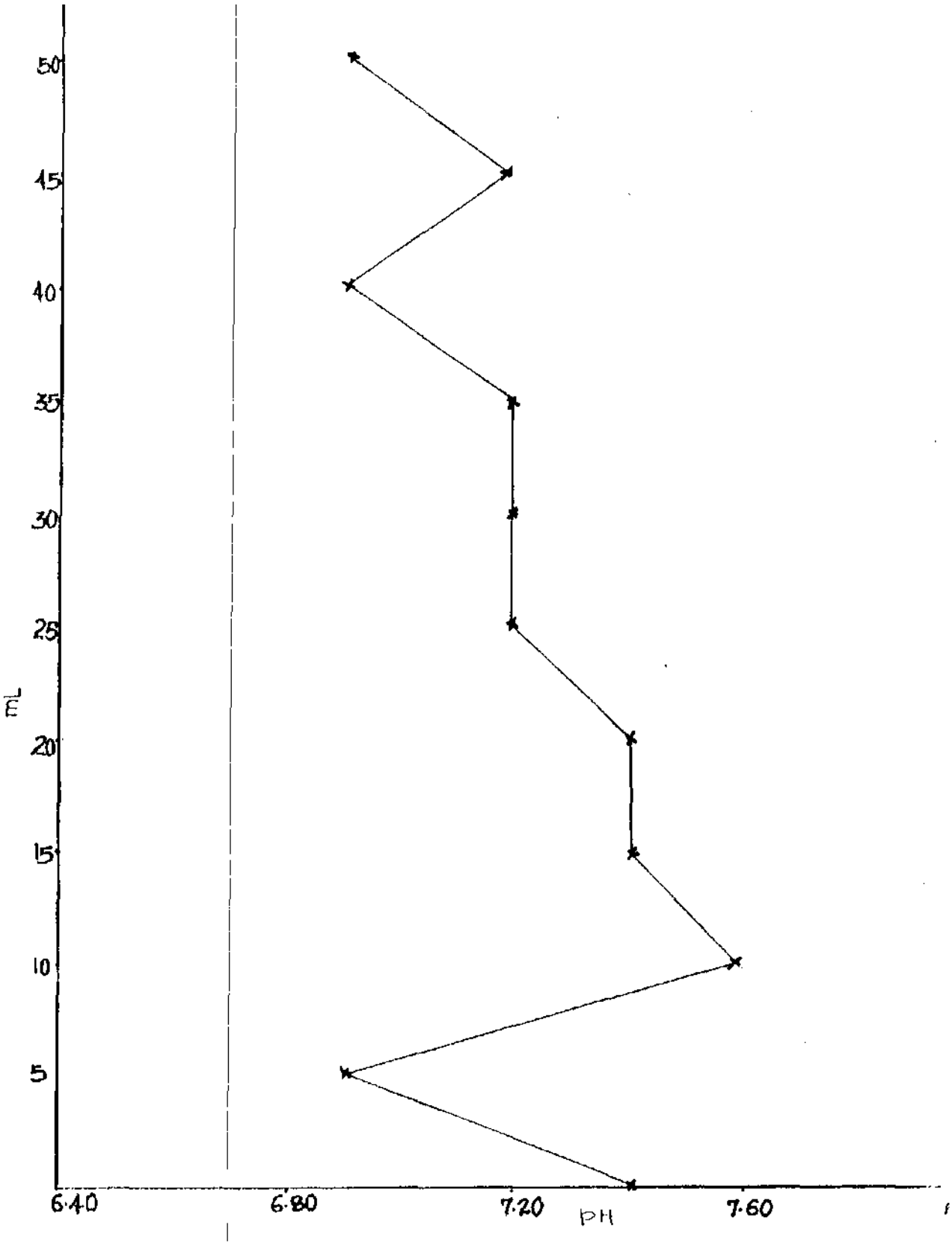


FIGURE 3·15 Curve for pH titrations of [Vanadate] = 1.08×10^{-4}M and [L-Tyrosine] = 0.02M; 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M

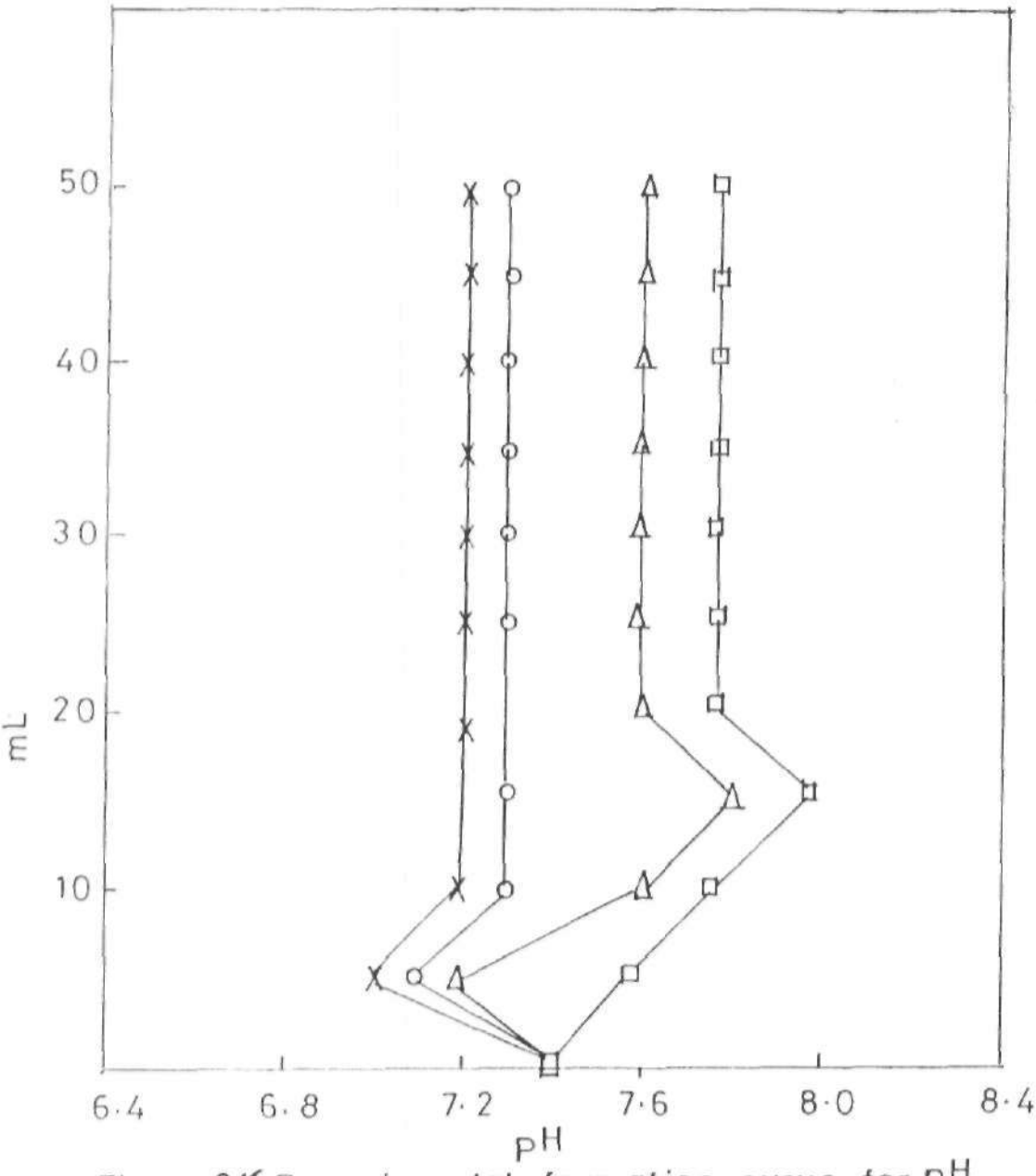


Figure 3·16. Experimental formation curve for pH titrations of [Vanadate] ≃ 1.08×15^{-4} M and [Acetate] ≃ 0·02 M x—x; [Ethylamine] ≃ 0·02 M, o—o; [Histamine] ≃ 0·02 M Δ—Δ; [2, 3 - DAPA] ≃ 0·02 M □—□; in 0·02 M Phosphate buffer, t = 29·5° ± 0·5°C, I = 0·02 M.

