Metal Ion Catalysis of the Transamination Reaction.

A thesis submitted to the University of London for the degree of

Doctor of Philosophy.

by

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Abstract

Part I of the thesis deals with the formation and transamination of Schiff's base complexes of copper, pyridoxal phosphate and glutamate, and the transamination of reaction mixtures containing copper, pyridoxamine phosphate and α-ketoglutaric acid.

The formation of the complex of copper,, pyridoxal phosphate and glutamate was found to be first order in both pyridoxal phosphate and glutamate and zero order in copper. Spectrophotometric studies showed isosbestic points when reaction mixtures were scanned over the range 20,000-35,000 cm^{-1} indicating that only one step is involved.

At high concentrations of copper (>16 mM) the initial reaction rate became slightly dependent upon the copper concentration, and the initial optical density of the reaction mixture departed from that of pyridoxal phosphate. These deviations are explained by the postulation of an intermediate carbinolamine complex.

The complex formed from copper, pyridoxal phosphate and glutamate transaminated to give pyridoxamine phosphate and α-ketoglutaric acid. The presence of metal ions appears to catalyse the transamination reaction, copper being the most active. If account was taken of the fact that in the absence of metal ions the Schiff's base of pyridoxal phosphate and glutamate is largely hydrolysed, however, the rate of transamination was found to be less in the presence of metal ions than in their absence.

The transamination of reaction mixtures containing copper, pyridoxamine phosphate and α-ketoglutaric acid took place without significant formation of the Schiff's base complex, due to the unfavourable

equilibrium constant for the formation of the Schiff's base in this system. Transamination was found to be very much more rapid than in the case of the copper complexes of the Schiff's base of pyridoxal phosphate and glutamate.

The transamination of pyridoxamine phosphate and α-ketoglutaric acid is first order in α-ketoglutaric acid (tailing off at higher concentrations of αKG) and exhibits a rate maximum at a copper concentration of twice that of pyridoxamine phosphate. This maximum is justified mathematically by assuming that the concentration of a complex of copper and the Schiff's base from pyridoxamine phosphate and α-ketoglutarate is very low, and that the complex accepts a further Cu^{2+} ion at high concentrations of copper.

The transamination of pyridoxamine phosphate was found to be preceded by what at first appeared to be an induction period, further study of which indicated that the very small change in absorbance was caused by a rate limiting dehydration of the carbinolamine of pyridoxamine phosphate and α-ketoglutarate. Low concentrations of carbinolamine; Schiff's base complex; Schiff's base and carbinolamine complex account for the first order nature of the reaction. The non-zero values of these concentrations probably cause the deviation from first order.

Part II of the thesis is concerned with the evaluation of the stability constants of the simple and mixed complexes of pyridoxal phosphate, pyridoxamine phosphate, glutamate and α-ketoglutarate.

The pK values of pyridoxal phosphate, pyridoxamine phosphate and α-ketoglutaric acid (necessary for stability constant determinations) were found by means of a potentiometric titration method. The stability constants of copper and nickel with pyridoxal phosphate; copper, nickel, cobalt and zinc with pyridoxamine phosphate phosphate; and copper, nickel, cobalt and zinc with α-ketoglutaric acid were also determined by a similar method.

The stability constants as normally defined are shown to be meaningless where the complex can accept protons at p[ligand anion] values of 0.5 and 1.5 and results obtained by the usual procedures have been converted to more meaningful results when the pK values of the complex are known.

A graphical method of determining the stability constants of the complexes of the Schiff's base of pyridoxal phosphate and glutamate has been developed. The method relies on absorbance readings taken at one wavelength only. A graph is plotted of Log(Stability Constant) against assumed values of the extinction coefficient of the complex. The intersection of these lines for several sets of experimental conditions gives the required value of the stability constant.

The equilibrium constant for the formation of the Schiff's base of pyridoxal phosphate and glutamate, required in the above, was found by the usual graphical method at several pH values.

A potentiometric titration method was used to evaluate the stability constants of complexes of the type MP_pG_g where P and G represent pyridoxal (or pyridoxamine) phosphate and glutamate (or α-ketoglutarate) respectively, and where p and g can take values up to 2. A computer was used to solve the complex equations which were derived to describe the system.

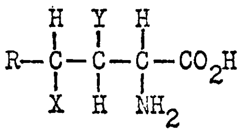
Acknowledgements

The author wishes to thank the Medical Research Council for the award of the Research Assistantship which made this work possible. He would also like to thank his supervisor, Dr. M.E.Farago, for her invaluable help and criticism during the production of this thesis.

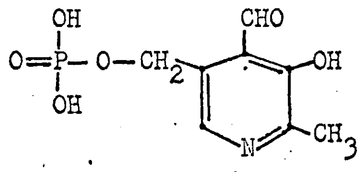
Contents

Introduction

Transamination is one of a large group of reactions of amino acids (I) which are catalysed by pyridoxal phosphate (II) containing enzymes*(1,2).



(I)



(II)

These include

(i) Elimination and replacement of substituents on the α-carbon, as in

(a) Transamination

(b) Dissociation of the α-hydrogen

(c) Racemisation of α-amino acids

(d) Oxydative deamination

(e) Decarboxylation of α-amino acids

(f) α,β clevage of β-hydroxyamino acids

(g) Condensation of glycine (or serine)

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* An enzyme is defined as a protein with catalytic propertities due to its powers of specific activation.

and

(ii) Elimination and replacement of substituents on β and γ-carbon atoms.

These apparently unrelated reactions have been rationalised by Metzler's mechanism (p 12).

Most of these enzymatic reactions have been duplicated in non-enzymatic 'model' systems (3-13), in which pyridoxal† (usually non-phosphorylated) or other appropriate aldehyde (4,8,17) and a suitable metal salt (6) serve as catalyst.

Snell (4) has suggested that the catalytic potentialities of pyridoxal phosphate containing enzymes are those of their prosthetic groups*, and that enzymatic and non-enzymatic reactions proceed by closely similar mechanisms.

Transamination is defined as the interchange of the functional groups $-NH_2$ and $=CO$ of an α-amino and an α-keto acid, as in equation (i).

$$R-\underset{\displaystyle CO_2H}{\underset{|}{CH}}-NH_2 \quad + \quad R'-\underset{\displaystyle CO_2H}{\underset{|}{CO}} \quad = \quad R-\underset{\displaystyle CO_2H}{\underset{|}{CO}} \quad + \quad R'-\underset{\displaystyle CO_2H}{\underset{|}{CH}}-NH_2 \qquad \text{(i)}$$

Biological transamination has been shown to require the presence of pyridoxal (14) or pyridoxal phosphate (15,16) which are known to

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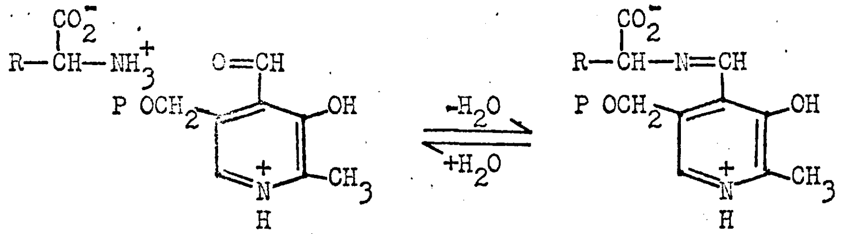
† For abbreviations of these terms see Appendix II

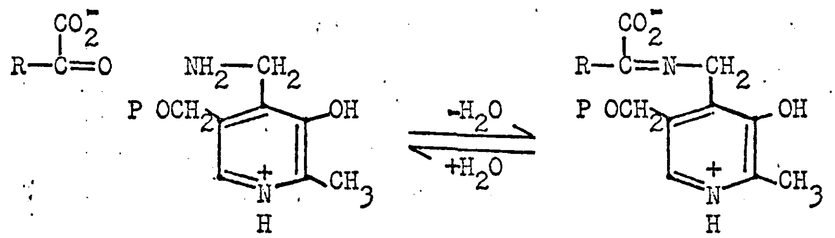
* The reactive groups attached to the enzyme. e.g. pyridoxal phosphate in transaminases.

form Schiff's bases with a variety of amino acids (18-22). It seemed probable, therefore, that transamination involved the steps:-



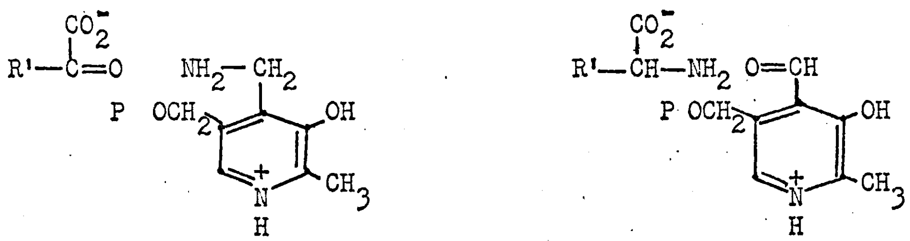
(III)

(ii)



(IV)

and by a similar mechanism



(iii)

Addition of equations (ii) and (iii) gives (i), the position of the equilibrium being decided by the thermodynamics of the individual steps. (The enzyme is not shown in the above equations but is believed to be attached to the phosphate group and the ring nitrogen (2)).

Non-enzymatic transamination of pyridoxal by an α-amino acid was first shown by Snell (23) in 1945. The reaction was followed at 100°C and the products estimated biologically by observing their effect on the rate of growth of certain bacteria. The retardation produced when non-enzymatic transamination was carried out in a chelating buffer (citrate) led Metzler and Snell to study the catalytic effect of added metal ions (6). They devised chemical methods for estimating pyridoxal and pyridoxamine in the presence of each other by adding an excess of ethanolamine to the reaction mixture. As the resulting pyridoxal-ethanolamine Schiff's base has a point of maximum absorption (at 24,000 cm^{-1}) where pyridoxamine does not absorb, the estimation of pyridoxal was relatively easy. Pyridoxamine, however, absorbs maximally only at points of non-zero absorption for both pyridoxal and pyridoxal-ethanolamine. Consequently the appearance of pyridoxal from reaction mixtures containing pyridoxamine and α-ketoglutaric acid was usually followed. Metzler and Snell found that the catalytic properties of the various metals decreased in the order Cu(11) $>$ Fe(11) $\simeq$ Fe(111) $\simeq$ Al(111) $>$ Ni(11) $>$ Co(11) $>$ other ions.

Similar work carried out by Longenecker and Snell (5) on the system pyridoxamine/α-ketoglutarate with Al(111) catalyst showed that the rate of appearance of pyridoxal was directly proportional to the concentration of metal ion at low metal ion concentrations but independent at high.

Matsuo (10) demonstrated the ready formation of, and tautomerism between, the Schiff's bases of pyridoxal phosphate-glutamate and pyridoxamine phosphate-α-ketoglutarate in alcoholic solution, in the absence of metal ions and at room temperatures. He recorded spectrophotometrically the appearance of a peak from pyridoxamine phosphate-α-ketoglutarate which he identified as being due to pyridoxal phosphate. As Schiff's base formation results in the elimination of a molecule of water, non-aqueous solvents such as alcohol should result in a displacement of the equilibrium to favour higher concentrations of Schiff's base. Matsuo suggested that this was the reason for the high rate of tautomerism in alcohol compared with water.

The tautomerism has, however, been shown to be comparably rapid in aqueous solution at room temperatures in the presence of metal ions, by Eichhorn and Dawes (26). They showed that the spectrum of a solution of metal ion/pyridoxamine/pyruvate became indistinguishable from that of metal ion/pyridoxal/alanine on standing. Banks et al. (18) showed that tautomerism took place in the same system in the absence of metal ions but only very much more slowly.

Fasella et al. (11,12) did not consider the spectrophotometric evidence of Eichhorn and Dawes sufficient to establish the participation of intermediate chelates in transamination, but they provided confirmation of the fact by repeating the work of Metzler and Snell (6) and analysing samples from the reaction mixtures by paper chromatography and electrophoresis. These techniques showed the existence of two intermediate chelates, C and C'. It was found that:-

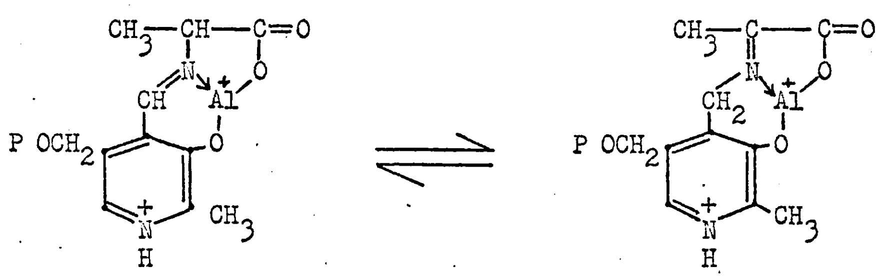
(a) For C and C' to be formed, all three reactants were necessary (metal, pyridoxal and alanine or metal, pyridoxamine and pyruvate).

(b) C and C' had no free $-NH_2$ or =CO groups, but that on heating they split up into pyruvate or alanine and the corresponding pyridoxine.

(c) C was formed first when starting from metal/pyridoxal/alanine, and C' when starting from metal/pyridoxamine/pyruvate.

These observations show that transamination in the presence of metal ions involves the appearance of two interconvertible chelate intermediates. These are most probably the chelates of the respective Schiff's bases of reactants and products of the transamination reaction.

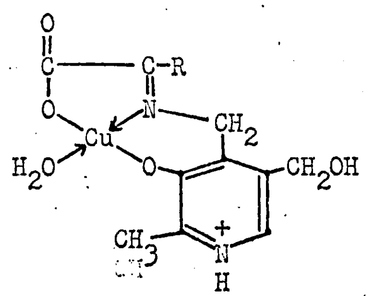
Fasella et al. postulated (V) and (VI) as probable formulae for C and C'. The difference is in the position of the labile proton.



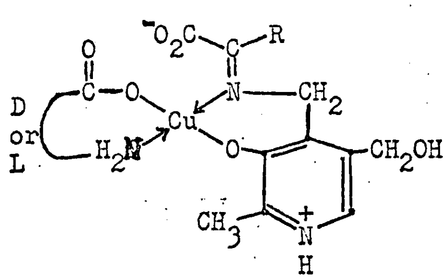
(V) (VI)

Similar work to that of Fasella has more recently been conducted by Cattaneo et al. (27) who investigated transamination between pyridoxal phosphate-glutamate and pyridoxamine phosphate-α-ketoglutarate at 37°C in the presence of Cu(11) ions. They showed the existence of an intermediate chelate by subjecting reaction mixturs to paper chromatography. This they did by viewing the developed chromatograms under ultra-violet light.

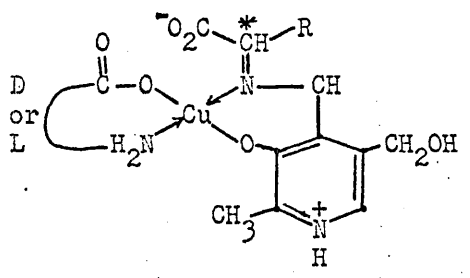
Longenecker and Snell (34) found that the model systems were slightly stereospecific. (Another similarity with the biological reactions which almost entirely favour the L-forms of optically active amino acids.) Using optically active alanine with Cu(11), pyridoxal and α-ketoglutaric acid they found about 4% retention of configuration in the resulting glutamate for both D and L alanine at 100°C and 37°C. With DL-alanine no preference for either configuration was found. Longenecker and Snell said that although (VIII) may participate in transamination, as it is not asymmetric there must be some other, optically active, intermediate formed. The mechanism would require at least a small proportion of (IX) which then transaminates to give optically active (X).



(VIII)

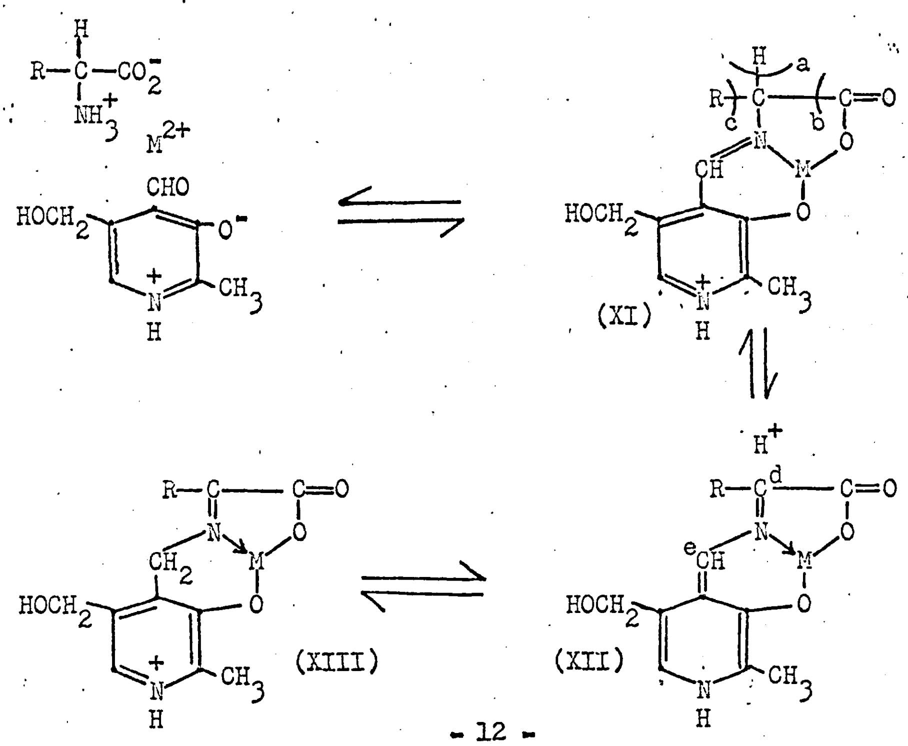


(IX)



(X)

Several mechanisms have been put forward to explain the catalysis of metal ions and pyridoxines in these reactions. The most important are those of Metzler et al. (4), Baddiley (24), Fasella et al. (12) and Braunstein et al. (29). The mechanism proposed by Metzler, Ikawa and Snell (4) is shown diagrammatically below.



Resonance stabilises the Schiff's base intermediates(XI) and (XII) and results in two nucleophilic centres, d and e in (XII), for subsequent proton attack. If protonation took place at d, hydrolysis would give the initial reactants, (if the amino acid were optically active the racemic product would be formed); whereas attack at e would give a second Schiff's base (XIII).

Pullman et al (30) concluded from considerations of the electron distribution over (XII) that e would be the marginally preferred site of protonation and hence the equilibrium would be expected to lie in favour of (XIII). This is contrary to what would be expected from the resonance energy of (XI) and (XII) which Pullman calculated as about 8 Kcals/Mole using the L.C.A.O. method of approximation. Pullman's findings qualitatively confirm Metzler's theory of the reaction mechanism, but quantitative comparisons are difficult as Pullman did not include the metal ion in his L.C.A.O. calculations.

Metzler's mechanism can be very simply adapted to explain other pyridoxal catalysed reactions some of which are summarised earlier (p 5). Thus labilisation of the bonds b and c in (XII) results in decarboxylation and α-β splitting respectively. The role of the metal ion is assumed to be four-fold:

It causes

(a) preliminary proton displacement facilitating Schiff's base formation;

(b) stabilisation of the imine formed;

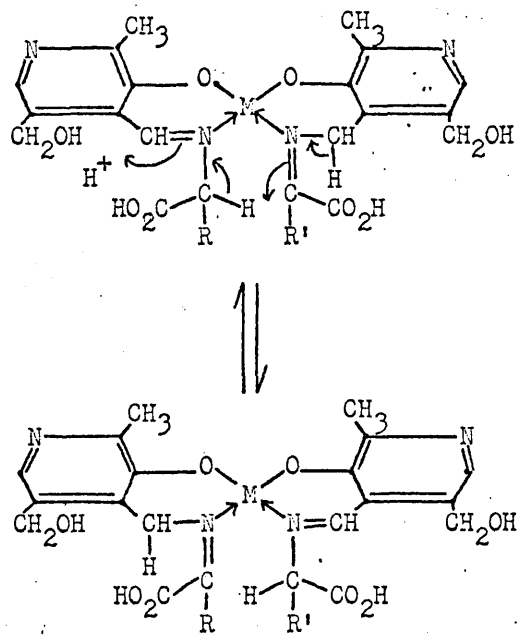
(c) the provision of a planar conjugated system;

(d) an increase in the inductive withdrawal of electrons from the α-carbon undergoing reaction, thereby labilising the groups attached to it.

The effect of the metal ion in stabilising (32) and destabilising (31) the imine double bond of the Schiff's bases has been discussed by Eichhorn et al. for salicylaldehyde-glycine and bis(2-thiophenal)-ethylenediamine respectively. They suggest that if the imine double bond can undergo hydrolysis without decreasing the degree of chelation, the result will be a destabilisation of the molecule; whereas if any chelate rings have to be broken during hydrolysis, the molecule will be stabilised. Although this does not help to predict which of the two possible Schiff's base intermediates (III) or (IV) would be favoured by chelation to a metal, it does predict that the presence of metal ions should increase the concentrations of Schiff's bases in equilibrium with their constituents.

The mechanisms for resonance stabilisation and subsequent prototropy of the intermediate Schiff's bases put forward by Braunstein (29), agree with those of Metzler. Braunstein, however, considers only the non-metal catalysed reaction and does not consider the effect of chelation.

Baddiley (24) suggested that two different forms of Schiff's base (a ketimine IV and an aldimine III) co-ordinated to the metal are necessary, transamination taking place between the two:-



Such a mechanism adequately explains the interconversion of two Schiff's bases in a situation which may arise biologically, but it does not explain transamination in a system containing only one form of a Schiff's base, as would initial be the case in usual model reaction mixtures. To overcome this difficulty Baddiley suggested that sufficient of the other form required is produced by 'spontaneous reaction' of the starting materials to give the complexes he describes, which then react as shown. This would require that R and R' were identical. Examination of the mechanism shows that if this is so, no provision is made for increasing the concentration of the second Schiff's base above that produced by 'spontaneous reaction'.

It is, however, conceivable that transamination occurs with increased facility in complexes containing more than one Schiff's base molecule per metal ion. The fact that a 1:1 complex may be thermodynamically preferred does not prevent the reaction proceeding via a minority reactive species. If this were so, it should be possible to find some relationship between the rate of transamination and the stability of the $M(SB)_2$ species. Such a relationship cannot be found at present because of the lack of information regarding the stabilities of the complexes. Qualitative considerations, such as steric factors, can however give an indication of the expected relative ease of $M(SB)_2$ formation. Thus,

(a) Valine - a fairly large molecule - forms mainly a 1:1 metal:Schiff's base complex (33,36) and has a very low rate of transamination (6).

(b) Glyoxylic acid - a small molecule - (nothing is reported on the nature of its complexes) transaminates comparatively easily (34).

(c) Pyridoxal, which tends to form 1:2 metal Schiff's base complexes (12), transaminates faster than pyridoxal phosphate (6) which prefers a 1:1 complex.

There are, however, numerous exceptions to the above generalisation. Thus, transamination with glycine, the reverse of (b), is reported to be very slow (6,35). The indications are that many of the observed rates of transamination are greatly affected by favourable or unfavourable Schiff's base formation equilibria.

The three equilibria of importance in transamination are

$$R\text{-}NH_2 + PyP \overset{K_1}{\rightleftharpoons} SB' \overset{K_2}{\rightleftharpoons} SB'' \overset{K_3}{\rightleftharpoons} R\text{-}CO + PamP \qquad \text{(iv)}$$

although the transamination equilibrium constant, K_2, would be expected to favour the more highly conjugated SB' and hence the L.H.S. of equation (iv), a sufficiently small value of K_3 or high value of $[RNH_2]$ could displace the equilibrium to the right. K_1 is reported for a variety of amino acids with pyridoxal (19) and its phosphate (10). It is usually such that appreciable quantities of SB' are formed only when the amino acid is present in considerable excess. K_3 is only reported for the system pyridoxamine-pyruvate (18) but this result, together with observations from the present work (p 75), indicates that K_3 is at least 10-fold less than K_1. Consequently, Metzler and Snell (6) found that conversion of pyridoxal and glutamate to pyridoxamine and α-ketoglutarate took place to about 50%, and that pyridoxal phosphate and glutamate resulted in almost complete conversion to pyridoxamine phosphate. The difference, they explained, was due to the low free aldehyde concentration in solutions of pyridoxal, where the predominant species is an internal hemi-acetal. The 'true' equilibrium they therefore took as that of the phosphorylated system. This equilibrium is then directed away from the expected resonance stabilised aldimine Schiff's base (III).

The reverse of the above situation occurs with glyoxalate and α-keto acids, where the equilibrium lies almost entirely on the side of pyridoxal and α-amino acid. The same preference for the aldehyde and the amino acid occurs in the system metal ion/pyridoxal/alanine investigated by Eichhorn and Dawes (26).

The Metal Schiff's Base Complexes

There is much evidence to suggest that the Schiff's base complexes investigated do not all have the same number of ligands associated to the metal. If the general formula is written as $M(SB)_n$, n has been shown to take the values 1 and 2 depending upon,

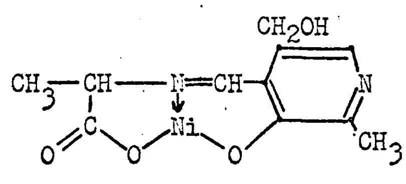
(a) the metal,

(b) the pyridoxine (whether or not phosphorylated),

(c) the amino or keto acid,

and (d) the ratio, in solution, of the concentrations of the metal ion and the Schiff's base constituents.

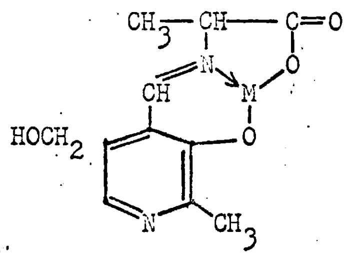
Eichhorn and Dawes (26) have shown by the method of continuous variation that the spectra they describe are due to a 1:2:2 complex of Ni(11):pyridoxal:alanine. Moreover, they suggest that each Schiff's base is bound as in (XIV).



(XIV)

They also found precipitates coming down in their reaction mixtures which they decided had the same ratio M:pyridoxal:alanine as the reactants in the solutions (1:2:2 for Ni and 1:1:1 for Cu). This conclusion was based on the fact that the spectra of the solutions underwent only a loss of intensity over the whole range and did not change in character.

Christensen (et al. 28) was able to prepare solid complexes of the 1:1:1 type only for M-pyridoxal-glycine (M being Cu(11), Ni(11), Mn(11), Zn(11) and Mg(11)), and complexes containing 2 pyridoxal molecules, only by using diaminobutyric acid. (See diagrams XV and XVI)



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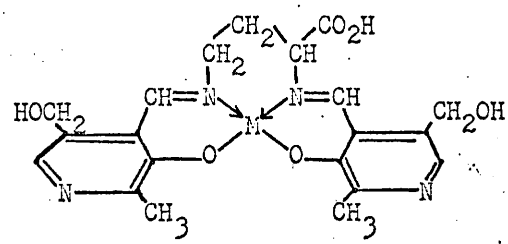
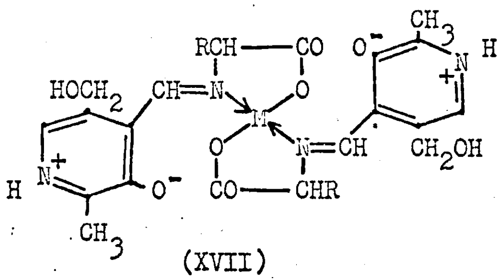


(XVI)

In a later paper (25) Christensen describes the preparation of numerous metal chelates of pyridoxylideneamino acids in crystalline form, usually with the composition $M(SB)_2$, (XVII), but in the case of copper, as Cu(SB), (XVIII).



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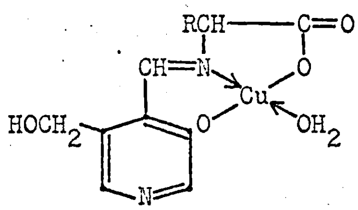
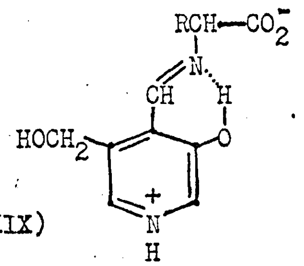


(XVIII)

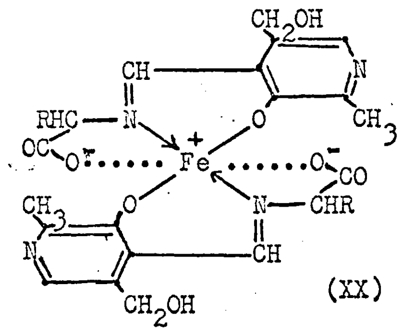
It was assumed that protons could associate freely with the chelate to give electroneutrality. Titration of the protons in the complexes showed the pK attributed to the ring nitrogen varied from metal to metal. Its magnitude was taken as a measure of the degree of co-ordination of the phenolic oxygen to the metal, by comparison with the pK's of the ring nitrogen in free pyridoxal and in the hydrogen bonded Schiff's base (XIX). (See p 139)



(XIX)

In the case of the catalytically more active Cu(11) and Fe(111) (see Metzler and Snell (6)) chelates, the pK of the ring nitrogen is lowered by more than 3 pH units from that of the Schiff's base (XIX). If the above criterion of phenolic bonding is correct, diagram (XVII) cannot describe the complexes of Fe(111) as a greater degree of bonding to the phenolic oxygen would be necessary to sufficiently lower the pK of the ring nitrogen. Christensen proposed the formula (XX) to overcome this difficulty. (Only one nitrogen is protonated for neutrality)

Although Christensen's work would indicate that 1:2 complexes are more stable than 1:1 types in the solid phase, Davies et al. (36) have shown that the predominant species in a solution containing metal ions, pyridoxal and amino acid is a 1:1 complex of M:SB (M being Cu(11), Ni(11)



(XX)

Mn(11), Mg(11) and Zn(11); and the amino acids including valine, glycine, alanine, threonine, isoleucine and glutamic acid). This is a direct contradiction of the results obtained by Eichhorn and Dawes (26), and, if correct, would cast doubt on the validity of Christensen's titrations of the 1:2 chelates carried out in aqueous solution.

Fasella et al. (12) used a method of continuous variation to find the ratio of M:SB for Al(111), alanine and phosphorylated as well as non-phosphorylated pyridoxal. They found that the non-phosphorylated Schiff's bases formed a 1:2 complex whereas the phosphorylated derivatives formed a 1:1 complex. This indicates that the phosphate group either co-ordinates itself, or that it sterically prevents a second Schiff's base from being added to the metal.

In conclusion, metal ion catalysed transamination involves two interconvertible chelates, an aldimine and a ketimine. It is not known why metals such as Cu(11) and Fe(111) have such a high catalytic ability compared with Ni(11), Co(11) and many other ions. It cannot be that Cu(11) and Fe(111) form higher concentrations of chelates in a given reaction mixture (although this may be largely responsible where the

the equilibrium constant of Schiff's base formation is very low, as in the case of pyridoxamine phosphate and α-ketoglutaric acid - see p 179 - as it is possible to produce almost complete conversion to chelate with most metals by increasing the concentration of amino acid. The only phenomenon which seems to parallel the catalytic properties of Cu(11) and Fe(111) is the ability of these metals to form very stable complexes with pyridoxamine, pyridoxamine phosphate, pyridoxal phosphate and Schiff's bases (33,36,37 and this thesis). Together with Christensen's titration results (25) this indicates a different kind of bonding in the Cu(11) and Fe(111) chelates.

In the present work it is proposed to investigate further the reactivity and stability of the complexes of pyridoxal phosphate-glutamate and pyridoxamine phosphate-α-ketoglutarate with special emphasis on the Cu(11) system.

Part I

Kinetics

of the

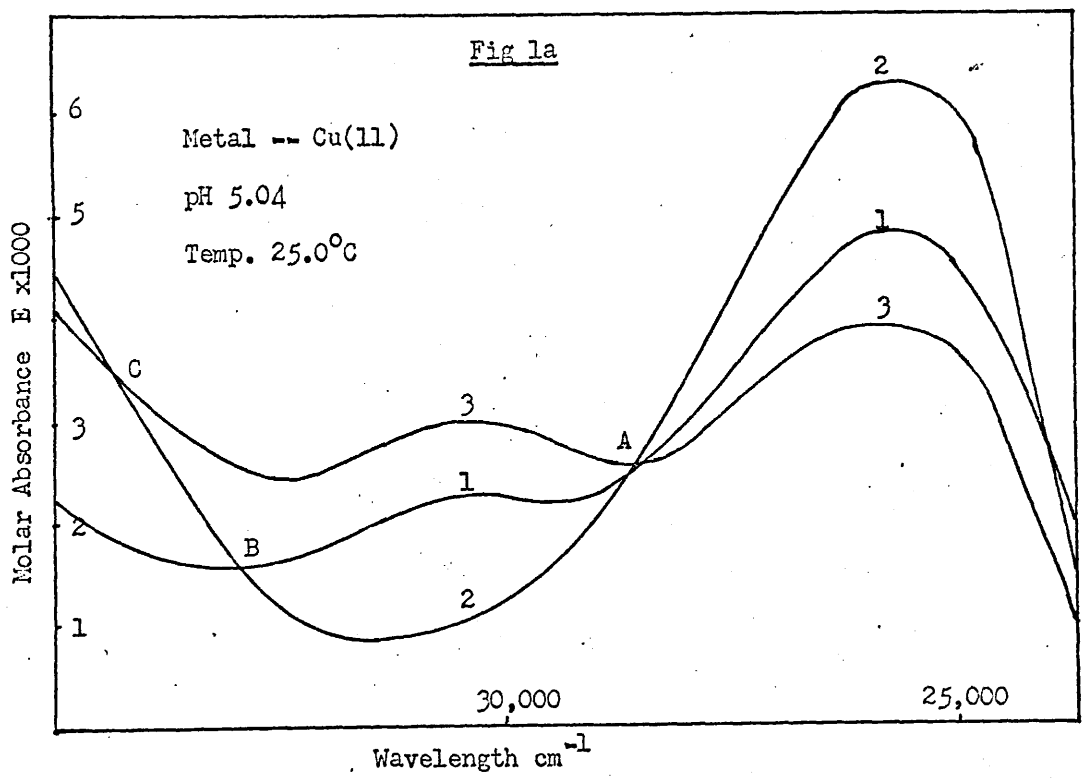
Transamination

Reaction

Reaction Between Pyridoxal Phosphate and Sodium Glutamate in the Presence of Divalent Metal Ions

It was found that the addition of pyridoxal phosphate solution to a buffered solution containing sodium glutamate and M(ll) ions in suitable concentrations (see experimental), caused two consecutive spectral changes to take place. (Fig 1a shows the spectra for Cu(ll), 1b for Ni(ll) and 1c for Co(ll) at pH 3.96 and 5.04). The first change was from the spectrum of pyridoxal phosphate alone (spectrum in Figs 1a, 1b and 1c; the spectrum includes a very intense absorption band from M(ll)-Glutamate charge transfer outside the region of interest), to that of a different species 2, and took place in about 30 minutes; the second change was from spectrum 2 to spectrum 3, and took place over a period

Fig 1a



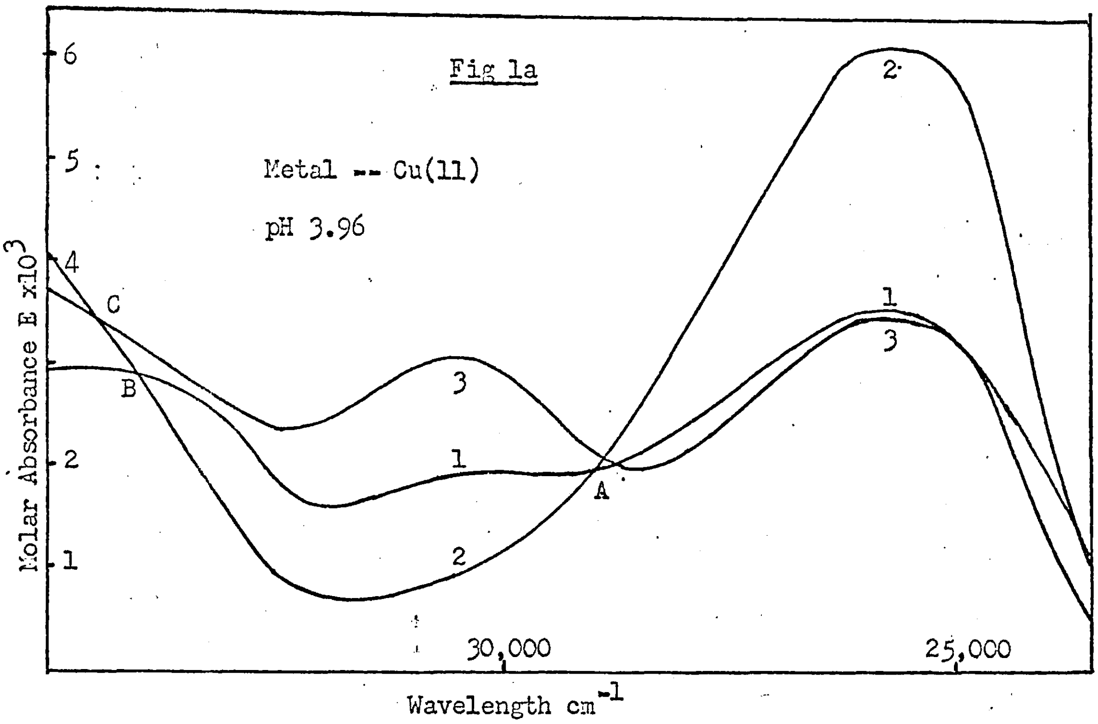


Fig 1a
Metal -- Cu(11)
pH 3.96
Molar Absorbance E x10^3
6
5
4
2
1
C
B
A
1
2
3
30,000
25,000
Wavelength cm^{-1}

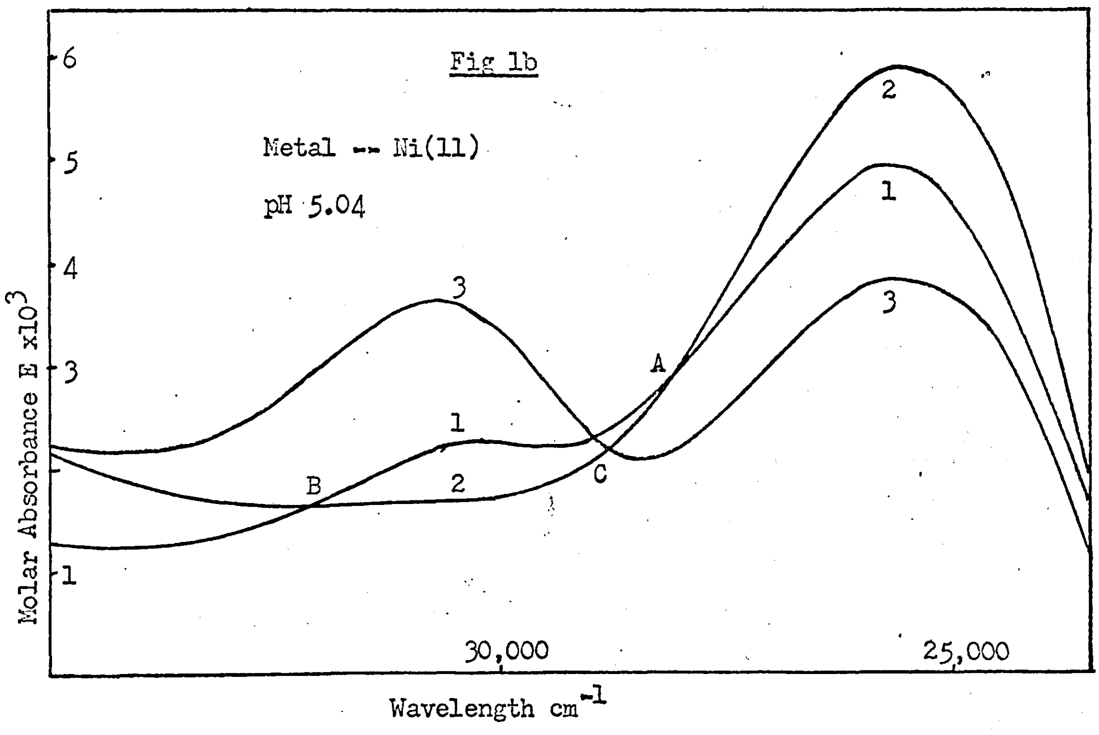
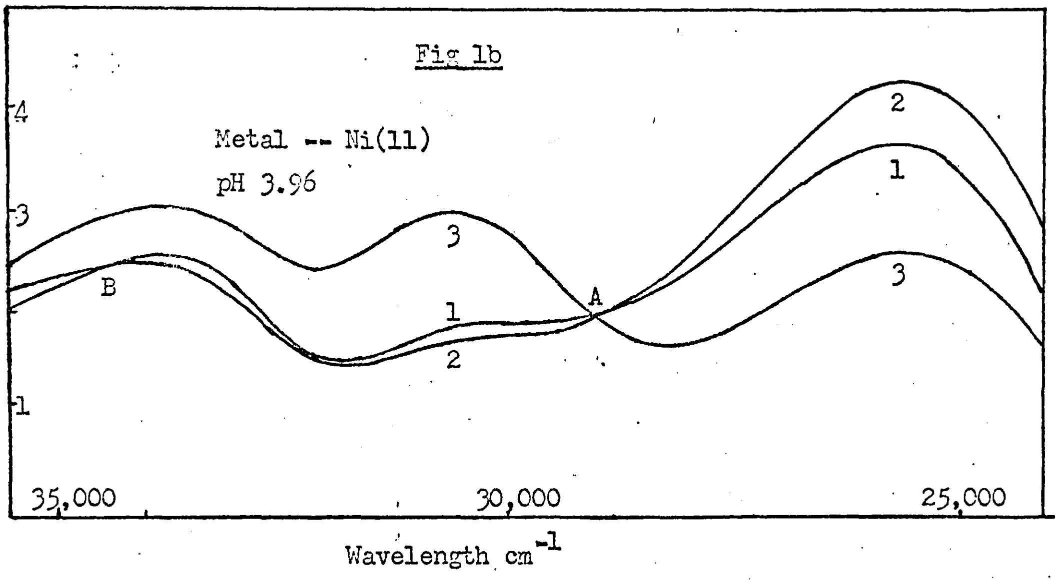
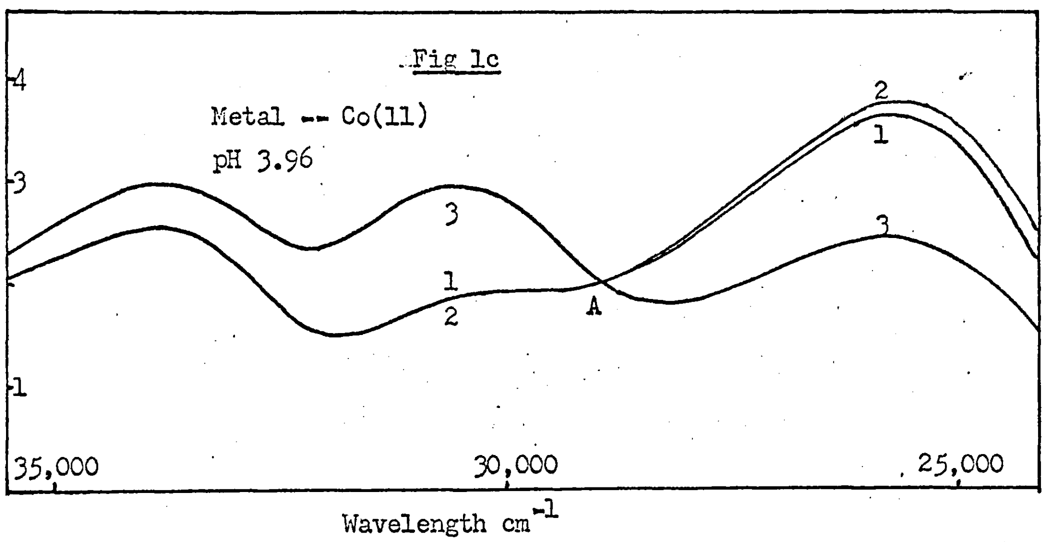


Fig 1b
Metal -- Ni(11)
pH 5.04
Molar Absorbance E x10^3
6
5
4
3
1
A
B
C
1
2
3
30,000
25,000
Wavelength cm^{-1}





The concentrations of these reaction mixtures were 0.2 mM in PyP, 8.0 mM in Glu and 0.4 mM in metal ion.

of about 4 days.

Sharp isosbestic points at A and B, and a slightly less sharp one at C indicate that the reactions can be considered as taking place in one step only (or involving only transient intermediates). As the second reaction was very slow compared with the first, each could be studied independently.

Single wavelength studies were carried out on the first reaction at the point of maximum change (25,500 cm^{-1}) and the effects on the initial reaction rate were observed of changes of:

(a) pH

(b) Sodium glutamate concentration

(c) Pyridoxal phosphate concentration

and (d) The concentration of $CuSO_4.5H_2O$

The initial reaction rate was measured by drawing tangents to an optical density/time trace on an external recorder, the signal from the spectrophotometer being magnified 20-fold. This caused an optical density difference of 0.1 OD units to give a full scale deflection of 10 inches on the recorder. Thus, for overall changes of about 1.0 OD units the trace was sensibly straight and tangents could be drawn at zero time with a repeatability of ca. 3%. This method obviates complicated trial integrals into which corresponding optical density and time reading would have to be substituted.

For greatest accuracy in the measurement of the gradients of these tangents the chart speed was adjusted to give a trace at about 45^{o} to the direction of motion of the paper.

As about 5 seconds usually elapsed between mixing the reactants and transferring the cell to the instrument, initial optical densities were measured by extrapolating the tangents back to zero time. Final optical densities were taken as the values when no further increase took place with time.

Experimental

The reaction was followed using a Unicam SP 800 recording spectrophotometer fitted with a scale expansion unit connected to an external Kent recorder. The instrument has a hollow cell housing through which water at 25.0°C was circulated from an external thermostat. The temperature of the cell housing was checked against standard thermometers and was found to be the same as that of the water in the thermostat to within ± 0.1°C.

The 1 cm quartz cells to be used were matched by filling them with distilled water and placing them in the appropriate beams of the instrument. Any differences between them were 'tuned out' by readjusting the external recorder to zero with the 'back-off' control, the reading of which was then marked on each cell.

2.0 ml of acetate buffer*, ionic strength 0.5, was pipetted into each of two cells. 1.0 ml of water was then added to the reference cell which was shaken and transferred to the instrument. Into the other cell was pipetted the required volumes of two of the three reactant solutions (usually the copper sulphate and the sodium glutamate solutions) and sufficient water to make the total volume, including

*See Appendix III for buffer specifications.

the third reactant, up to 3.0 ml. The cell was then placed in the instrument for 10 minutes to thermally equilibriate when the third reactant was pipetted in and the mixture shaken to start the reaction. The recorder was started simultaneously.

Results

(a) Dependence of the reaction rate on pH

The changes of the following were studied for the pH range 2 to 6:

(i) The initial gradient of the reaction (Fig 2)

and (ii) The initial and final optical densities of the reaction mixture (Figs 3 and 4)

The concentrations of the reactants were kept constant at 0.2 mM in pyridoxal phosphate, 0.4 mM in copper sulphate and 8.0 mM in glutamate.

The initial reaction rates (also shown in Fig 2) were obtained by dividing the initial gradients of the reaction by the differences in the molar absorbancies of the products and reactants, obtained from Figs 3 and 4.

The initial rates were not recorded above pH 6 as the molar absorbancies of the reactants and the products became too close for the initial gradients to be measured accurately.

The initial molar absorbance of the reaction mixture is compared with that of pyridoxal phosphate alone in Fig 3. The similarity suggests that pyridoxal phosphate does not undergo any reaction before that being followed.

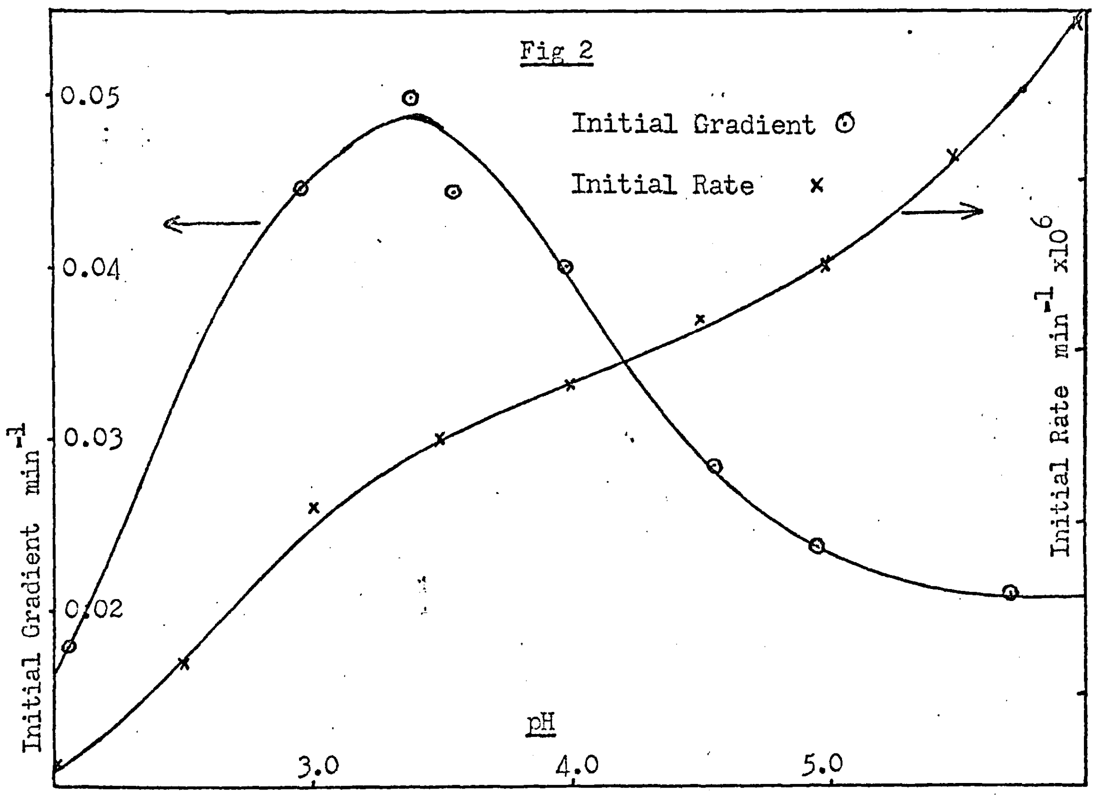


Fig 2
Initial Gradient ⊙
Initial Rate ×
0.05
0.04
0.03
0.02
Initial Gradient min-1
20
15
10
5
Initial Rate min-1 x106
pH
3.0
4.0
5.0

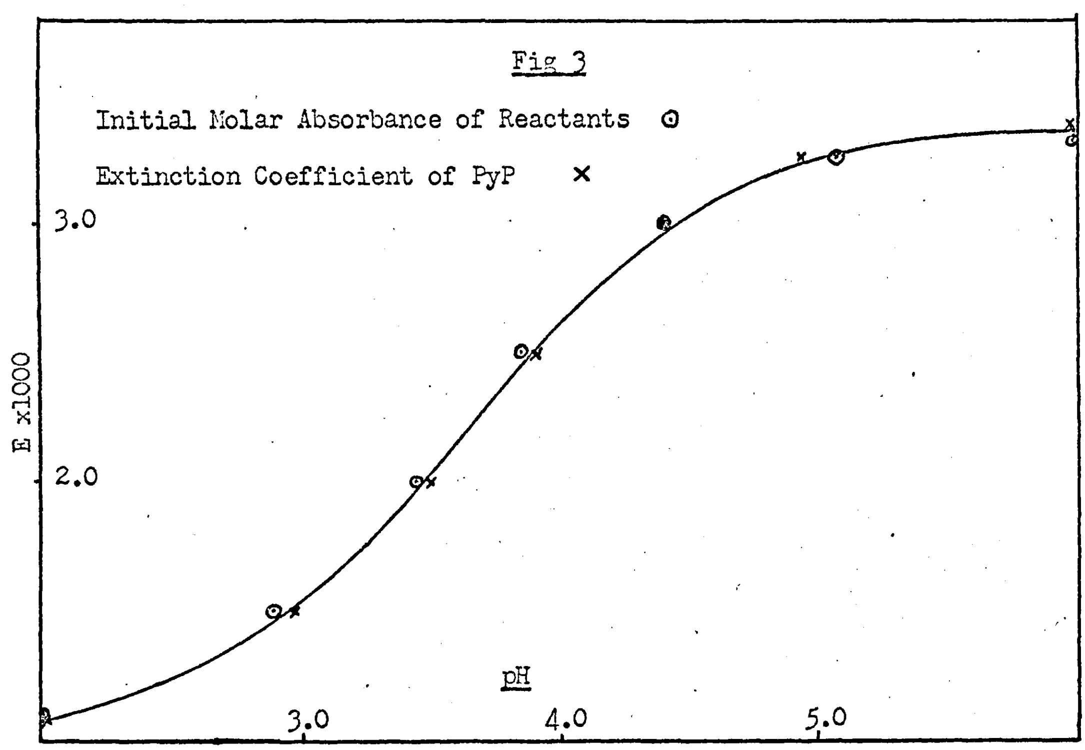


Fig 3
Initial Molar Absorbance of Reactants ⊙
Extinction Coefficient of PyP ×
3.0
2.0
E x1000
pH
3.0
4.0
5.0

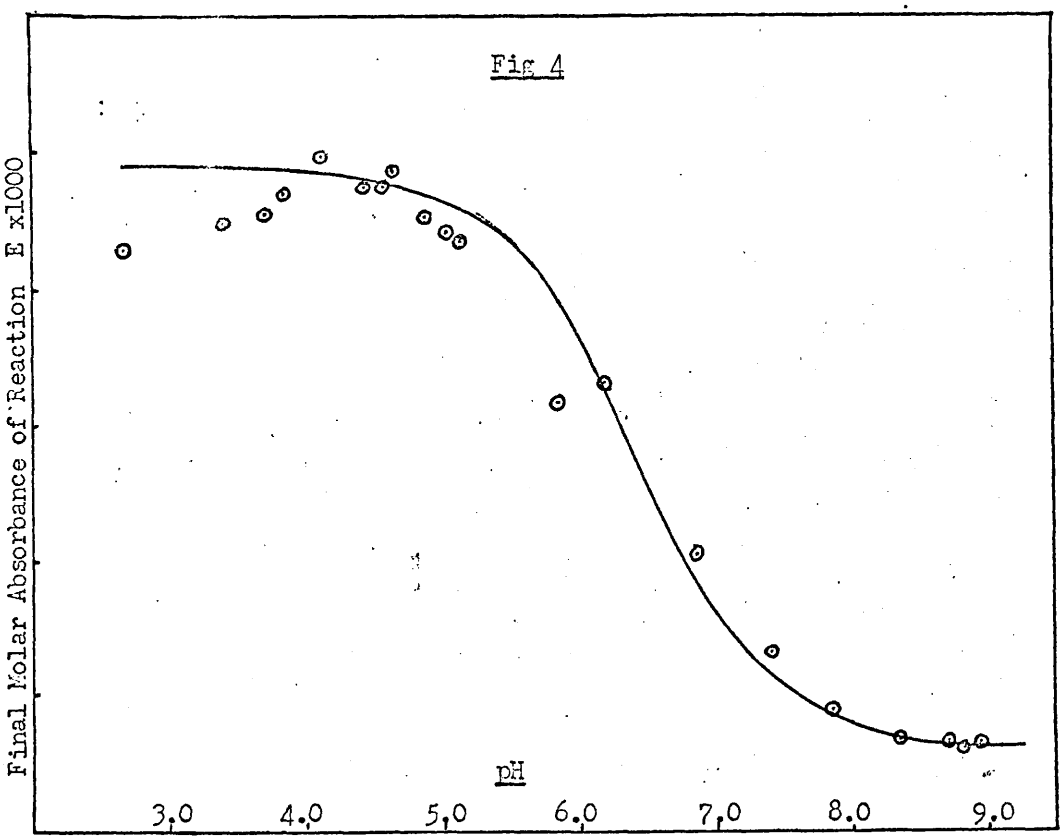


Fig 4

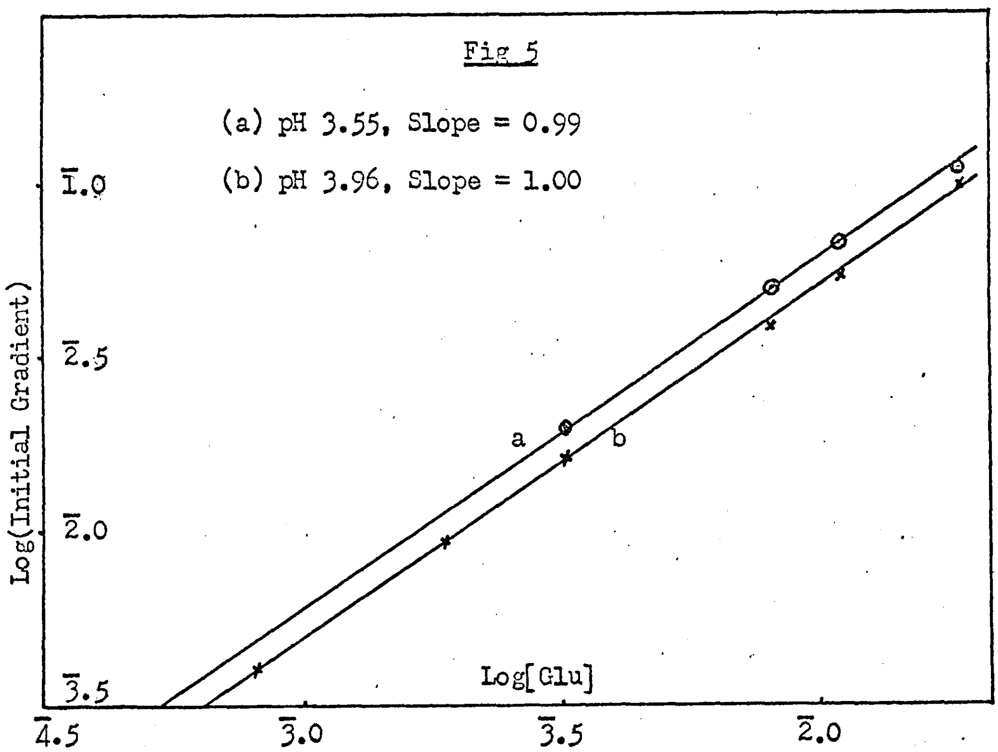
Fig 4 shows the final optical density of the reaction mixture over a wider range of pH. If this represents the absorbance of the completely, or almost completely formed Cu-Schiff's base complex (the results of p 129 and Fig 60 indicate that this is so), then the spectral change associated with the change of pH can be attributed to the ionisation of a proton. The points in Fig 4 are experimental and the line is theoretical for such an ionisation of pK=6.4. The fall-off of points below pH 4 is probably due to incomplete complex formation. According to Davies et al. (36) and Christensen (25), the dissociation can be attributed to the ring nitrogen of the Schiff's base in the complex.

(b) Dependence of reaction rate on the concentration of glutamate

The initial gradient of the reaction was measured in a series of runs in which the concentration of sodium glutamate was varied. The concentrations of the other reactants were kept constant (0.2 mM in pyridoxal phosphate, 0.4 mM in copper sulphate). The kinetic runs were carried out at pH 3.55 and 3.96.

As high concentrations of glutamate tended to alter the pH of the buffered solution by a small amount, the pH of each run was measured and the gradient corrected empirically where necessary using the previously obtained graph of (initial gradient)/pH. Fig 4 shows the resulting graph of Log(Initial gradient)/Log[Glu].

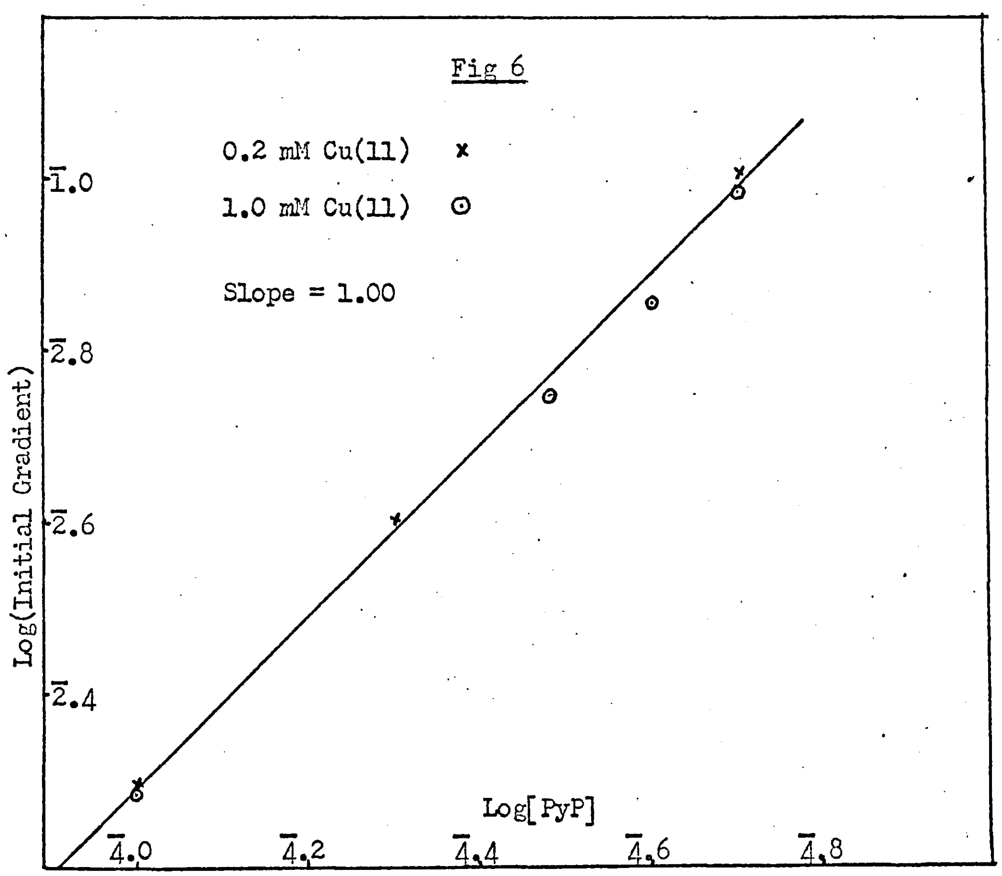
Fig 5



(c) Dependence of reaction rate on the concentration of pyridoxal phosphate.

Because the absorption of the pyridoxal phosphate itself is very intense, its concentration could be varied only between fairly narrow limits. The runs were carried out at pH 3.99 and at Cu(11) concentrations of 0.2 mM and 1.0 mM. The concentration of sodium glutamate was 8.0 mM in each case.

Graphs of Log(Initial gradient)/Log[PyP] are shown in Fig 6.

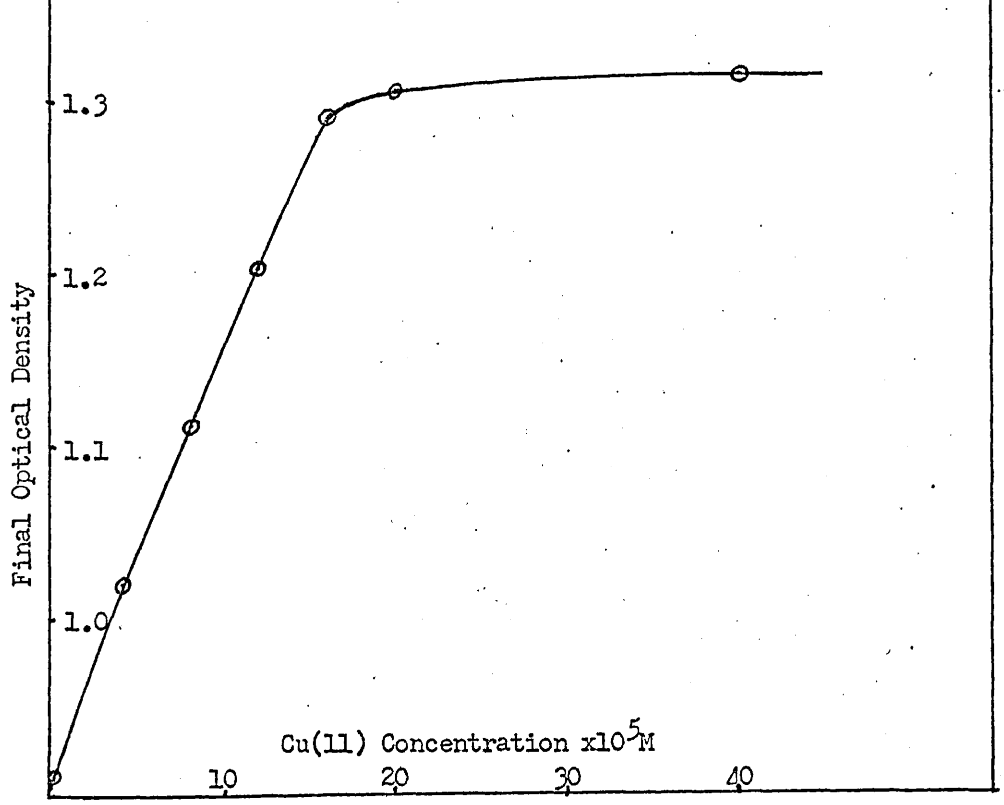


(d) The effect of variation of the Cu(11) concentration

The runs were carried out at pH 3.99 and at concentrations of 0.2 mM in pyridoxal phosphate and 8.0 mM in glutamate.

The initial optical density of the reaction mixture and the initial gradient were found to be unaffected by the concentration of the Cu(11) ions when this was less than ca. 1.6 mM. The final optical density was found to be almost directly proportional to the concentration of Cu(11) at concentrations below that of pyridoxal phosphate, and almost independent at concentrations above (Fig 7). The departures

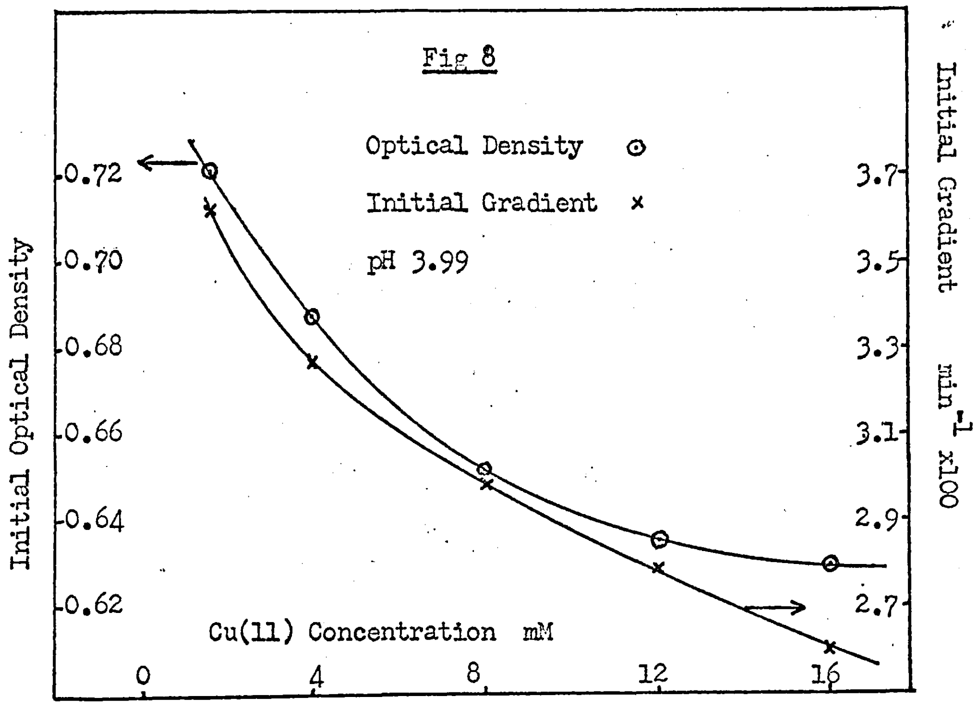
Fig 7



from ideal behaviour were utilised in attempts to find the stability of the complexes formed. (See p 123).

In the absence of Cu(11) ions there was only a very slight (ca. 0.01 OD units) increase in optical density. This was caused by the formation of a small amount of Schiff's base, the molar absorbance of which is only a little greater than that of pyridoxal phosphate at 25,500 cm^{-1}.

For Cu(11) concentrations above 1.6 mM and up to 16 mM, the highest concentration studied, a very different behaviour was observed. As the concentration of copper was increased the initial gradient decreased and the initial optical density fell below that due to pyridoxal phosphate alone (Fig 8). Because of the rapidity of the



initial fall in optical density preceding the normal rise, only the latter stages were observable. The initial gradient of the subsequent rise in optical density was recorded as usual. The apparent initial optical density was determined as before, by producing the tangents back to zero time.

The data for Fig 8 are reproduced in Table 1

Table 1

$[Cu].10^3$ M	Initial O.D. Of Reaction D	$D - D_o$	$dD/dt .10^2$ min^{-1}
1.6	0.721	0.006	3.62
4.0	0.687	0.040	3.27
8.0	0.651	0.076	2.98
12.0	0.635	0.092	2.79
16.0	0.629	0.098	2.59

Optical density of 0.2 mM PyP alone $D_o = 0.727$

Concentration of glutamate $= 8.0$ mM

The figures in column 4 have been adjusted to pH 3.96 (the pH of the buffer) as high concentrations of Cu(11) made the reaction mixture more acid.

Defining the degree of retardation as

$$A = (dD/dt)_{at\ [Cu]=1.6\ mM} - (dD/dt)_{at\ [Cu]>1.6\ mM}$$

and the degree of lowering of the initial optical density as

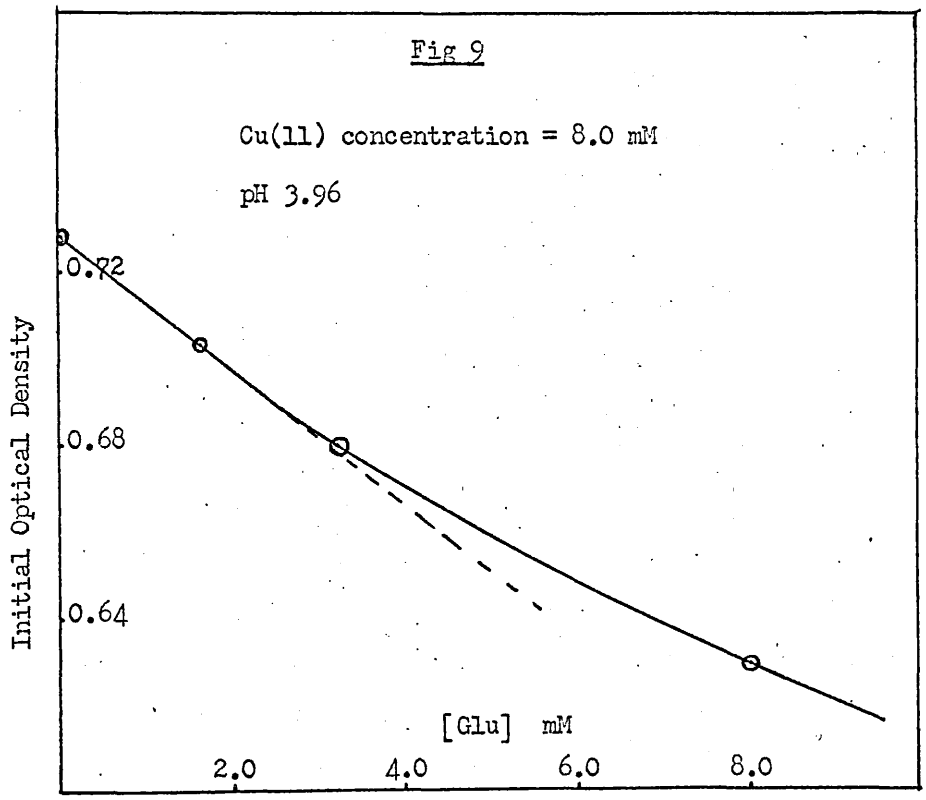
$$B = (D - D_o)_{at\ [Cu]=1.6\ mM} - (D - D_o)_{at\ [Cu]>1.6\ mM}$$

gives Table 2. The ratio A/B has a mean value of $0.101 \pm 0.007\ min^{-1}$.

Table 2

D - D_o	B OD units	dD/dt min^{-1}	A.10 min^{-1}	A/B min^{-1}
0.006		3.62		
0.040	0.034	3.27	0.035	0.103
0.076	0.070	3.98	0.064	0.091
0.092	0.086	2.78	0.083	0.097
0.098	0.092	2.59	0.103	0.112

The effect of altering the concentration of sodium glutamate on this lowering of the initial optical density was studied at high Cu(11) concentrations. A graph of (D - D_o)/[Glu] is shown in Fig 9. This



shows that $(D - D_o)$ is proportional to [Glu] at low concentrations, but 'tails off' at high.

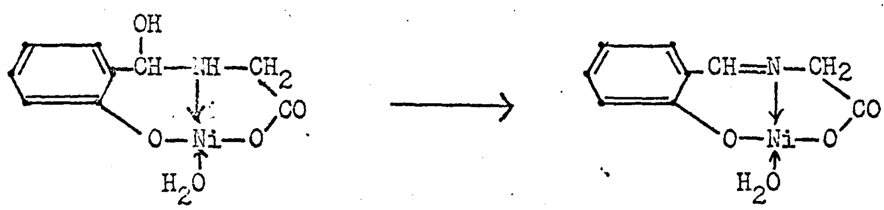
Discussion :

Considering first of all the results obtained at low concentrations of copper, the independence of the reaction rate on copper concentration and the results from sections (b) and (c) indicate that the rate determining step of the reaction is the combination of pyridoxal phosphate and sodium glutamate. Figs 5 and 6 show that this is first order in both reactants. This step can only be the formation of Schiff's base. The second, rapid step would then be the chelation of the Schiff's base to the copper ion. Because of the dependence of the final optical density on the copper concentration only for values of [Cu] less than [PyP] (Fig 8), it would appear that the complex formed has a Cu:Schiff's base ratio of 1:1.

At high concentrations of copper, the evidence of Tables 1 and 2 and Fig 9 indicates that some other complex, C, of the three reactants can exist. Fig 9 shows that glutamate must be present, so C cannot simply be a complex of Cu(11) and pyridoxal phosphate.

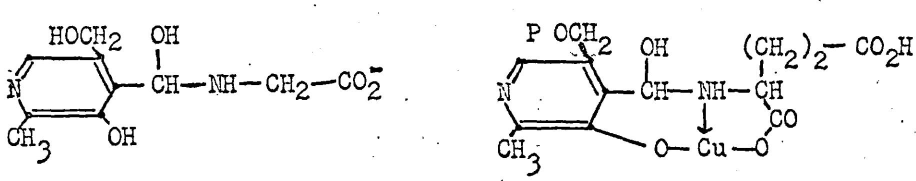
The removal of free pyridoxal phosphate in forming C, as measured by the drop in initial optical density, accounts for the concomitant decrease in reaction rate (the effect of removing an equal amount of glutamate would not be noticed as it is present in a large excess) if it is assumed that C cannot be converted directly to the Cu-Schiff's base complex without prior dissociation to its constituents.

Nunez and Eichhorn (8), in order to explain similar phenomena in the system nickel(11)/salicylaldehyde/glycine, suggested that a carbinolamine complex (i) existed. They, however, assumed that complex (i) could decompose to give (ii) directly, where (ii) is the equivalent metal-Schiff's base complex.



(i) (ii)

It is in fact most probable that carbinolamines of the type (iii) are formed as intermediates during Schiff's base formation (19,22,38,39). Metzler (22) estimated that about 11% of the Schiff's base formed from glycine and pyridoxal exists as carbinolamine. Chelation of the carbinolamine to the copper ion would then give a complex (iv) similar to that proposed by Nunez and Eichhorn (8) above.



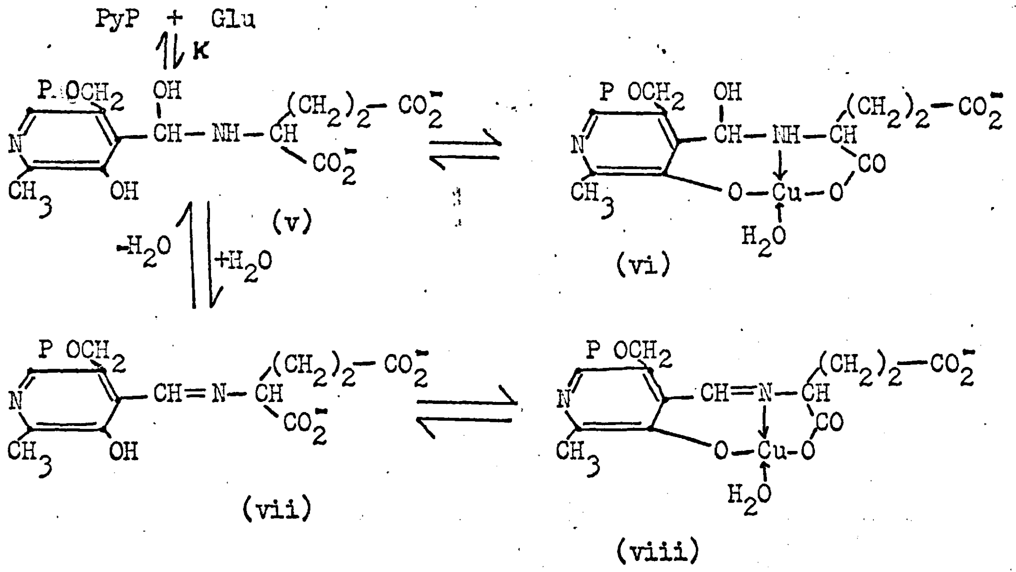
(iii) (iv)

When Cu(11) is present in small quantities it would preferentially co-ordinate to the glutamate and not be available to form complexes of the type (iv) unless these had a comparable stability to the metal-Schiff's base complexes. When Cu(11) is present in an excess, complexes of the type (iv) would be expected to form more readily. This would explain why the initial optical density of the reaction mixture was lower than expected only when Cu(11) was present in high concentrations.

However, if the rate limiting step were the reaction of pyridoxal phosphate with glutamate, no explanation of the very rapid appearance of the carbinolamine could be found. Such a concentration build-up of carbinolamine would only be possible if the rate limiting step were the dehydration of the carbinolamine to give the Schiff's base. This would seem contrary to the evidence of sections (b) and (c).