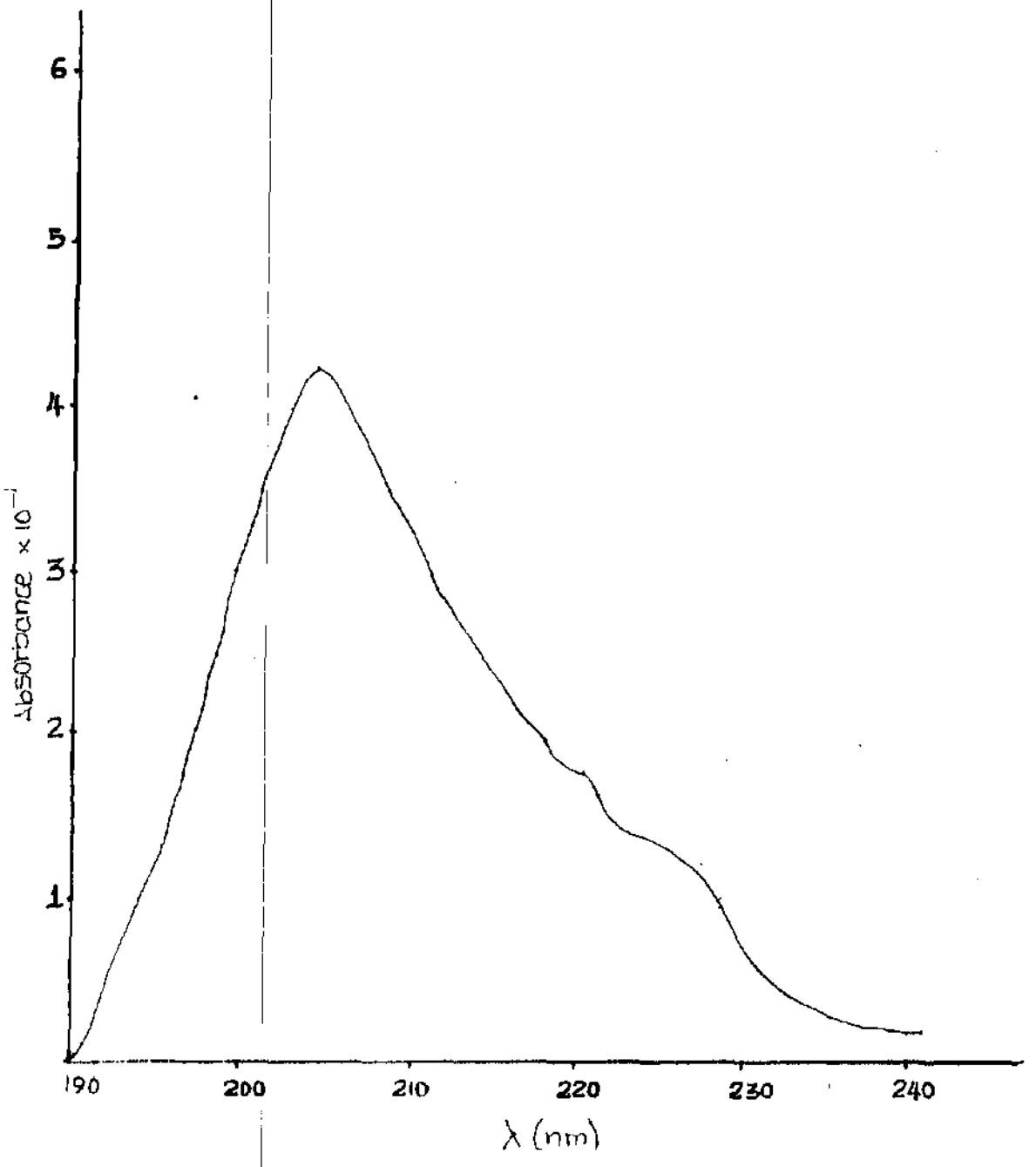


FIGURE 3.17. **Absorption Spectra of L-Alanine - Vanadate Complex,3:1, pH 7.40, 0.02M Phosphate buffer, t = 29.50 ± 0.50°C, I = 0.02M**

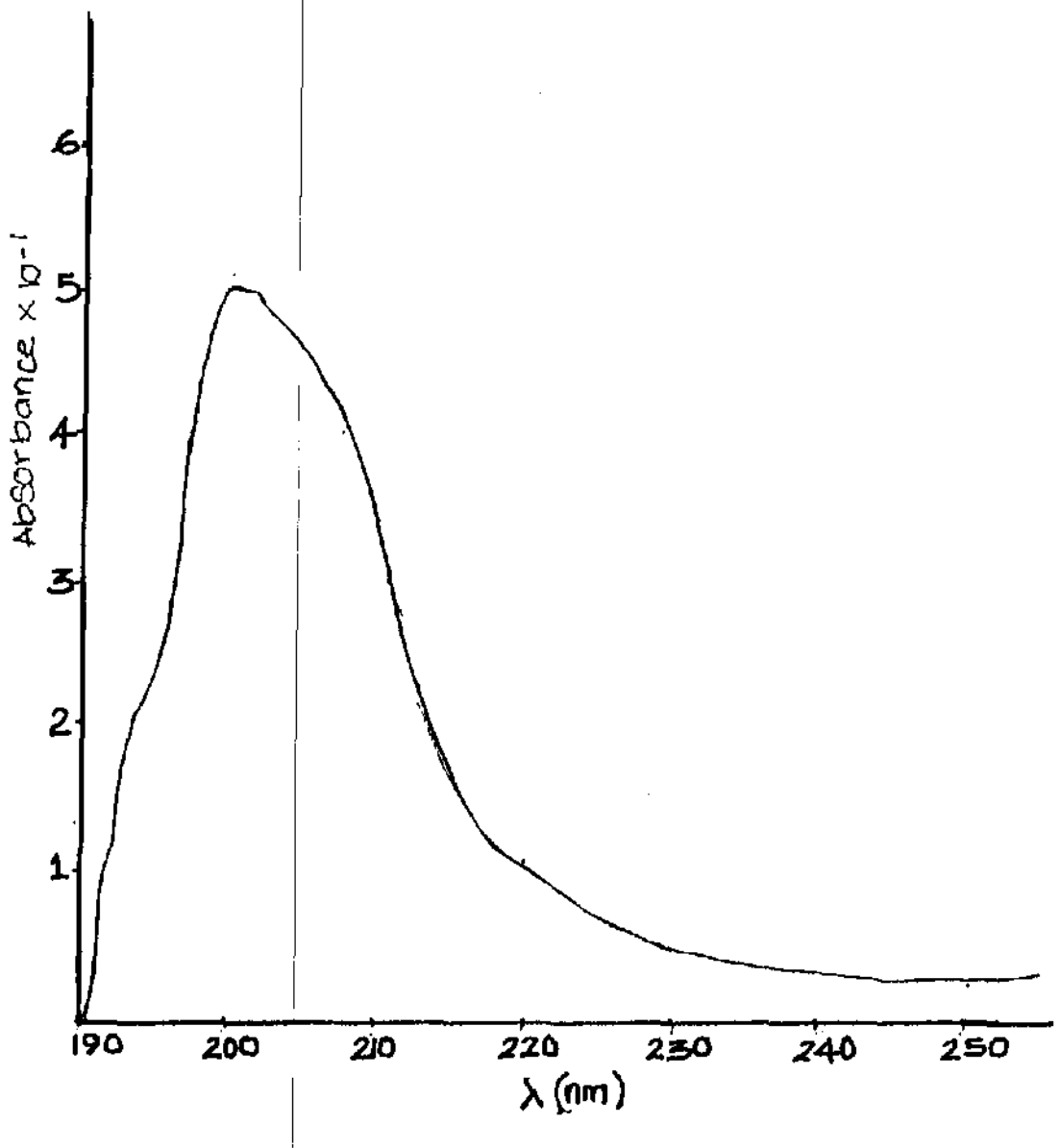


FIGURE 3.18 Absorption Spectra of L-Ascorbic Acid - Vanadate Complex, 1:1, pH 7.40, 0.02M Phosphate buffer, t = 29.50± 0.50°C, I = 0.02M