That both rate limiting attack of amine on aldehyde, and dehydration of carbinolamine are possible has been shown by Cordes and Jencks (39) and French and Bruice (38) who suggest that the former takes place at low pH and the latter at neutral and high pH. It would seem that the conditions of the present study favour dehydration of the carbinolamine as the rate determining step. The observed order of the reaction with respect to pyridoxal phosphate and glutamate (Sections b and c) can then be explained in the following manner. If pyridoxal phosphate and glutamate are involved in a very rapidly attained equilibrium with the carbinolamine, which then undergoes rate determining dehydration, the reaction would appear to be first order in both reactants if the equilibrium constant of carbinolamine formation K were very low (i.e. if the carbinolamine concentration were proportional to the concentrations of pyridoxal phosphate and glutamate). For the observed rate/pH graph (Fig 2) to be true, a more rapid increase in K with pH than decrease in acid catalysed carbinolamine dehydration would be required. Secondly, the phenomenon should be more noticeable at higher pH values as greater concentrations of complex (iv) would be produced. The reactions were carried out at pH 5 and this last requirement was found to be satisfied.

The proposed mechanism is shown diagrammatically below.



The carbinolamine (v) is assumed to be rapidly formed but present only in a low stationary concentration before rate determining dehydration to give the Schiff's base (vii). The carbinolamine complex (vi) acts as a 'reservoir' when an excess of copper ions are present and removes the reactive carbinolamine (v) to cause a decrease in the observed rate of Schiff's base formation. The chelation of the Schiff's base (vii) to the copper is rapid and almost quantitative, and prevents significant reversal of the dehydration step (v to vii).

Transamination of the Complexes Formed from Pyridoxal Phosphate and Sodium Glutamate

The spectra of the complexes formed from pyridoxal phosphate and sodium glutamate (of the type viii - p 37) were found to change slowly over a period of a few days to spectra 3 (Figs 1a, b and c pp 24 to 26). These spectra were identified as being due to a mixture of CuSB' and pyridoxamine phosphate for the Cu(11) system. The identification was carried out by preparing reaction mixtures similar to those described earlier (p 28) with pyridoxamine phosphate, α-ketoglutaric acid and copper sulphate as reactants. These reaction mixtures transaminate comparatively rapidly (without prior spectral change associated with metal-Schiff's base formation - see p 60) to give the metal-Schiff's base complex derived from pyridoxal phosphate and glutamate (MSB', see diagram viii, p 41). Excess α-ketoglutaric acid drives the reaction to completion. When the peaks which appeared at 25,500 cm^{-1} reached the height of the corresponding peaks of spectra 3 (Fig 1), the complete spectra of the reaction mixtures were recorded. These are compared with spectra 3 for Cu(11) at pH 3.96 and 5.04 (Fig 10). Fig 10 shows that complexes of pyridoxal phosphate-glutamate Schiff's bases reversibly transaminate to pyridoxamine phosphate and α-ketoglutarate.

The kinetics of the reaction were studied at 25,500 cm^{-1} and 25.0°C.

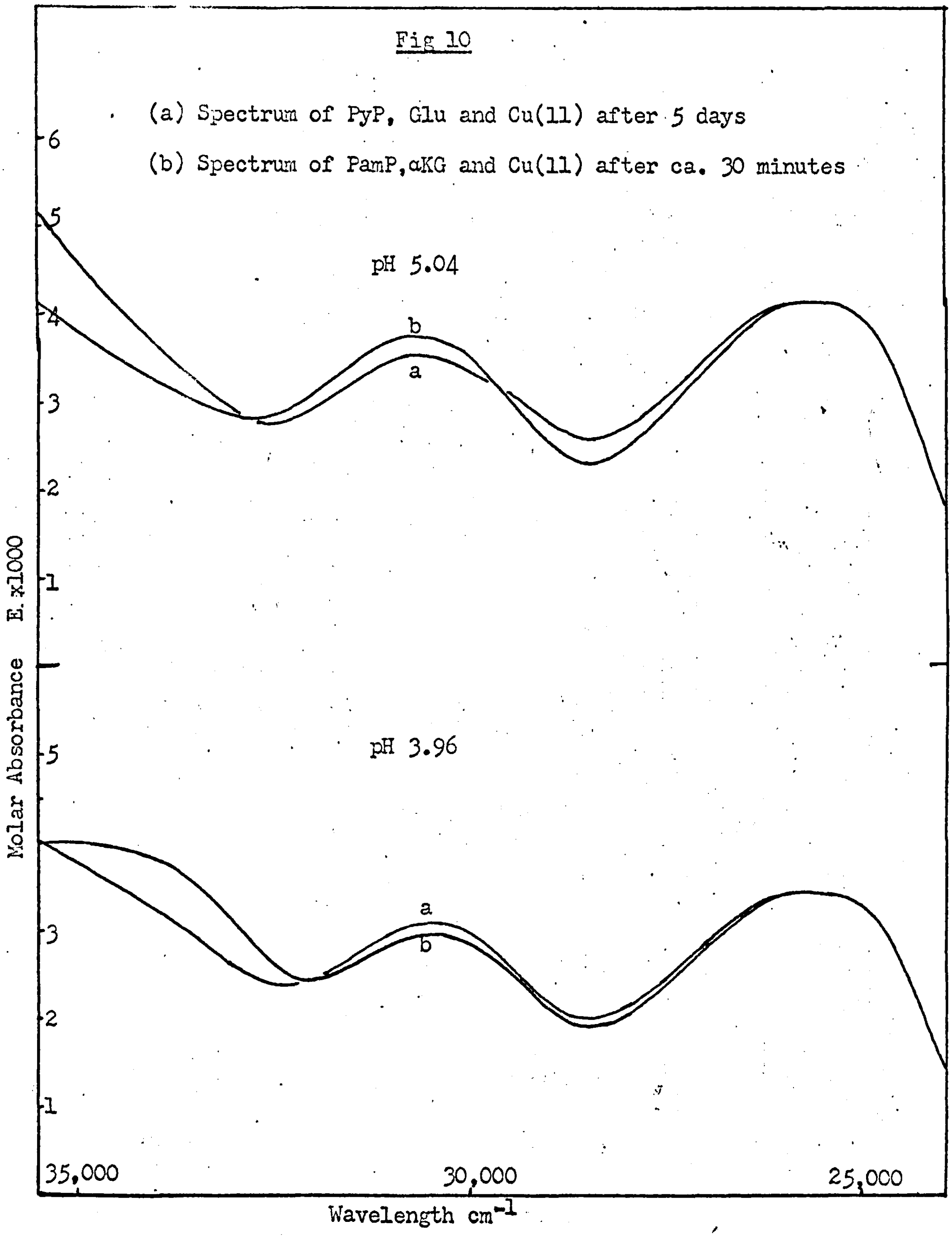


Fig 10
(a) Spectrum of PyP, Glu and Cu(11) after 5 days
(b) Spectrum of PamP, αKG and Cu(11) after ca. 30 minutes
pH 5.04
pH 3.96
a
b
6
5
4
3
2
1
Molar Absorbance E.x1000
35,000
30,000
25,000
Wavelength cm-1

Experimental

Reaction mixtures similar to those already described (p 28) were made up and allowed to react until the first step (metal-Schiff's base formation) was complete. The SP 800 spectrophotometer was then set to take optical density readings at fifteen minute intervals for a period of about 12 hours.

The effect on the rate of transamination was studied of variation of:

(a) the metal ion

(b) the concentration of metal ion

(c) the pH

(d) the concentration of sodium glutamate

Theoretical

For a first order reaction of the type

$$MSB' \longrightarrow \text{Products}$$

the rate of disappearance of MSB', $-dx/dt$, is proportional to its concentration, x.

or $$dx/dt = -kx$$

Integration between the boundary conditions $x = a$ when $t = 0$ and $x = x$ when $t = t$, gives

$\ln x = \ln a - kt$ where $\ln = \log_e$

The optical density of the reaction mixture, D, is given by

$D = lEx$ where l is the path length (1 cm.)

E is the extinction coefficient of the reactive species.

Then $\ln D = \ln D_o - kt$ (i)

A graph of log D / t should give a straight line of gradient -0.434k.

For a reversible reaction the relationship is more complicated and no simple graph will define k alone, but will include a constant k' for the reverse reaction. As k' is also an unknown, evaluation of k is difficult. In the present case readings were confined to the first 10% of reaction where the reverse reaction was assumed to have a negligible effect. If the reaction products had absorbed at 25,500 cm^{-1} equation (i) would have had to be altered to

$$\ln(D - D_p) = \ln(D_o - D_p) - kt$$

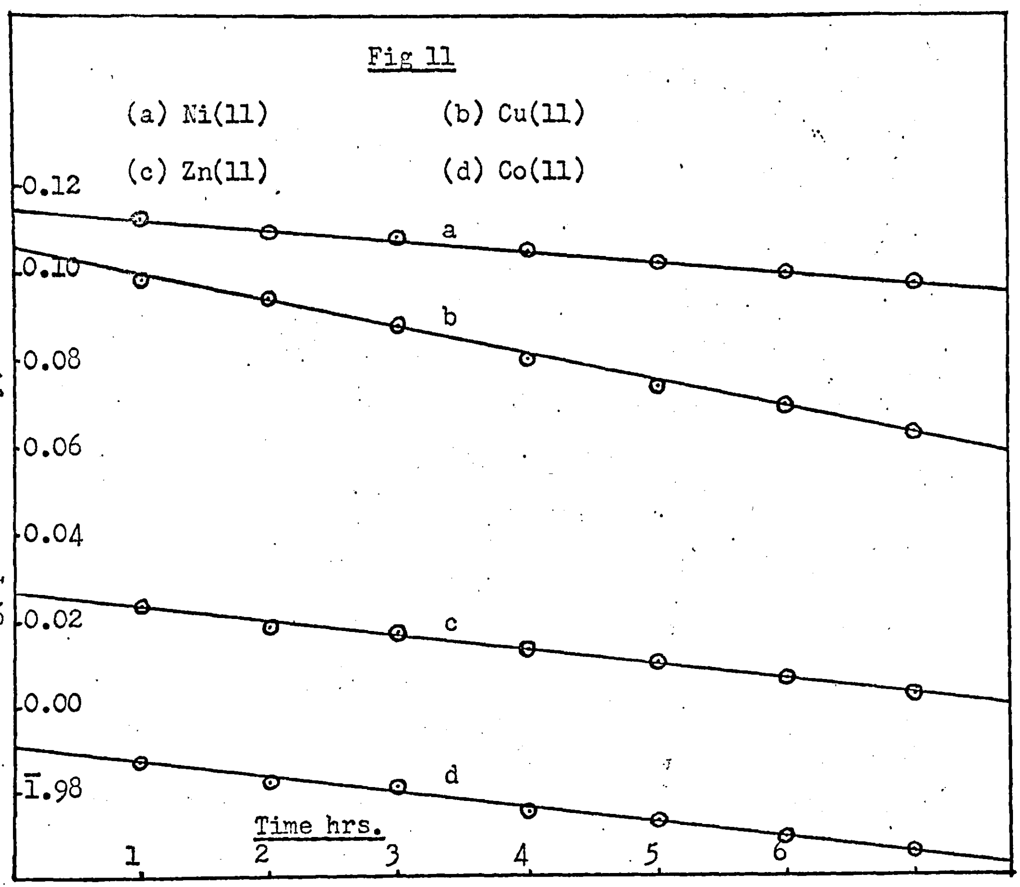
where D_p is the optical density of the completely reacted mixture. The value of D_p was taken as zero as neither pyridoxamine phosphate nor α-ketoglutarate absorb at 25,500 cm^{-1}. The non-zero value of 'infinity' readings, taken up to 1 week later, was explained by by the presence of an equilibrium concentration of MSB' necessarily present through the reversible nature of the reaction.

The straight lines produced when log D was plotted against time indicate that transamination of the complexes MSB' is a first order reaction. The slope of these graphs gives the first order rate constant, $k/2.303$ min^{-1}.

Results

(a) Comparison of the first order rate constants for the reaction in the presence of different metals.

The results of first plots of logD/Time are shown in Fig 11 for the metals Cu(11), Ni(11), Co(11) and Zn(11). The concentrations of the reactants were 0.333 mM in metal ion, 0.2 mM in pyridoxal phosphate and 8.0 mM in glutamate. The pH was 5.04.



The gradients of these graphs are shown in Table 3.

Table 3

Metal	Cu(11)	Zn(11)	Ni(11)	Co(11)
k_1 x10^5 min^{-1}	32.9	12.6	9.20	13.6
Infinity OD.	0.408	0.330	0.634	0.292

(Infinity readings were taken after 5-7 days)

The low value of k_1 for Ni(11) may be due, in part, to some interference from the reverse reaction, the importance of which is indicated by the high infinity readings.

(b) The effect on the first order rate constant of variation of the concentration of metal ion.

Because of the obvious differences in the behaviour of Cu(11) and the other metals, as shown in (a) above, these experiments were carried out with both Cu(11) and Ni(11) to give a comparison between the two.

The concentrations of the other reactants were 0.2 mM in pyridoxal phosphate and 8.0 mM in glutamate. The pH was 5.04.

The first order plots for Cu(11) are shown in Fig 12 and for Ni(11) in Fig 13. The results are summarised in Table 4 and a graph of $k_1/[Cu^{2+}]$ is shown in Fig 14.

The maximum which appears in the final optical density (Table 4) at $[Cu^{2+}]$ = 0.4 mM (i.e. a 1:2 ratio of PyP:Cu) may be a reflection of the rate maximum which occurs at this ratio of concentrations in the reverse reaction (see p 70).

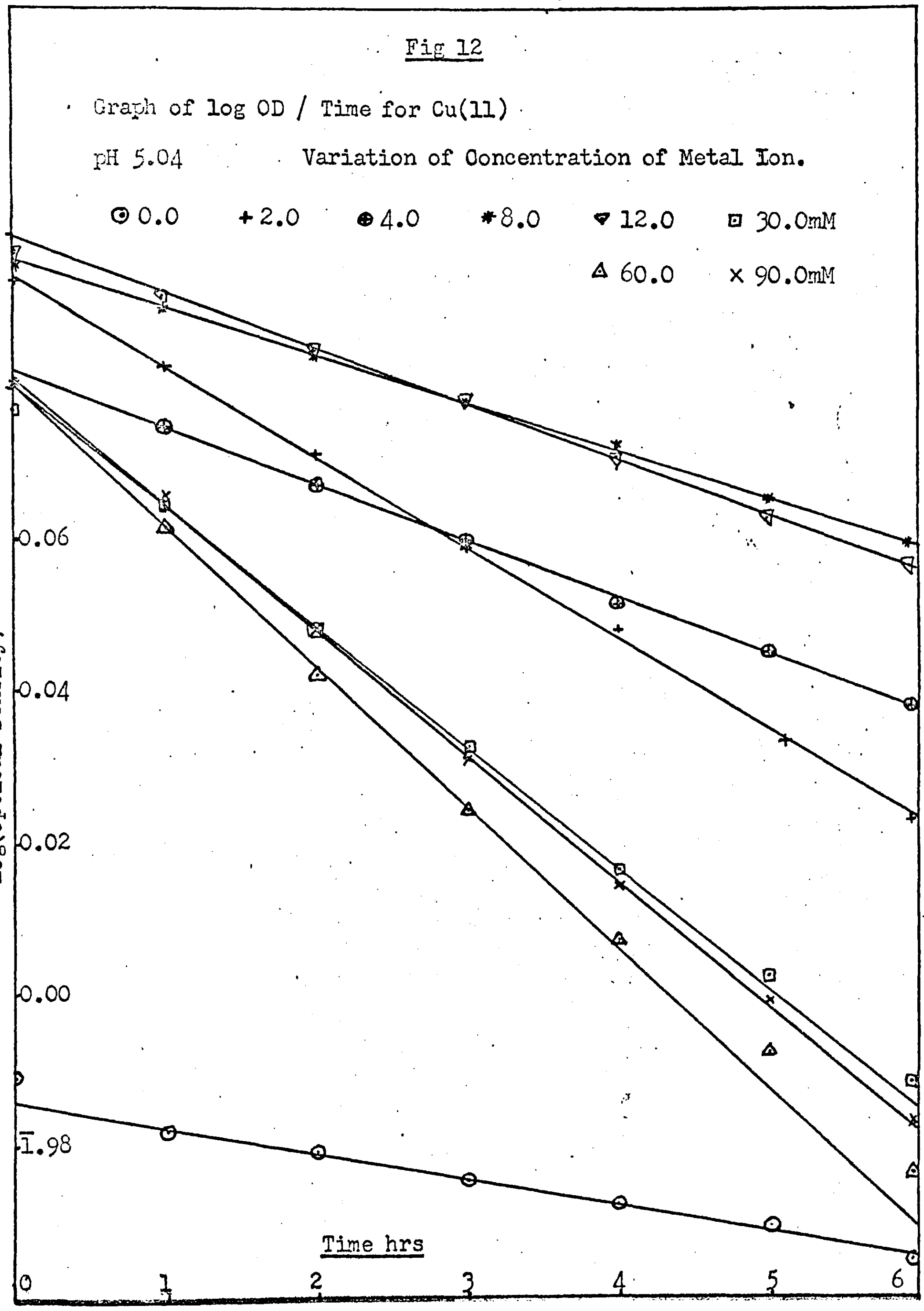
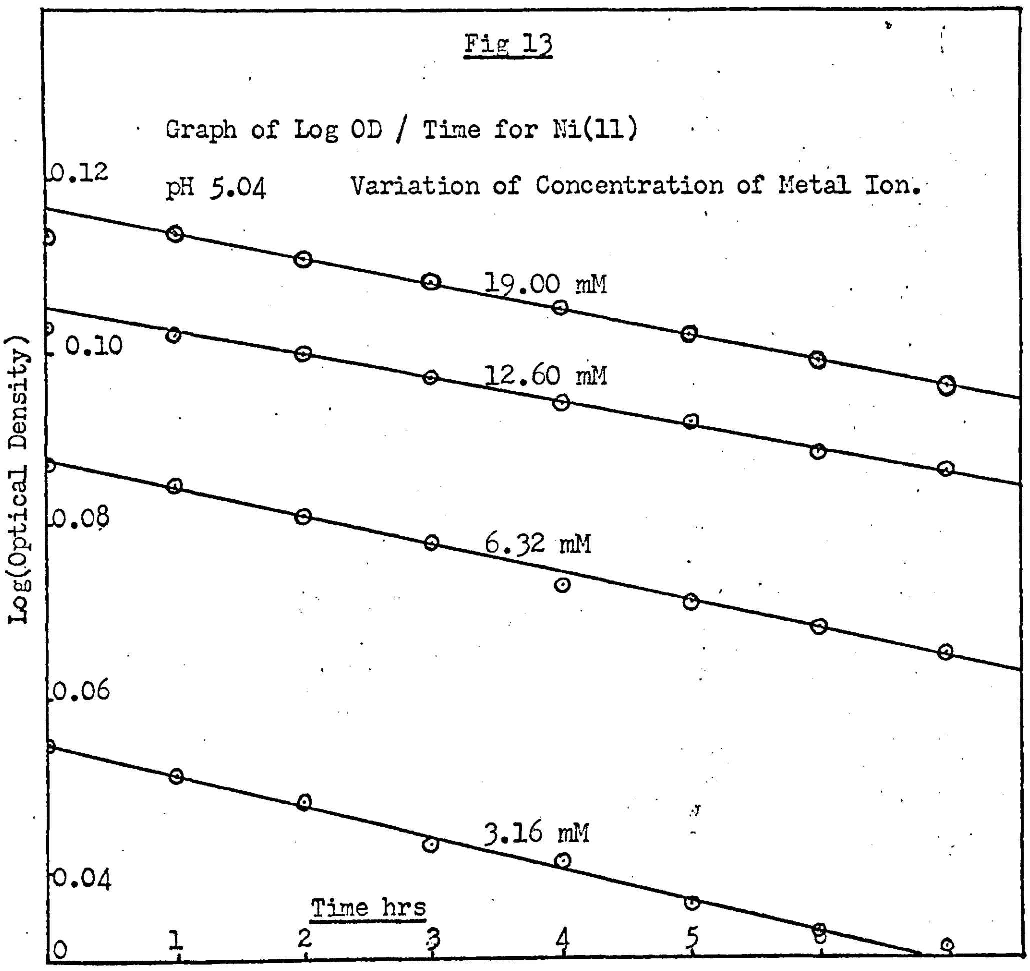


Fig 12
Graph of log OD / Time for Cu(11)
pH 5.04 Variation of Concentration of Metal Ion.
0.0
+2.0
4.0
8.0
12.0
30.0mM
60.0
× 90.0mM
0.06
0.04
0.02
0.00
1̄.98
Log(Optical Density)
Time hrs
0
1
2
3
4
5
6

The value for k_1 of 1.31 $\times 10^{-4}$ min^{-1} at zero copper concentration (Table 4) is not very realistic as it does not take into account that the freeSchiff's base undergoing transamination is largely dissociated. To correct for this, the above rate constant must be multiplied by the factor $(1 + 1/Kb)$, where K is the equilibrium constant for Schiff's base formation at pH 5.04 (taken from Table 19, p 143) and b is the concentration of glutamate (which must be present in a large excess for the above factor to be exact). This correction makes $k_1 = 2.37 \times 10^{-3}$ min^{-1}.

This indicates that transamination of pyridoxal phosphate and



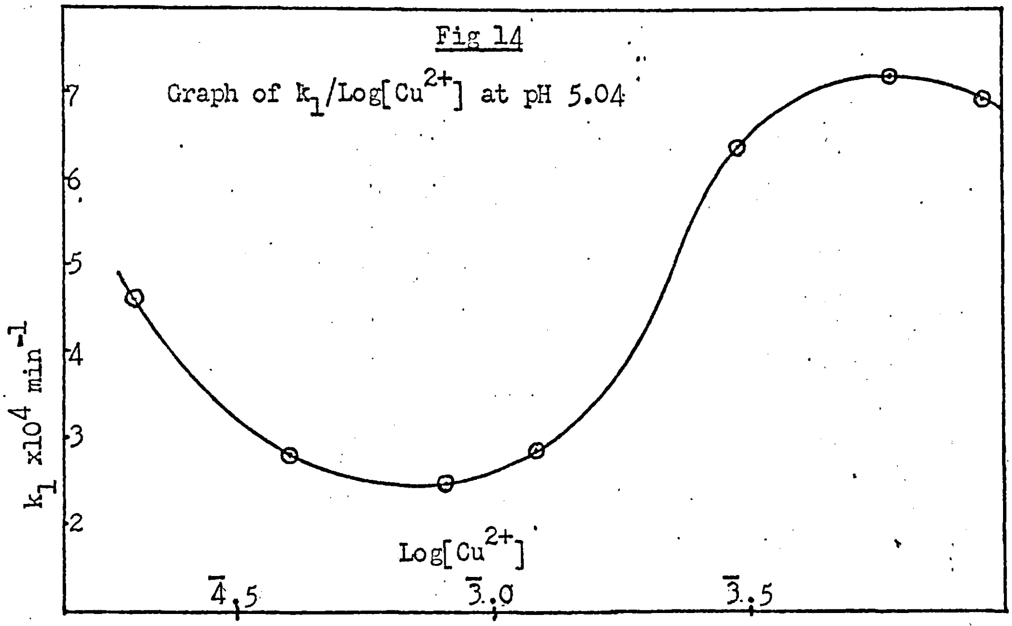


Table 4

Cu			Ni		
[Cu] $M.10^4$	$k_1\ min^{-1}$ $x10^4$	Final OD.	[Ni] $M.10^4$	$k_1\ min^{-1}$ $x10^4$	Final OD.
0.0	1.31	0.135	3.16	1.37	0.520
2.0	4.60	0.408	6.32	1.28	0.635
4.0	2.85	0.648	12.60	1.10	0.650
8.0	2.44	0.335	19.00	1.15	0.588
12.0	2.90	0.200			
30.0	6.35	0.070			
60.0	7.22	0.064			
90.0	6.90	0.058			

glutamate to pyridoxamine phosphate and α-ketoglutarate proceeds faster in the absence of metal ions. Column 3 of Table 4 indicates that transamination is also more complete when metal ions are absent (see Introduction).

(c) The effect on the first order rate constant of variation of pH

The concentrations of the reactants were 0.2 mM in pyridoxal phosphate, 16.0 mM in glutamate and 0.4 mM in Cu(11). The pH was varied between 3 and 6.

Plots of log D / Time are shown in Fig 15 and a graph of log k_1/pH is shown in Fig 16.

(d) The effect on k_1 of variation of the concentration of glutamate.

These experiments were again carried out with both Cu(11) and Ni(11) for comparison. The concentrations of metal ion and pyridoxal phosphate were 0.333 mM and 0.2 mM respectively. The concentration of glutamate was varied between 1.6 and 22.4 mM. The pH was 5.04.

First order plots for the Ni(11) and Cu(11) systems are shown in Figs 17 and 18 respectively. The results are summarised in Table 5.

The final optical density of the reaction mixture containing Cu(11) can be seen to increase with the concentration of glutamate. This may be caused by the formation of mixed complexes (see pp 136 and 145) of the type MSB'-Glu, which would stabilise the reactants on the L.H.S. of the equation

$$\text{MSB'GLU} \xleftarrow[\text{Glu}]{} \text{MSB'} \longrightarrow \text{Products}$$

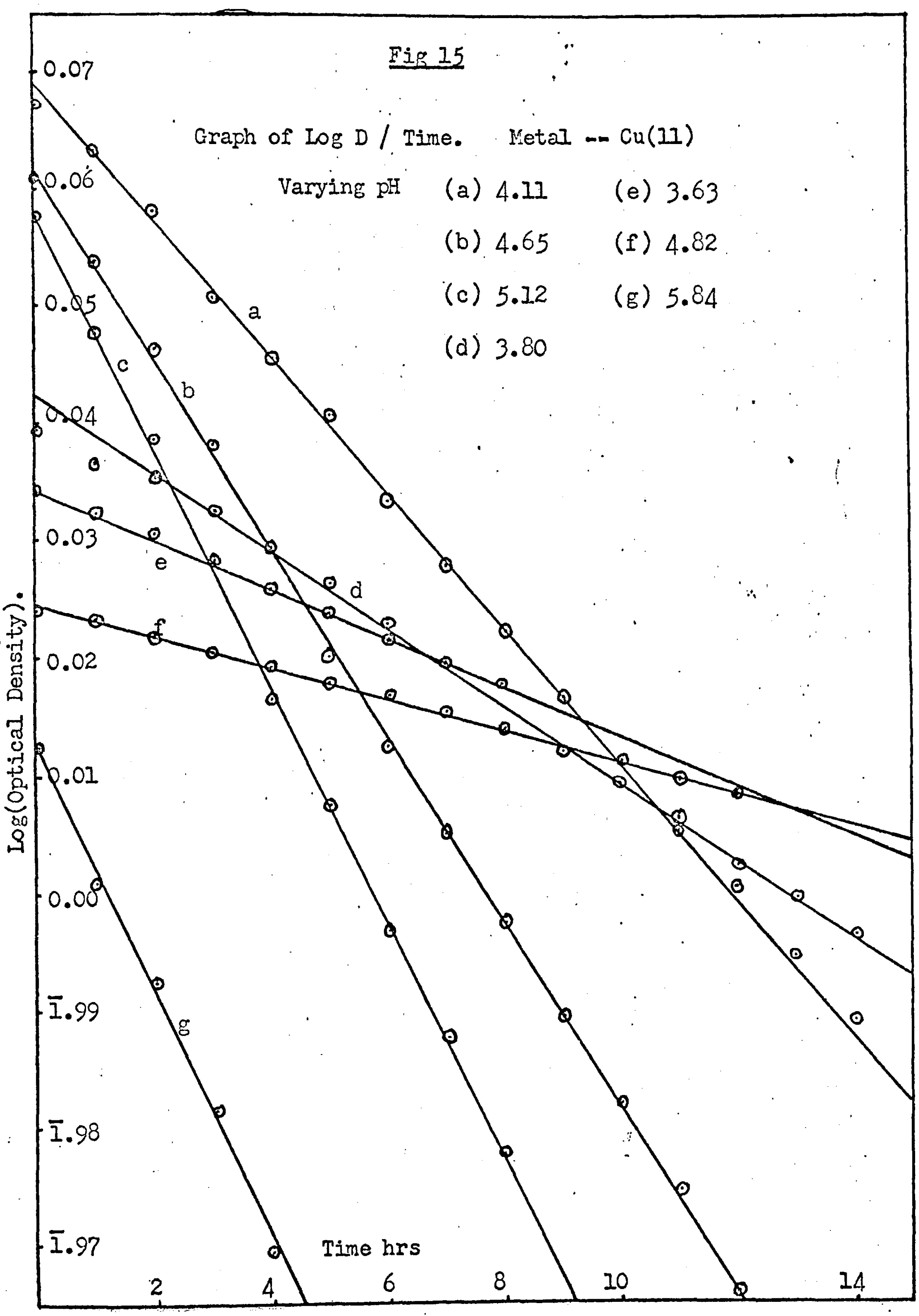


Fig 15
Graph of Log D / Time. Metal -- Cu(11)
Varying pH (a) 4.11 (e) 3.63
(b) 4.65 (f) 4.82
(c) 5.12 (g) 5.84
(d) 3.80
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
1̄.99
1̄.98
1̄.97
Log(Optical Density).
Time hrs
2
4
6
8
10
14
a
b
c
d
e
f
g

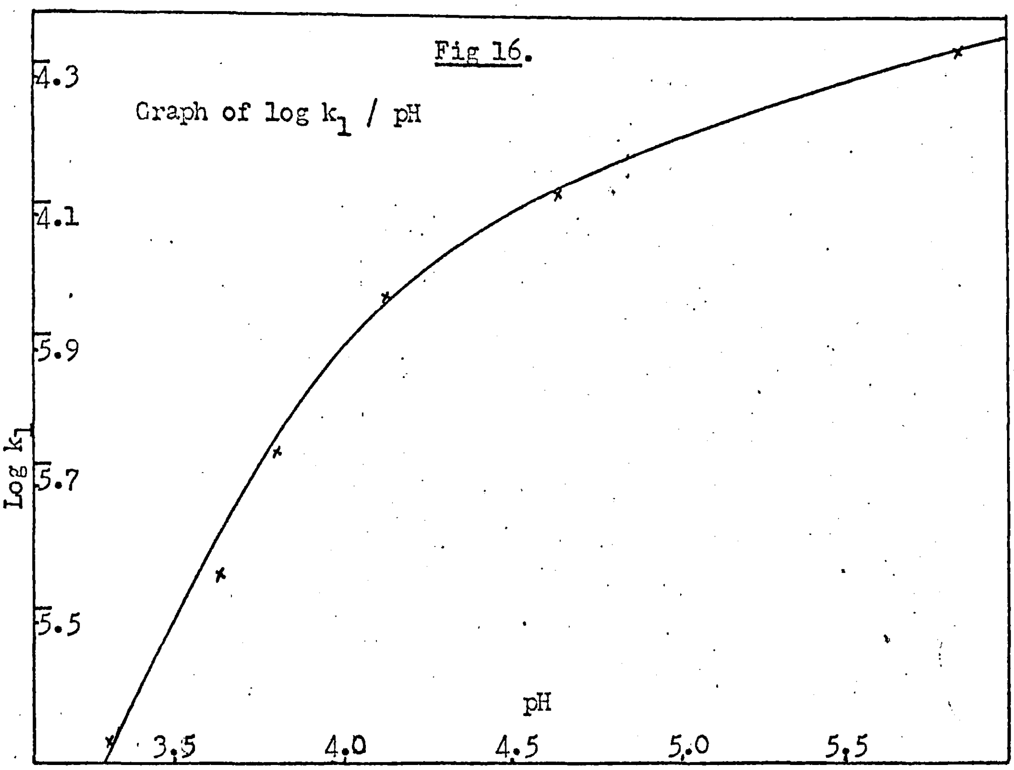


Fig 16.
Graph of log k_1 / pH
Log k_1
$\bar{4}.3$
$\bar{4}.1$
$\bar{5}.9$
$\bar{5}.7$
$\bar{5}.5$
pH
3.5
4.0
4.5
5.0
5.5

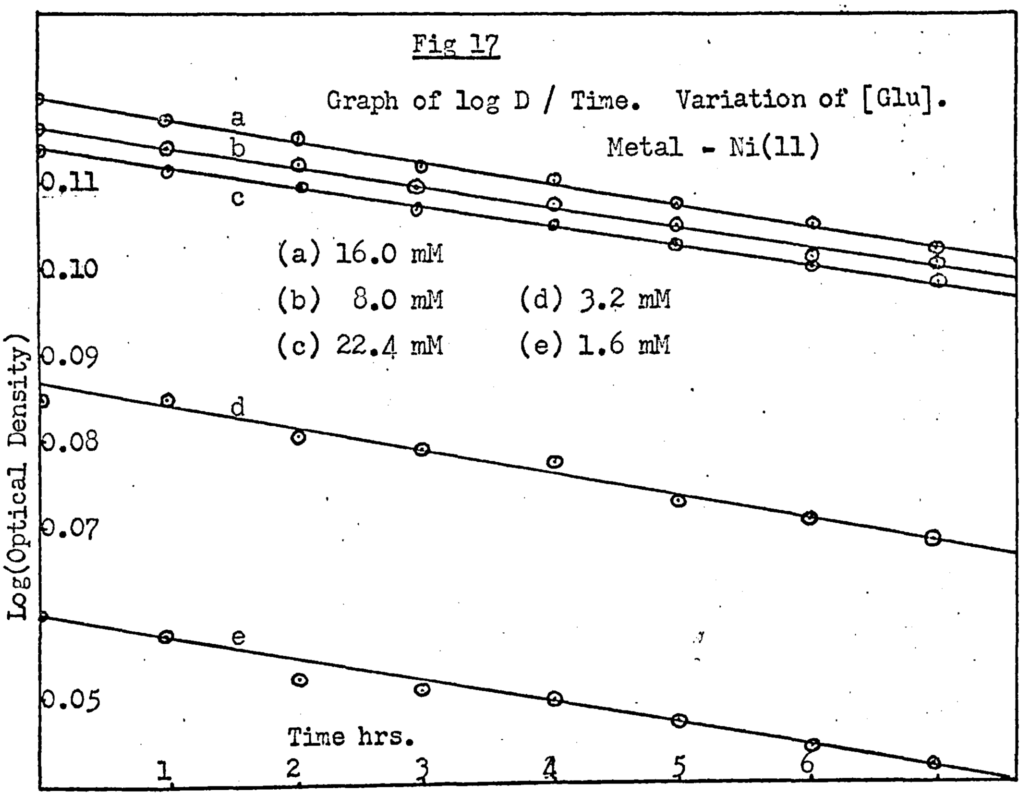


Fig 17
Graph of log D / Time. Variation of [Glu].
Metal - Ni(11)
(a) 16.0 mM
(b) 8.0 mM
(c) 22.4 mM
(d) 3.2 mM
(e) 1.6 mM
a
b
c
d
e
Log(Optical Density)
0.11
0.10
0.09
0.08
0.07
0.05
Time hrs.
1
2
3
4
5
6

Fig 18

Graph of Log D / Time for Cu(11)

Variation of [Glu].

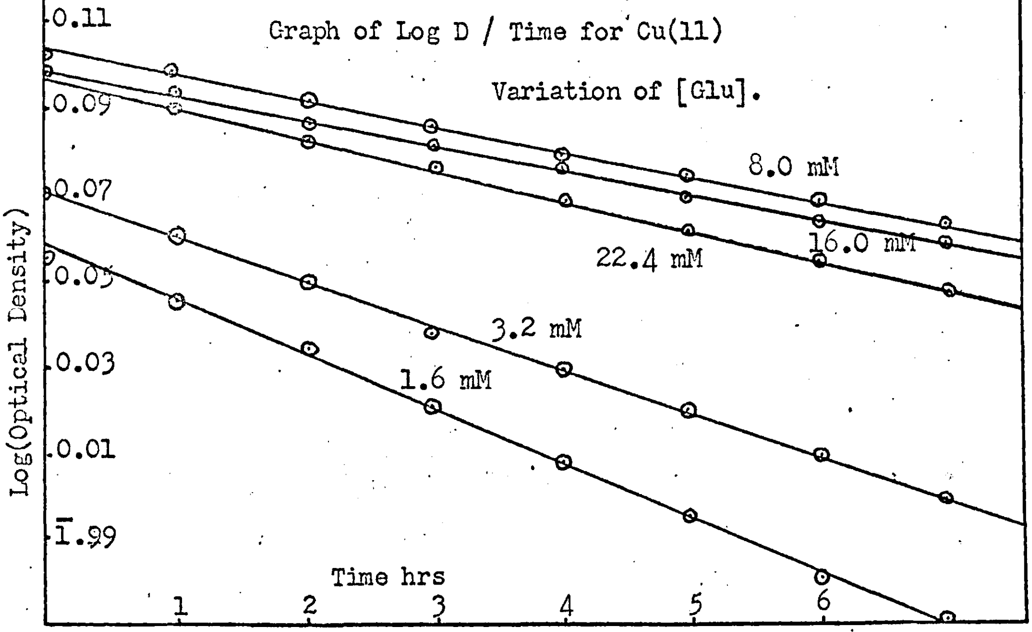


Table 5

[Glu]	Cu		Ni	
	k_1 min^{-1}	Final	k_1 min^{-1}	Final
M.10^3	x10^4	OD.	x10^4	OD.
1.6	5.00	0.142	1.04	0.601
3.2	4.55	0.162	1.04	0.607
8.0	3.29	0.408	0.92	0.634
16.0	2.37	0.776	0.96	0.682
22.4	2.78	0.667	0.90	0.679

by removing the reactive MSB' species. This would also account for the decrease in k_1 with increased concentration of glutamate.

On the basis of this explanation, and by comparison of the stabilities of the complexes of copper and nickel glutamates, it would be expected that the system containing Ni(11) should not exhibit such large changes in final optical density and k_1 as the system containing Cu(11). Examination of Table 5 shows this to be so. It would also be expected that these changes should be less marked the lower the pH. The reactions were carried out at pH 3.96 and it was found that k_1 was more constant throughout the experimental range although the final optical density still varied considerably.

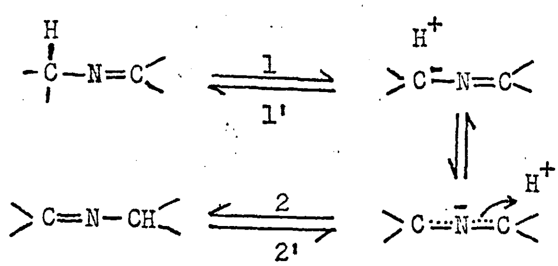
The first order constants for the system containing Cu(11) at pH 3.96 are shown in Table 6. The concentrations of the other reactants were 0.333 mM in Cu(11) and 0.2 mM in PyP.

Table 6.

[Glu] $M.10^3$	k_1 min^{-1} $x10^4$	Final OD.
1.6	1.20	0.320
3.2	1.17	0.553
16.0	1.15	0.747
22.4	1.18	0.654

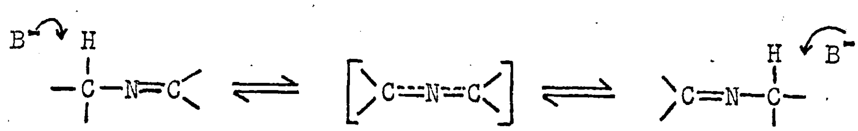
Discussion.

The rate determining step of transamination can depend upon the molecularity of the reaction. The mechanism for a unimolecular prototropic shift is:-



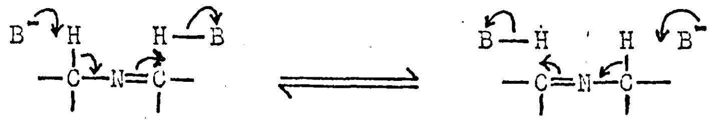
If step one is the rate determining step, the reaction rate would be expected to be influenced only by the polarity of the solvent, which would affect the ease of the initial proton loss, and not by the pH of the solution.

Ingold (40) envisaged the unimolecular mechanism as taking place by the steps:-



where B can be either the solvent or a stronger base. Such a mechanism as this could account for the observed pH dependence of the transamination reaction (Fig. 16) by the introduction of a term [B] in the rate equation.