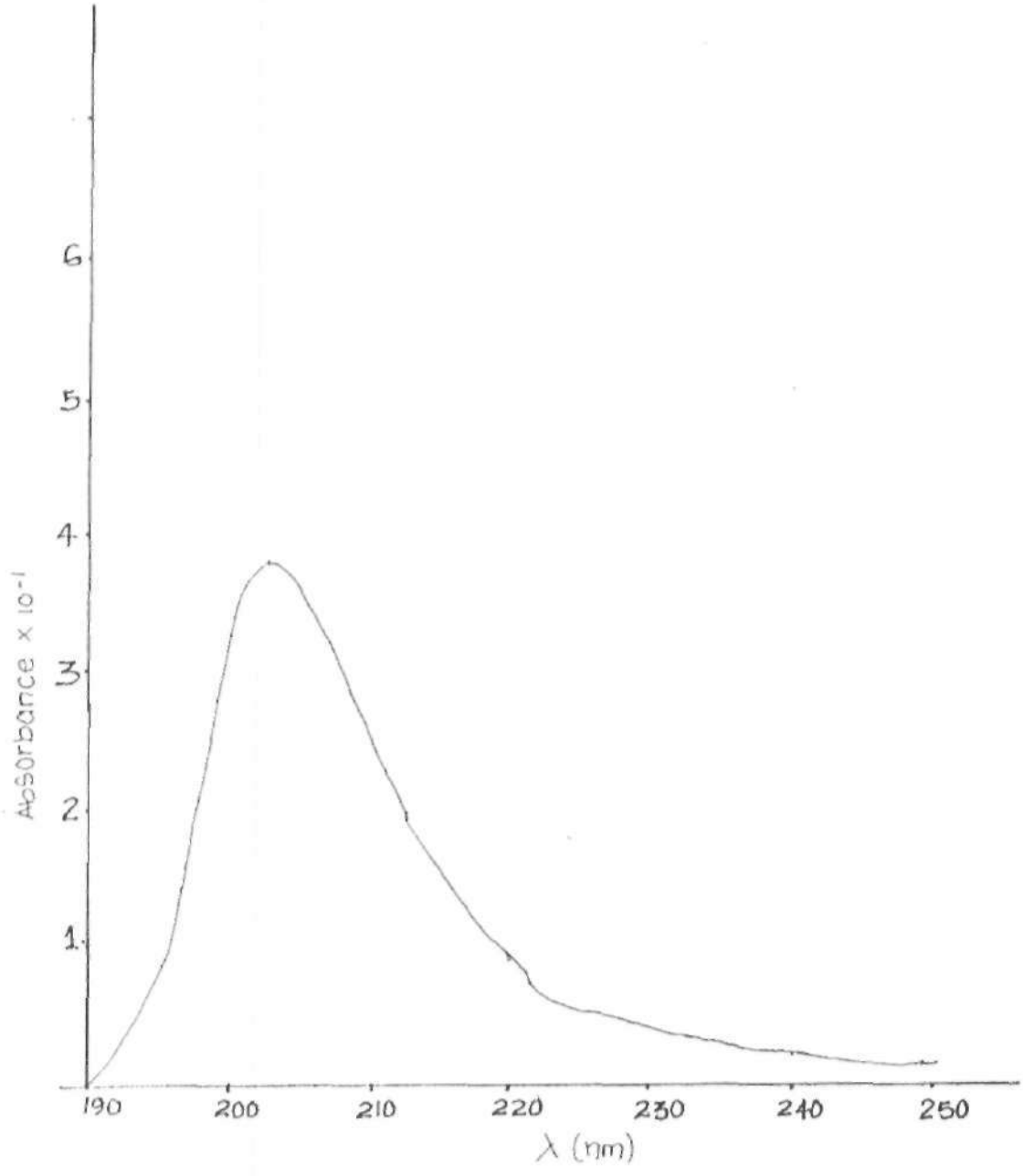


FIGURE 3.19 Absorption Spectra of L-Aspatic Acid – Vanadate Complex, 3:1, pH 7.40, 0.02M Phosphate buffer, t = 29.50 ± 0.50 °C, I = 0.02M

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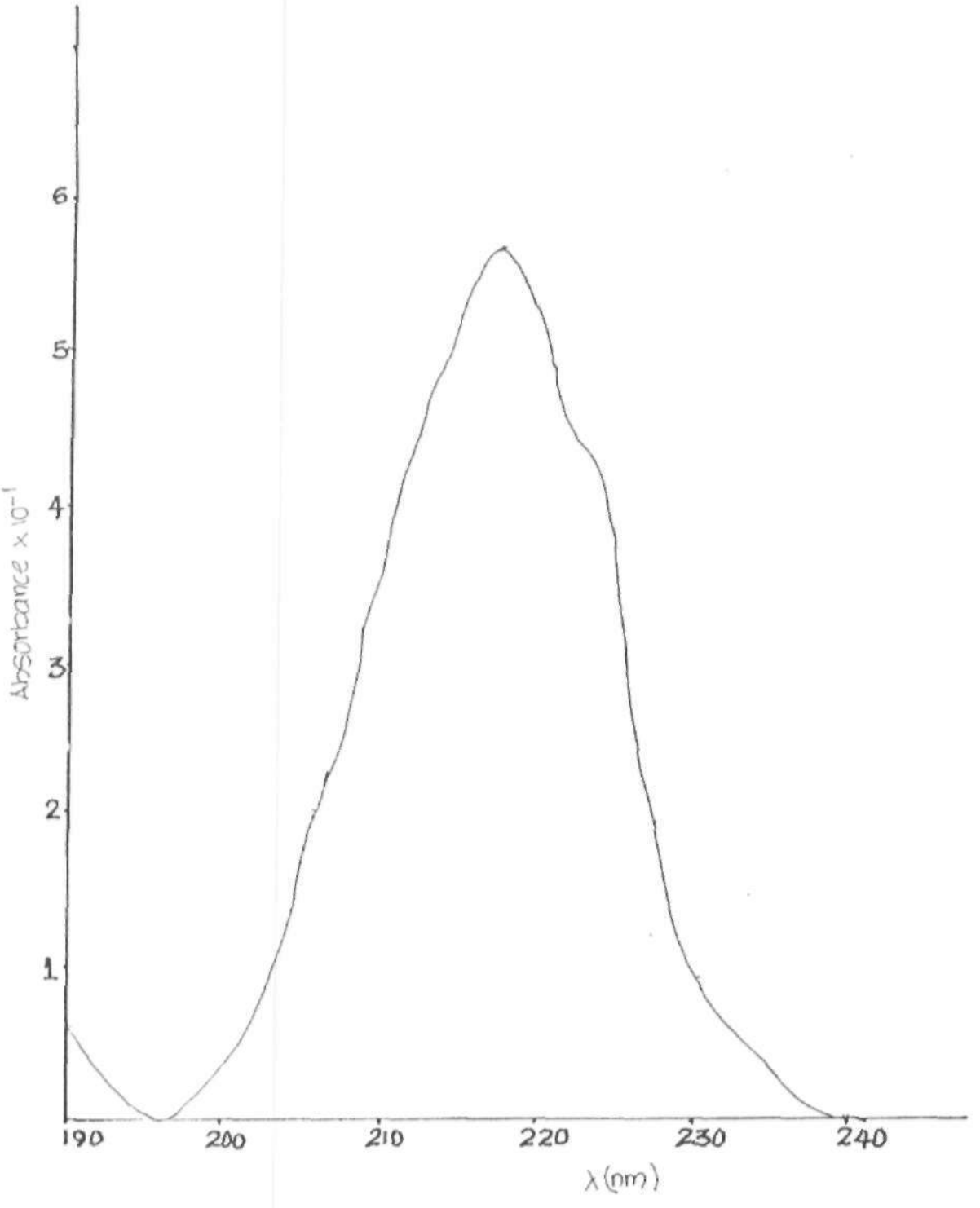


FIGURE 3.20 **Absorption Spectra of L-Histidine - Vanadate Complex (1:1, pH 7.48, 0.02M Phosphate buffer, t = 29.5±0.5°C, I = 0.02M**

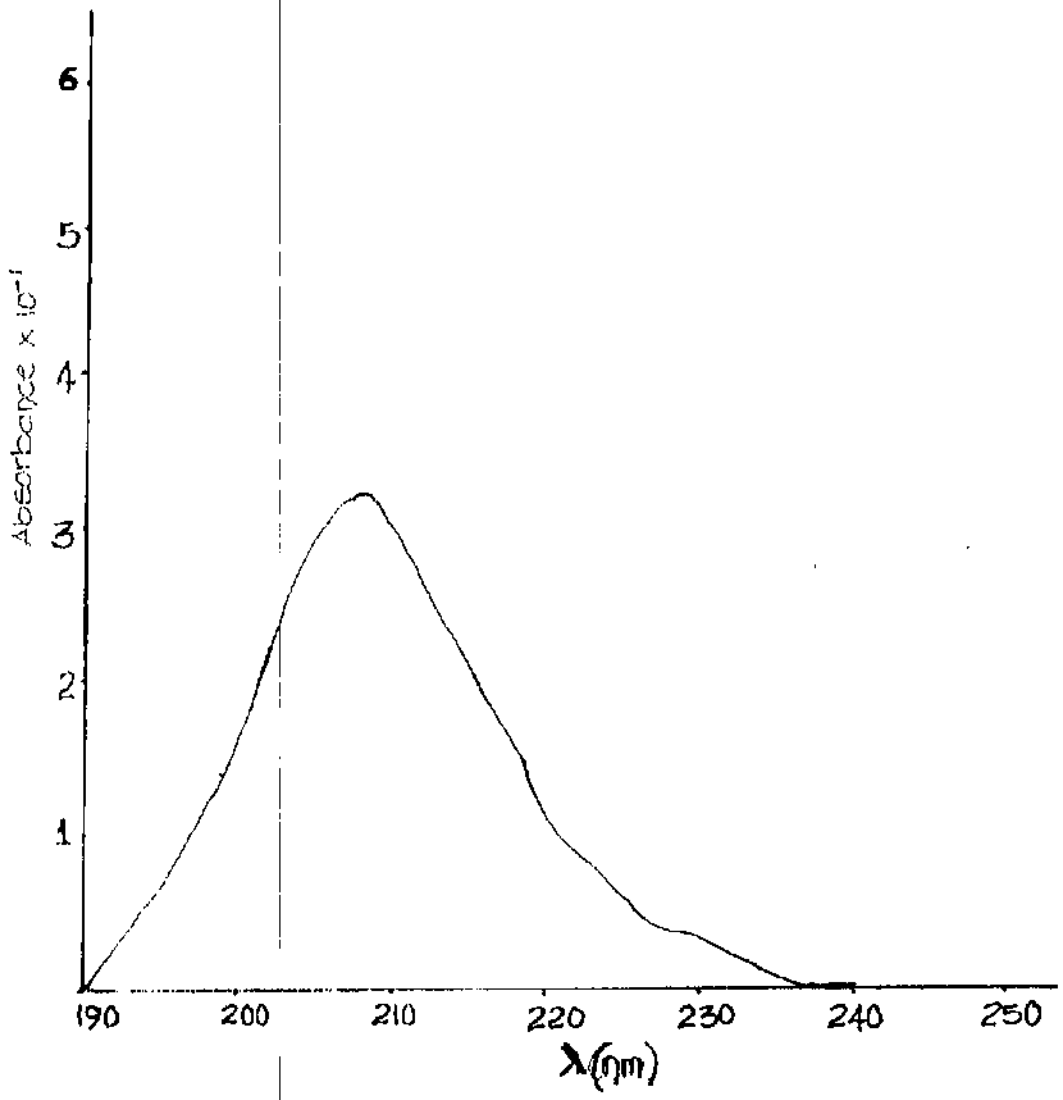


FIGURE 3.21 Absorption Spectra of L-Proline - Vanadate Complex, 2:1, pH 7.40, 0.02M Phosphate buffer, t = 29.50± 0.50°C, I = 0.02M

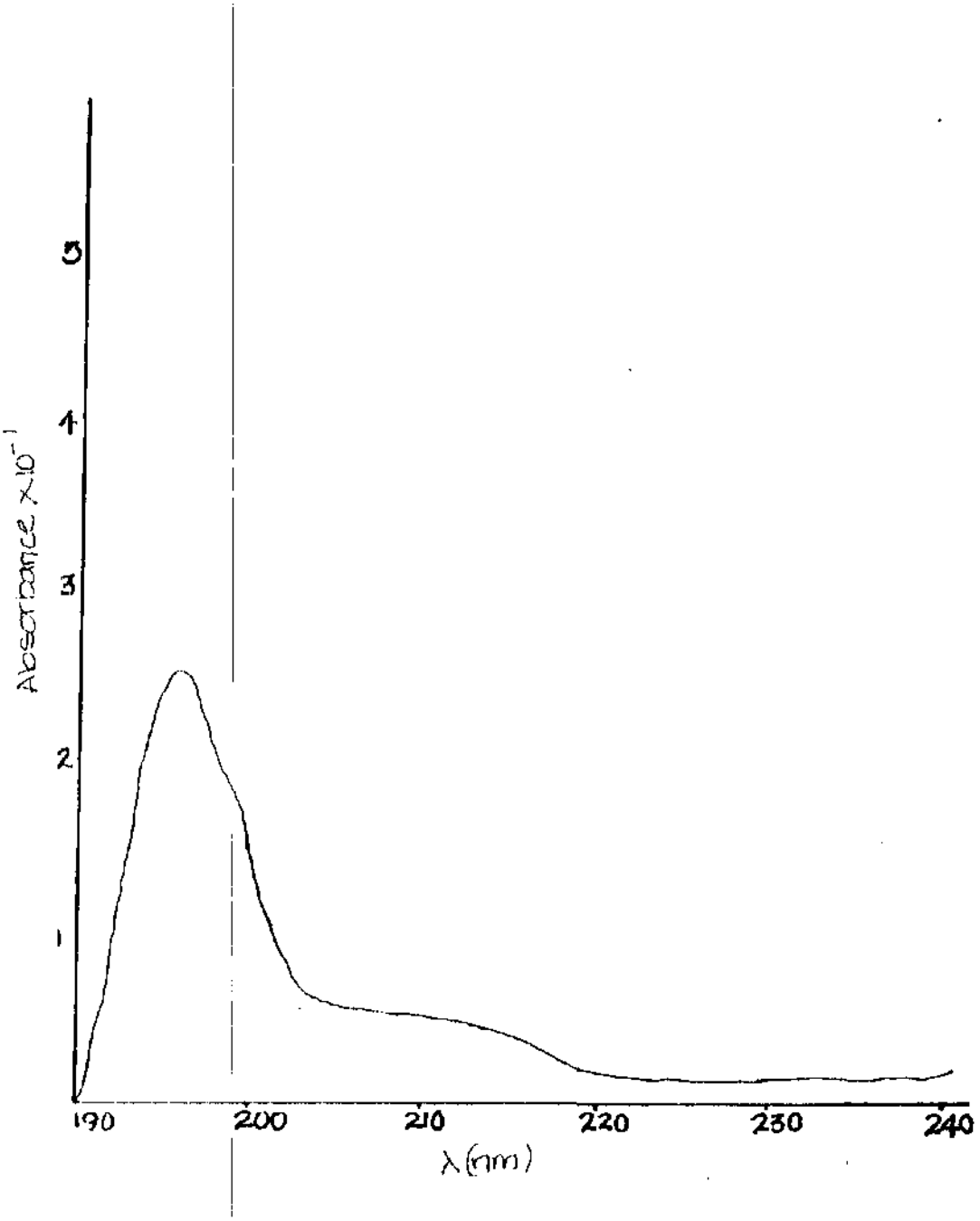


FIGURE 3.22 Absorption Spectra of L-Threonine - Vanadate Complex, 4:1, pH 7.40, 0.02M Phosphate buffer, t = 29.50±0.50°C, I = 0.02M

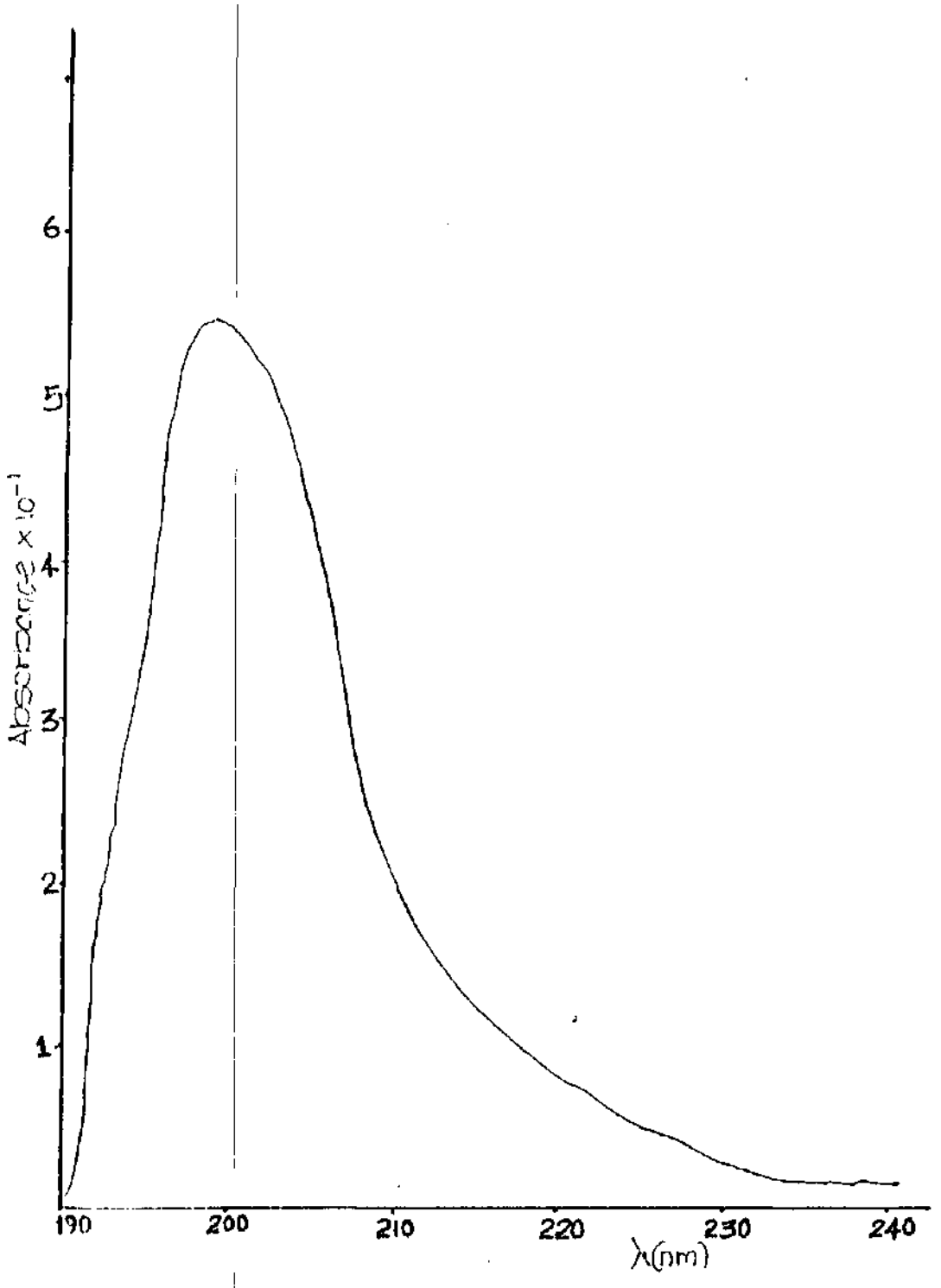


FIGURE 3.23 Absorption Spectra of L-Tyrosine - Vanadate Complex, 3:1, pH 7.40, 0.02M Phosphate buffer, T = 29.50 ± 0.50°C, I = 0.02M