Ingold (41) also proposed a bimolecular base catalysed reaction as follows:-



This mechanism requires that the terms $[B^-]$ and $[HB]$ appear in the raté equation. Bruice and Topping (7) found that these terms appeared in the rate equation for the system pyridoxal/phenylglycine in the absence of metal ions and using imidazole as base catalyst. It is unlikely that this type of bimolecular reaction contributes significantly to the kinetics of the present system as the rate of transamination would be expected to be more dependent upon the concentration of the glutamate ion than was the case (Figs 17 and 18). The independence of the rate of transamination on the concentration of glutamate also suggests that OH^- (and possibly to a lesser extent H_2O) acts as a base catalyst.

The slope of Fig 16 deviates from the value of unity expected from the above 'unimolecular' mechanism. This may be accounted for by the fact that the electronic state of the complexes can change through the dissociation of 'non-tautomeric' protons from other acidic groups present in the complexes. Dissociation of these groups will result in several possible intermediates $[R_1R_2C{=\!=}N{=\!=}CR_3R_4]$ (differing in electronic structure of R_1, R_2 etc.), each of which will have a different reactivity. As the concentration of each of these intermediates will be governed by more complex equilibria (by including the dissociations within the groups R_1, R_2 etc.) than those shown in Ingold's unimolecular mechanism, the dependence of the rate of reaction on $[OH^-]$ would not be expected to be linear.

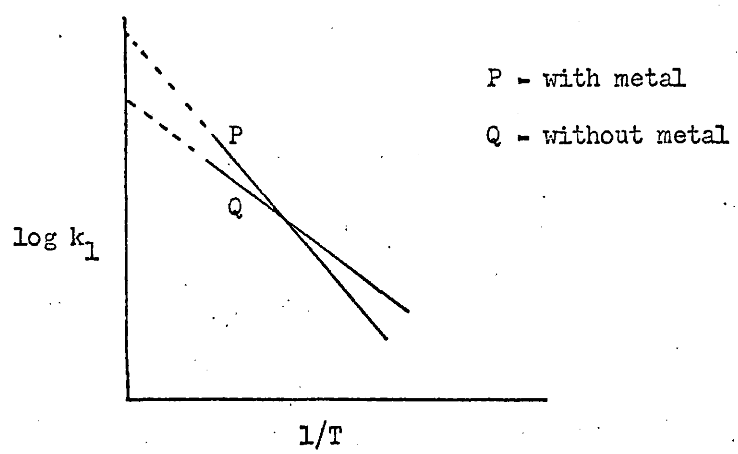
The observed retardation of the transamination of the Schiff's base SB' in the presence of metal ions is contrary to the results obtained at high temperatures (100^{o}C) by Snell (6) and others (see Introduction). This could be that Snell, in presenting his results, did not take into account that Schiff's bases are highly dissociated in the absence of metal ions.

Even so, this may not be sufficient to account for the differences in rate for 'catalysed' and uncatalysed reactions found by Snell. An explanation can be found by considering Arrhenius' equation,

$$k_1 = Ae^{-E_o/RT}$$

where E_o is the energy of activation and A is a constant which can be related to the entropy of activation, ΔS^* (42). Then, if the addition of metal ions to a solution of the Schiff's base SB' caused an increase in both ΔS^* and E_o, the change over from metal catalysed reaction at high temperatures to metal retarded reaction at lower temperatures is possible. This can be illustrated by plotting log k_1 against 1/T (Fig 19).

Fig 19.



The slope of graph P for the metal 'catalysed' reaction is E'_o and for the metal free reaction E_o. The intercepts on the ordinate are log A' and log A for metal catalysed and metal free systems respectively.

The increase in the energy of activation of the reactant species in the presence of metal ions is understandable in terms of the high thermodynamic stability of the complexes formed. The interpretation of the increase in the entropy of activation is less clear, however. A possible explanation is in terms of the changes in the number of degrees of freedom in going from the reactants to the transition state. Even a qualitative analysis of the number of degrees of freedom is difficult in the present case because of the complexity of the system.

Reaction Between Pyridoxamine Phosphate and α-Ketoglutaric Acid in the Presence of Cu(11) Ions.

On mixing solutions of pyridoxamine phosphate, α-ketoglutaric acid and copper sulphate, spectral changes ocurred similar to those discussed earlier (p 24). These are recorded in Fig 20a and b.

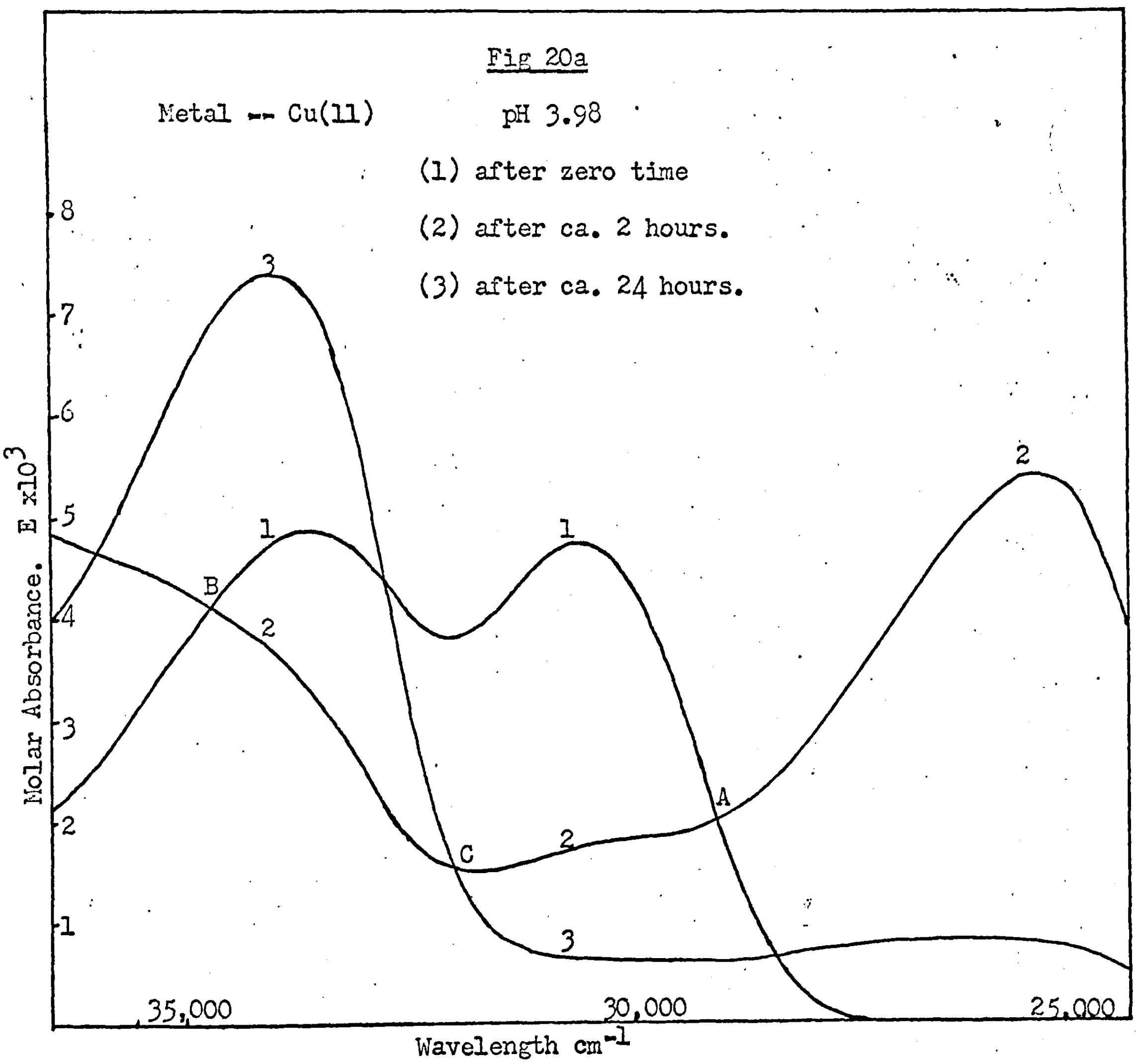
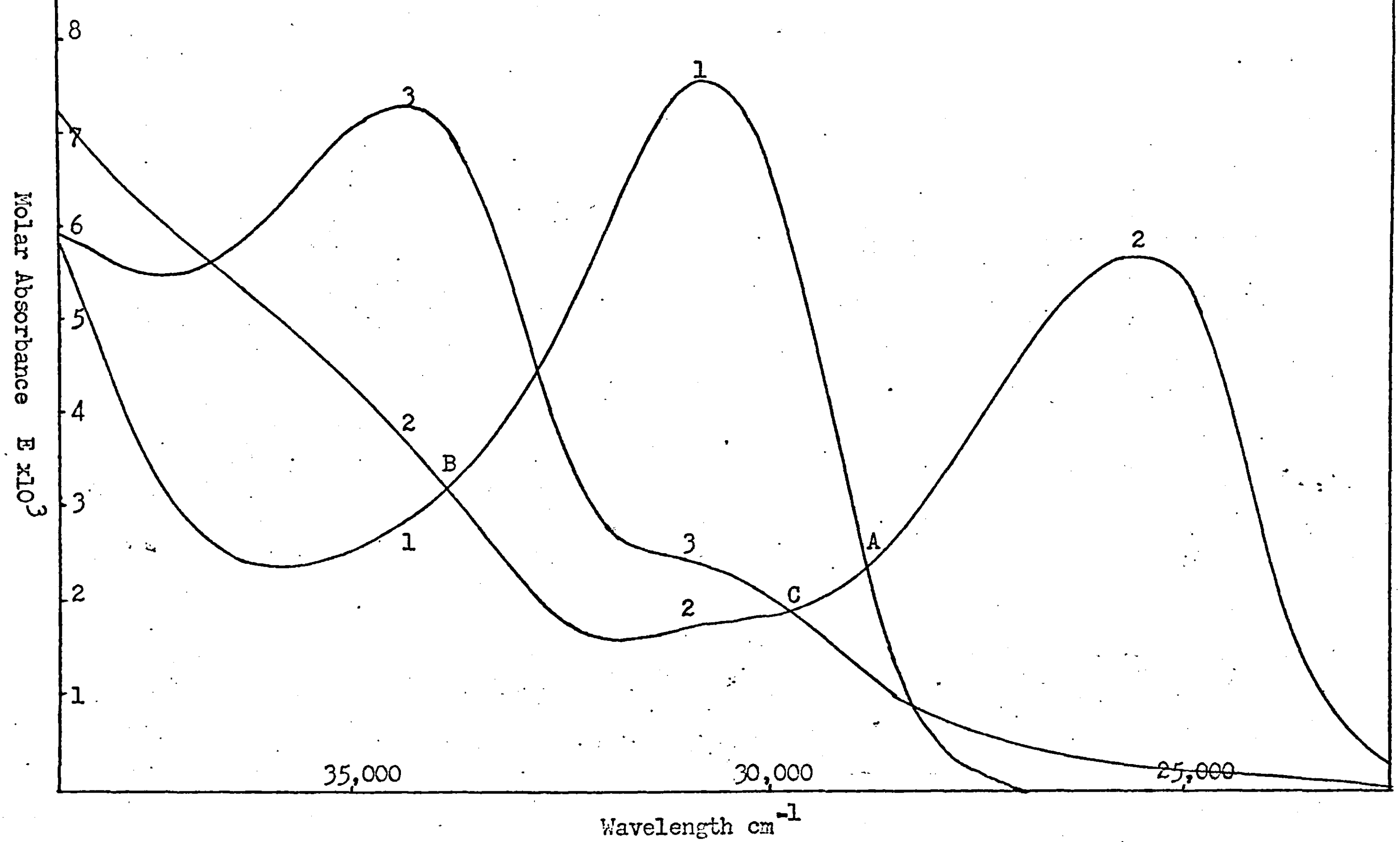


Fig 20b

Metal -- Cu(11)   pH 5.04



The spectrum changes from that of pyridoxamine phosphate alone (1) to a species (2) which can be identified with (2) in Fig. 1a (p 24). This was true for the pH values from 3 to 6 as well as those recorded here (3.9[illegible] and 5.04). With metals other than copper the extent of the reaction is insufficient for comparision of spectra (2) with those recorded earlier (p 25), but the characteristics of the spectra indicate that the reactions with copper and with other metal ions are the same, (Fig. 21a and b).

This initial reaction is followed by a slower one to give spectrum (3), (Fig. 20). The species responsible for spectrum (3) is, as yet, unidentified. Possible interpretations are discussed later (p 88). No spectra comparable to (3) appear in reaction mixtures containing metal ions other than Cu(11), (Fig. 21).

The reference cell for the above spectra was made up to contain the same concentration of α-ketoglutarate as in the reaction mixtures in order to compensate for its slight absorption.

Apparently sharp isobestic points were observed at A and B, and another less sharp one at C (Fig. 20).

<u>Experimental.</u>

Single wavelength studies carried out on the first reaction showed two main differences from the pyridoxal phosphate-glutamate reaction. The first was an 'induction period' during which spectrum (1) in Fig. 1 remained almost constant for a period of a few minutes before changing to (2). The second was an unusual dependence of the reaction rate on

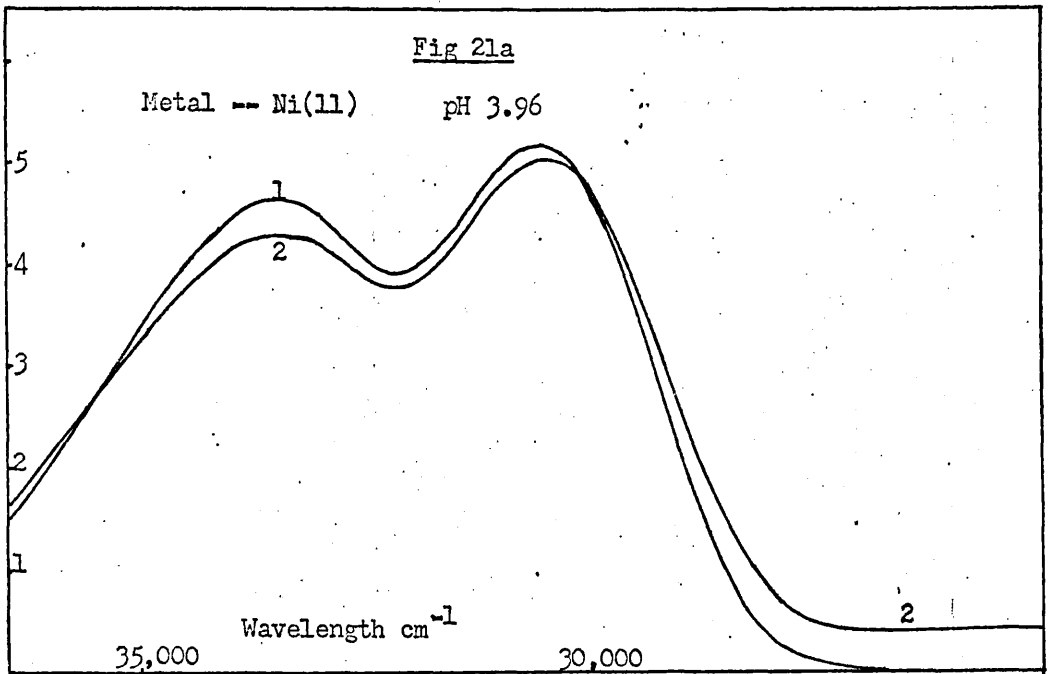


Fig 21a
Metal -- Ni(11)
pH 3.96
Molar Absorbance E x10^3
5
4
3
2
1
Wavelength cm^-1
35,000
30,000

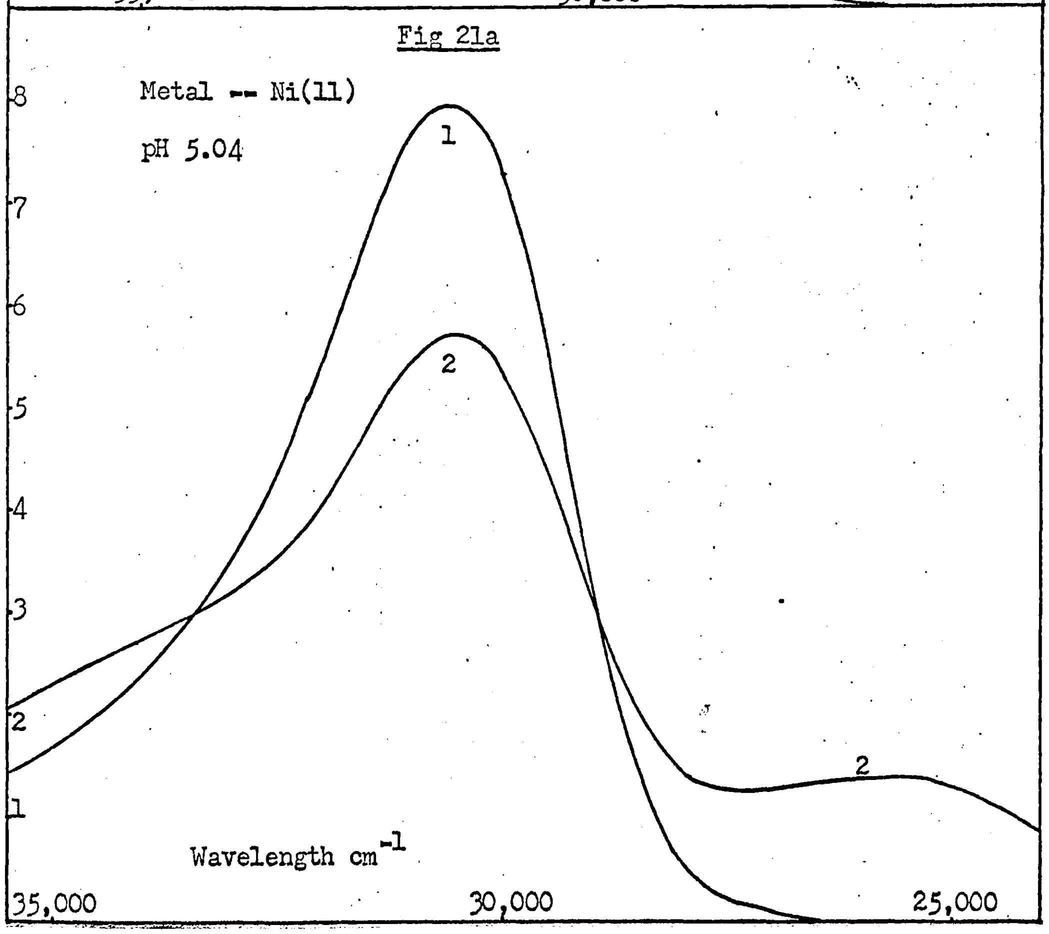
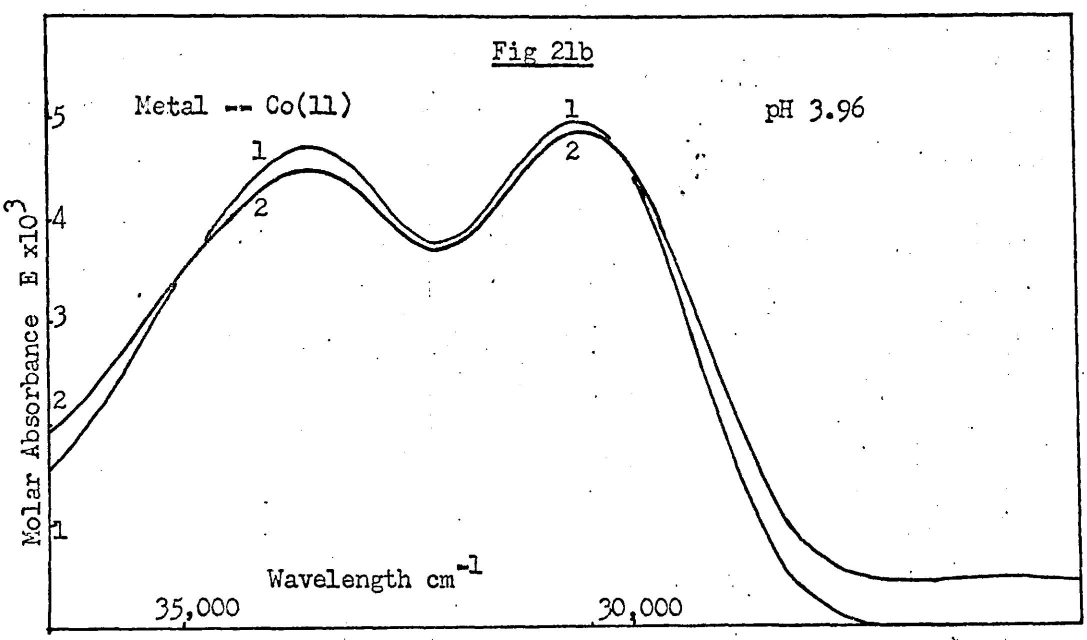


Fig 21a
Metal -- Ni(11)
pH 5.04
Molar Absorbance E x10^3
8
7
6
5
4
3
2
1
Wavelength cm^-1
35,000
30,000
25,000



the concentration of copper ions.

Because of the 'induction period' the initial gradient of the reaction could not be measured directly as before, so for each run a set of gradients dD/dt were plotted against their corresponding optical densities D. The straight line portion of these graphs was extrapolated back to zero optical density - the initial optical density of the reaction mixture - and the intercept on the ordinate taken as the initial gradient of the reaction.

The 'induction period' was found to be present no matter in which order the reactants were mixed, but it was possible to arrange experimental conditions to minimise its effect in some of the experiments.

The effects on the initial reaction rate were studied of variations of:-

(a) pH

(b) the concentration of α-ketoglutarate

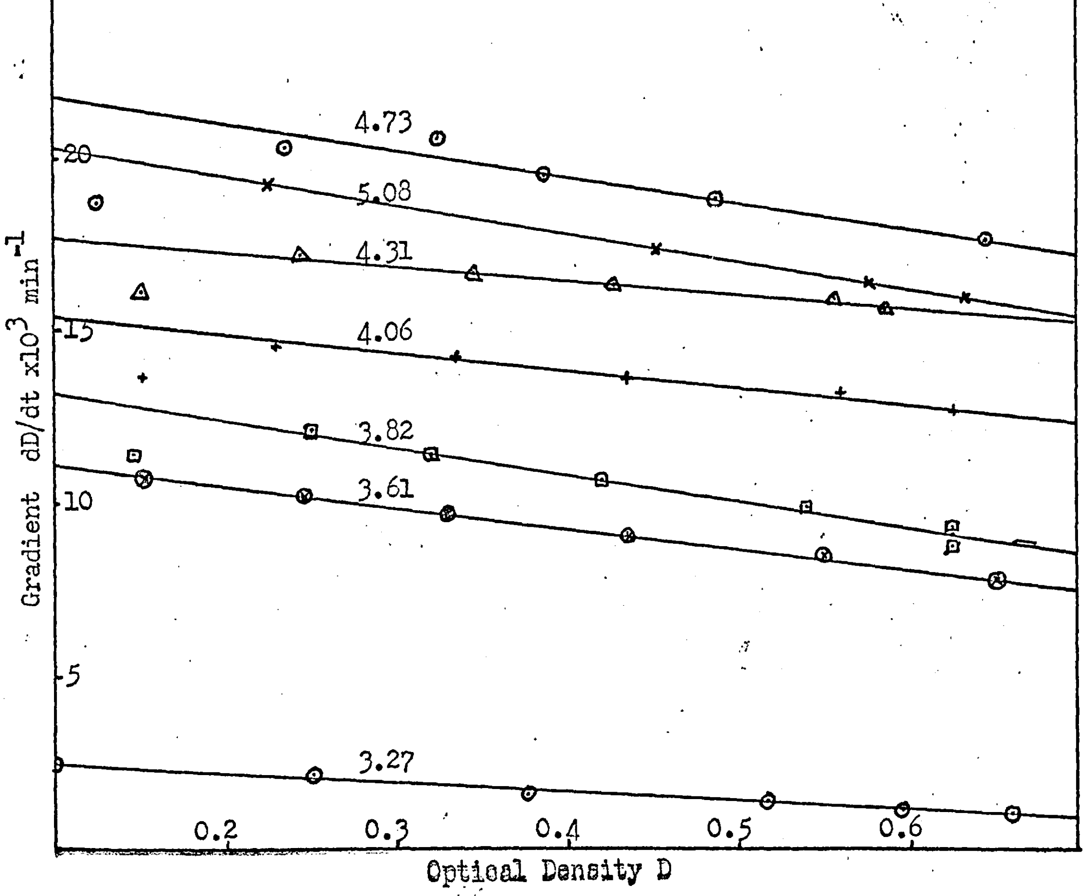
and (c) the concentration of Cu(11).

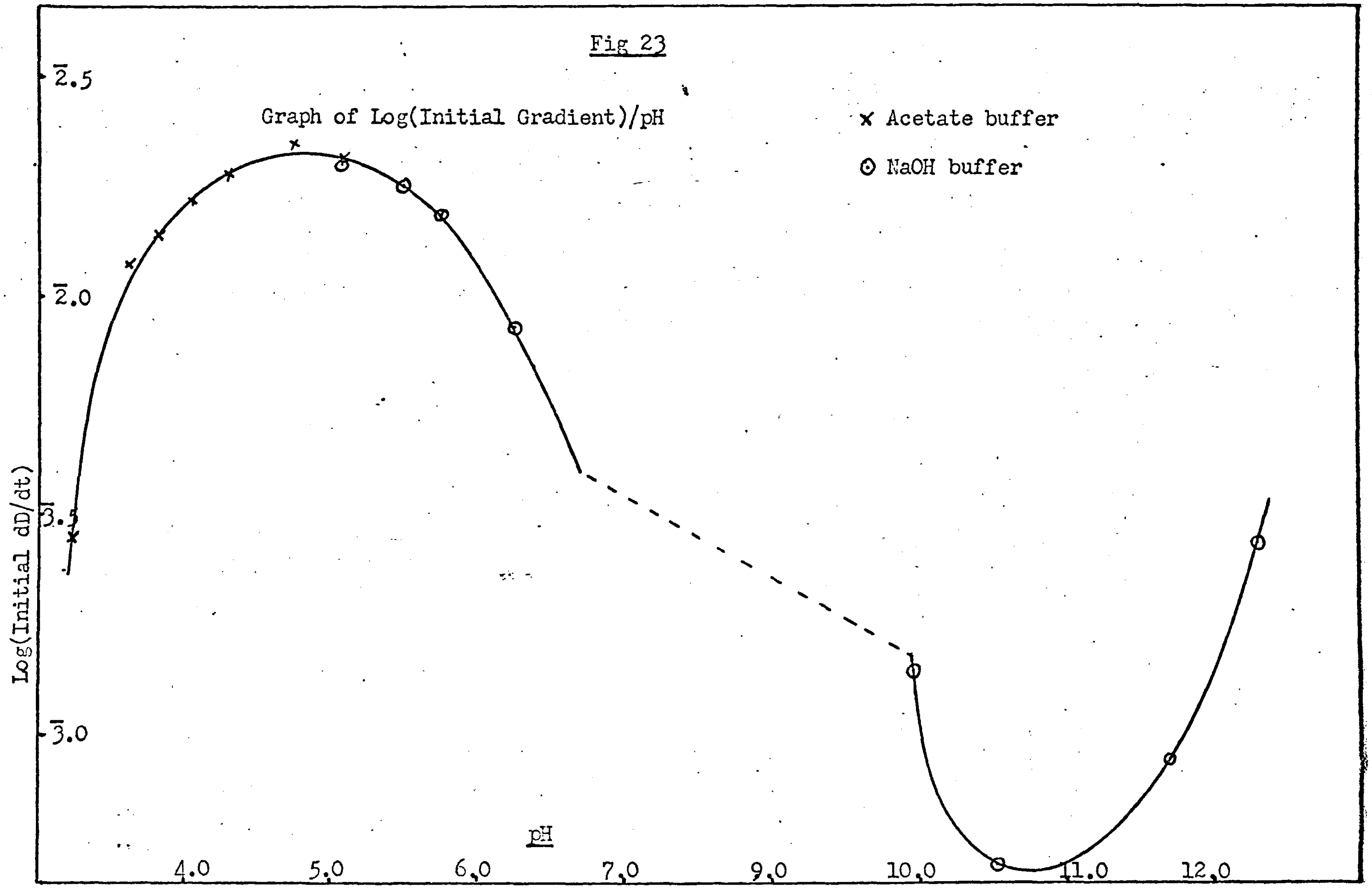
(a) The effect of variation of the pH on the initial reaction rate.

The concentrations of the reactants were kept constant at 16mM. in αKG, 0.3mM. in PamP and 0.6mM. in Cu(11).

Fig 22

Graph of dD/dt against D at different pH values





Graph of Log(Initial Gradient)/pH
× Acetate buffer
⊙ NaOH buffer
Log(Initial dD/dt)
$\bar{2}.5$
$\bar{2}.0$
$\bar{3}.5$
$\bar{3}.0$
pH
4.0
5.0
6.0
7.0
9.0
10.0
11.0
12.0

Fig 23

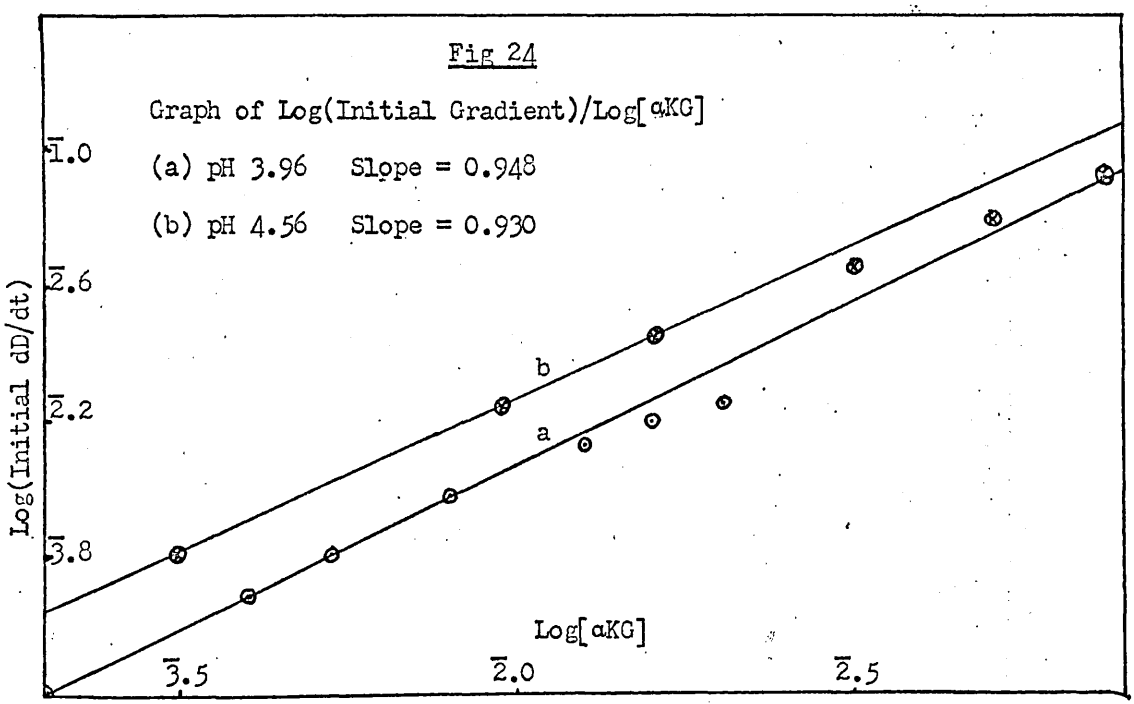
The pH was varied from 3.2 to 5.2 using acetate buffer, and from 5.0 to 12.5 using the reactants and sodium hydroxide as buffer.

Typical plots of dD/dt against D are shown in Fig 22, the intercepts of which gave the initial reaction gradients. These were plotted against pH in Fig 23.

A rate maximum was observed at about pH 5, followed by a further increase above pH 10.5.

(b) The effect of variation of the concentration of αKG on the initial reaction rate.

The concentrations of pyridoxamine phosphate and Cu(11) were maintained at 0.3 mM and 0.6 mM respectively, and the concentration of αKG was varied between 2.0 and 20.8 mM at pH 3.96, and between 3.2

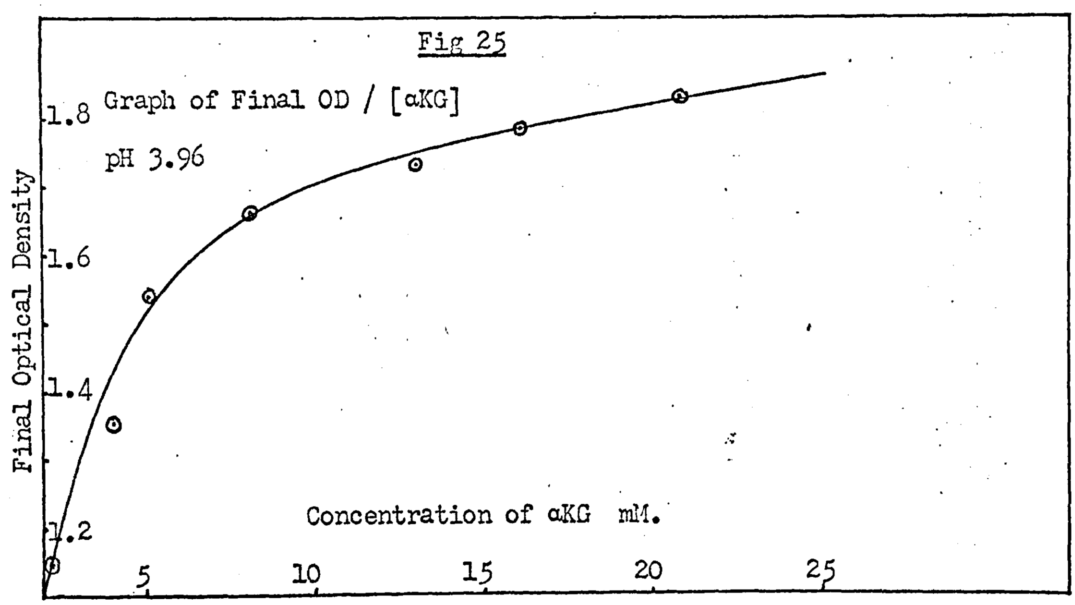


and 105.6 mM at pH 4.56. (In the former case the pH of the αKG solution had not been adjusted to that of the buffer and so in some reaction mixtures the pH was found to have changed. In these cases the initial gradient was corrected to pH 3.96 using Fig 23).

A graph of Log(Initial gradient)/Log[αKG] was plotted at each pH (Fig 24).

The slopes of these graphs were 0.95 at pH 3.96, and 0.93 at pH 4.56. The points at higher concentrations can be seen to fall increasingly short of the straight line relationship for those at low concentrations of αKG. The significance of this is discussed later.

The final optical density of the reaction mixture was very dependent on the concentration of α-ketoglutarate indicating that an equilibrium is involved. A graph of final optical density/[αKG] is shown in Fig 25 for the runs at pH 3.96. The points are experimental and the curve is theoretical for an equation of the type:

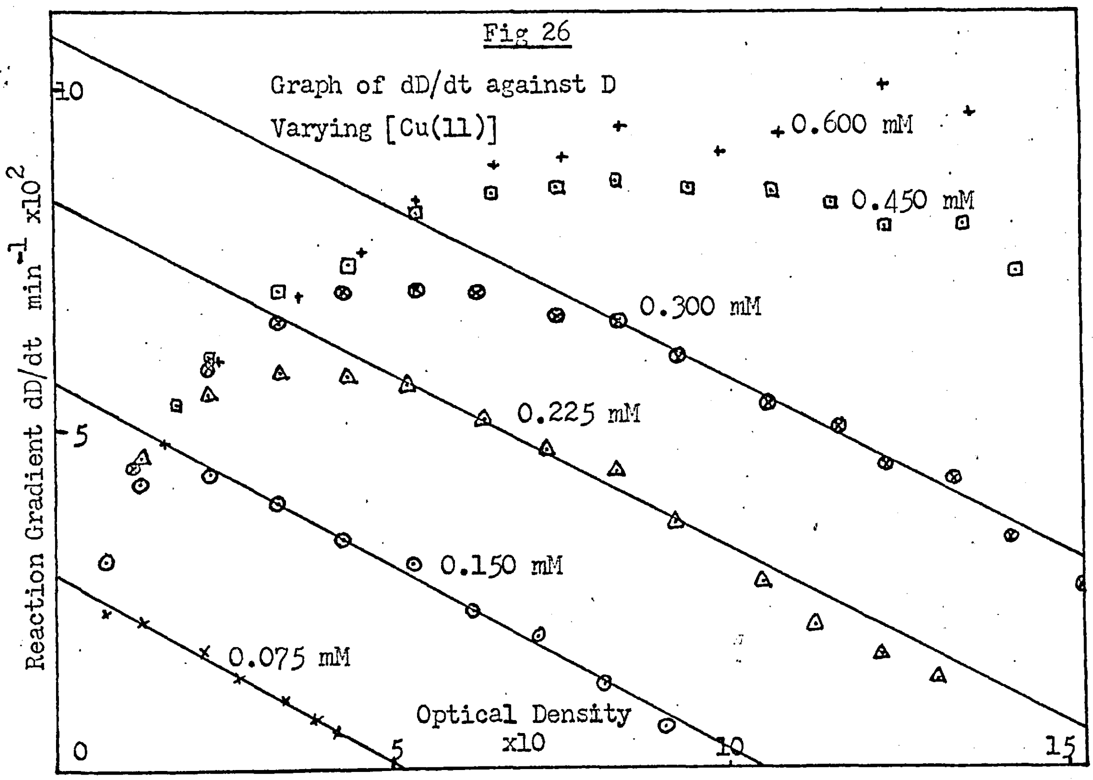


$$PamP + \alpha KG \overset{K}{\rightleftharpoons} Products$$

if the value of K is taken as 710 M^{-1}. The influence of the metal ion is ignored.

(c) The effect of variation of the concentration of Cu(11).

It was difficult at first to find experimental concentrations for pyridoxamine phosphate and α-ketoglutarate which gave a minimum of interference from the 'induction period' over a wide range of Cu(11) concentrations. The effect on the graphs of dD/dt against D of too high a concentration of pyridoxamine phosphate (3.0 mM.) is shown in Fig. 26. The concentration of α-ketoglutarate was 8.0 mM. and the pH 4.45.



At higher concentrations of Cu(11) than 0.3 mM. the induction period became so important that no straight portion of the graph dD/dt against D was observed.