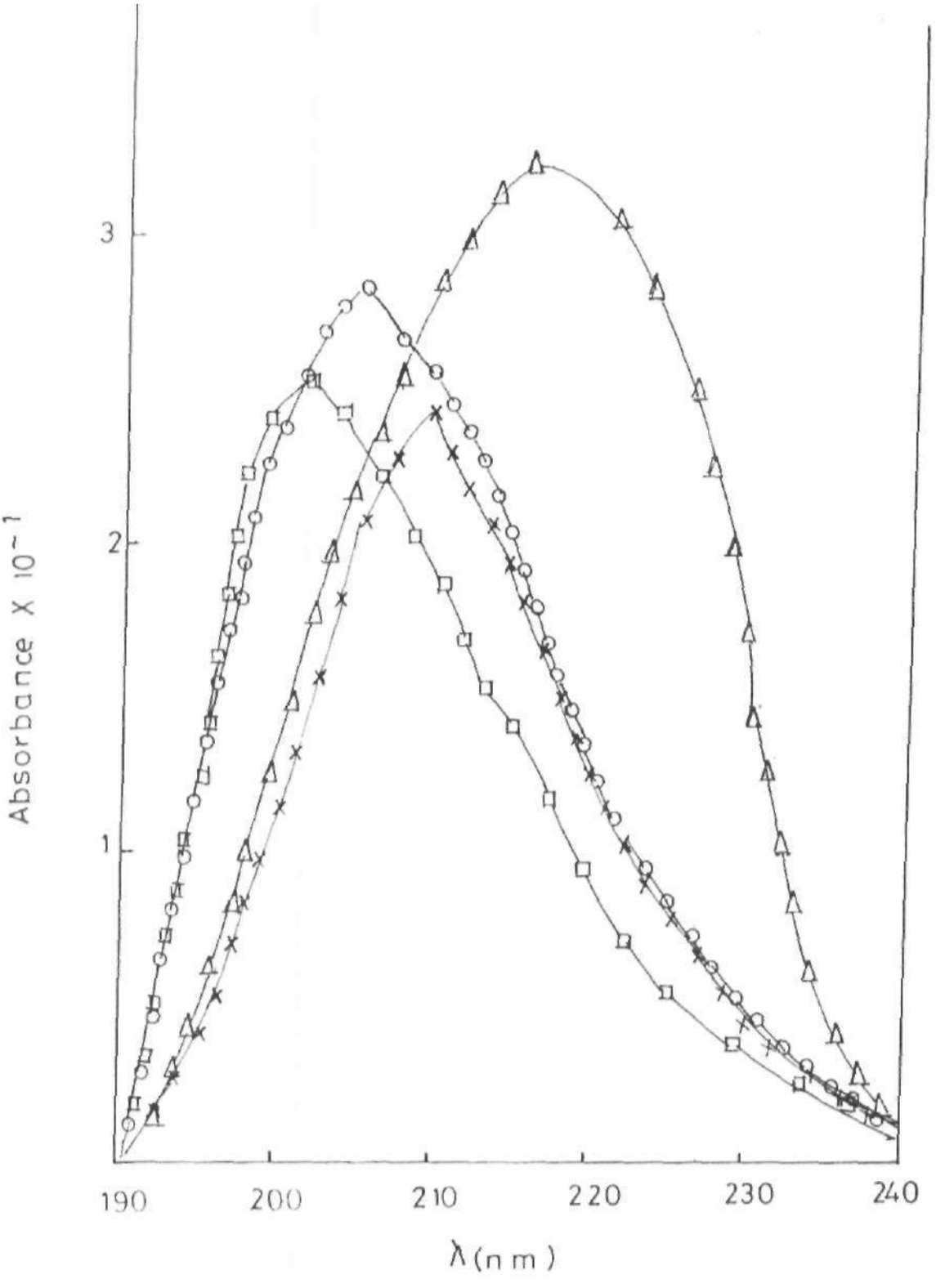


Figure 3.24. Absorption spectra of model ligands showing:

Δ—Δ = (Histamine - Vanadate), 2:1

o—o = (Ethylamine - Vanadate), 2:1

x—x = (Acetate - Vanadate), 2:1

□—□ = (2,3- DAPA - Vanadat), 3:1

0·02 M phosphate buffer, pH 7·40, t = 29·5 ± 0·5°C.

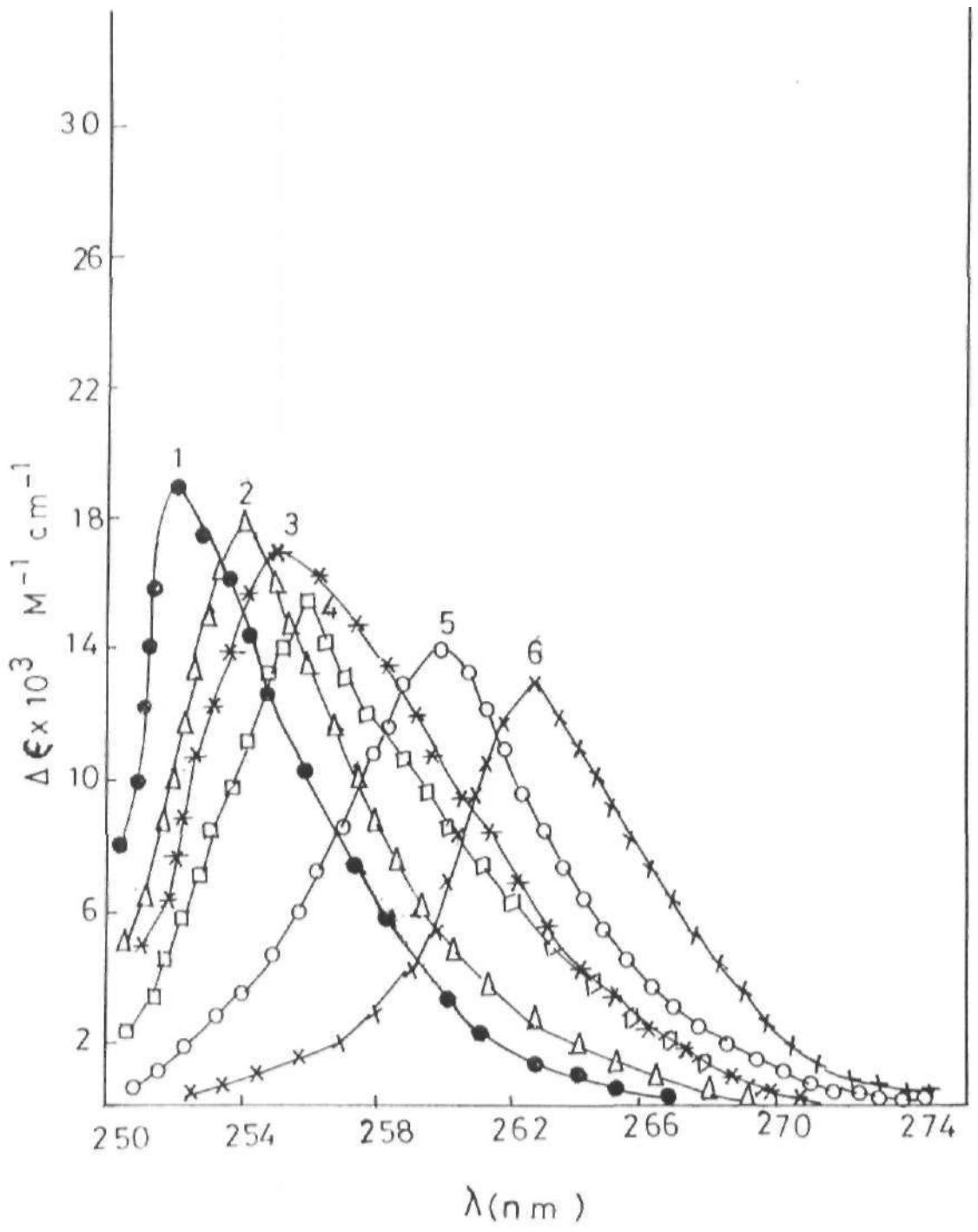


Figure 3.25. Difference UV spectra of 1:1 Vanadium(V) - thrombin complex as a function of pH. 1, pH 6.20; 2, pH 6.75; 3, pH 7.00; 4, pH 7.40; 5, pH 8.60; 6, 9.20.

TABLE 3.4 pH Metric titrations of 10mL [vanadate] = 1.08×10^{-4} M pH 7.40 with the various ligands as shown.

Volume of Ligand added(mL)	Corresponding pH changes on addition of [Ligand] = 0.02M, pH 7.40										
	L-Ala	L-Asc	L -Asp	L-His	L-pro	L-Thr	L-Tyr	Ethy	Acet	Hist	DAPA
0	7.40	7.40	7.40	7.40	7.40	7.40	7.40	7.40	7.40	7.40	7.40
5	7.40	7.10	7.20	7.00	7.20	7.40	6.90	7.95	7.00	7.20	7.10
10	7.70	6.40	7.00	7.40	7.40	7.60	7.60	7.75	7.20	7.60	7.30
15	7.65	6.40	7.00	7.60	7.60	7.60	7.40	7.90	7.40	7.80	7.30
20	7.70	6.40	7.60	7.40	7.40	7.80	7.40	7.75	7.20	7.60	7.50
25	7.70	6.40	7.40	7.40	7.40	7.40	7.20	7.75	7.20	7.60	7.30
30	7.40	6.40	7.40	7.40	7.40	7.45	7.20	7.75	7.20	7.60	7.30
35	7.40	6.40	7.40	7.40	7.40	7.45	7.20	7.75	7.20	7.60	7.30
40	7.40	6.45	7.40	7.40	7.40	7.40	7.00	7.75	7.20	7.60	7.30
45	7.40	6.40	7.40	7.40	7.40	7.40	7.25	7.75	7.20	7.60	7.30
50	7.40	6.40	7.40	7.40	7.40	7.40	7.00	7.75	7.20	7.60	7.30

Table 3.6 Charge transfer spetra at the equivalence points cosidered for the vanadate-Ligand complexes.