However, satisfactory results were obtained for the two sets of experimental conditions

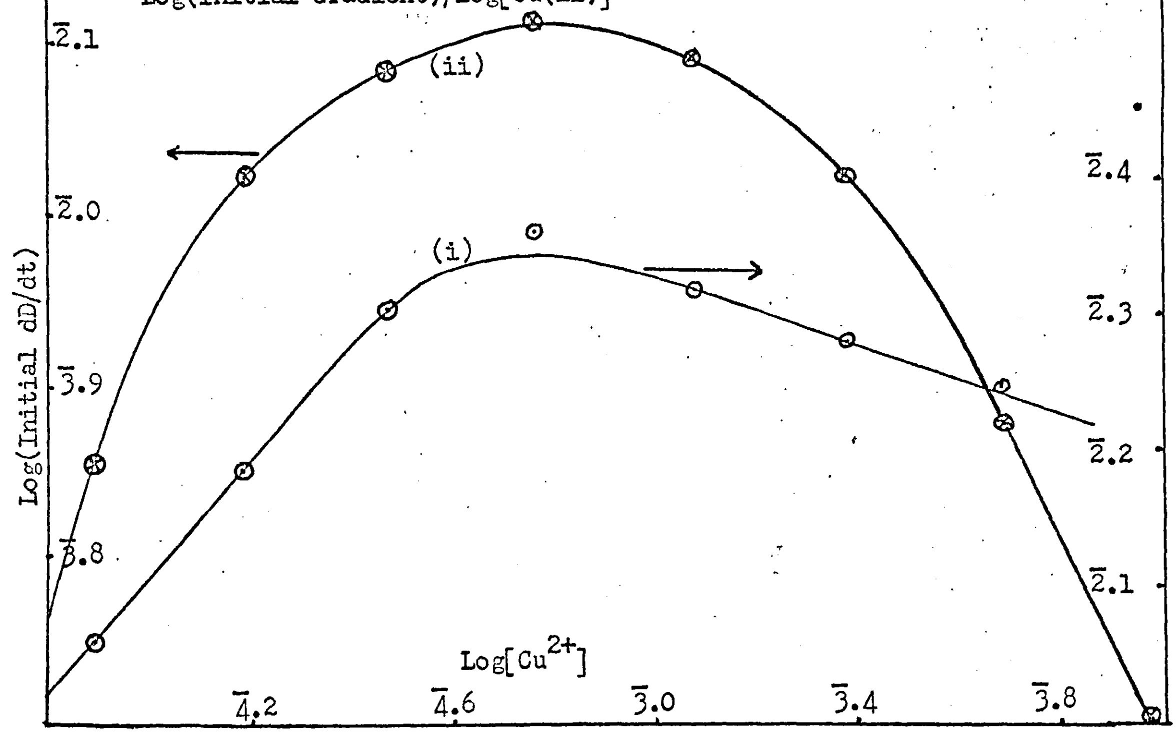
(i) 0.3 mM. PamP and 16mM. αKG at pH 4.22

and (ii) 0.3 mM. PamP and 8 mM. αKG at pH 4.42.

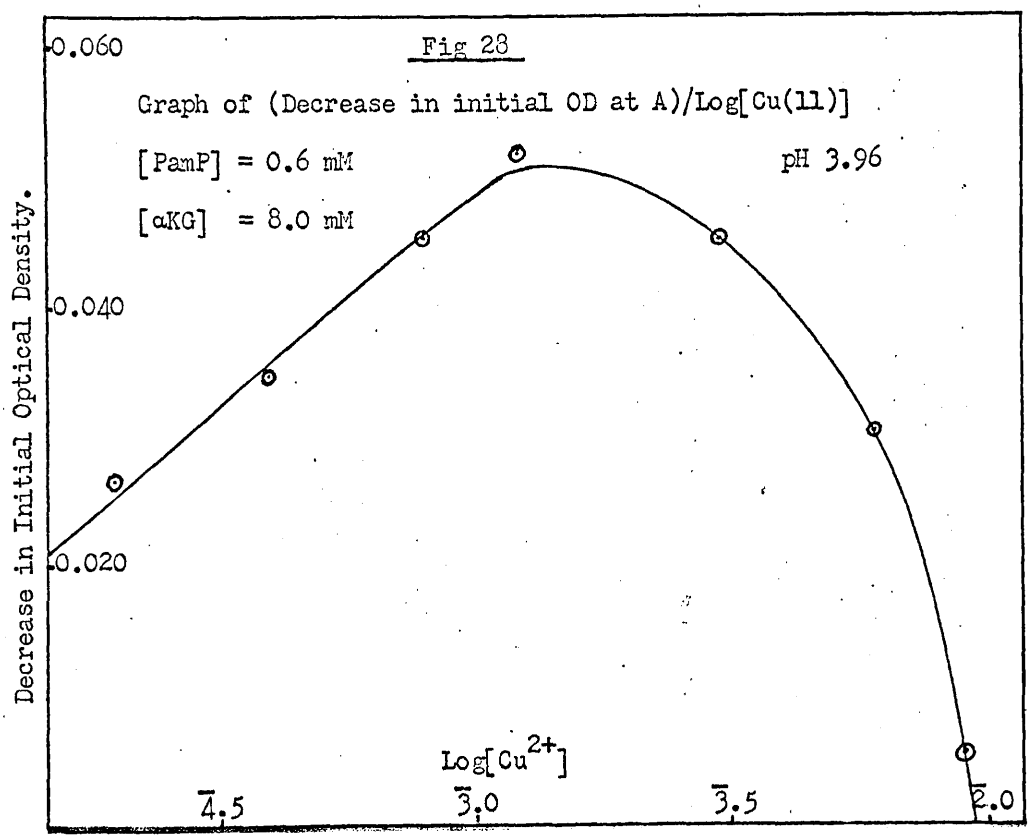
Graphs of Log(Initial gradient)/Log[Cu(11)] are shown in Fig. 27. Maxima occur at Cu(11) concentrations corresponding to a ratio of PamP:Cu(11) of 1:2. This was the ratio at which the runs in sections (a) and (b) were carried out.

Fig 27

Log(Initial Gradient)/Log[Cu(11)]



It was assumed initially that the isosbestic points at A and B in Figs 20a and b were sharp. This was apparently true when the flat-bed recorder of the SP 800 spectrophotometer was used, especially at the low concentrations of pyridoxamine phosphate used when the spectra of Fig 20 were recorded. When, however, isosbestic point A was studied more closely at a single wavelength unsing the scale expansion unit, it was found that the optical density first decreased by a small amount before finally becoming steady. The time taken for this initial decrease corresponded closely to that of the induction period, indicating some relationship between the two. The extent of the decrease depended on the concentration of Cu(11). The relationship is shown in Fig 28.



There is an obvious similarity between Figs 27 and 28. In both cases maxima occur at a ratio of Cu:PamP of 2:1.

It was found that the extent of the decrease was also dependent upon the concentration of α-ketoglutarate. Attempts were made to study the change in absorbance at point A during the 'induction period' as a function of αKG concentration. It was found, however, that changing the concentration of α-ketoglutarate moved the isosbestic point slightly so that there was interference from the transamination step. The quantitative interpretation of these results was therefore prevented.

The measurements at isosbestic point A were carried out as described below.

Experimental

The concentrations of pyridoxamine phosphate and α-ketoglutarate were kept constant at 0.6 mM and 8.0 mM respectively, and the concentration of Cu(11) was varied between 0.2 mM and 9.0 mM.

The isosbestic point was found approximately from Fig 20. A reaction mixture was then placed in the cell compartment and allowed to react to a stage well past the induction period; i.e. to a stage where no further change in optical density with time should occur at A. A change in either direction would indicate that the instrument were not set exactly on A. Furthermore, the direction of this change would indicate on which side of A the instrument was set. The wavelength was then finally adjusted until no change in optical density took place with time.

Results

The results are shown in Table 7. Also recorded are the initial optical densities of the reaction mixtures. These are compared with the calculated initial optical densities found from a set of readings on solutions 0.6 mM pyridoxamine phosphate and various concentrations of Cu(11). To these readings was added the optical density of an 8.0 mM solution of α-ketoglutaric acid.

Table 7

pH = 3.96, OD of 8.0 mM αKG = 0.134

[Cu] $M.10^4$	Initial OD. D_o	Final OD. D	D_o - D	Calc. Init. OD. D_o'	D_o' - D_o
0.0	1.667	1.665	0.002	1.713	0.046
2.0	1.651	1.624	0.027	1.695	0.044
4.0	1.636	1.599	0.037	1.666	0.030
8.0	1.602	1.556	0.046	1.622	0.020
12.0	1.574	1.520	0.054	1.588	0.014
30.0	1.448	1.411	0.037	1.428	-0.020
60.0	1.325	1.301	0.024	1.277	-0.048
90.0	1.249	1.243	0.006	1.170	-0.079

A maximum occurs in D_o - D at a Cu(11):PamP ratio of 2:1 (Fig 28). By comparison with Fig 27 it would seem that this ratio gave the maximum concentration of the reactive species. The concentration of this reactive species must be a function of the equilibrium constant of its formation and of the concentrations of the reactants. Also, this equilibrium constant (and hence the concentration of the reactive species) must be small to account for the observed linear relationship between the reaction rate and the concentration of α-ketoglutarate (Fig 24). The departure from linearity at higher concentrations of αKG supports this view.

The problem is to find out which type of reactive species would be expected to give the observed dependence of the reaction rate on the concentration of Cu(11). If, as has been suggested (see Introduction), the reactive species is the metal chelate of the Schiff's base derived from pyridoxamine phosphate and α-ketoglutarate, CuSB", the following equations should describe the overall reaction:

$$\text{PamP} + \alpha\text{KG} \overset{K_1}{\rightleftharpoons} \text{SB}'' \qquad \text{(i)}$$

$$\text{SB}'' + \text{Cu} \overset{K_2}{\rightleftharpoons} \text{CuSB}'' \qquad \text{(ii)}$$

and

$$\text{CuSB}'' \overset{K_3}{\rightleftharpoons} \text{CuSB}' \qquad \text{(iii)}$$

If these are the only equilibria to be considered, either equation (i) or (ii) must account for the observed behaviour at A preceding the reaction shown in equation (iii).

The absence of any appreciable spectral change before that due to transamination (Fig 20) would indicate either that the species CuSB" were present in low concentrations, or that its spectrum was similar to that of pyridoxamine phosphate.

It is unlikely that the attainment of equilibrium in equation (ii) would be slow enough to be observed. Consequently the changes observed at A cannot be caused by the reaction shown in equation (ii). The rate determining step for the changes observed at the isosbestic point must therefore be that shown in equation (i). This would be followed by the rapid and almost complete reaction (ii) with which the spectral change is probably associated. If the reactive species, CuSB", were present in only low concentrations, as was concluded above, this must be caused by an unfavourable equilibrium constant K_1 in equation (i).

The equilibrium constant K_1 for Schiff's base formation from pyridoxamine phosphate and α-ketoglutarate is unknown. Attempts to evaluate it spectrophotometrically (as in p 141) were unsuccessful because of the small changes in optical density. This indicates again either that K_1 is very low or that the spectra of SB" and pyridoxamine phosphate are similar. The equilibrium constants for Schiff's base formation from pyridoxamine and pyruvate, evaluated by Banks et al. (18), suggest that the former suggestion is correct. The presence of Cu(11) ions in the system would not favour as great a stabilisation of the Schiff's base from PamP and αKG as it did for the Schiff's base from PyP and Glu because of the high stability of complexes of Cu(11) and PamP, one of the reactants (see p 108). It may be expected, therefore, that some optimum concentration of Cu(11) exists at which [CuSB"] has a maximum value.

Defining the equilibrium constants K_1 and K_2 from equations (i) and (ii)

as $$K_1 = [SB'']/[P^{3-}][G^{2-}] \quad \text{(iv)}$$

and $$K_2 = [CuSB'']/[Cu^{2+}][SB''] \quad \text{(v)}$$

or, multiplying together (iv) and (v),

$$K_1K_2 = [CuSB'']/[Cu^{2+}][P^{3-}][G^{2-}] \quad \text{(vi)}$$

Rearranging (vi) gives,

$$[CuSB''] = K_1K_2[Cu^{2+}][G^{2-}][P^{3-}] \qquad \text{(viii)}$$

and differentiating w.r.t the total Cu concentration, C_m,

$$d[CuSB'']/dC_m = K_1K_2[G^{2-}]\left\{[P^{3-}].d[Cu^{2+}]/dC_m + [Cu^{2+}].d[P^{3-}]/dC_m\right\} \qquad \text{(ix)}$$

As αKG was in considerable excess derivatives of $[G^{2-}]$ were taken as zero. For a maximum concentration of CuSB", $d[CuSB'']/dC_m = 0$

$$\text{or } [P^{3-}].d[Cu^{2+}]/dC_m = -[Cu^{2+}].d[P^{3-}]/dC_m \qquad \text{(x)}$$

The successive stability constants of Cu(11) and PamP are given by;

$$k_1 = [CuP^-]/[Cu^{2+}][P^{3-}] \qquad \text{(xi)}$$

$$\text{and} \quad k_2 = [CuP_2^{4-}]/[CuP^-][P^{3-}] \qquad \text{(xi)}$$

Summing the concentrations of all the species,

$$C_p = [CuP^-] + 2[CuP_2^{4-}] + A[P^{3-}] + [CuSB''] \qquad \text{(xii)}$$

where A is the ratio (Total free PamP)/Anionic PamP).
The concentration of free SB" is taken as zero in equation (xii).

$$C_m = [CuP^-] + [CuP_2^{4-}] + [Cu^{2+}] + [CuSB''] \qquad \text{(xiii)}$$

Substituting equations (iv), (v), (vi), (viii) and (xi) in (xii) and (xiii):

$$C_p = k_1Cu.P + 2k_1k_2Cu.P^2 + AP + K_1K_2Cu.P.G$$

$$= P(k_1Cu + 2k_1k_2Cu.P + A + K_1K_2Cu.G) \qquad \text{(xiv)}$$

$$\text{and} \quad C_m = k_1Cu.P + k_1k_2Cu.P^2 + Cu + K_1K_2Cu.P.G$$

$$= Cu(k_1P + k_1k_2P + 1 + K_1K_2P.G) \qquad (xv)$$

where $P \equiv [P^{3-}]$ etc. for simplicity.

Differentiating (xiv) and (xv) w.r.t. C_m gives:

$$0 = (k_1Cu + 2k_1k_2Cu.P^2 + K_1K_2Cu.G + 1).dP/dC_m$$
$$+ P([k_1 + 2k_1k_2P + K_1K_2G].dCu/dC_m + 2k_1k_2Cu.dP/dC_m) \qquad (xvi)$$

$$1 = (k_1P + k_1k_2P^2 + K_1K_2P.G + 1).dCu/dC_m$$
$$+ (k_1 + 2k_1k_2P + K_1K_2G).Cu.dP/dC_m \qquad (xvii)$$

Putting $-P.dCu/dC_m$ for $Cu.dP/dC_m$ (equation x) in (xvii):

$$dCu/dC_m = 1/(1 - k_1k_2P^2) \qquad (xviii)$$

and, from (x)

$$dP/dC_m = -P/(1 - k_1k_2P^2).Cu \qquad (xix)$$

Substituting (xviii) and (xix) into (xvi) gives,

$$A/Cu + 2k_1k_2P = 0$$

This condition cannot be fulfilled for positive concentrations of Cu and P. The physical interpretation of this is that the concentration of the species CuSB" cannot have a maximum value at finite reactant concentrations if the only equilibria involved are those shown in equations (i) to (iii). This view is supported by the figures in column 6 of Table 7 which show the differences between the theoretical and experimental optical densities at A immediately upon mixing the reactants. These results would indicate the presence of another species, the concentration of which increased with that of Cu(11) throughout the experimental range.

The mathematical treatment above indicates that at least one other equilibrium must exist in the reaction mixtures for it to be possible for [CuSB"] to have a maximum value. The two most probable equilibria are (a) $CuSB'' + SB'' \rightleftharpoons Cu(SB'')_2$

and (b) $CuSB'' + Cu^{2+} \rightleftharpoons Cu_2SB''$

Of these, suggestion (a) seems the most probable in view of the number of species of the type $M(SB)_2$ reported in the literature (see Introduction) and the absence of any reports of species of the type M_2SB. Mathematical treatment of suggestion (a) shows that the addition of this extra equilibrium does indeed cause the term [CuSB"] to go through a maximum value, but at an expected ratio Cu(11):PamP of 1:2 and not 2:1 as required.

Similar treatment of suggestion (b) is rather more complex unless several assumptions are made. These are,

(i) that a negligible amount of Cu-PamP co-ordination takes place at the pH of the experiment (3.96);

and (ii) that the concentrations of CuSB" and Cu_2SB'' are small compared with the total concentrations of Cu(11) and PamP.

The first of these assumptions is justifiable by inspection of the stability constant results of p 121. These show that at pH 3.96 the average number of pyridoxamine phosphate ligands associated to each copper ion is only ca. 0.1. Assumption (ii) is merely a restatement of what was said earlier (p 74).

Then, introducing K_4:

where $K_4 = [Cu_2SB'']/Cu][CuSB'']$

the total concentrations of Cu(11) and PamP become

$$C_m = Cu.(1 + K_1K_2P.G + 2K_1K_2K_4Cu.P\ G) \qquad \text{(xx)}$$

and

$$C_p = P.(A + K_1K_2Cu.G + K_1K_2K_4Cu^2G) \qquad \text{(xxi)}$$

Differentiating (xx) w.r.t. C_m gives,

$$0 = (K_1K_2Cu.G + K_1K_2K_4Cu^2G + A).dP/dC_m + (K_1K_2G + 2K_1K_2K_4Cu.G).P.dCu/dC_m \qquad \text{(xxii)}$$

Substituting equation (x) into equation (xxii) and simplifying gives,

$$A - K_1K_2K_4Cu.G = 0$$

or

$$Cu = A/K_1K_2K_4G \qquad \text{(xxiii)}$$

Substituting equation (xxiii) into (xx) and (xxi), and dividing equation (xx) by (xxi):

$$\frac{C_m}{C_p} = \frac{Cu + A.P/K_4 + 2Cu.A.P}{A.P + A.P/K_4 + Cu.A.P} \qquad \text{(xxiv)}$$

The desired ratio of C_m/C_p is 2, therefore this in equation (xxiv) and simplifying gives,

$$Cu = A.P.(2 + 1/K_4)$$

If K_4 is sufficiently large, this becomes

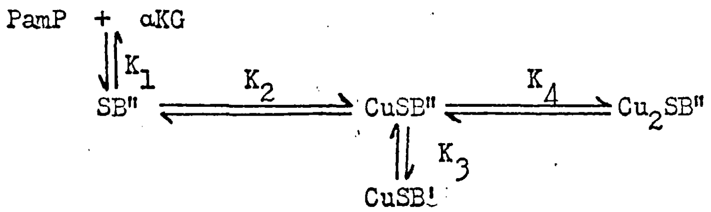
$$Cu = 2A.P \qquad \text{(xxv)}$$

Now A.P is the total concentration of free PamP, therefore if the concentration of free copper ions (Cu in the above derivation) is comparable to the total concentration of copper, C_m, (see assumption ii above) then equation (xxv) becomes

$$C_m = 2C_p$$

This means that, if the above assumptions hold, the concentration of the species CuSB" has a maximum value at a ratio of $C_m/C_p = 2$. It also means that the concentrations of the proposed complexes are small compared with the concentrations of the other reactants. (This is presumably only true at low pH values).

The proposed mechanism is shown diagrammatically below.



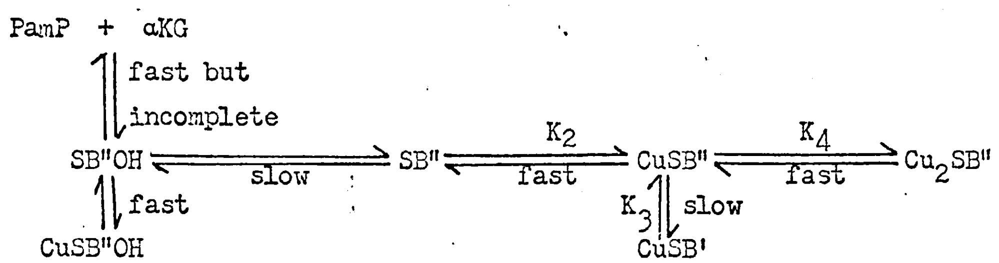
K_1 is assumed to be very small and K_2 and K_4 are assumed to be very large. Then the mechanism implies that the steps

$$\text{PamP} + \alpha\text{KG} \rightleftharpoons \text{SB}'' \rightleftharpoons \text{CuSB}''$$

are responsible for the decrease in optical density observed at the isosbestic point A.

However, these equilibria alone do not account for all the experimental observations. Column 6 in Table 7 exhibits a steady change with increasing Cu(11) concentration which cannot be caused by the species Cu_2SB'' postulated here as the values of $D_o' - D_o$ are those for the reaction mixture immediately upon mixing, whereas the complexes CuSB" and Cu_2SB'' are not formed until after the slow SB" formation step. This phenomenon is very similar to that found in the PyP/Glu system (p 37-42) where carbinolamine complexes were proposed. Such complexes could possibly exist in the present system without the need for drastic alteration of the mechanism already put forward. These carbinolamine complexes could not themselves be the reactive species as column 6 (Table 7) exhibits no maximum or minimum to parallel the maximum which appears in column 4.

The revised mechanism is then:-



Further Reaction of Pyridoxamine Phosphate and α-Ketoglutaric Acid in the Presence of Cu(11).

The products of the transamination reaction of pyridoxamine phosphate and α-ketoglutarate with Cu(11) were found to react further to give the species with a spectrum 3 in Fig 20 (p 60). This reaction took 1-2 days for completion.

Examination of Fig 21 (p 63) shows that the species only appears in the system containing Cu(11) and not with the other metals used. Its absence from reaction mixtures of pyridoxal phosphate, glutamate and Cu(11) even after a period of 1 week (after which time the same species should be present as in a reaction mixture of pyridoxamine phosphate, α-ketoglutarate and Cu(11)) would indicate that a large excess of α-ketoglutarate is necessary for its formation.

A series of single wavelength runs on the disappearance of the peak at 25,500 cm^{-1} was carried out to investigate the dependence of the rate of disappearance on:

(a) the pH

(b) the concentration of Cu(11)

and (c) the concentration of αKG

First order plots of logD/Time were found to give straight lines indicating a first order (or pseudo first order) reaction.

Experimental

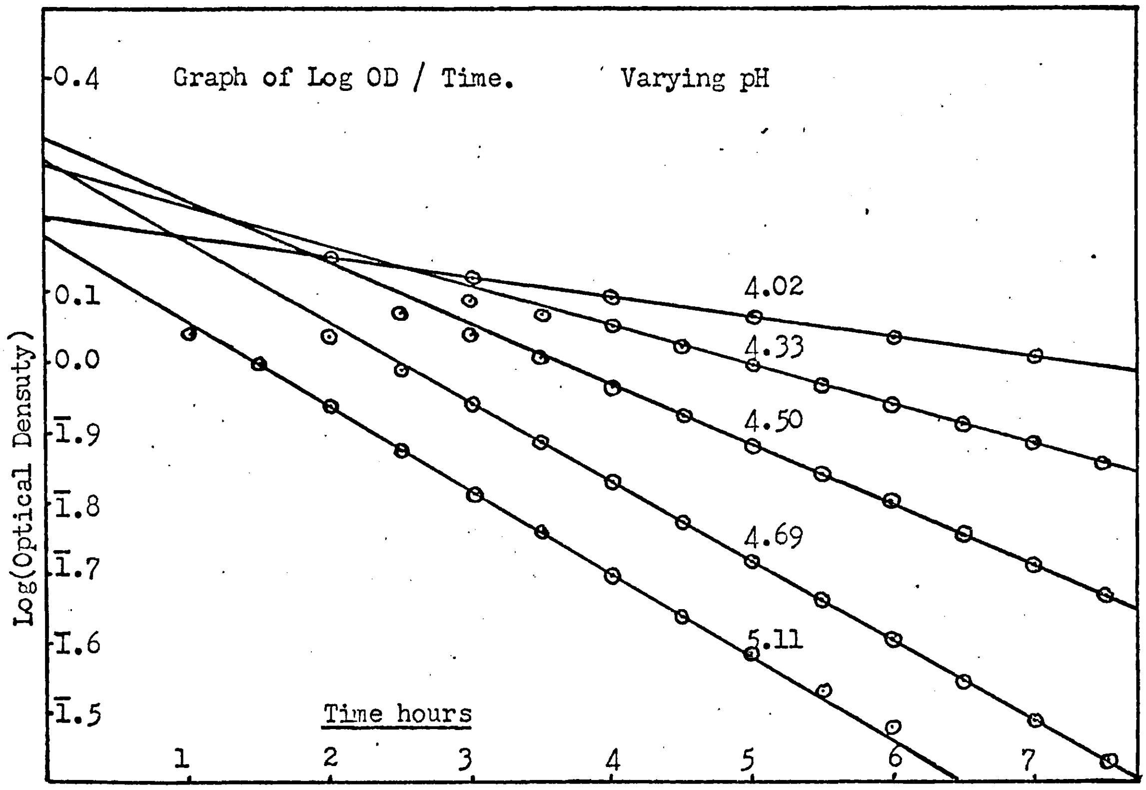
Reaction mixtures (similar to those described in p 65) were made up and allowed to react to the point corresponding to spectrum 2 (Fig 20) before readings were taken. The SP 800 spectrophotometer was then set to take optical density readings every 15 minutes for a period of about 12 hours.

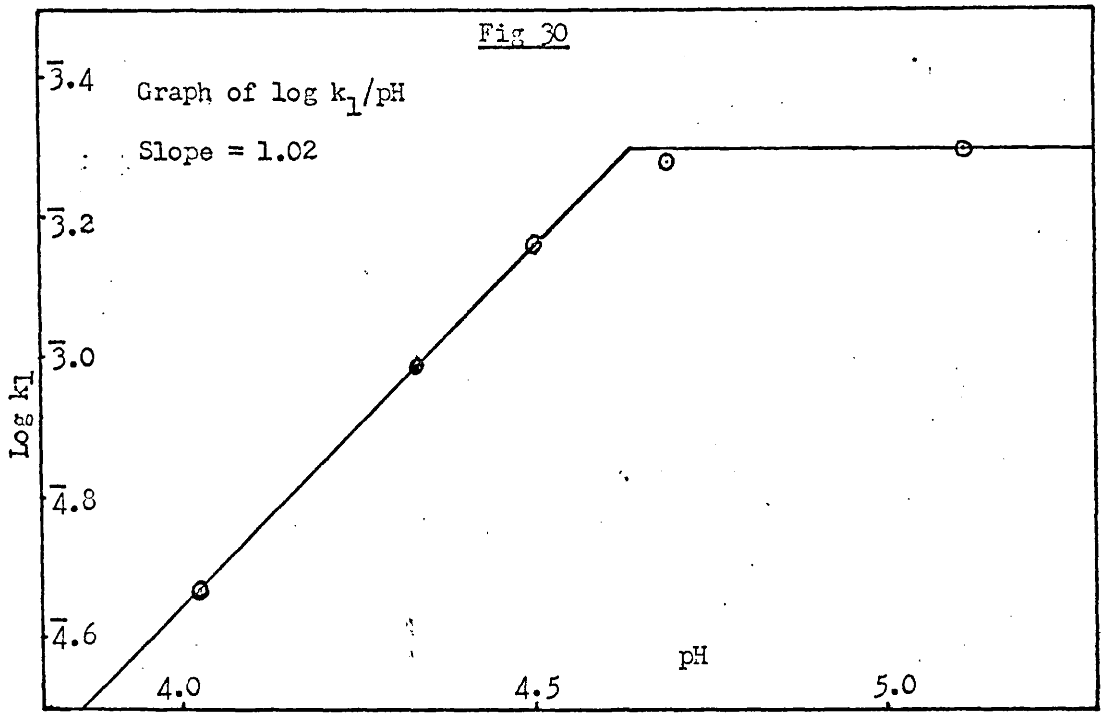
(a) The effect on the first order rate constant of variation of the pH

The concentrations of the reactants were 16.0 mM in αKG, 0.3 mM in pyridoxamine phosphate and 0.6 mM in Cu(11). The pH was varied between 4.0 and 5.1.

First order plots are shown in Fig 29 and a graph of log k_1/pH is shown in Fig 30. The gradient of unity for log k_1/pH below pH 5.6 indicates that the reaction is base catalysed.

Fig 29





(b) The effect on k_1 of variation of the concentration of Cu(11)

The concentrations of the other reactants were 0.3 mM and 16.0 mM in pyridoxamine phosphate and α-ketoglutarate respectively. The runs were carried out at pH 4.02 and pH 4.50. The concentration of Cu(11) was varied between 0.36 and 0.60 mM. at pH 4.02 and between 0.36 and 1.5 mM. at pH 4.50.

First order plots are shown in Figs 31 and 32. Graphs of log k_1 / log$[Cu^{2+}]$ are shown in Fig 33.

Fig 31

Graph of Log OD / Time. Varying [Cu] mM

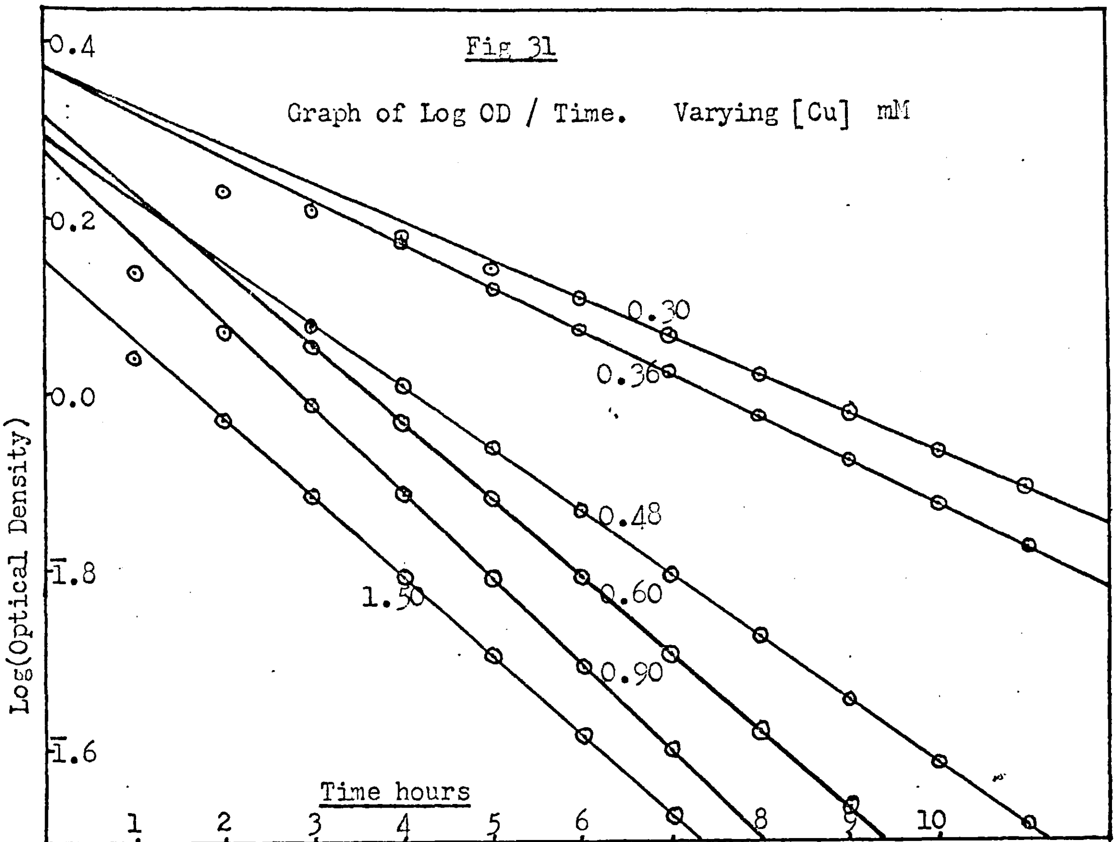
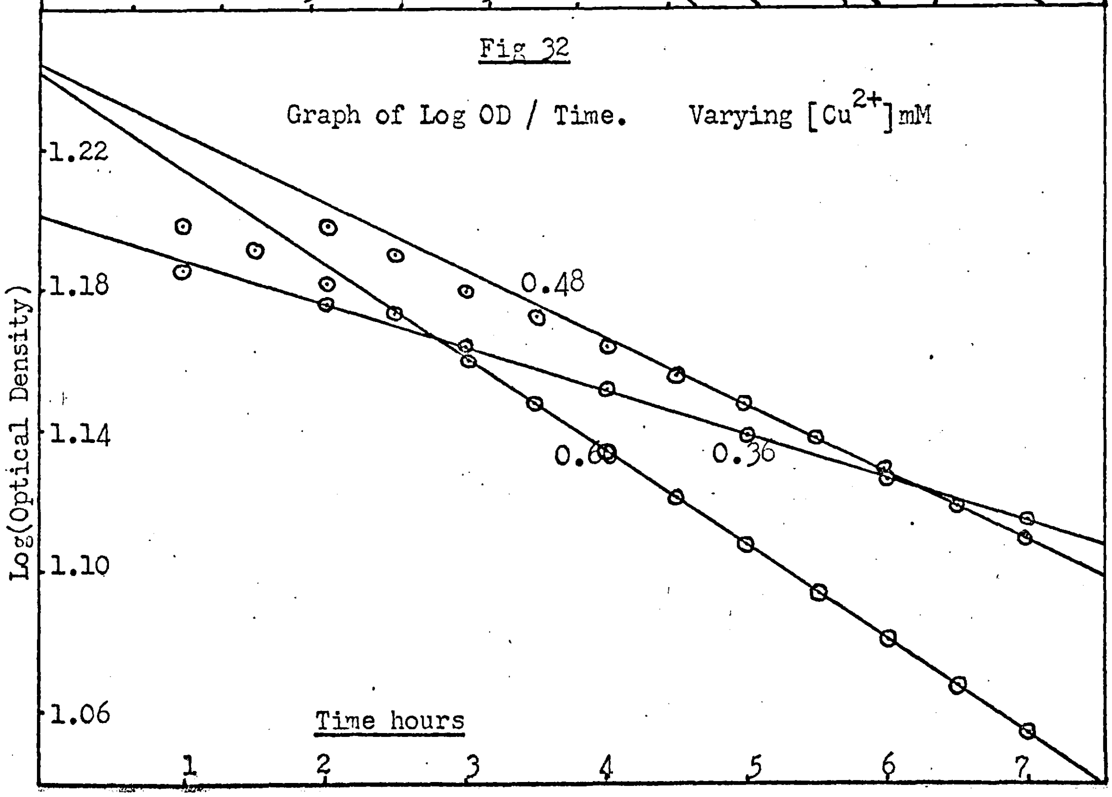
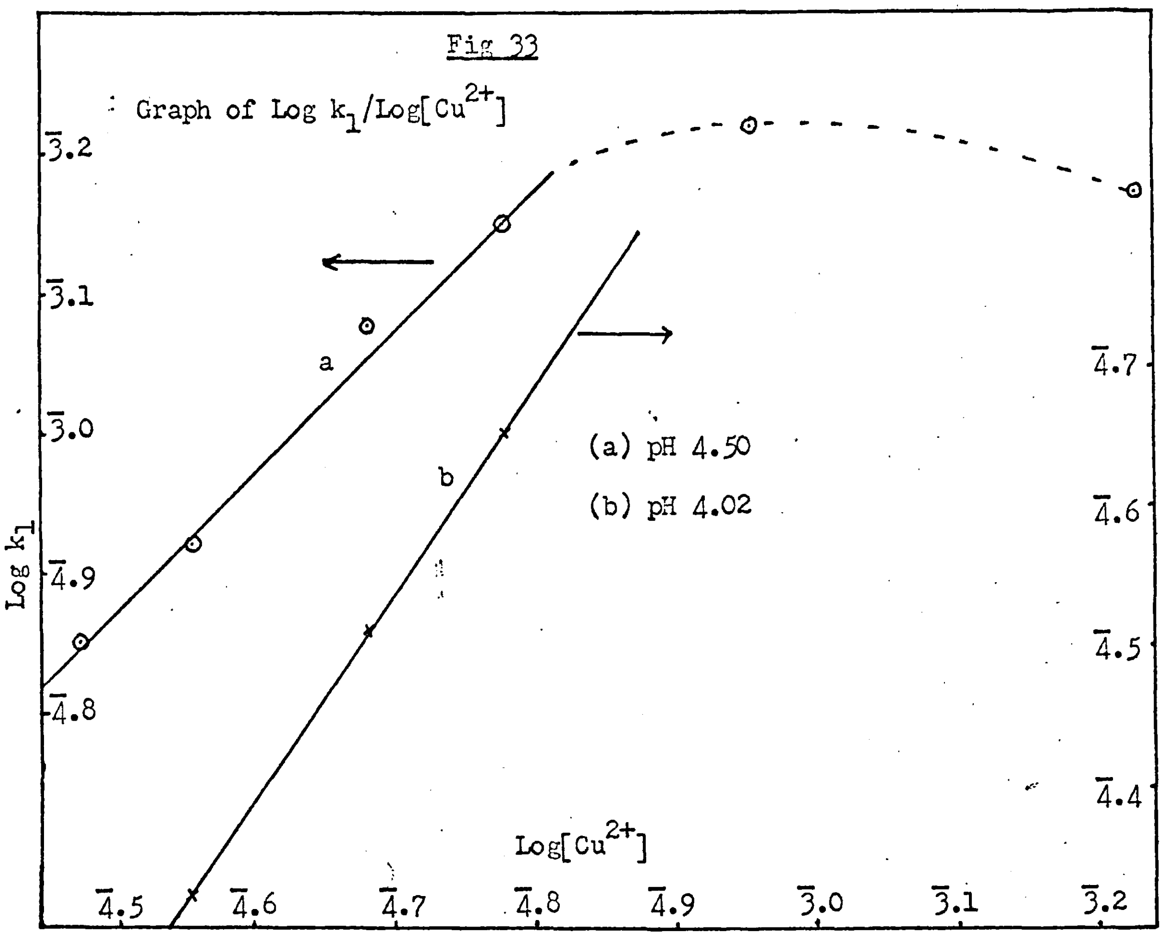


Fig 32

Graph of Log OD / Time. Varying [Cu^{2+}] mM





(c) The effect on k_1 of variation of the concentration of αKG

The concentrations of pyridoxamine phosphate and Cu(11) were 0.3 and 0.6 mM respectively and the pH was 4.56. The concentration of αKG was varied between 3.2 and 105.6 mM.

First order plots are shown in Fig 34 and a graph of log k_1/log[αKG] is shown in Fig 35.