		λ_{MAX}			
LIGAND	M:L	1:1	1:2	1:3	1:4
L- Alanine		-	-	204nm	-
L-Ascorbic acid		200nm	-	-	-
L-Aspartic acid		-	-	202nm	-
L-Histidine		-	216nm	-	-
L-Proline		-	208nm	-	-
L-Threonine		-	-	-	196nm
L-Tyrosine		-	-	198nm	-
Acetate		-	209nm	-	-
Ethylamine		-	205nm	-	-
Histamine		-	215nm	-	-
2,3 Diamino-Propionic acid		-	-	201nm	-

CHAPTER FOUR

4.0 DISCUSSIONS

4.1 Ammonium Vanadate pH Dependent Spectra

Much work has been devoted to clarifying the nature of the vanadium(V) species on solutions at varying hydrogen ion concentrations. However, even though the existence of VO_2^+ ion at low concentration has been well established the strong tendency to the formation of polymerization reactions has made it difficult to determine the composition of those hydroxo -complexes that are formed in solution between pH 1 and 11 and many different formulae have been published (Rossotti and Rossotti, 1956; Ingri and Brito, 1960).The study of the hydrolysis of vanadium(V) of varying pH in 0.02M per chlorate medium using 0.02M phosphate buffer in the pH range 10.55 to 5.60 at 29.5°C and assigned the following equilibria:

$$2HVO_4^{2-} \quad HV_2O_7^{3-} + OH^- \quad \ldots\ldots 4.1$$

$$3HVO_4^{2-} \quad V_3O_9^{3-} + 3OH^- \quad \ldots\ldots 4.2$$

$$HVO_4^{2-} \quad VO_3^- + OH^- \quad \ldots\ldots 4.3$$

$$HVO_4^{2-} + OH^- \quad VO_4^{3-} \quad \ldots\ldots 4.4$$

The vanadate ion has its own pH dependent UV spectra as shown in Figure 3.1. The HV_4^{2-} species has a λ_{max} at 266nm in this work aprotonation constant for HVO_4^{2-} of $10^{8.12}$ was obtained, which is in agreement with the literature value $10^{8.50}$ (Smith, 1976).

4.2 Nature of Vanadium(V) Bound to Thrombin

The biological activity of vanadium is obviously dependent upon the oxidation state (Boyd et al. 1984; Chasteen et al. 1986). Although the results

presented here clearly indicate that thrombin binds two equivalents of vanadium(V), figure 3.4, the question arises as to how the vanadium is binding to the protein. Two classes of thrombin binding sites have been reported as high and low affinity binding sites (Lin et al. 1979); trypsin shows the same site specificity with thrombin and plasmin (Sherry, 1954; Elmore, 1963). Therefore from trypsin used as a model ligand in these studies, one might deduce some evidence about the binding site of thrombin in the interaction of vanadate with thrombin by making judicious comparisons. Trypsin shows only one metal ion binding site and that consists of one or more carboxyl side chains. This study agree with Epstein (1974)that at the metal binding site of trypsin, the carboxyl chains come together and form a cluster of negative charges which interact with metal ion. As a corollary only cationic species of metal ions are expected to bind at this site. From these studies, it was established that the vanadate species is binding as a cation, hence binding to the protein took place at the protein-metal binding site. This conclusion was drawn from the reversibility studies carried out with iron (IV) ions (figure 3.5)and gallium(III) ions (figure 3.6) which displaced the vanadate from the vanadate thrombin complex. Trypsin is reported to form octahedral complexes with some metal cations (Epstein et al., 1974).Vanadium also exhibits propensity towards forming complexes with high coordination numbers. The bisoxalate complex of vanadate is reported to be octahedral and absorbing in the UV region (Tracey et al., 1987). At pH 7.40, the vanadate-trypsin complex (1:1) has molar absorptivity greater than $10692 M^{-1} cm^{-1}$ in the difference UV, figures 3.7 and 3.8; the vanadium(V)-thrombin complex (1:1) metal to protein ratio hasmolar absorptivity of about $10082 M^{-1} cm^{-1}$ per metal binding site. The free vanadate

itself has an absorptivity of only $1944M^{-1}$ cm^{-1} at pH 7.40. The intensity of the vanadium(V)-thrombin absorbance and particularly the match with vanadium(V)-trypsin spectrum, suggests a reasonably strong interaction between the vanadium and the carboxyl groups at the metal binding sites of thrombin and trypsin.

Some, of the coordinated vanadium(V) for example VO_2^+ ,$VO(OH)^{2+}$, VO^{3+}, the unhydrolysed V^{5+}, $VO(O_2)^+$ ions are known (Orhanovic and Wilkins, 1967; Boyd and Kustin, 1984; Djordjevic et al. 1985, 1988; Chasteen et al. 1986). In these studies intense absorbance bands are occurring at λ_{max} 272nm for the model complex and at 256nm for the thrombin complex. This has been assigned as Ligand- to-metal charge transfer (LMCT) transitions from π electrons of the carboxyl oxygen of glutamine residue to d_π empty orbital of vanadium(V). The differences in the transition could probably be due to the strongly π-donating ligands at the metal binding sites of the protein that are capable of having exclusive share of one d_π orbital each (d_{xz},d_{yz}) and a third one (d_{xy}) OR share two orbitals and leave one unused (Cotton and Wilkinson, 1987; Sharpe, 1989).

Studies have shown V^{5+}, VO^{3+} and $VO(O_2)^+$ ions when coordinated in proteins are expected to produce intense bands in the visible region (Cooper, 1982; Carrano, 1984; Djordjevic, 1985); the lack of any significant visible absorbance spectrum for vanadium(V)-thrombin, strongly suggested that the vanadium(V)is coordinating as VO_2^+ or $VO(OH)^{2+}$. The pH dependence of the difference UV spectrum probably represents a shift in the equilibrium between these two forms of vanadium(V). It is possible that the vanadium is binding as a dioxovanadium(V) ion. As the pH is lowered, one of the oxo groups is protonated which shifts the

λ_{max} in the difference spectra and raises the $\Delta\in$ value from about 13000 to 19000 per vanadium over the pH range 9.20 to 6.20 studied, table 4.1.

TABLE 4.1 MOLAR ABSORPTIVITIES OF VANADIUM(V) - THROMBIN AS A FUNCTION OF pH

pH	λ_{max} (nm)	$\Delta\epsilon$ (M^{-1} cm^{-1})
6.20	252	19226
6.75	254	18193
7.00	255	16774
7.40	256	15354
8.60	260	14322
9.20	263	13161

The absorbance of the vanadate-thrombin complex is still much less than the ones observed for authentic trivalent metalions such as gallium(III) which has a value of 20986 per metalion (Table 4.2).

The vanadium(V) in VO_2^+, with two of its coordination sites occupied by oxygen atoms probably has an octahedral configuration with four available coordination sites. Hence, the donor atoms of the metal binding site of the protein will compete for these available coordination sites. From studies that vanadium(V)binds to protein in the absence of a synergistic anion (Schlabach and Bates, 1975) led to the possibility that there was retention of two oxo ligands (equation 4.3) OR an oxo and hydroxo ligands(equation 4.4), on the vanadium(V) in the vandadate-thrombin complex.

VO^+ + Thrombin = (O_2 V-Thrombin) 4.3

$VO(OH)^{2+}$ + Thrombin = ({OH}OV - Thrombin) 4.4

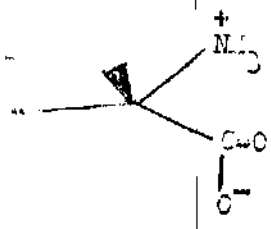
In some metal-protein chemistry studied, one theory holds that an anion is necessary to shield the incoming cation from a positively charged arginine or lysine near the protein metal binding site (Campbell and Chasteen, 1977). It is possible that an oxo or a hydroxo group bound to the central vanadium ion may in effect, act as the synergistic anion by assuming an orientation in the complex such that it is interposed between the vanadium(V) ion and the positively charged protein side groups.

TABLE 4.2 VALUES OF VANADATE-THROMBIN (1:1), VANADATE-TRYPSIN (1:1), Fe(III)-THROMBIN, Ga(III)-THROMBIN, COMPLEXES, AT pH 7.40

Complex	$\Delta\epsilon$ (M^{-1} cm^{-1})
Vanadate - Thrombin	10082
Vanadate - Trypsin	10692
Fe(III) - Thrombin	15197
Ga(III) - Thrombin	20986

4.3 Vanadate - Ligand, ML_n Type Complexes.

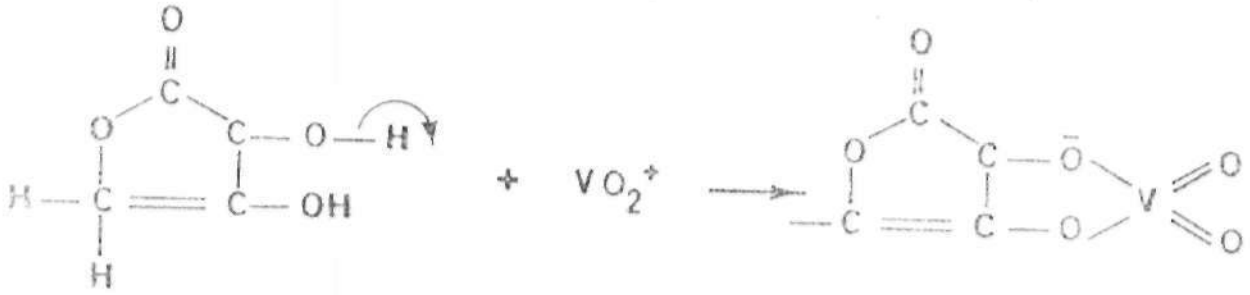
The vanadate complexes of the ligands studied -representative pH metric to titration curves for ligands and vanadate system are as shown in figure 3.9 to 3.16, while the charge transfer spectra at the metal-ligand ratio which the stable complex is formed are as represented in figures 3.19 to 3.24. Amino acids display the zwitterion in scheme 1 at physiological pH (Murray et al, 1990).