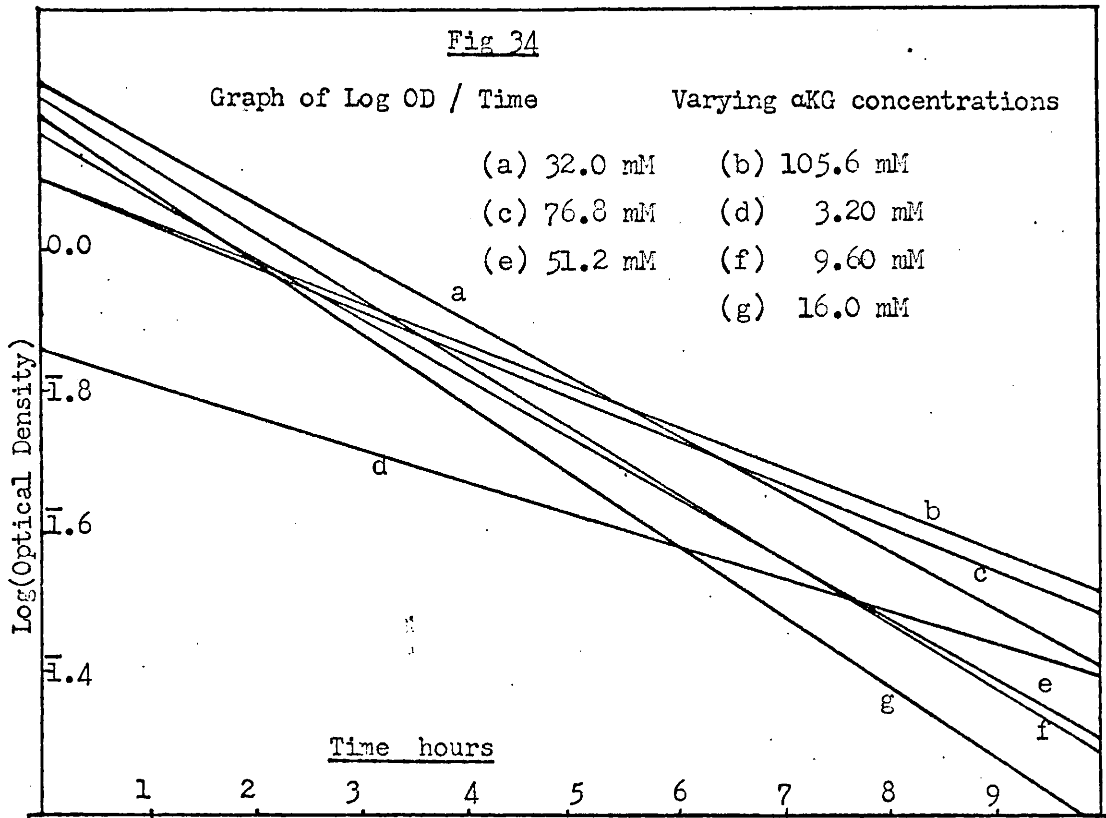


Fig 34
Graph of Log OD / Time
Varying αKG concentrations
(a) 32.0 mM
(b) 105.6 mM
(c) 76.8 mM
(d) 3.20 mM
(e) 51.2 mM
(f) 9.60 mM
(g) 16.0 mM
Log(Optical Density)
0.0
1̄.8
1̄.6
1̄.4
a
b
c
d
e
f
g
Time hours
1
2
3
4
5
6
7
8
9

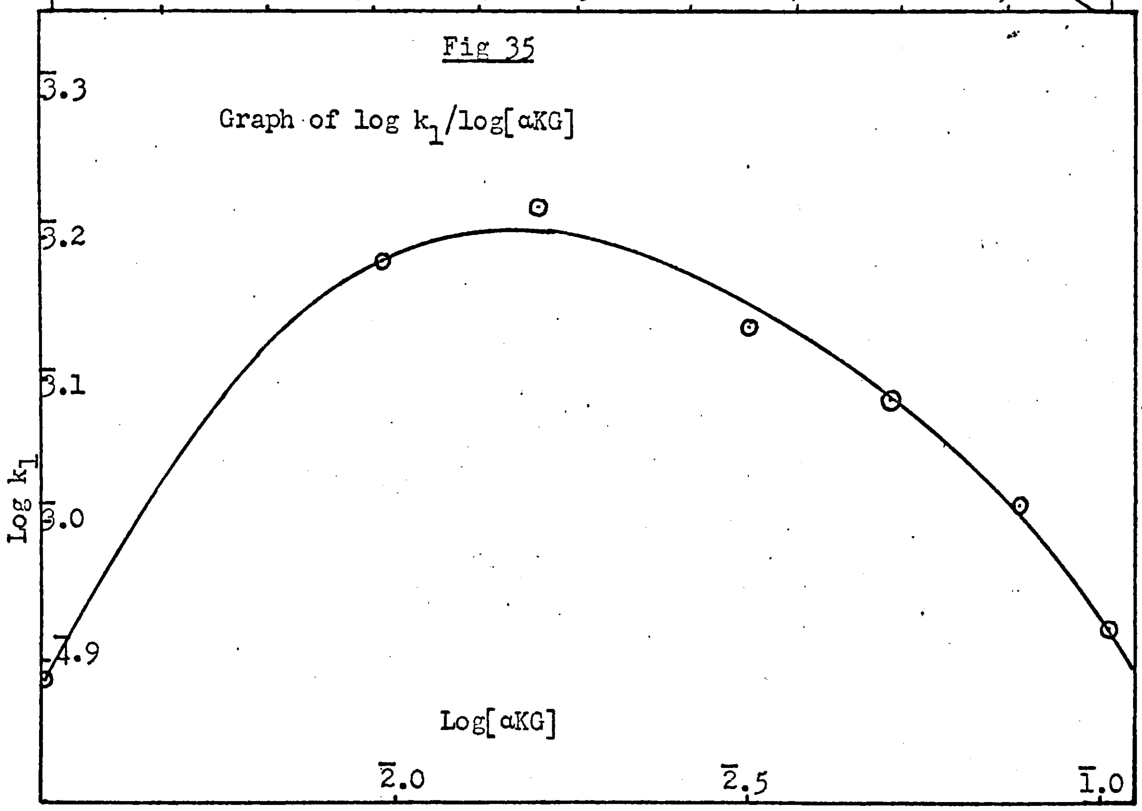


Fig 35
Graph of log k1/log[αKG]
Log k1
3̄.3
3̄.2
3̄.1
3̄.0
4̄.9
Log[αKG]
2̄.0
2̄.5
1̄.0

Discussion.

The species responsible for the spectrum 3 (Fig. 20) is as yet unidentified. The absence of any similar spectrum when any one of the reactants was omitted from the reaction mixture indicates that all three are necessary -Cu(11), pyridoxamine phosphate and α-ketoglutarate. The dependence of the rate of formation of the species on the concentration of Cu(11) also indicates that the copper acts as a catalyst. If so, Cu(11) is very much more active than any other metal ions used as no spectrum comparable to 3 appeared when Ni(11), Co(11) andZn(11) were present.

If the species is a reaction product from some further reaction of CuSB', an excess of αKG must be present as is shown by the fact that CuSB' formed directly from pyridoxal phosphate, glutamate and Cu(11) does not give a comparable spectrum even after 7 days. The suggestion is that the complex CuSB' reacts with αKG by some base catalysed mechanism (Fig. 30) to give the unidentified species.

The reaction is considered incidental to the transamination reactions being studied and was not pursued further.

Part 11.

The Stability Constants of the Complexes of Pyridoxal Phosphate, Pyridoxamine Phosphate, α-ketoglutarate and Schiff's Bases with Several Metals.

Transamination has been shown to proceed via the metal chelates of the respective Schiff's bases (see Introduction). Many of these chelates have been prepared as solids and their empirical formulae determined (25), but this does not necessarily mean that the species of these formulae predominate in aqueous solution, nor that they are the reactive species which undergo transamination. Stability measurements on the various possible complexes in aqueous solution are therefore desirable in order to try to discover some parallel between the kinetic behaviour of reaction mixtures and the concentrations of the various species in them.

A potentiometric titration technique was employed to measure the stability constants not recorder in the literature of several complexes of pyridoxal and pyridoxamine phosphates and α-ketoglutaric acid. These were needed during calculations to find the stabilities of their respective Schiff's base chelates, (p 145).

It was first necessary to determine the pK values of the various ligands. These determinations were also carried out by means of potentiometric titration the theory of which (below - adapted from that of Bjerrum (43)) is equally applicable to both pK and stability constant determinations.

Where reference is made to pK values or stability constants it is understood that these are apparent values for ionic strength 0.1. The activities of the various species are assumed to be identical to their concentrations except for H^+ to which a correction was made where necessary, (see p 97).

The only distinction is that stability constants are association constants whereas the corresponding constants for acids are dissociation constants; (i.e. each is the reciprocal of the other).

Theory

During the titration of a solution of pyridoxal phosphate (the theory can be extended to acids of any basicity), the average number of protons associated to the ligand is, according to Bjerrum (43)

$$\bar{n}_a = \frac{\text{Total no. of replaceable protons} - OH^- \text{ added} - \text{dissociated } H^+}{\text{Total molar ligand}}$$

or

$$\bar{n}_a = \frac{C_h - m - [H^+]}{C_a} \qquad \text{(i)}$$

For abbreviations see Appendix II.

Taking into account the dilution caused by adding v ml. of C_{oh} molar alkali to 150 ml of solution, (i) becomes,

$$\bar{n}_a = \frac{150C_h - (v - v')C_{oh} - (150 + v)[H^+]}{150C_a} \qquad \text{(ii)}$$

where v' is the volume of titrant required to give the same pH in the absence of ligand or added acid. (v' is only of importance above pH 8, becoming very important above pH 10). It was found from a graph of pH/v for a blank titration on the solvent alone.

$[H^+]$ was calculated at each pH from the relationship:

$$[H^+] = a^{0.98} \qquad \text{(see p 101)}$$

$\bar{n}_a$ was calculated at each pH from equation (ii) and plotted against pH (Fig 38).

The average number of protons associated to the ligand anion can also be expressed as:

$$\bar{n}_a = \frac{[HA^{2-}] + 2[H_2A^-] + 3[H_3A^o] + 4[H_4A^+]}{[A^{3-}] + [HA^{2-}] + [H_2A^-] + [H_3A^o] + [H_4A^+]}$$

$$= \frac{\frac{[H^+]}{k_1} + \frac{2[H^+]^2}{k_1k_2} + \frac{3[H^+]^3}{k_1k_2k_3} + \frac{4[H^+]^4}{k_1k_2k_3k_4}}{1 + \frac{[H^+]}{k_1} + \frac{[H^+]^2}{k_1k_2} + \frac{[H^+]^3}{k_1k_2k_3} + \frac{[H^+]^4}{k_1k_2k_3k_4}} \qquad \text{(iii)}$$

Where $k_1 = [H^+][A^{3-}]/[HA^{2-}]$

$k_2 = [H^+][HA^{2-}]/[H_2A^-]$

$k_3 = [H^+][H_2A^-]/[H_3A^o]$

and $k_4 = [H^+][H_3A^o]/[H_4A^+]$

If the difference between any two successive dissociation constants were caused by purely statistical effects, their ratio would be:

$$\frac{k_{n+1}}{k_n} = \frac{(n + 1)(N - n + 1)}{n(N - n)}$$

N is the total number of dissociable protons.

To account for the deviations from this due to electrostatic, saturation and steric effects etc., Bjerrum introduced a spreading factor x such that,

$$\frac{k_{n+1}}{k_n} = \frac{(n+1)(N-n+1)}{n(N-n)} x^2$$

Thus in the present case

$$k_2/k_1 = 8x^2/3$$

$$k_3/k_2 = 9x^2/4 \qquad \text{(iv)}$$

and $$k_4/k_3 = 8x^2/3$$

Defining an 'average' constant k as

$$k = (k_1k_2k_3k_4)^{\frac{1}{4}}$$

and substituting into equations (iv) gives

$$k_2^{\frac{1}{2}} = (8x^2/3)^{\frac{1}{4}}k/(k_3k_4)^{\frac{1}{4}} \qquad \text{(v)}$$

$$k_1^{\frac{1}{2}} = k/(8k_3k_4x^2/3)^{\frac{1}{4}} \qquad \text{(vi)}$$

$$k_3^{\frac{1}{2}} = (9x^2/4)^{\frac{1}{4}}k/(k_1k_4)^{\frac{1}{4}} \qquad \text{(vii)}$$

$$k_2^{\frac{1}{2}} = k/(9x^2k_1k_4/4)^{\frac{1}{4}} \qquad \text{(viii)}$$

$$k_4^{\frac{1}{2}} = (8x^2/3)^{\frac{1}{4}}k/(k_1k_2)^{\frac{1}{4}} \qquad \text{(ix)}$$

$$k_3^{\frac{1}{2}} = k/(8k_1k_2x^2/3)^{\frac{1}{4}} \qquad \text{(x)}$$

Dividing (v) by (viii) $\quad k_3 = 6x^4k_1 \quad$ (xi)

" (x) (vii) $\quad k_4 = 6x^4k_2 \quad$ (xii)

" (vi) (ix) $\quad k_4 = 16x^6k_1 \quad$ (xiii)

Substituting (xi), (xii) and (xiii) into (v) and (vi)

$$k_2 = 2k/3x \qquad \text{(xiv)}$$

and $$k_1 = k/4x^3 \qquad \text{(xv)}$$

From (xiii) and (xiv), $k_4 = 4x^3k$ (xvi)

(xi) (xv), $k_3 = 3kx/2$ (xvii)

Substituting the values from (xiv) to (xvii) into (iii):

$$\bar{n}_a = \frac{4k^3x^3[H^+] + 12k^2x^4[H^+]^2 + 12kx^3[H^+]^3 + 4[H^+]^4}{k^4 + 4k^3x^3[H^+] + 6k^2x^4[H^+]^2 + 4kx^3[H^+]^3 + [H^+]^4} \quad \text{(xviii)}$$

The value required from the experimental $\bar{n}_a$/pH curve is that value of $\bar{n}_a$ at which the pH is the same as pk. i.e. substituting k for $[H^+]$ in (xviii) and simplifying gives $\bar{n}_a = 2$ as a rigorous solution.

Similarly the values of $\bar{n}_a$ at which the pH has the same values as pk_1, pk_2 etc. can be found by substituting equations (xiv) to (xvii) into (xviii) and equating $[H^+]$ with k_1, k_2 etc.

This gives:-

for k_2

$$\bar{n}_a = \frac{81x^6/2 + 18x^4 + 2}{27x^6 + 177x^4/16 + 1}$$

$$\doteq \frac{81/2}{27} \quad \text{for large x}$$

$$= 1.50$$

for k_1

$$\bar{n}_a \doteq \frac{(4x^3)^4 + 12x^4 16x^6 + 48x^6 + 4}{2(4x^3)^4 + 96x^{10} + 16x^6 + 1}$$

$$= 0.50$$

for k_3

$$\bar{n}_a = \frac{32/27 + 40x^2/3 + 4}{16/81x^4 + 32/27 + 16x^2/3 + 1}$$

$$= 2.50$$

and for k_4 $\quad \bar{n}_a = \dfrac{1/16x^6 + 3/4x^2 + 7}{1/(4x^3)^4 + 1/16x^6 + 3/8x^6 + 2}$.

$= 3.50$

Hence approximate values of k_1, k_2 etc. (depending upon the magnitude of x; i.e. the spread of the pk values) were determined from the experimental $\bar{n}_a$/pH curve at $\bar{n}_a$ values of 0.5, 1.5, 2.5 and 3.5 respectively.

The procedure was repeated for pyridoxamine phosphate and α-ketoglutaric acid.

When metal ions are present during the titration, these compete with the hydrogen for the ligand anion. According to Bjerrum (43), the average number of ligand molecules attached to each metal, $\bar{n}$, is given by:-

$$\bar{n} = \frac{[MA^-] + 2[MA_2^{4-}]}{[M^{2+}] + [MA^-] + [MA_2^{4-}]}$$

If the successive stability constants are K_1 and K_2,

$$K_1 = [MA^-]/[M^{2+}][A^{3-}]$$

and

$$K_2 = [MA_2^{4-}]/[MA^-][A^{3-}]$$

$$\bar{n} = \frac{K_1[A^{3-}] + 2K_1K_2[A^{3-}]^2}{1 + K_1[A^{3-}] + K_1K_2[A^{3-}]^2} \qquad \text{(xix)}$$

The values of K_1 and K_2 are determined experimentally by measuring the pH of the protons displaced by the metal ion. Then the average number of protons associated to each ligand anion is given by:-

$$\bar{n}_a = \frac{\text{Total bound protons}}{\text{Total free ligand}}$$

$$= \frac{C_h - m - [H^+]}{C_a - \bar{n}C_m}$$

Hence

$$\bar{n} = \frac{C_a\bar{n}_a - C_h + m + [H^+]}{\bar{n}_a C_m}$$

Accounting for dilution as before,

$$= \frac{150C_a\bar{n}_a - 150C_h + (v - v')C_{oh} + (150 + v)[H^+]}{150\bar{n}_a C_m}$$

$\bar{n}_a$ was read from the previous graphs of $\bar{n}_a$/pH and hence $\bar{n}$ was found at each experimental pH.

But,

$$\bar{n} = \frac{\text{Total bound ligand}}{\text{Total metal}}$$

$$= \frac{C_a - (A)}{C_m}$$ (A) is the total free ligand

In the case of pyridoxal phosphate this gives;

$$\bar{n} = \frac{C_a - [A^{3-}] - [HA^{2-}] - [H_2A^-] - [H_3A^o] - [H_4A^+]}{C_m}$$

$$\bar{n} = \frac{C_a - \left\{1 + \frac{[H^+]}{k_1} + \frac{[H^+]^2}{k_1k_2} + \frac{[H^+]^3}{k_1k_2k_3} + \frac{[H^+]^4}{k_1k_2k_3k_4}\right\}[A^{3-}]}{C_m}$$

or

$$\frac{1}{[A^{3-}]} = \frac{\left\{1 + \frac{[H^+]}{k_1} + \frac{[H^+]^2}{k_1k_2} + \frac{[H^+]^3}{k_1k_2k_3} + \frac{[H^+]^4}{k_1k_2k_3k_4}\right\}}{C_a - \bar{n}C_m}$$

Values of $p[A^{3-}]$ were calculated for each $\bar{n}$, and graphs of $\bar{n}/p[A^{3-}]$ were drawn for each titration.

By similar reasoning to that above (p 92) it can be shown that approximate values of K_1 and K_2 can be obtained from these graphs at $\bar{n}$ values of 0.5 and 1.5. More accurate values were determined by successive approximations in equation (xix). One iteration was found to be sufficient to give results within experimental error.

In order to carry out the calculations described above it was necessary to know the relationship between the hydrogen ion concentration $[H^+]$ and activity a. This was determined by titration of standard acetic acid with sodium hydroxide.

The acetic acid and water present can be regarded as competing for the added OH^-. If b moles are taken by the acetic acid and d moles by the water, then

$[HA] = C_a - (b + c)$

$[A^-] = b + c$

$[H^+]$ from HA alone $= c$.

Similarly for water $[OH^-] = d + e$

and $[H^+]$ from H_2O alone $= e$

Then total $[H^+] = c + e$

c is the contribution from the dissociation of the acid, and e from that of water.

The equilibrium constants are given by:

$$K = [H^+][A^-]/[HA]$$

and $$k = [OH^-][H^+]$$

Substituting for $[A^-]$, $[HA]$ and $[OH^-]$ gives,

$$K = \frac{(b + c)[H^+]}{C_a - (b + c)}$$

and $$k = (d + e)[H^+]$$

or $$c = KC_a/(K + [H^+]) - b \qquad \text{(xx)}$$

and $$e = k/[H^+] - d \qquad \text{(xxi)}$$

Adding equations (xx) and (xxi),

Total concentration of hydrogen ions

$$[H^+] = k/[H^+] + KC_a/(K + [H^+]) - (d + b) \qquad \text{(xxii)}$$

but $d + b = m$ total moles of OH^- added

Hence from (xxii)

$$m = k/[H^+] + KC_a/(K + [H^+]) - [H^+]$$

If C_b is the concentration of the NaOH added, then taking into account the dilution upon adding m moles of alkali of volume v:-

$$\frac{C_b v}{(150 + v)} = \frac{k}{[H^+]} + \frac{KC_a.150/(150 + v)}{K + [H^+]} - [H^+]$$

or

$$v = \frac{150C_aK[H^+] + 150(Kk - [H^+]^3 - K[H^+]^2 - k[H^+])}{C_b([H^+]^2 + K[H^+]) - (Kk - [H^+]^3 - K[H^+]^2 - k[H^+])}$$

Values of $[H^+]$ were substituted in the above equation to find their corresponding values of v. $p[H^+]$ was plotted against v alongside the experimental curve.

K, the 'concentration' dissociation constant of acetic acid was obtained from a graph of K/ionic strength for each value of v, (Fig 36). Data for Fig 36 was taken from Harned and Owen (45).

Experimental

(i) Determination of the relationship between a and $[H^+]$.

10.0 ml of 1.5 M KCl and 10.0 ml of acetic acid (approximately 0.025 M) were pipetted into a three-necked, round-bottomed flask and the volume made up to 150 ml. The flask was placed in a water thermostat at 25.0°C. Into one neck of the flask was inserted a combined glass and calomel electrode while nitrogen saturated with water vapour at 25°C was passed in through another. The third neck housed a glass stirrer together with the jet of a burette containing standard NaOH. (Standardised by previous potentiometric titration against potassium hydrogen phthalate.)

The pH of the solution was measured on a Pye Dynacap pH meter after each addition of NaOH. The measurements were read to 0.005 pH units and 0.01 ml of titrant.

The concentration of the acetic was determined from the end point of the titration by plotting pH against volume of titrant. The end point was taken as the point of maximum slope.

A similar graph was plotted in the pH range 4 to 6 and compared with the theoretical curve calculated in terms of $-\text{Log}[H^+]$. These are shown in Fig 37.

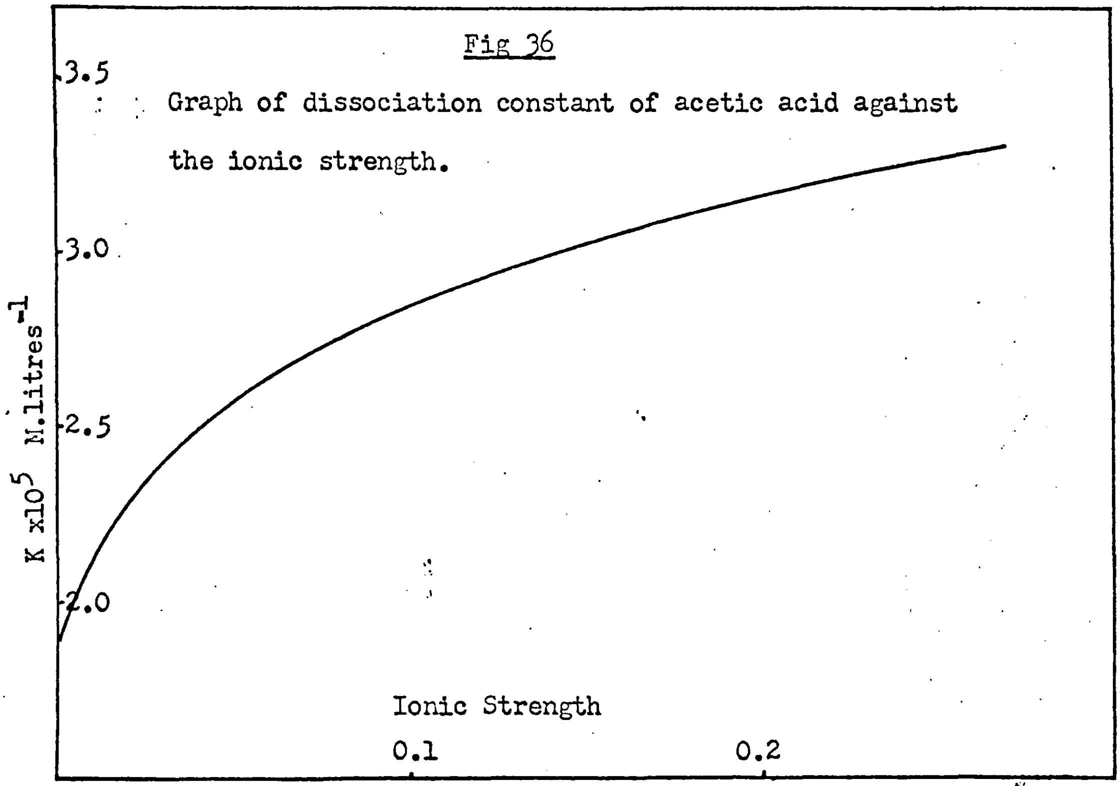


Fig 36
Graph of dissociation constant of acetic acid against the ionic strength.
3.5
3.0
2.5
2.0
K x10^5 M.litres^{-1}
Ionic Strength
0.1
0.2

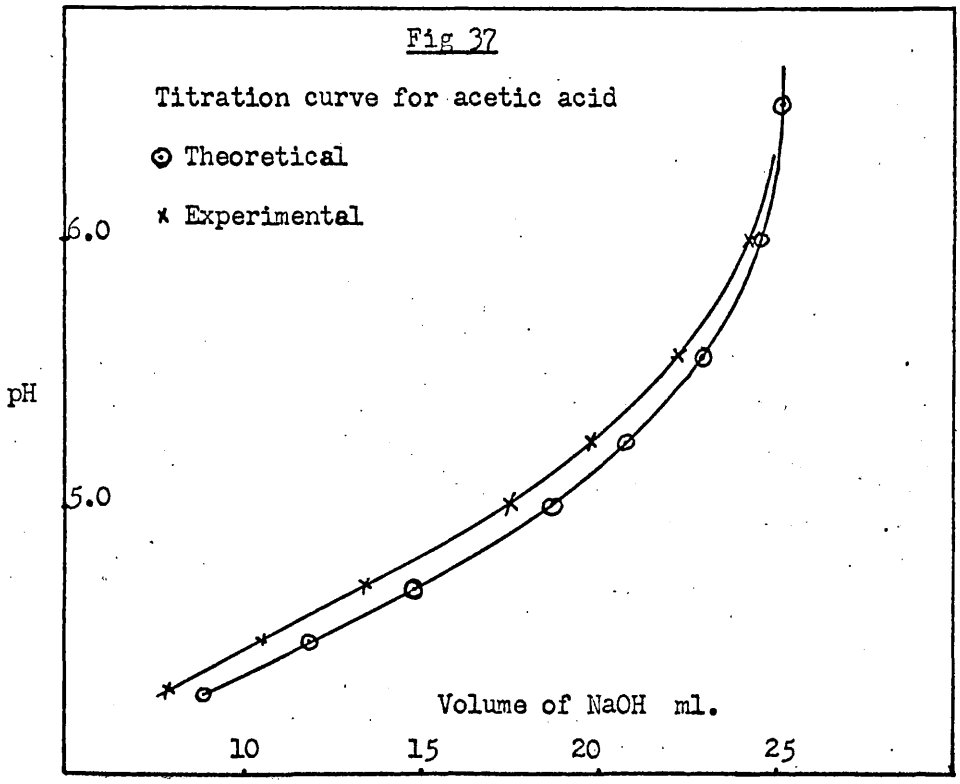


Fig 37
Titration curve for acetic acid
Theoretical
Experimental
6.0
5.0
pH
Volume of NaOH ml.
10
15
20
25

Results

The relationship between the activity and concentration of the hydrogen ion was found by dividing theoretical values from Fig 37 by the experimental ones at several points along the curves. From the mean ratio obtained the following relationship was found to hold:-

$$[H^+] = a^{0.98}$$

(ii) Determination of the pK values of pyridoxal phosphate, pyridoxamine phosphate and α-ketoglutaric acid.

10.0 ml of 1.5 M KCl, 10.0 ml of 0.0100 M PyP and 10.0 ml of standard (0.01974 N) HCl were pipetted into the three-necked flask described above. The volume was made up to 150 ml with CO_2-free (boiled) distilled water and the solution titrated with 0.0200 N KOH. The pH was noted after each addition of titrant.

The experiment was repeated for pyridoxamine phosphate and α-ketoglutaric acid. Graphs of $\bar{n}_a$/pH were plotted (Figs 38-40).

Results

Typical data taken from the titration of pyridoxal phosphate with KOH are shown in Table 8.

Table 8

v	pH	$p[H^+]$	v'	$\bar{n}_a$
0.00	2.800	2.744	-	2.953
10.50	3.615	3.543	-	2.558
15.64	5.685	5.571	-	1.843
19.28	7.260	-	0.12	1.142
22.20	8.535	-	0.17	0.510
23.83	9.480	-	0.35	0.264

The approximate values obtained from the graphs of $\bar{n}_a$/pH were substituted in turn into equation (iii). One iteration was found to be sufficient in most cases to give a constant result. The values obtained for pyridoxal phosphate, α-ketoglutaric acid and pyridoxamine

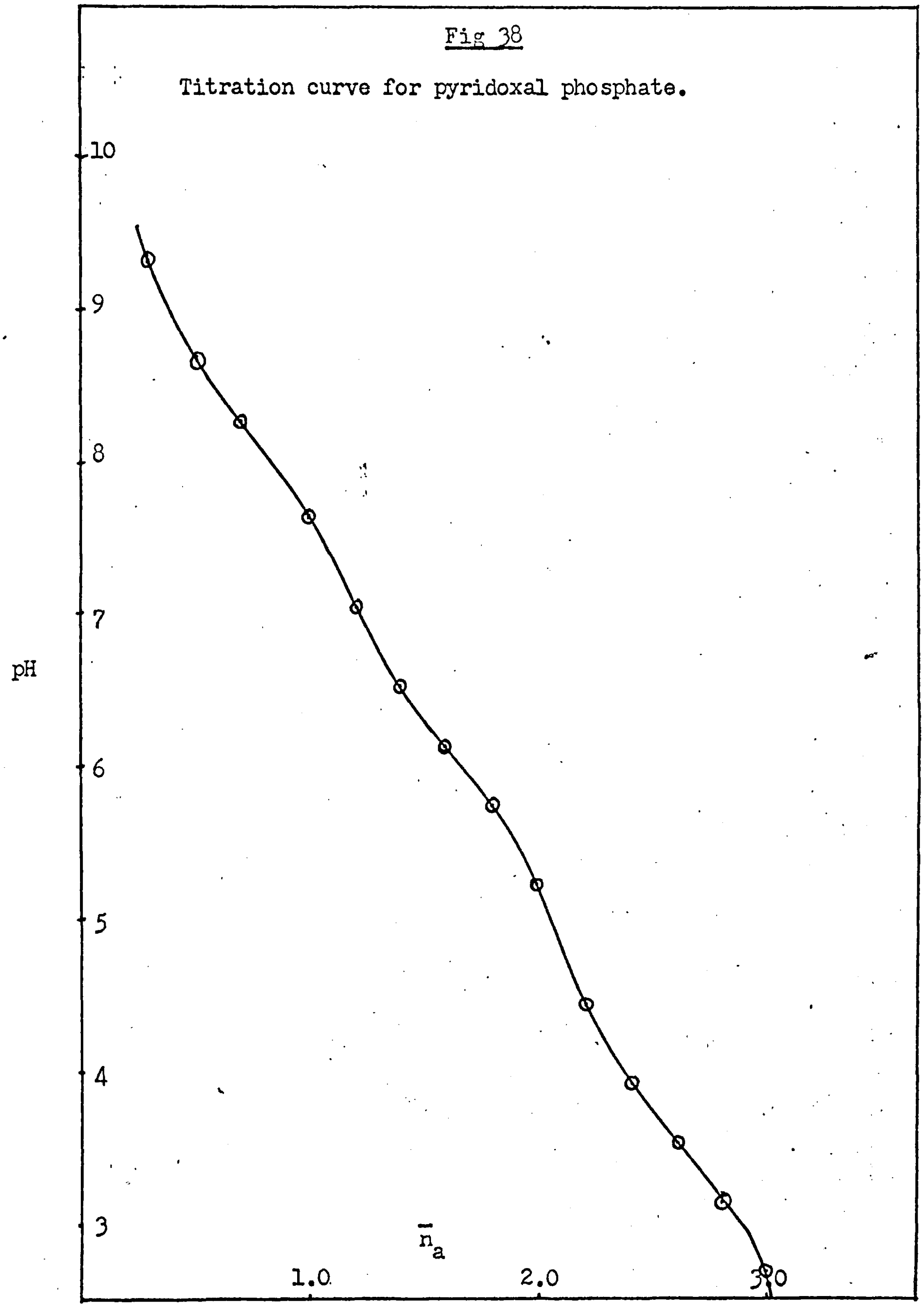


Fig 38
Titration curve for pyridoxal phosphate.
pH
10
9
8
7
6
5
4
3
$\bar{n}_a$
1.0
2.0
3.0

Fig 39

Titration curve for pyridoxamine phosphate

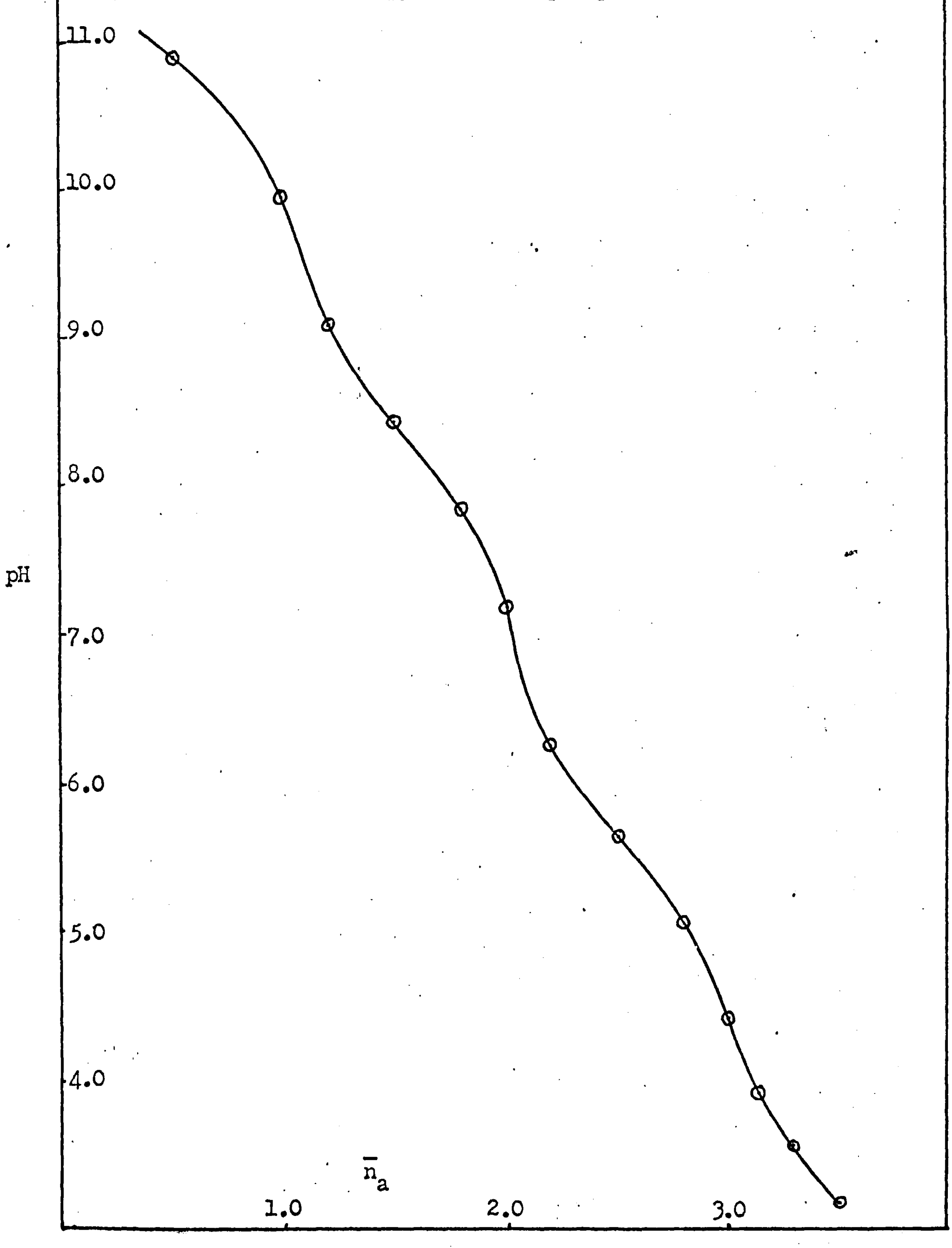
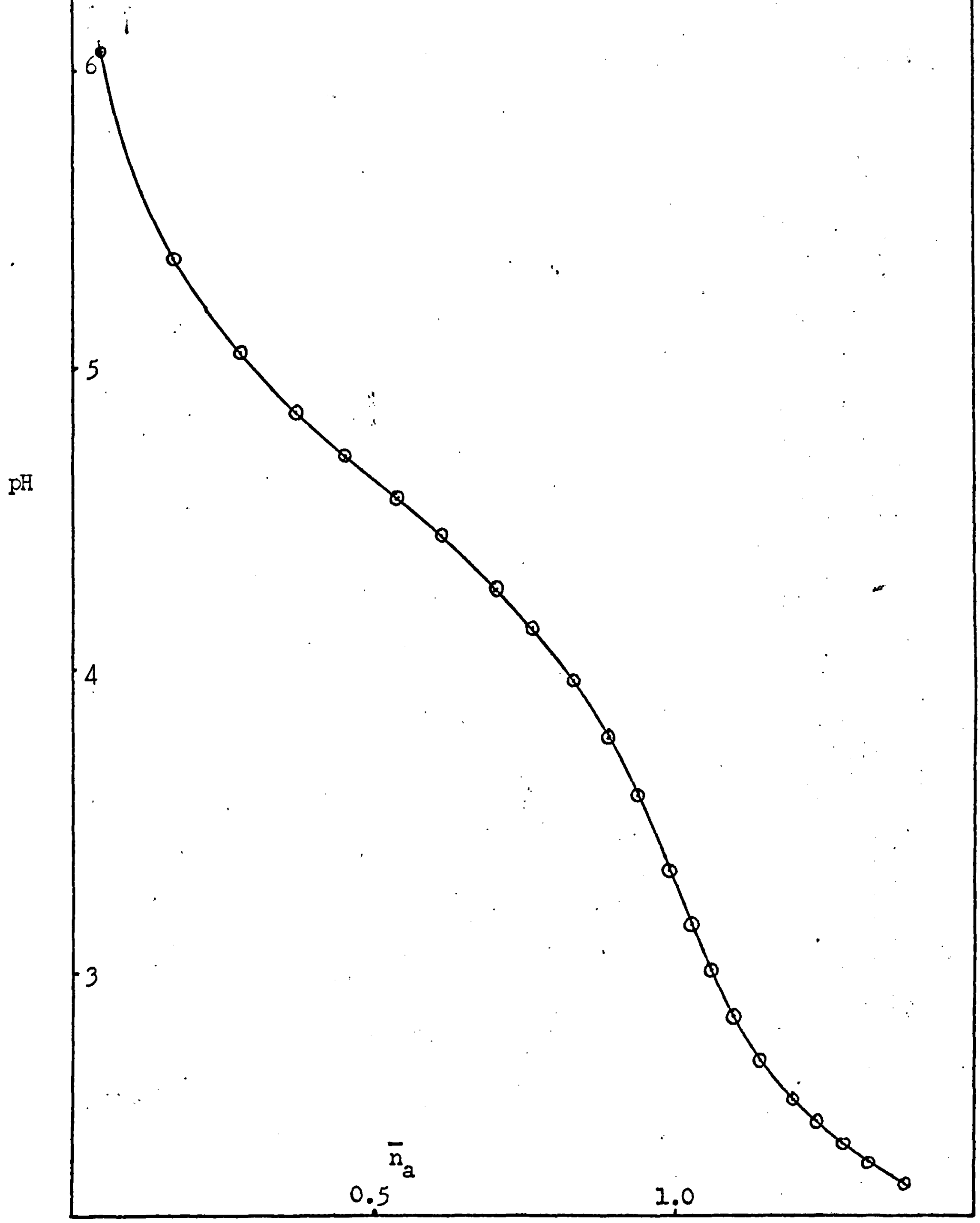


Fig 40

Titration curve for α-ketoglutaric acid



phosphate are recorded in Table 9.

Table 9

	Amine N pk_1	Pyridine N pk_2	Sec. PO_4 pk_3	Phenol OH pk_4	Prim. PO_4 pk_5
PyP		8.69	6.32	3.73	1.89
		(8.69)	(6.20)	(4.14)	(<2.5)
PamP	10.70	8.41	5.72	3.32	-
	(10.92)	(8.61)	(5.76)	(3.69)	(<2.5)

	pk_1	pk_2
αKG	4.74	2.06

Figures in parentheses are due to Williams and Neilands (44).

Approximate values of pk_5 for pyridoxal phosphate and pk_2 for αKG could not be determined directly from the graphs as the pH of the solutions was never low enough for $\bar{n}_a$ to reach 3.5 and 1.5 respectively. Values were taken from the graphs as near as possible to the required readings and substituted into equation (iii). Consequently the resulting values may reflect errors in adjacent pk's which contribute to the pH at these points than would otherwise be the case.

(iii) Determination of the stability constants of pyridoxal and pyridoxamine phosphates and α-ketoglutaric acid with various metals.

The titrations described in section (ii) were repeated in the presence of suitable concentrations of metal ions (Cu(11), Ni(11), Co(11) and Zn(11) were used). The ratio of ligand to metal was usually 2:1.