Scheme 1

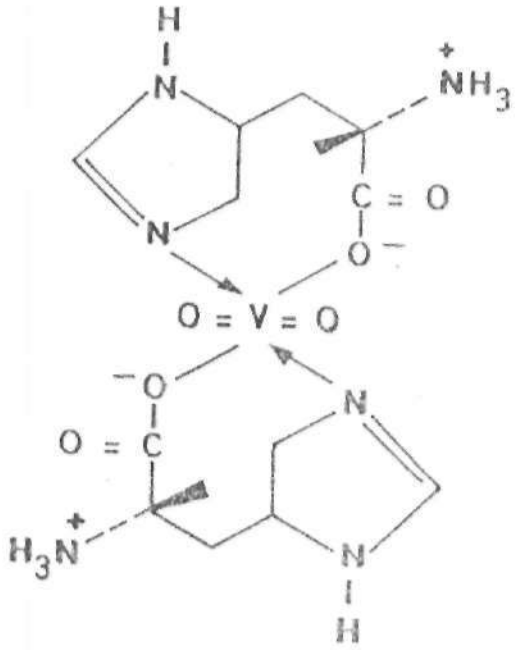
4.3.1 Vanadate - Ligand (1:1), ML Types Complex

Vanadium(V) formed a stable 1:1 metal-ligand complex with L-Ascorbic acid at pH 6.40. Figure 3.10 shows the profiles of aliquot addition in the pH metric titration. A λ_{max} of 200nm for the vanadate-ascorbate, 1:1 complex interaction at the physiological pH is being reported as shown in figure 3.18. The ascorbate anion has the ability to form complexes with metallic cations (Lewin, 1976). Several mechanisms are possible as to the nature of vanadate binding to the ascorbate anion; there is the possibility that the vanadate forms a complex, with the ascorbate coordinating through two oxygen atoms of the dihydroxy groups thus shown in scheme 2.

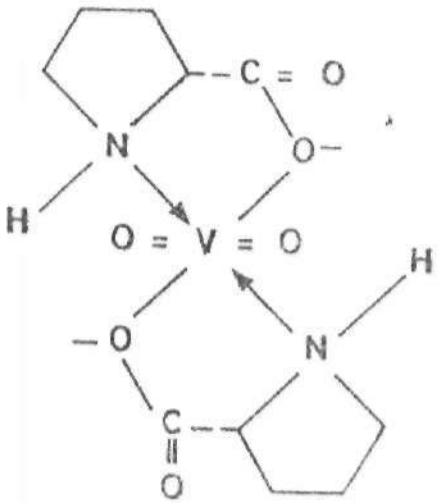


Ascorbic acid

Scheme 2



Scheme 3

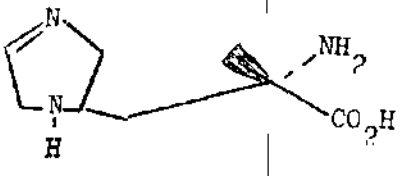


Scheme 4

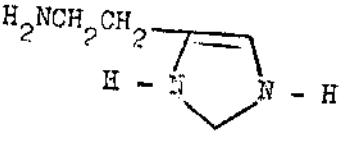
4.3.2 Vanadate - Ligand (1:2), ML_2 Type Complexes

Vanadate-L-Histidine complex (V- His_2)

L-Histidine ((S) -α- amino-1H-imidazole-4-propanoic acid) has the structure as shown in (I) and, its model ligand, Histamine as shown in (II) thus:



(I)

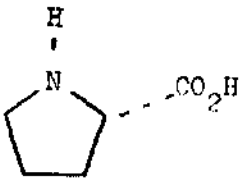


(II)

Vanadium(V) formed a stable 1:2, ML_2 type complex with L-Histidine and the model ligand histamine. Table 3.5 is summary of the relationship between the stable metal-ligand ratio complexes and the λ_{max} at which they absorb. The near identity in the λ_{max} of the histidinato-vanadate complex, to the λ_{max} of the histamino-vanadate complex; and the similarity in the potentiometric equivalence point, indicate that the imidazole group is involved in coordination to the metal centre. All the three donor atoms of tridentate ligands such as histidine, sterically could simultaneously occupy planar positions about a single metal ion (Einstein et al. 1975; Vuletic et al. 1973);since each of the three donor groups may be placed in a unique apical position, scheme 3 shows possible binding mode of L-histidine in the vanadate-L-histidine, ML_2 type complex, pH 7.40, as a bidentate ligand.

Vanadate-L-Proline complex (V-Pro_2)

L-proline (carboxypyrrolidine) has the structure as shown in(III) thus:



(III)

The results summarized in table 3.4 shows an absorption maximum at 208nm for the 1:2 vanadate-L-proline (ML) type complex.

The vanadate-L-proline complex showed an 8nm less in λ_{max} compared to the vanadate-L-histidine complex ($\Delta\epsilon$ = 15900M^{-1} cm^{-1}), this shows that there is greater interaction due to the imidazole group of the L-histidine, to the pyrrolidine group of L-proline (9300 M^{-1} cm^{-1}), table 4.3. The charge-transfer spectra of the vanadate-L-proline complex when compared with those of the vanadate-model ligand complexes i.e ethylamino-vanadate and acetato-vanadate (figure 3.24) shows that the pyrrolidine group is coordinating with the metal centre together with the oxygen of the carboxylic group. Scheme 4 shows possible binding mode, from the pH metric titration stoichiometry.

TABLE 4.3 MOLAR ABSORPTIVITIES OF THE COMPLEXES OF VANADATE(M)WITH THE FOLLOWING LIGANDS(L)

L	M:L RATIO	λ_{max} (nm)	$\Delta\epsilon$ (M^{-1} cm^{-1})
Acetate	1:3	209	9074
Alanine	1:3	204	15741
Ascorbic acid	1:1	200	9259
Aspartic acid	1:3	202	14259
Histidine	1:3	216	15972
Histamine	1:2	215	9028
Proline	1:2	208	9306
Elthylamine	1:2	205	7917
2,3-diamino Propaionic acid	1:3	201	9444
Threonine	1:4	196	11574
Tyrosine	1:3	198	20370

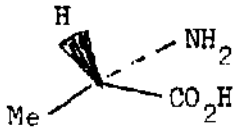
4.3.3 Vanadate - Ligand (1:3), ML_3 Type Complexes

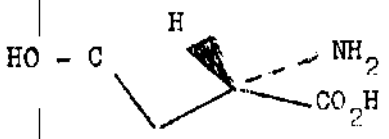
The ML_3 type stable complexs were observed for the vanadate-L-alanine, vanadate-L-aspartic acid and vanadate-L-tyrosine systems.

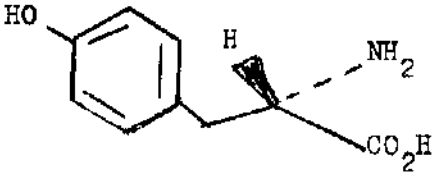
L-Alanine ((S)-2-amino propanoic acid),

L-Aspartic acid ((S)- α -amino butanedioic acid) and

L-Tyrosine ((S)- α -amino-4-hydroxybenzene propanoic acid) have basic structures as represented by (IV), (V) and (VI)respectively thus:







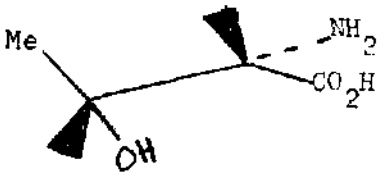
(IV) (V) (VI)

From the results summarized in table 3.6 and table 4.3, the λ_{max} of these three ML_3 type stable complexes compared to that of the model ligand complex vanadate-2,3-diaminopropaionic acid which absorbs at the same equivalence ratio, λ_{max} 201nm suggests the same binding mode. From the physical methods employed, the pH metric titrations suggests a 1:3 metal-ligand stable ratio complex formed, while the ultraviolet charge-transfer spectra indicate a neighbouring group effect. The λ_{max} for the three complexes increase in the order L-tryrosine<L-aspartic acid<L-

alanine. This trend thus observed shows that as the chain moves closer to the α-carbon atom the λ_{max} increases - 198nm <202nm <204nm respectively. This group of vanadate-L-amino acid complexes formed at pH 7.40 exhibited the most complex binding mode. Based on the observation from these studies, complexes with lower wave numbers are characteristic donor nitrogen interaction while higher wave number and increasing number of coordinating ligands tends to implicate the oxygen donor atom of the carboxylic group, table 3.6. The limiting structures for five-coordination are the trigonal bipyramid and the square pyramid. Further studies would be required to ascertain binding mode as vanadates favour octahedral structures. From these studies, a generalized possible binding mode for a five coordination is as suggested in scheme 5, where R is the respective substituent group on the α-amino carboxylic carbon atom of L-alanine (R_1 = Me); L-asparticacid (R_2 = HO_2 CCH_2) and L-tyrosine (R_3 = HOphCH).

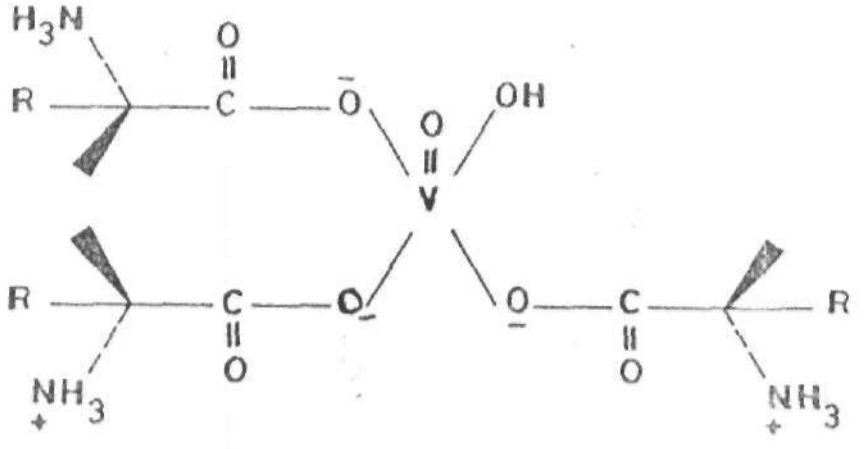
4.3.4 Vanadate - Ligand (1:4), ML_4 Type Complex

L-Threonine (([R-(R,*S*)]-2-amino-3-hydroxybutanoic acid))represented as in (VII) formed a stable ML_4 type complex with the vanadate system at the physiological pH.



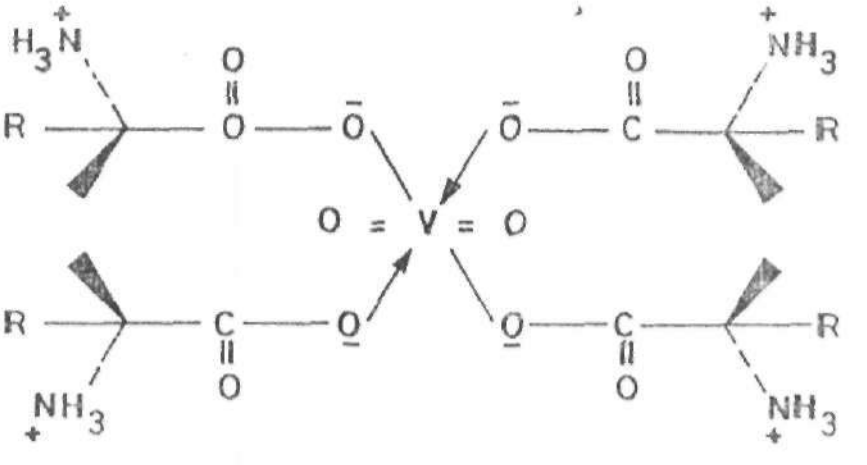
(VII)

The highest energy band was exhibited by the vanadate-L-theronine, ML_4 type complex, figure 3.22. The of 196nm for the stable ML_4 type complex at the physiological pH is being reported. Scheme 6 shows possible binding mode, with the ligand coordinating as a monodentate ligand with the oxygen atom of the carboxylic group, R = MeH(C)OH.



[R = Me, HO_2CH_2, or $HOPhCH_2$]

Scheme 5



Scheme 6

4.4 Summary and Conclusion

Human thrombin was complexed by vanadate at various pH with emphasis on physiological pH. It was observed in this work that thrombin binds with two equivalent of vanadium(V) at pH 7.40. In the thrombin studies, reversibility studies supports the conclusive remark that dioxo vanadium(V) was bounded at the protein-metal binding site.

It can be deduced that the electron transfer is tuned by the composition and the stereochemistry of ligand spheres. It has been observed that in aqueous solutions reduction of V(V)occurs at different rates in the presence of a number of bioligands such as ascorbic acid and α -amino acids.

The physical methods employed- ultraviolet charge-transfer spectra and pH metric titrations indicate that the vanadium(V) complexes of, threonine, alanine, aspartic acid and tyrosine are bound through the carboxylate oxygen in charged forms; histidine - bound through the carboxylate oxygen and ω -nitrogen of the imidazole group. Proline - bound through carboxylate oxygen and nitrogen of the pyrrolidine group. The ascorbate-vanadate system, binds both oxygen of the ascorbate anion (one ionized and the other as a hydroxy).

An extensive study of ultraviolet charge-transfer, visible circular dichroism (CD) and absorption spectra, titrations, and formation constants would be exploited to ascertain the mode of vanadium(V) binding to these variety of ligands.

4.5 Suggestion for Further Works

A lot of studies are still being carried out on blood samples towards understanding and eradicating blood borne diseases such as the dreaded Acquired Immune Deficiency Syndrome(A.I.D.S), high cholesterol level in adults,

thrombosis to mention a few. Vanadium - α -amino acid complexes are of great interest from yet another point of view, regarding the interaction of vanadium with transferrin. Bioligands like α -amino acid ligands are supposed to compete in the cell with transferrin for iron and aluminum ions, with which they form stable complexes. Analogous ligand competition between amino acids and transferrin proposed for iron may well operate for vanadium, and the character of the vanadium - α -amino acid bonds would be of great interest in this respect.

In order to give more support to these findings on the:

1. The nature of vanadium(V) bound to thrombin, it is suggested that NMR of the one or two equivalent vanadate complex be determined to ascertain the binding sites towards better understanding of eventual biological applications at V(V) metal ion centre.

2. Ascorbic acid is known to have detoxification effect and also enhance cellular uptake of iron and copper, with recent findings of vanadium based enzymes, further insights into whether the ascorbic acid would flush out excess vanadium or enhance its cellular uptake is suggested.

3. The formation constants of vanadate and α -amino acids have not been documented at time of this work. It is suggested that further work in this area be carried out as they will have utility as probes into the biochemistry of amino acids which in some instances function as transition-state analogues.

4. Literature survey carried out revealed little information concerning the ligation of amide (Peptide) functionality to vanadium. It is suggested that structurally characterized vanadium(V) - protein species be studied.

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APPENDIX

Structure of Thrombin Molecule (Magnusson, 1971)
Chain A has the following amino acid residues joined together thus:

Thr-Sr-Glu-Asn-His-Phe-Glu-Pro-
Phe-Phe-Asn-Glu-Lys-Thr-Phe-Gly-
Ala-Gly-Glu-Ala-Asp-Cys-Gly-Leu-
Arg-Pro-Leu-Phe-Glu-Lys-Lys-Gln-
Val-Gln-Asp-Glu-Thr-Gln-Lys-Glu-
Leu-Phe-Glu-Ser-Tyr-Ile-Glu-Gly-Arg-

Chain B has the following amino acid residues joined together as thus:

ILE-Val-Glu-Gly-Gln-Asp-Ala-Glu-Val-Gly-Leu-
Ser-Pro-Trp-Glu-Val-Met-Leu-Phe-Arg-Lys-Ser-
Pro-Gla-Glu-Leu-Leu-Gys-Gly-Ala-Ser-Leu-Ile-
Ser-Asp-Arg-Trp-Val-Leu-Thr-Ala-Ala-His-Cys-
Leu-Leu-Tyr-Pro-(Trp,Pro,Asx,Lys,Asx-CBH,Phe)-
Thr-Val-Asx-Asx-Leu-Leu-(Ser,Val,Val,Glx,Trp)-
Arg,Ile-Gly-Lys,His-Ser-Arg-Thr-Arg-Tyr-Glu-
Arg-Lys-Val-Glu-Lys-Ile-Ser-Net-Leu-Asp-
Lys-Ile-Tyr-Ile-His-Pro-Arg-Tyr-Asn-Trp-
Lys-Glu-Asn-Leu-Asp-Arg-Asp-Ile-Ala-Leu-
Leu-Lys-Leu-Lys-Arg-Pro-Ile-Glu-Leu-Ser-
Asp-Tyr-Ile-His-Pro-Val-Gys-Leu-Pro-Lys-
Ala-Ser-Thr-Arg-,Leu-Leu-His-Ala-Gly-Phe-
Lys-Gly-Arg-Val-Thr-Gly-Trp-Gly-Asn-Arg-
Thr-Thr-Ser-Val-Ala-Glu-Val-Gln-Pro-Ser-
Val-Leu,Gln-thr-Ala-Ala-Lys-Leu,Gln-Val-
Val-Asn-Leu-Pro-Leu,Val-Glu-Arg-Pro-Val-
Cys-Lys,Arg-Ile-Arg-Ile-Thr-Asx-Asx-Net-
Phe-Cys-Ala-Gly-Tyr-Lys-Pro-Gly-Glu-Gly-
Lys-Arg-Gly-Asp-Ala-Cys-Glu-Gly-Asp-SER-
Gly-Ile-Var-SER-Trp-Gly-Glu-Gly-Cys-
Asp-Arg-Asn-Gly-Lys-Tyr-Gly-Phe-Tyr-
Thr-His-Val-Phe-Arg-Lys-Ley-Lys-Trp-
Ile-Gln-Lys-Val-Ile-Asp-Arg-Arg-Leu-Gly-Ser