The concentrations of the Co(11) and Ni(11) stock solutions were determined by complexometric titrations using the method due to Schwarzenbach (46). The metal solution was titrated with E.D.T.A. using murexide as indicator. Solutions of Cu(11) and Zn(11) of the required concentrations were made up by weight.

Results

Several representative results are shown in Table 10. These are taken from the titration of pyridoxamine phosphate and Ni(11). (13.3×10^{-4} and 6.5×10^{-4} M respectively).

Table 10

v	pH	$p[H^+]$	v'	$\bar{n}_a$	$\bar{n}$	p[A]
10.20	5.445	5.353	-	2.650	0.002	11.930
15.36	6.790	6.688	-	2.084	0.112	8.875
19.25	7.870	-	0.05	1.848	0.429	6.840
32.33	8.720	-	0.10	1.437	0.735	5.569
26.40	9.510	-	0.23	1.123	1.046	4.740
29.28	10.215	-	1.60	0.970	1.430	4.271

Graphs of $\bar{n}$/p[A] were plotted (Figs 41-50) and the values of p[A] at $\bar{n}$ = 0.5 and 1.5 taken as the approximate values of the logarithms of the respective stability constants. These were then substituted in turn into equation (xix) until constant values were obtained. The results are summarised in Table 11.

Table 11

	Cu(11)		Ni(11)		Co(11)		Zn(11)
	K_1	K_2	K_1	K_2	K_1	K_2	K_1
PyP	6.07	4.01	3.51	-			
PamP	10.96	5.86	6.50	4.23	5.69	4.52	7.13
αKG	2.90	-	2.81	-	2.83	-	2.83

Fig 41

Graph of p[A]/$\bar{n}$ for pyridoxal phosphate and Ni(11)

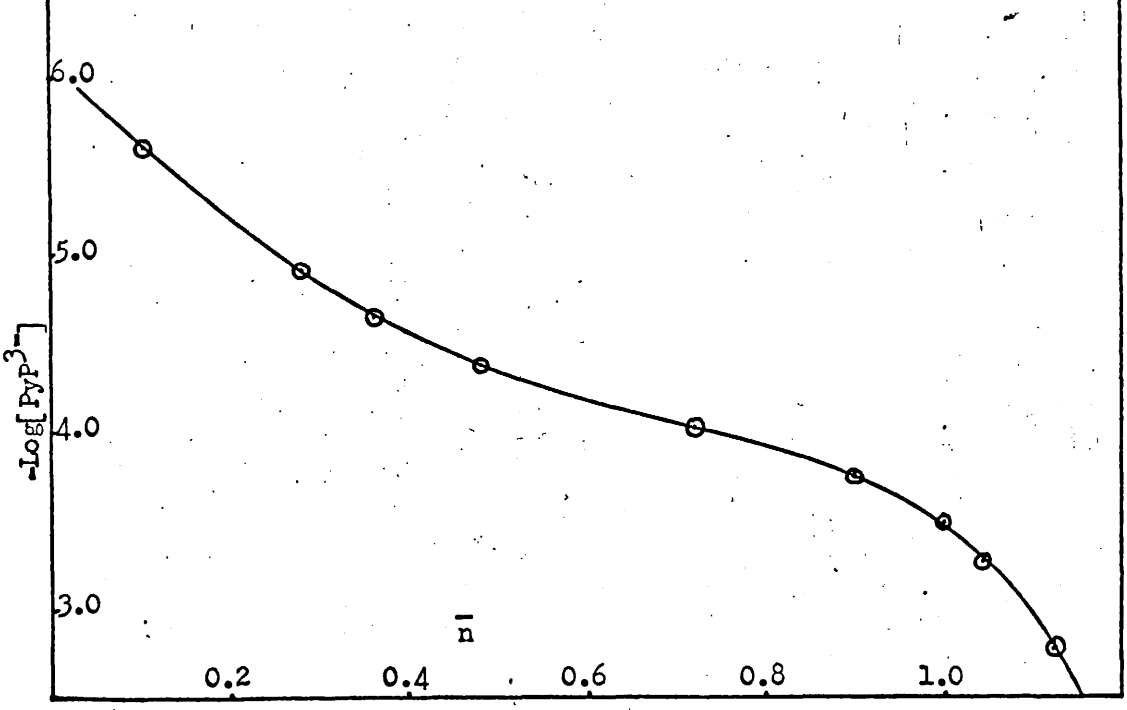
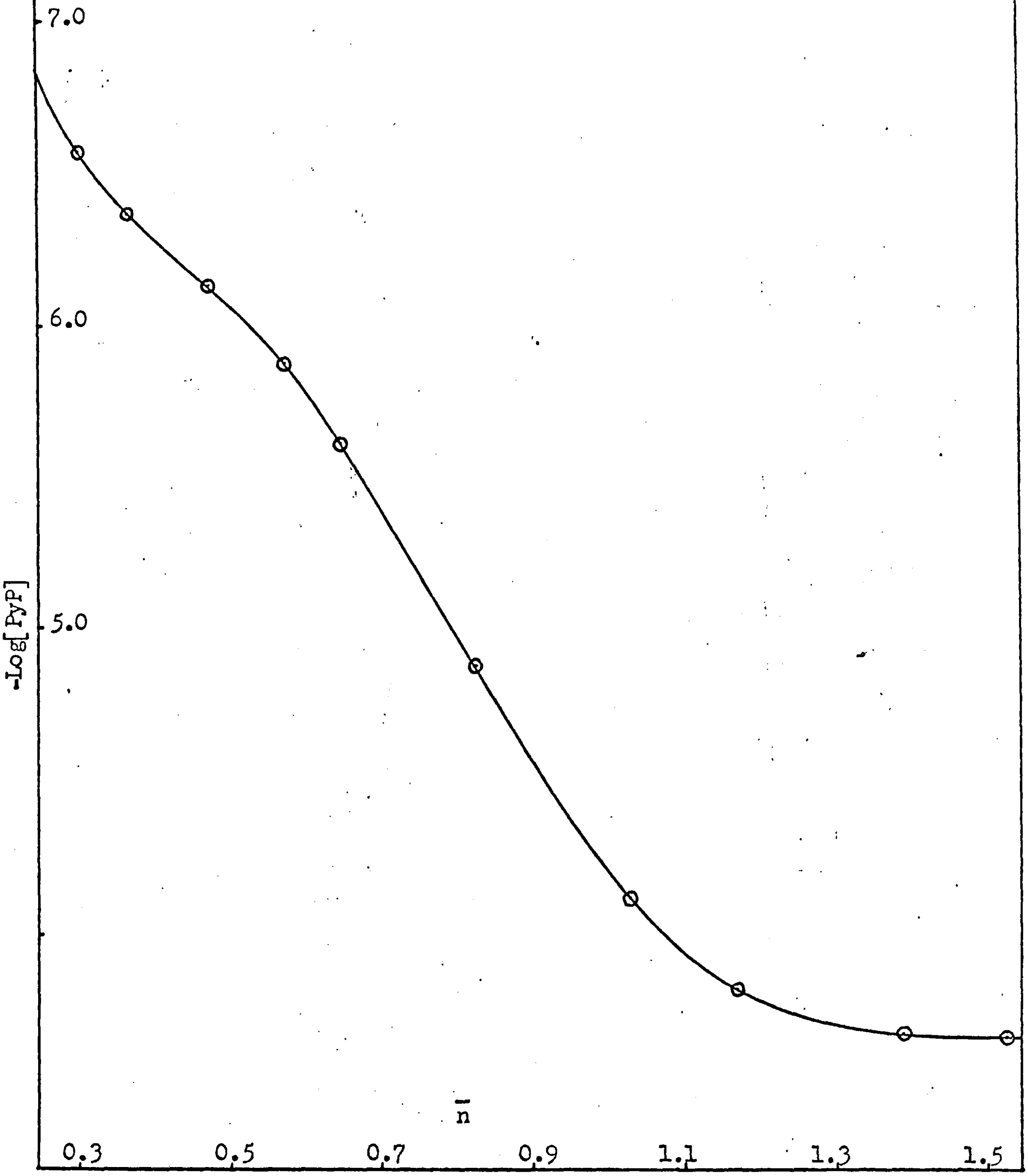


Fig 42

Graph of p[A]/$\bar{n}$ for pyridoxal phosphate and Cu(11)



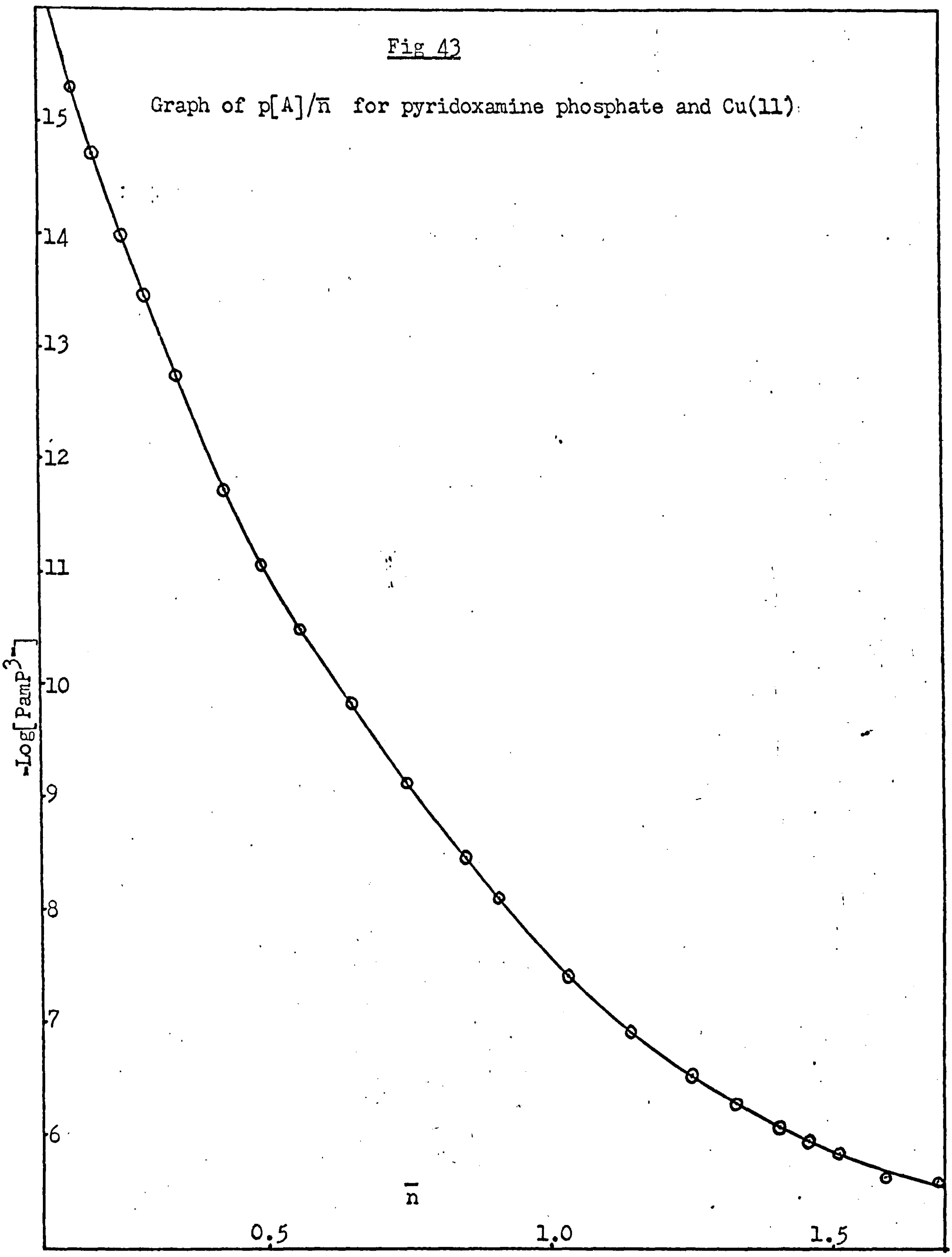


Fig 43
Graph of p[A]/n̄ for pyridoxamine phosphate and Cu(11)
-Log[PamP^3-]
15
14
13
12
11
10
9
8
7
6
n̄
0.5
1.0
1.5

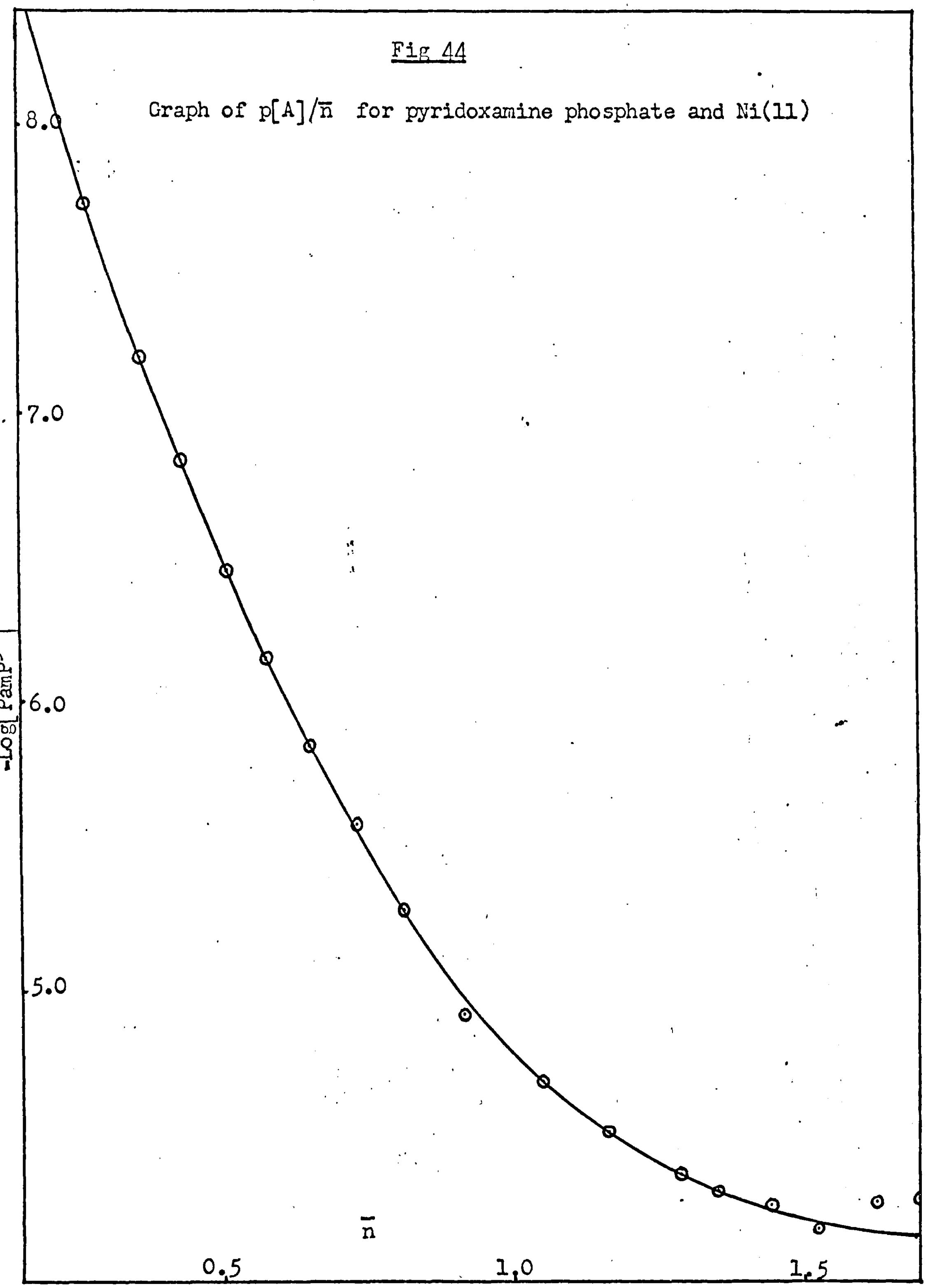


Fig 44
Graph of p[A]/n̄ for pyridoxamine phosphate and Ni(11)
8.0
7.0
6.0
5.0
-Log[PamP3-]
n̄
0.5
1.0
1.5

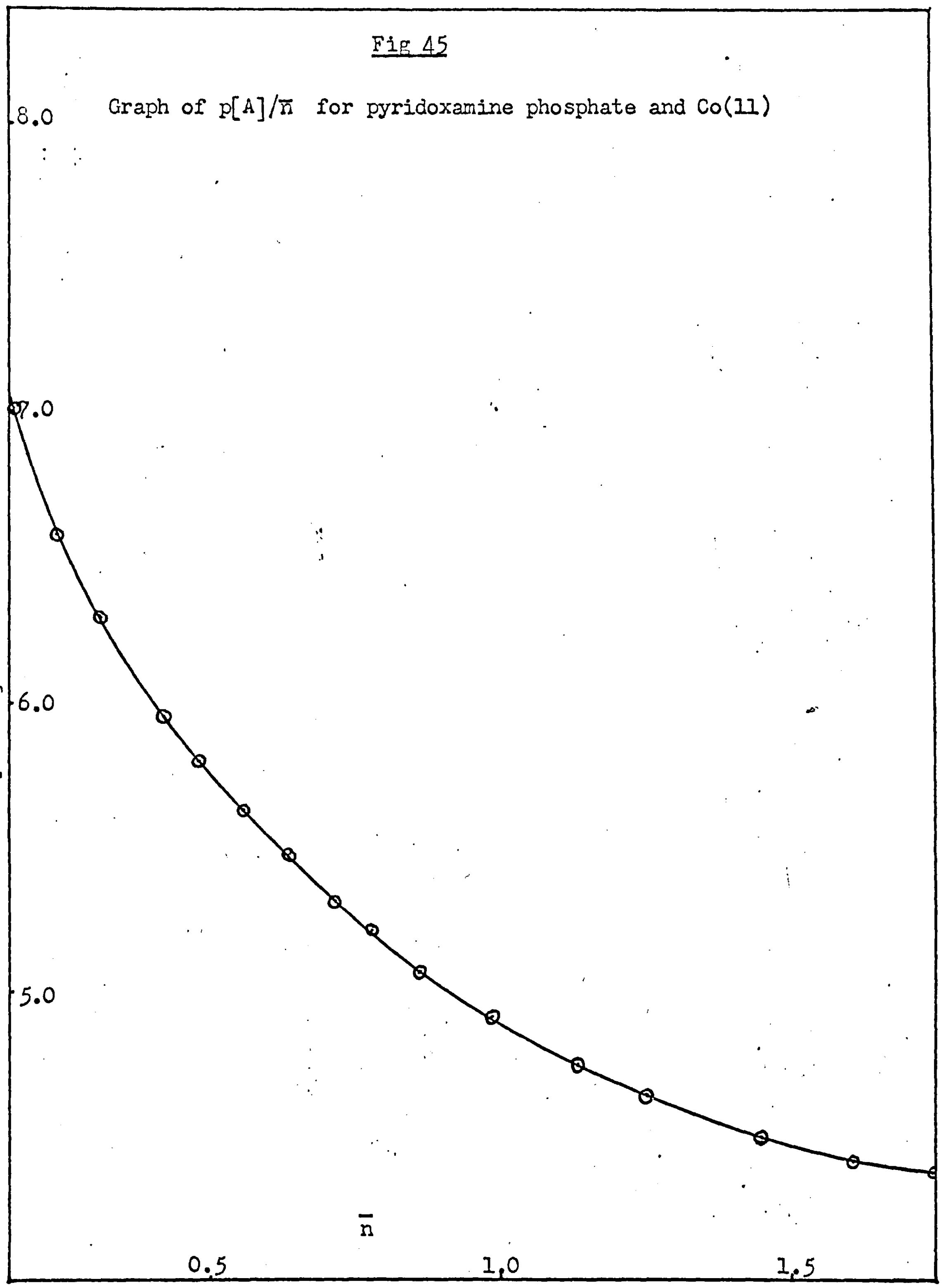


Fig 45

Graph of p[A]/$\bar{n}$ for pyridoxamine phosphate and Co(11)

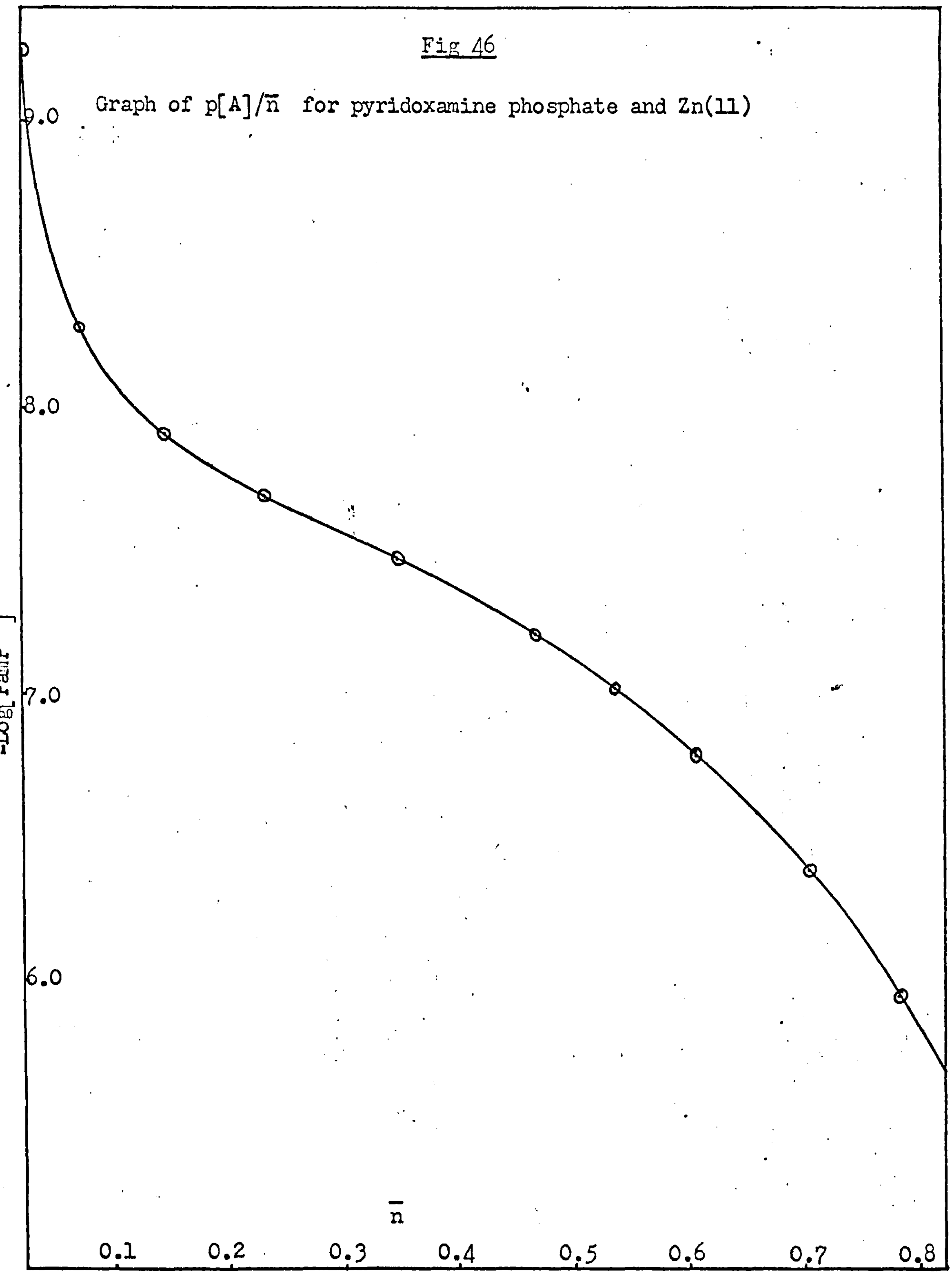


Fig 46
Graph of p[A]/n̄ for pyridoxamine phosphate and Zn(11)
9.0
8.0
7.0
6.0
-Log[PamP3-]
n̄
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Fig 47

Graph of p[A]/$\bar{n}$ for α-ketoglutarate and Zn(11)

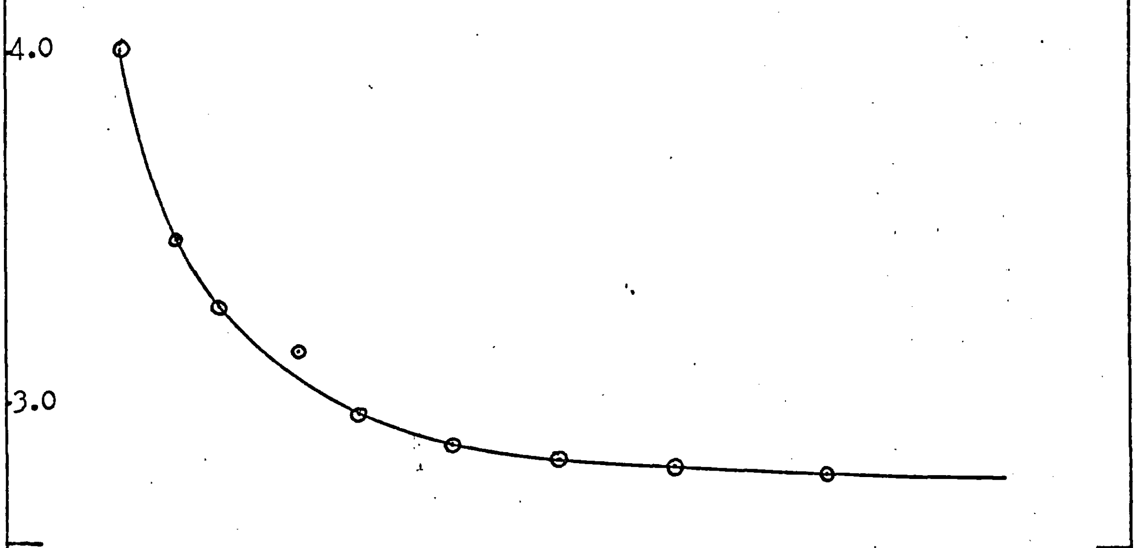
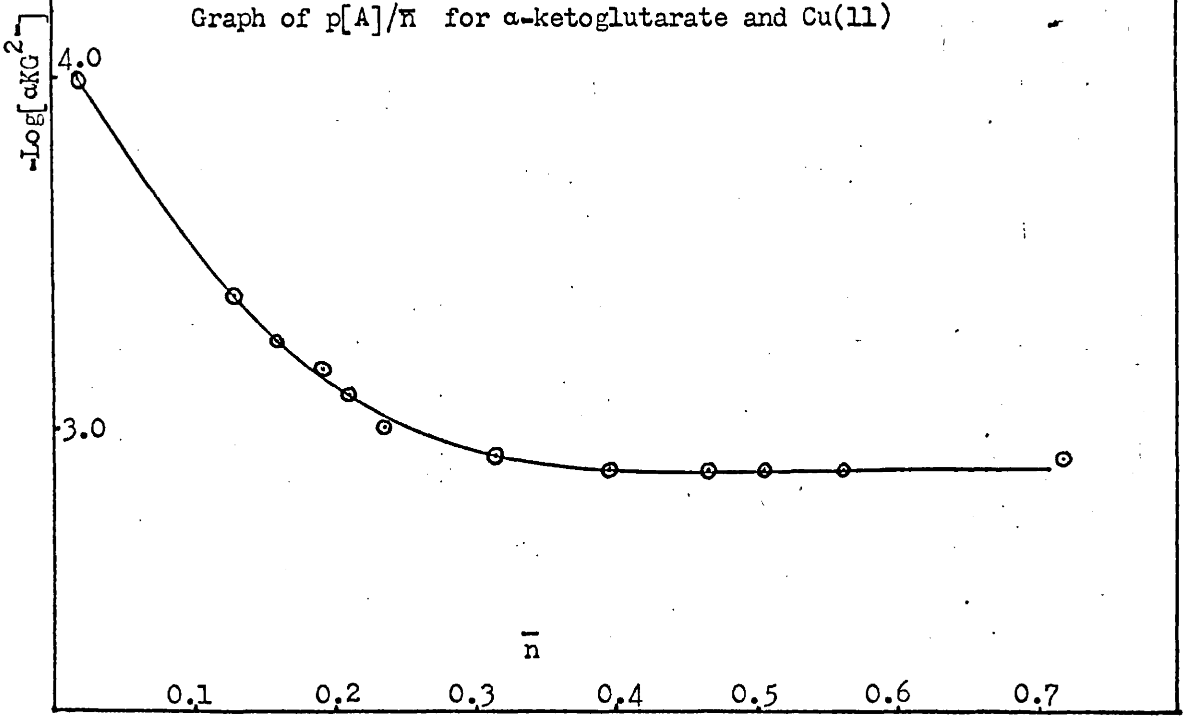


Fig 48

Graph of p[A]/$\bar{n}$ for α-ketoglutarate and Cu(11)



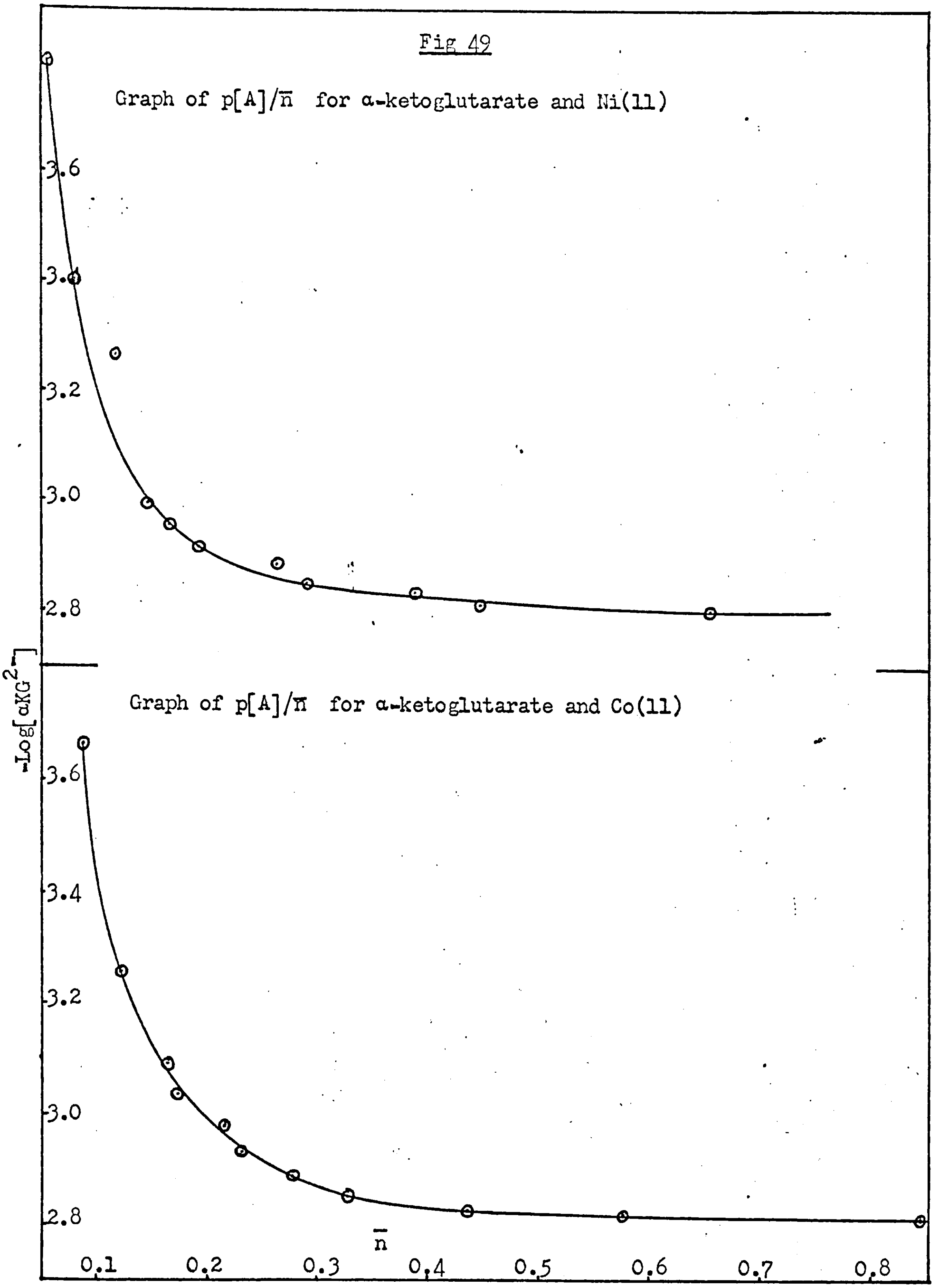


Fig 49
Graph of p[A]/n̄ for α-ketoglutarate and Ni(11)
Graph of p[A]/n̄ for α-ketoglutarate and Co(11)
-Log[αKG²⁻]
3.6
3.4
3.2
3.0
2.8
3.6
3.4
3.2
3.0
2.8
n̄
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Discussion

The theory behind the determination of stability constants implies that at $\bar{n} = 0.5$ (and 1.5 although this is not so important, as will be seen) all the available protons are associated to the free ligand (or exist as dissociated H^+) and that non is associated to the complex. If it is assumed that the metal co-ordinates to the amine nitrogen and the phenolic oxygen of pyridoxamine phosphate, and that co-ordination does not alter the pK's of the remaining groups, then Table 10 shows that for Ni(11) and PamP, $\bar{n} = 0.5$ at a pH (ca. 8.1) which is below the pK of an unco-ordinated group (the ring nitrogen with a pK of 8.4 - Table 9). The same is more or less true for Co(11) and Zn(11) with PamP where $\bar{n} = 0.5$ at pH $\sim$ 8.4 and $\sim$ 7.7 respectively. With Cu(11) and pyridoxamine phosphate the pH is ca. 5.8, which means that two groups could be almost completely protonated while co-ordination took place, the ring nitrogen and the secondary phosphate (with pK values of 8.4 and 5.7 respectively).

Similarly with pyridoxal phosphate and Cu(11), if it is assumed that co-ordination takes place only through the ring nitrogen, $\bar{n} = 0.5$ at a pH (of ca. 6) which is less than the pK for the secondary phosphate of 6.3 (Table 9).

This means that the theory developed on p 96 should read:-

Total bound protons = protons bound to free ligand + protons bound to co-ordinated ligand.

or $$C_h - m - [H^+] = \bar{n}_a(C_a - \bar{n}'C_m) + \bar{n}_a'\bar{n}'C_m \qquad \text{(xxiii)}$$

where $\bar{n}'_a$ is the average number of protons bound to each co-ordinated ligand molecule, and $\bar{n}'$ is the average number of ligand molecules associated to the metal (including protonated molecules).

$\bar{n}'_a$ can be calculated if, as was assumed above, the metal co-ordinates to the centres suggested and if co-ordination leaves the pK's of the remaining groups unaffected. Then,

$$\bar{n}'_a = \frac{[H^+]/k'_1 + 2[H^+]^2/k'_1k'_2 + 3[H^+]^3/k'_1k'_2k'_3}{1 + [H^+]/k_1 + [H^+]^2/k'_1k'_2 + [H^+]^3/k'_1k'_2k'_3}$$

where k'_1, k'_2 etc. are the dissociation constants of the remaining groups. $\bar{n}'_a$ was calculated over the desired pH range for the complexes of both pyridoxal and pyridoxamine phosphates and graphs of $\bar{n}'_a$/pH were plotted. These are shown in Fig 51.

Then, rearranging equation (xxiii) gives,

$$\bar{n}' = \frac{C_a\bar{n}_a - C_h + m + [H^+]}{(\bar{n}_a - \bar{n}'_a)C_m} \qquad \text{(xxiv)}$$

$\bar{n}'$ was calculated from equation (xxiv). In Table 12 are shown some values of $\bar{n}'$ compared with the corresponding values of $\bar{n}$ calculated before. The data are taken from the titration of Cu(11) and pyridoxal phosphate with KOH (p 107). Interpolation of this data gives $\bar{n}' = 0.5$ and 1.5 at the pH values of 5.40 and 6.66 respectively. These correspond to p[A] values of 11.69 for K'_1 and 9.37 for K'_2 (cf. Table 11 p 108).

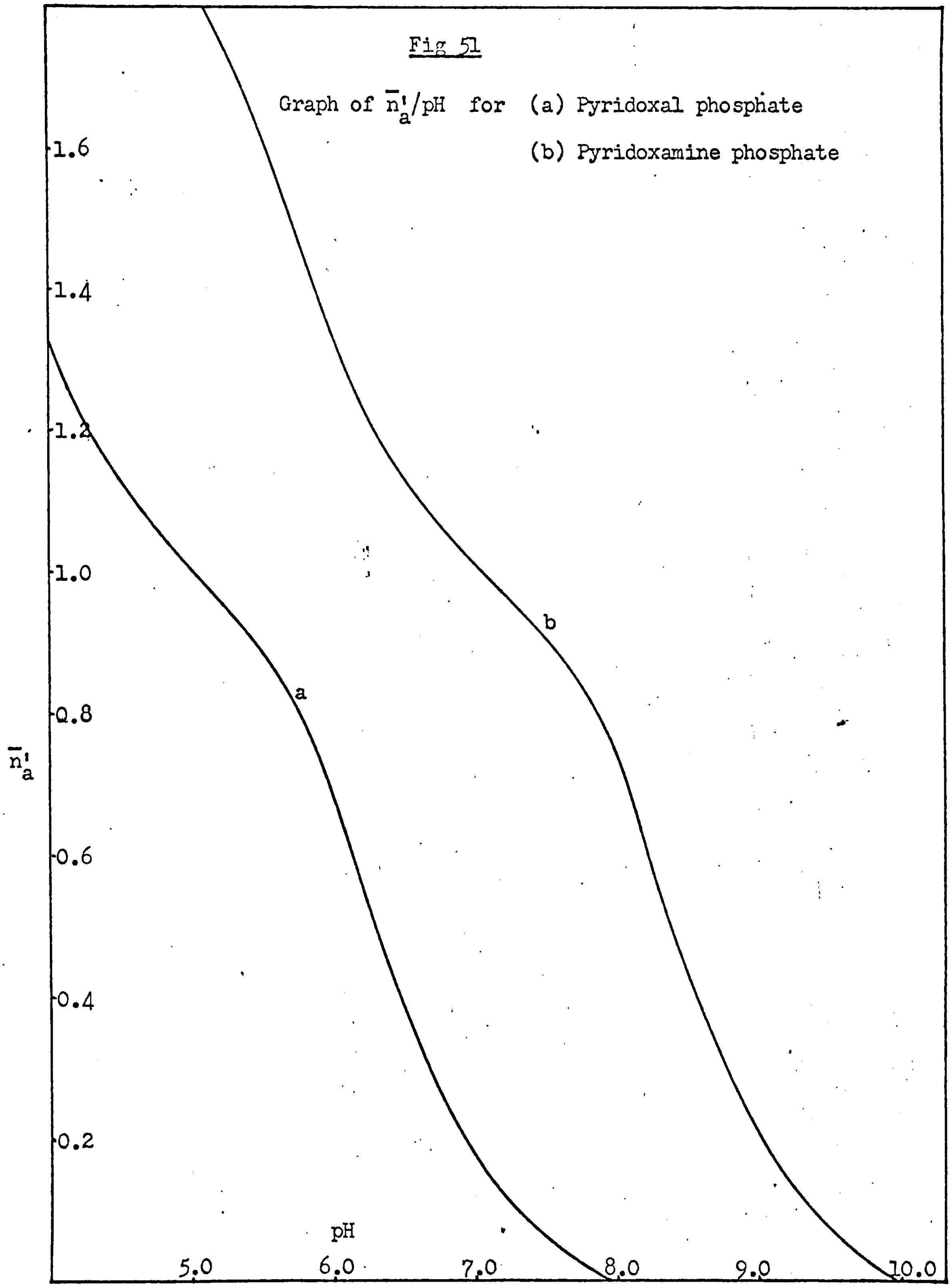


Fig 51
Graph of $\bar{n}'_a$/pH for (a) Pyridoxal phosphate
(b) Pyridoxamine phosphate
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
$\bar{n}'_a$
a
b
pH
5.0
6.0
7.0
8.0
9.0
10.0

Table 12

$\bar{n}_a$	pH	$\bar{n}$	$\bar{n}'_a$	$\bar{n}'$
2.729	5.300	0.3679	1.718	0.4059
2.653	5.440	0.3939	1.648	0.5212
2.588	5.565	0.4254	1.590	0.5508
2.140	6.545	0.6916	1.120	1.451
2.101	6.695	0.7339	1.084	1.516
2.063	6.900	0.7853	1.035	1.576
1.850	7.840	1.092	0.760	1.854
1.668	8.285	1.251	0.566	1.893
1.430	8.725	1.456	0.333	1.893

As $\bar{n}'$ is the average of all the protonated and unprotonated forms of pyridoxamine phosphate, the constants K'_1 and K'_2 are defined by:

$$K'_1 = [\text{CuP - all forms}]/[Cu^{2+}][P^{3-}]$$

and $$K'_2 = [\text{CuP}_2\text{ - all forms}]/[\text{CuP - all forms}][P^{3-}]$$

In order to obtain K_1 and K_2 in the forms

$$K''_1 = [CuP^-]/[Cu^{2+}][P^{3-}]$$

and $$K''_2 = [CuP_2^{4-}]/[CuP^-][P^{3-}] \qquad \text{(xxv)}$$

the following relationships must be used:-

$$[\text{CuP- all forms}] = [CuP^-](1 + [H^+]/k'_1 + [H^+]^2/k'_1k'_2 + [H^+]^3/k'_1k'_2k'_3)$$

and

$$[\text{CuP}_2\text{- all forms}] = [CuP_2^{4-}](1 + [H^+]/k'_1 + [H^+]^2/k'^2_1 + [H^+]^3/k'^2_1k'_2 + [H^+]^4/k'^2_1k'^2_2 + [H^+]^5/k'^2_1k'^2_2k'_3 + [H^+]^6/k'^2_1k'^2_2k'^2_3)$$

Substituting the hydrogen ion concentrations at $\bar{n}' = 0.5$ and 1.5 into the above equations and correcting K_1 and K_2 gave,

$$K_1' = 8.19 \text{ and } K_2' = 5.81$$

(cf. the original values of 10.96 and 5.86). K_2 would not be expected to alter very much by this correction as the pH at which $\bar{n}' = 1.5$ is comparatively high, and more of the protons which would have been associated to the complexes have been neutralised. In view of the assumptions made the agreement between K_2 and K_2' is good.

These calculations were also carried out on data from the titrations of pyridoxamine phosphate-Ni(11) and pyridoxal phosphate-Cu(11). The results are summarised in Table 13. The values of K_1' and K_2' in Table 13 are the 'all forms' constants calculated from the corrected values of $\bar{n}$; i.e. $\bar{n}'$.

Table 13

	K_1	K_2	K_1'	K_2'	K_1''	K_2''
Ni(11)-PamP	6.50	4.23	8.40	4.23	7.34	4.23
Cu(11)-PyP	6.07	4.01	6.83	4.01	6.00	4.01
Cu(11)-PamP	10.96	5.86	11.69	9.37	8.19	5.81

In this case the constants K_1 and K_2 are meaningless and must be replaced by either K_1' and K_2' or K_1'' and K_2'' as required.

Two factors probably account for the differences in the stability constants of the complexes of pyridoxal and pyridoxamine phosphates.

These are:

(i) The different hybrid states of the donor nitrogen;

and (ii) The bidentate nature of pyridoxamine phosphate compared with the monodentate pyridoxal phosphate.

The stability constants of several complexes of pyridine and ethylamine are shown in Table 14 for comparison with the present results.

Table 14

	H	Cu(11)		Ni(11)	
	K_1	K_1	K_2	K_1	K_2
Pyridine	5.18	2.52	1.86	1.78	1.05
Ethylamine	11.52	11.90	9.97		

(Data from ref 47)

The low values for pyridine are probably caused by the low pK (5.18) compared with the pK for the ring nitrogen of pyridoxal phosphate (8.69 - Table 9,p 106). An approximate correction for this effect can be obtained by adding the difference between the two pK values onto the stability constants shown above. The orders of magnitude of K for the complexes of pyridine and pyridoxal phosphate are then similar.

Conclusions

The recognised potentiometric titration method of determining the stability constants of complexes which are capable of accepting protons has been shown to be unsatisfactory for cases in which the stabilities are such that appreciable concentrations of complex are formed at low pH values. The method can be adapted for these cases if it is known to how many centres the metal is co-ordinated, and if the pK values of the complexes are known.

Determination of the Stability Constants of the Metal Schiff's Base Complexes.

Two methods were used to determine the stability constants of the complexes derived from the Schiff's bases of pyridoxal phosphate-glutamate and pyridoxamine phosphate-α-ketoglutarate. These were

(a) A spectrophotometric method

and (b) A potentiometric titration method.

(a) The final optical density of a reaction mixture containing pyridoxal phosphate, glutamate and Cu(11) ions was found to be almost directly proportional to the concentration of Cu(11) for a ratio of Cu(11):PyP less than 1, and almost independent for a ratio greater than 1, (p 34). The final optical density also varied with the glutamate concentration, sometimes even decreasing when the glutamate concentration became high.

Christensen (33) reported that when a 0.1 mM solution of the Schiff's base-metal complex of Cu(11), pyridoxal and valine at pH 8.5 was suddenly made 0.1 M in valine, the copper became equally distributed between the Schiff's base and the valine in a period of about 2 minutes. By measuring the absorbance at three different wavelengths he was able to estimate the concentrations of pyridoxal, Schiff's base and chelate present and thereby obtain a value for log K of 14.4, where the stability constant K is defined as

$$K = [CuPyVal^{o}]/[Cu^{2+}][PyVal^{2-}]$$

Davies et al. (36) later obtained a value of 14.5 for the same system using a similar method.

It would seem, therefore, that a similar competition between an excess of glutamate and the Schiff's base were being observed in the present study; a balance being obtained between increased Schiff's base formation and decreased copper availability on raising the concentration of glutamate. If so, it should be possible to obtain an estimate of the stability of the complex formed.

The method of Christensen (33) and Davies (36) was tried, but without success. This was because:-

(i) Small changes in optical density were involved at all wavelengths other than 25,500 cm^{-1}. (Even these changes were quite small for metals other than Cu(11) and Ni(11).);

(ii) The other two species absorbed to a certain extent at the wavelength chosen to measure the concentration of the third. This made successive approximations necessary to obtain satisfactory results;

(iii) The process accumulated errors incurred at all three wavelengths.

In this work a method was applied which utilised measurements at one wavelength only. The point chosen was that which corresponded to the peak of an absorption band due to the metal-Schiff's base at 25,500 cm^{-1}.

Theory

Consider the equilibria

$$PyP + Glu \overset{K_1}{\rightleftharpoons} SB'$$

and

$$SB' + M^{2+} \overset{K_2}{\rightleftharpoons} MSB'$$

then

$$K_1 = [SB']/[PyP]C_g \qquad \text{(i)}$$

and

$$K_2 = [MSB']/[SB'][M^{2+}] \qquad \text{(ii)}$$

where C_g is the total glutamate concentration - also taken as the total free glutamate concentration because of its large excess. Square brackets represent the concentrations of free species.

From (i) and (ii), $K_1K_2 = [MSB']/[PyP][M^{2+}]C_g \qquad \text{(iii)}$

The value required is that of K_2.

The dissociation constants of glutamic acid are given by:-

$$\begin{aligned} k_{a1} &= [H^+][Glu^{2-}]/[HGlu^-] \\ k_{a2} &= [H^+][HGlu^-]/[H_2Glu] \\ k_{a3} &= [H^+][H_2Glu]/[H_3Glu^+] \end{aligned} \qquad \text{(iv)}$$

and the stability constants of the metal-glutamate complexes by:-

$$\begin{aligned} k_1 &= [Mglu]/[M^{2+}][Glu^{2-}] \\ k_2 &= [MGlu_2^{2-}]/[MGlu][Glu^{2-}] \end{aligned} \qquad \text{(v)}$$

Summing the concentrations of all the reactants gives,

Total concentration of metal,

$$C_m = [M^{2+}] + [MGlu] + [MGlu_2^{2-}] + [MSB'] \qquad (vi)$$

The species of M-PyP are ignored. This is justifiable by comparisons of the stability constants, pK values and concentrations of PyP and Glu which show that in the most favourable cases only about 1% of the metal exists in the form of M-PyP complexes, the percentage usually being much lower.

Total concentration of PyP,

$$C_p = [PyP] + [SB'] + [MSB'] \qquad (vii)$$

Summing the contributions of all the absorbing species towards the optical density:

$$D = [PyP]E_p + [SB']E_{sb} + [MSB']E_{msb} \qquad (viii)$$

Substituting (iii) and (v) in (vi) gives:

$$C_m = [M^{2+}](1 + k_1[Glu^{2-}] + k_1k_2[Glu^{2-}]^2 + K_1K_2[PyP]C_g) \qquad (ix)$$

Substituting (i) and (iii) in (vii) gives:

$$C_p = [PyP](1 + K_1C_g + K_1K_2C_g[M^{2+}]) \qquad (x)$$

Substituting (i) and (iii) in (viii) gives:

$$D = [PyP](E_p + K_1C_gE_{sb} + K_1K_2C_gE_{msb}[M^{2+}]) \qquad (xi)$$

Solving equation (x) for [PyP] and substituting in (ix) gives:

$$C_m = [M^{2+}]\left\{ F + \frac{K_1K_2C_gC_p}{1 + K_1C_g + K_1K_2C_g[M^{2+}]} \right\} \qquad \text{(xii)}$$

where $F = 1 + k_1[Glu^{2-}] + k_1k_2[Glu^{2-}]^2$

Eliminating [PyP from (x) and (xi) gives:

$$D(1 + K_1C_g + K_1K_2C_g[M^{2+}]) = C_p(E_p + K_1C_gE_{sb} + K_1K_2C_gE_{msb}[M^{2+}]) \qquad \text{(xiii)}$$

Solving (xiii) for $[M^{2+}]$ and substituting in (xii) gave an equation which, when rearranged in terms of K_2 became:

$$K_2 = \frac{AF}{K_1C_g\left\{C_mB - \frac{C_pA}{1 + K_1C_g + A/B}\right\}} \qquad \text{(xiv)}$$

where

$$A = C_p(E_p + K_1C_gE_{sb}) - D(1 + K_1C_g)$$

and $B = D - C_pE_{msb}$

The only constants in equation (xiv) not independently determinable are K_2 and E_{msb}. These were found by plotting a graph of log K_2 against assumed values of E_{msb} for each set of experimental values of D and C_g. The graphs should intersect in a point giving the correct values of K_2 and E_{msb}.

Experimental

1.0 ml of acetate buffer and 1.75 ml of water were pipetted into a 3 ml quartz 1 cm cell. This was followed by 0.25 ml of pyridoxal phosphate solution to give a concentration of 0.2 mM. The optical density of the resulting solution was measured (at 25,500 cm^{-1}) against a blank of buffer and water using the scale expansion unit. The procedure was repeated until results were consistent to within 0.001 OD units. The extinction coefficient of pyridoxal phosphate E_p at that pH and wavelength was then $D/2.10^{-4}$.

The above procedure was repeated using 1.75 ml of the sodium glutamate solution (previously adjusted to the pH of the buffer with NaOH solution) instead of water. The concentration of the glutamate in the sample was then 0.112 M. Using the equilibrium constant for Schiff's base formation (see p 143), the concentration of Schiff's base in the sample was calculated and hence its extinction coefficient E_{sb}.

Optical density measurements were taken on solutions 0.2 mM in pyridoxal phosphate, approximately 0.4 mM in metal (the actual concentrations were determined by complexometric titration on the stock solutions as reported in p 107) and from 32 to 73 mM in glutamate. The ionic strength was kept constant with NaCl. The experiments were repeated at each concentration of glutamate until results were consistent to within 0.003 OD units.

The values of D and C_g were substituted into equation (xiv) together with the values of E_p, E_{sb} and K_1. K_2 was calculated as discussed above.

Results

Graphs of $\log K_2/E_{msb}$ are shown in Figs 52-57 for the various metals at several pH values. The results are summarised in Table 15.

Table 15

pH		3.94	4.57	5.04	6.37
K_1		6.4 ± 0.2	7.4 ± 0.5	7.3 ± 0.1	16.1 ± 0.2
K_2	Ni	a	4.76 ± 0.02	5.01 ± 0.01	5.58 ± 0.04
	Co	b	3.97 ± 0.04	3.65 ± 0.20	b
	Cu	6.1 ± 0.4	a	a	a
	Zn	b	a	a	b

a - graphs did not intersect

b - not measured

The probable errors represent the mean deviation of the intersections from the average value of the intersections. These are usually much less than the corresponding probable errors in K_1 indicating that the method is quite insensitive to the value of K_1 - as expected.

It was found that, with the exception of copper at pH 3.94, the results could be divided into two clearly defined categories; those which intersected in a point (or over a small area), and those in which no intersections were observed, the curves running 'parallel'. In the latter case even random intersections were rare. The lines on the graph for copper at pH 3.94 were found to intersect, but to do so only over a comparatively large area.

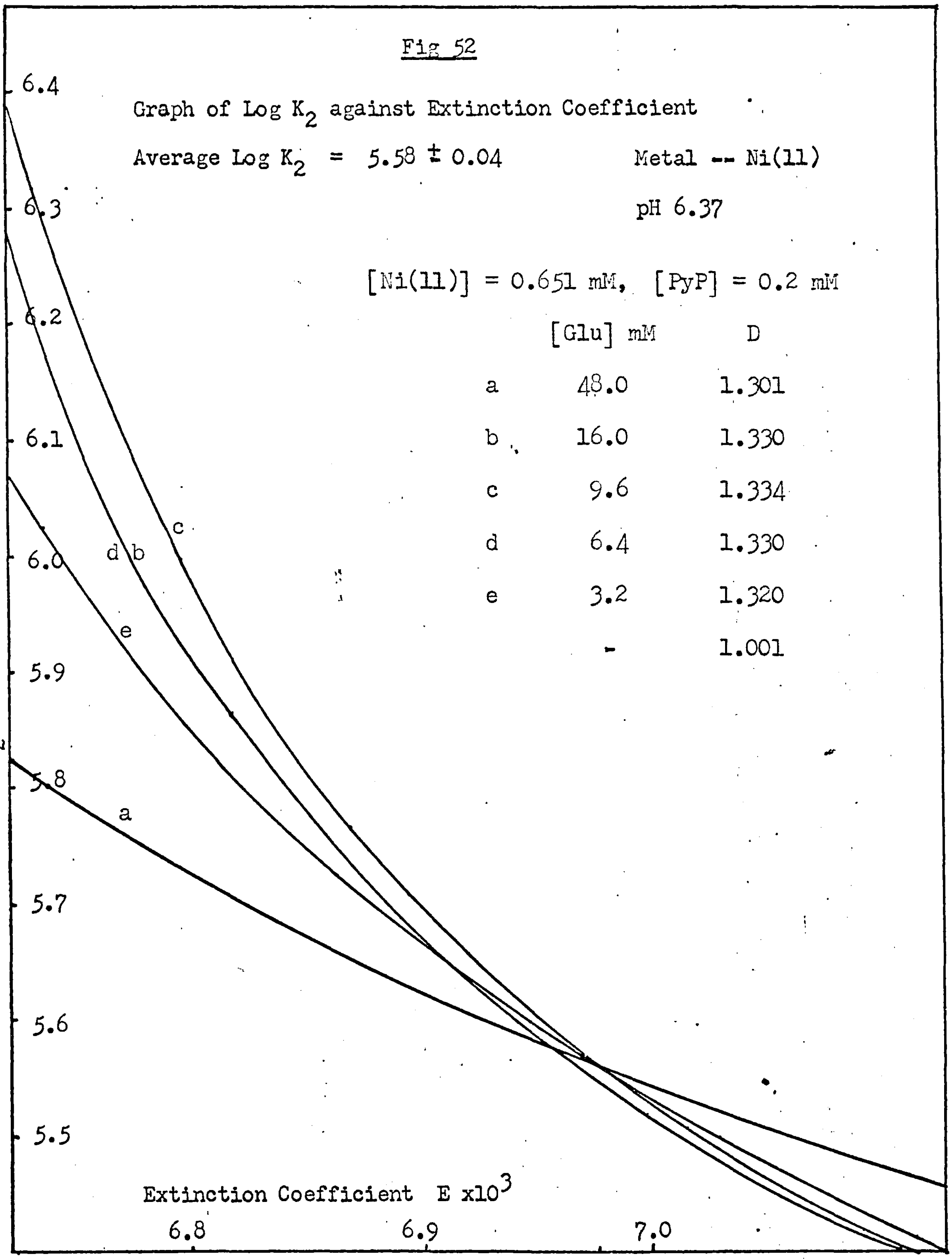


Fig 52
Graph of Log K_2 against Extinction Coefficient
Average Log K_2 = 5.58 ± 0.04
Metal -- Ni(11)
pH 6.37
[Ni(11)] = 0.651 mM, [PyP] = 0.2 mM
[Glu] mM D
a 48.0 1.301
b 16.0 1.330
c 9.6 1.334
d 6.4 1.330
e 3.2 1.320
- 1.001
Log K_2
6.4
6.3
6.2
6.1
6.0
5.9
5.8
5.7
5.6
5.5
a
b
c
d
e
Extinction Coefficient E x10^3
6.8
6.9
7.0

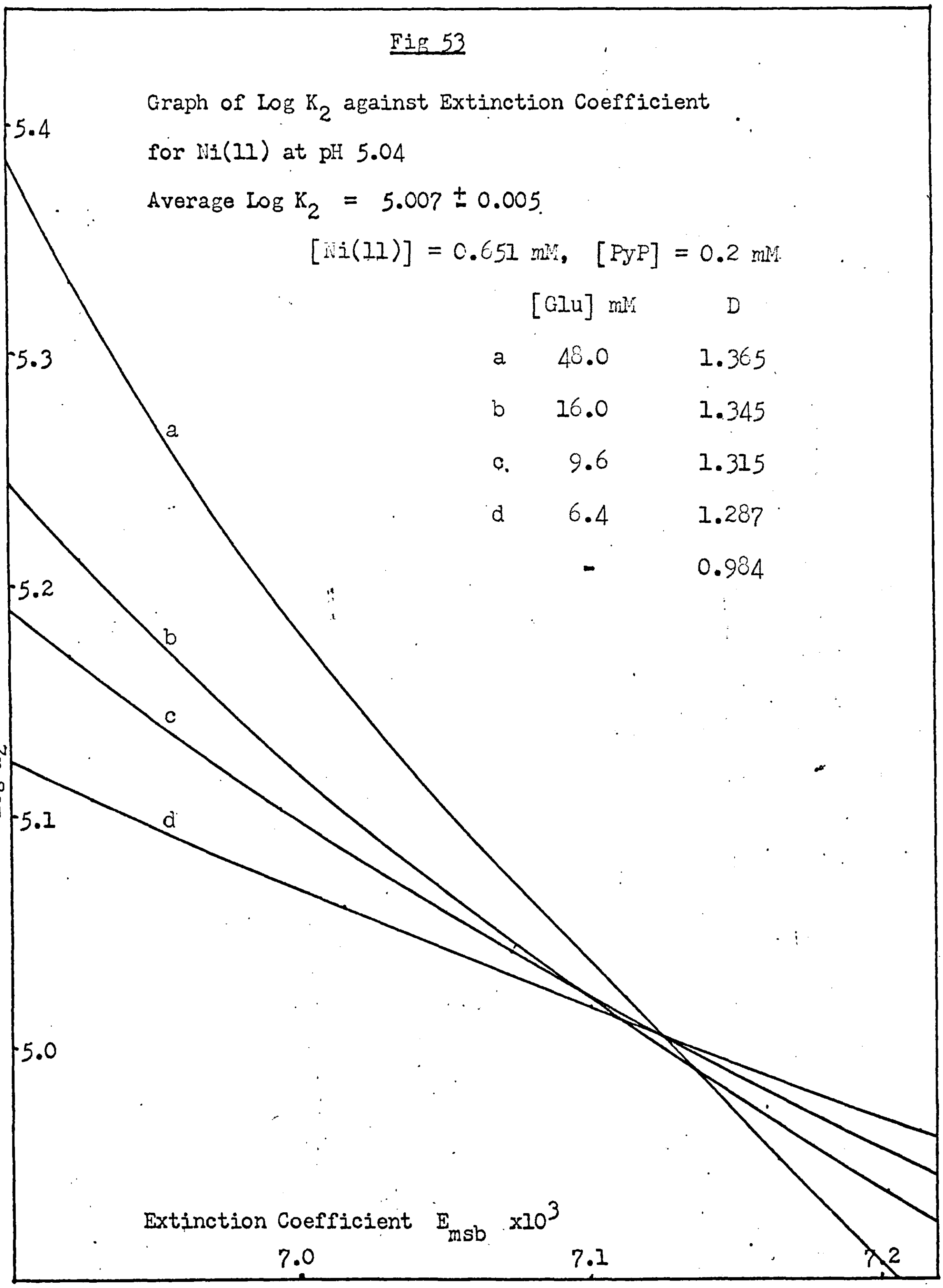


Fig 53
Graph of Log K_2 against Extinction Coefficient
for Ni(11) at pH 5.04
Average Log K_2 = 5.007 ± 0.005
[Ni(11)] = 0.651 mM, [PyP] = 0.2 mM
[Glu] mM D
a 48.0 1.365
b 16.0 1.345
c 9.6 1.315
d 6.4 1.287
- 0.984
5.4
5.3
5.2
5.1
5.0
Log K_2
a
b
c
d
Extinction Coefficient E_{msb} x10^3
7.0
7.1
7.2

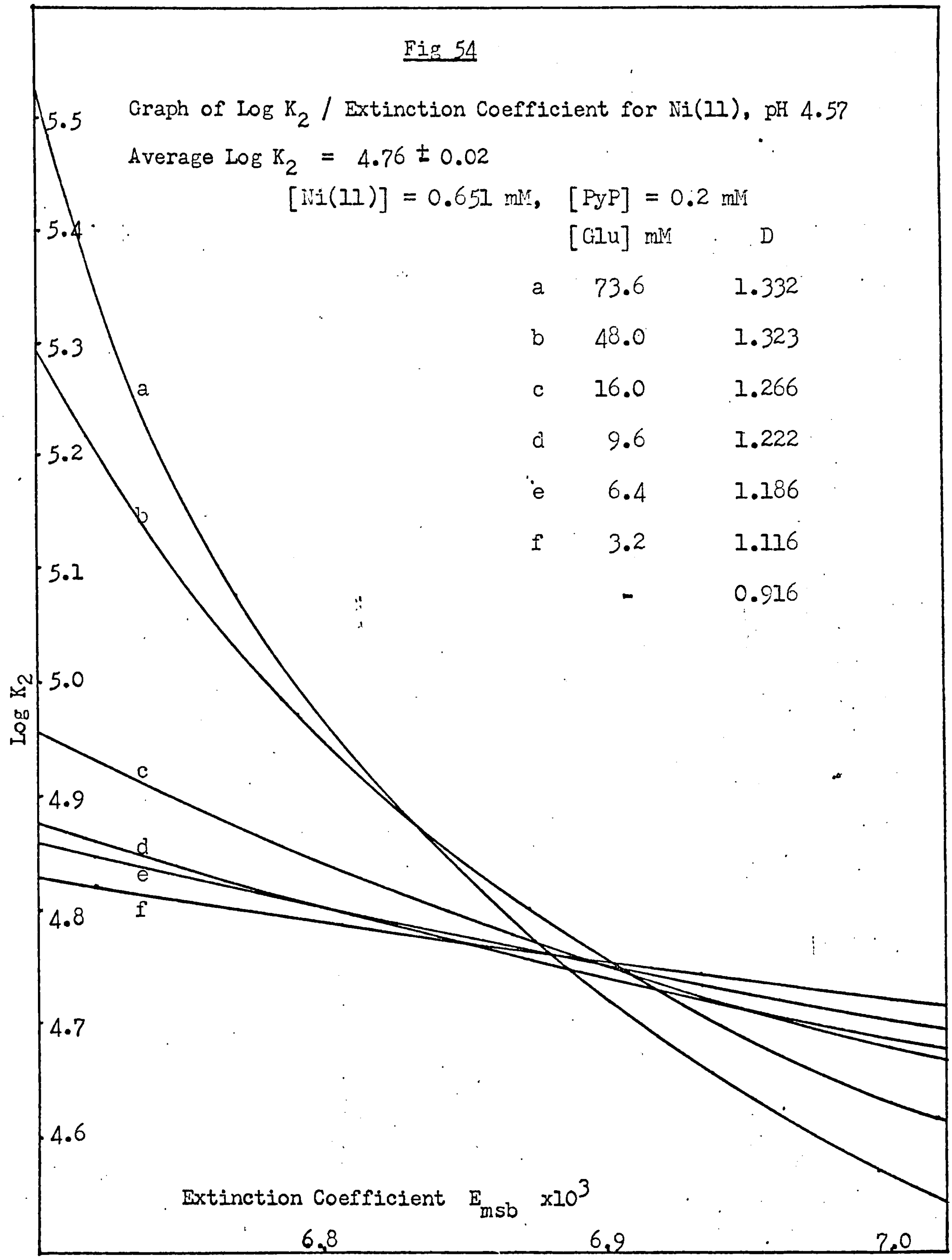


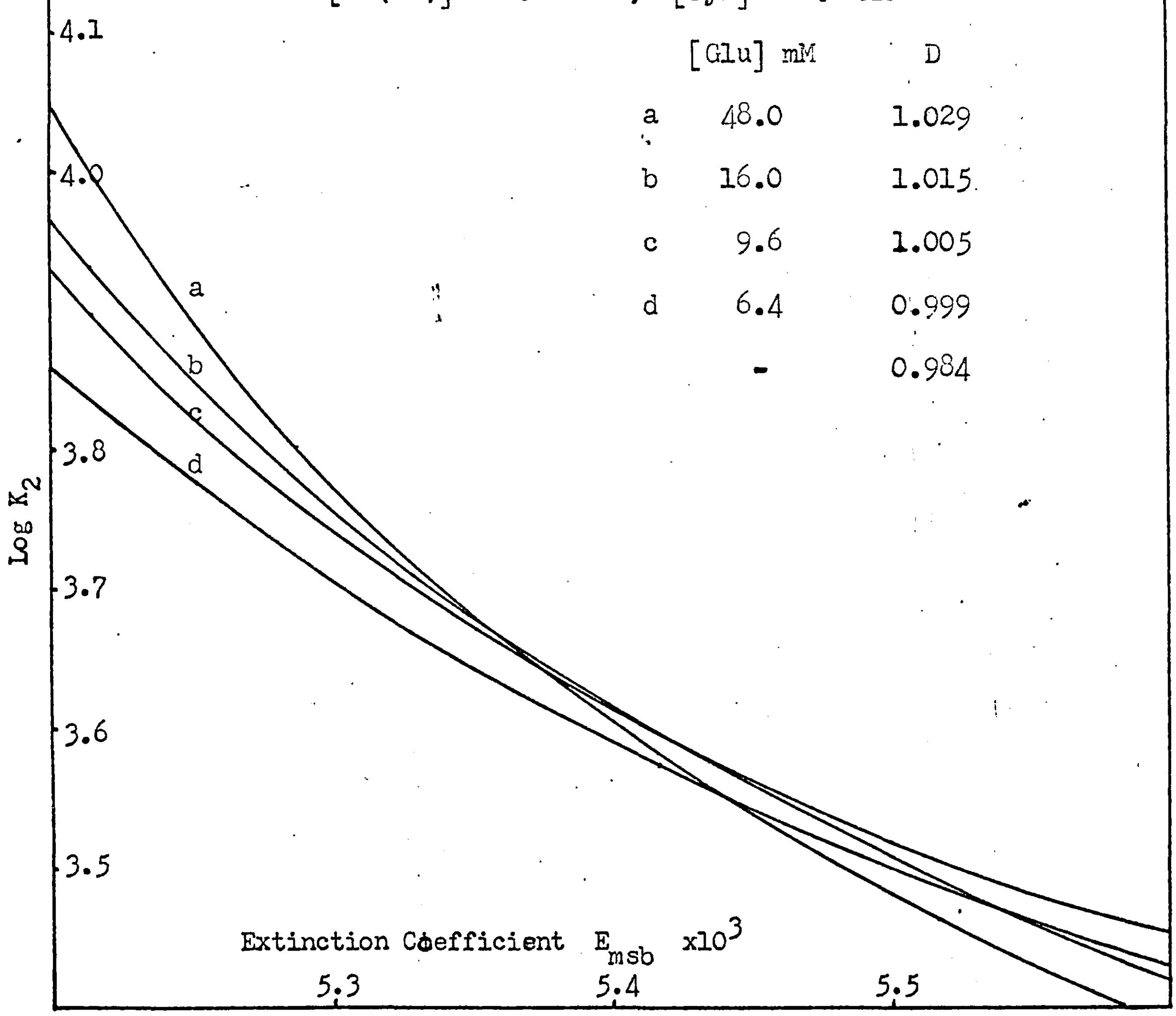
Fig 54
Graph of Log K_2 / Extinction Coefficient for Ni(11), pH 4.57
Average Log K_2 = 4.76 ± 0.02
[Ni(11)] = 0.651 mM, [PyP] = 0.2 mM
[Glu] mM D
a 73.6 1.332
b 48.0 1.323
c 16.0 1.266
d 9.6 1.222
e 6.4 1.186
f 3.2 1.116
- 0.916
Log K_2
5.5
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
a
b
c
d
e
f
Extinction Coefficient E_{msb} x10^3
6.8
6.9
7.0

Fig 55

Graph of Log K_2 / Extinction Coefficient for Co(11) at pH 5.04

Average Log K_2 = 3.65 ± 0.2

[Co(11)] = 0.610 mM, [PyP] = 0.2 mM



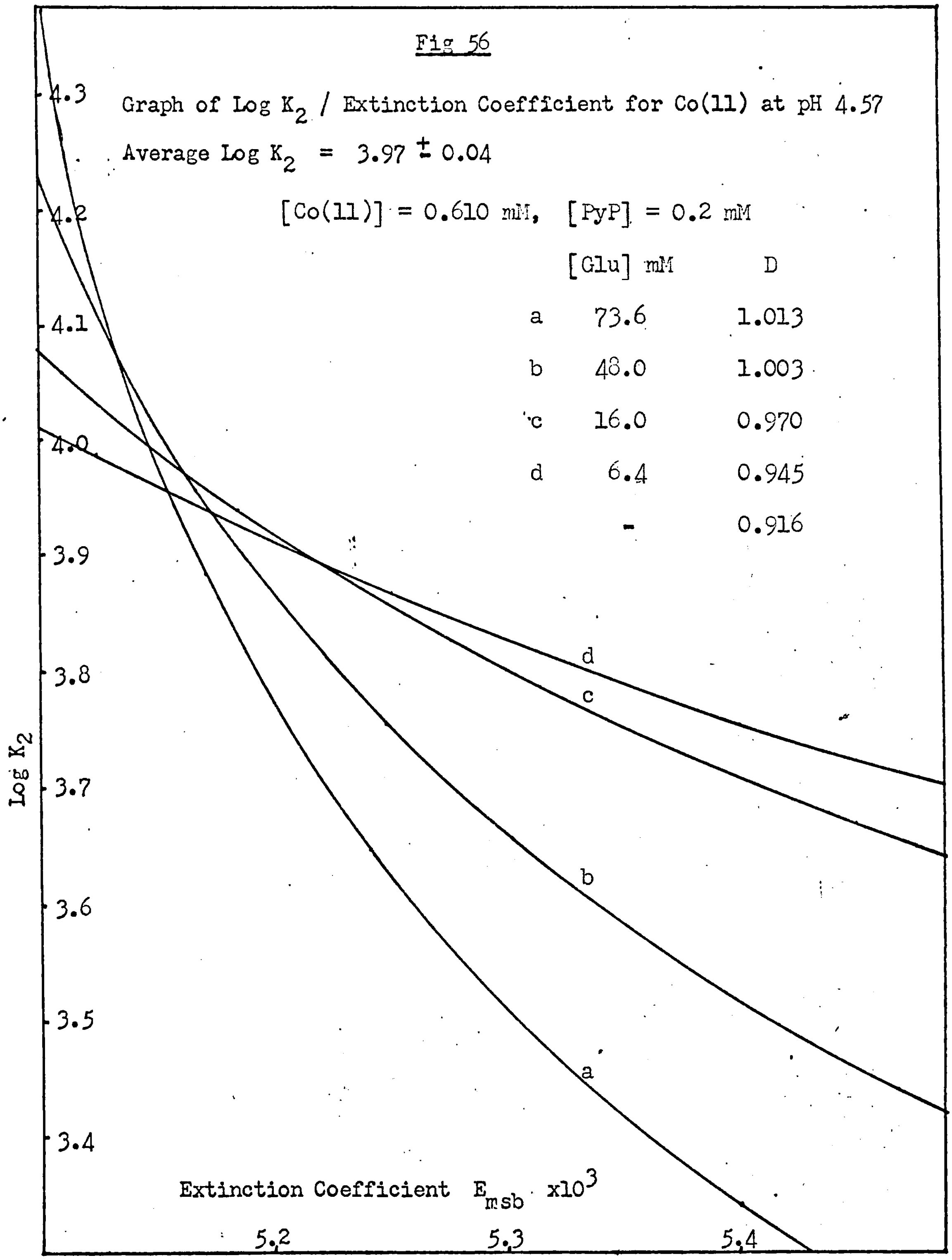


Fig 56
Graph of Log K_2 / Extinction Coefficient for Co(11) at pH 4.57
Average Log K_2 = 3.97 ± 0.04
[Co(11)] = 0.610 mM, [PyP] = 0.2 mM
[Glu] mM D
a 73.6 1.013
b 48.0 1.003
c 16.0 0.970
d 6.4 0.945
- 0.916
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
Log K_2
a
b
c
d
Extinction Coefficient E_{msb} x10^3
5.2
5.3
5.4

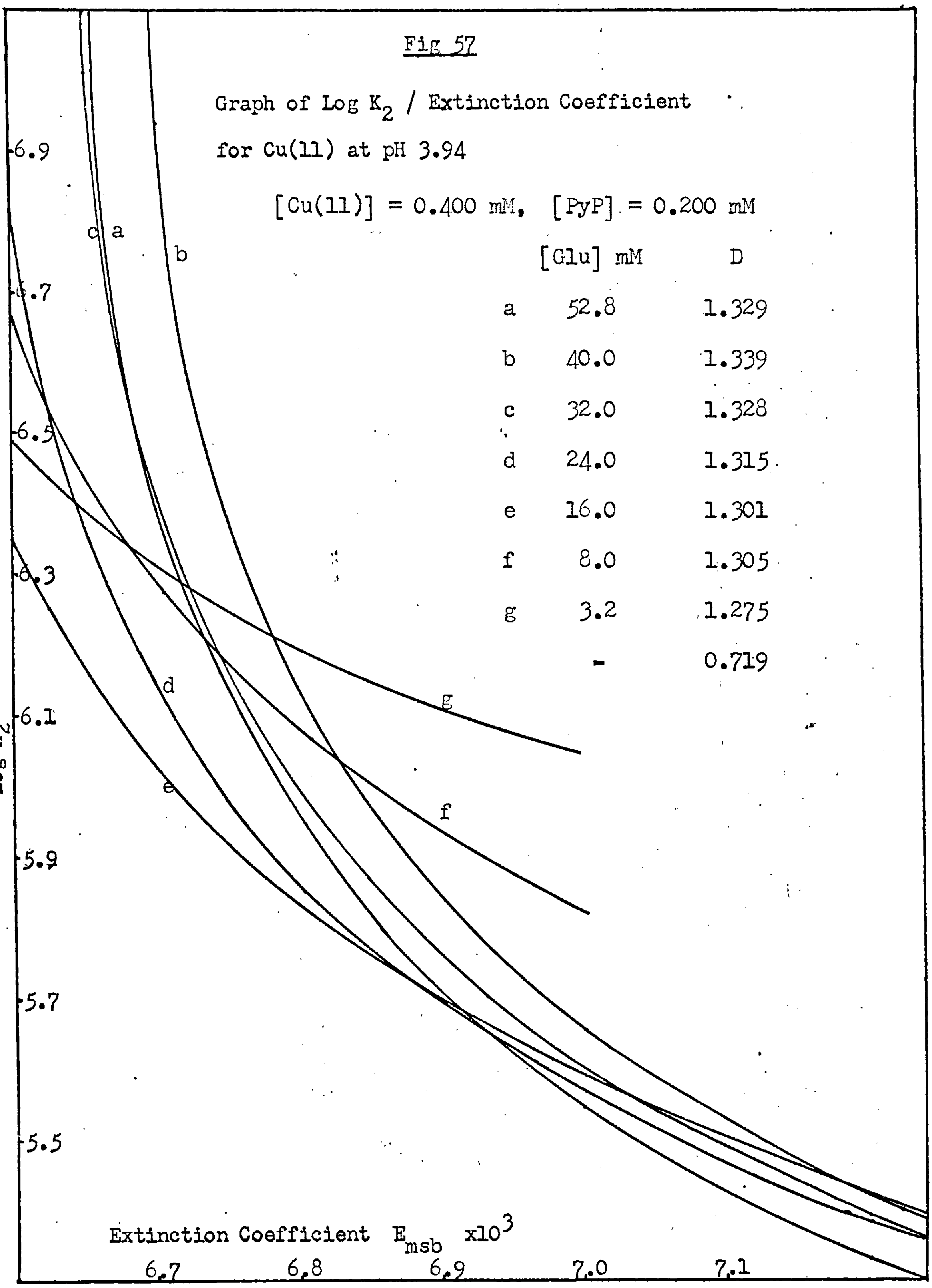


Fig 57

Graph of Log K_2 / Extinction Coefficient for Cu(11) at pH 3.94

[Cu(11)] = 0.400 mM, [PyP] = 0.200 mM

	[Glu] mM	D
a	52.8	1.329
b	40.0	1.339
c	32.0	1.328
d	24.0	1.315
e	16.0	1.301
f	8.0	1.305
g	3.2	1.275
	-	0.719

The physical reason for non-intersection would be either that that the extinction coefficient changed with increasing glutamate concentration, or that K_2 as defined in equation (ii) were not a constant.

Either or both of these possibilities would be true if:

(i) more glutamate co-ordinates with the metal already complexed to the Schiff's base (resulting in a'mixed' complex). Such co-ordination, whether or not accompanied by a spectral change, may by expected to give rise to deviations from the theoretical similar to those observed, as the equations derived above (p 125-7) would no longer hold;

(ii) the metal were still effectively available for MSB' formation even when co-ordinated to glutamate. Although this is theoretically improbable, it was noticed that experimental conditions which favoured a high F value (in effect a measure of the degree of metal-glutamate complexing - see p 127), the further away the curves were from intersecting. This was usually the case for copper.

If the total amount of metal were assumed to be always available for MSB' formation, whether or not already co-ordinated to glutamate, (equivalent to making F unity) lines which had not previously intersected were found to do so.

This seems to support suggestion (ii) even though it is not theoretically justifiable. However, conditions which would be expected to favour (ii) - e.g high F - would also facilitate the addition of one or more extra glutamate ions to the metal already bound to the Schiff's base. This would satisfy suggestion (i) which seems the more likely.

Zinc was found to be an exception to these considerations. Although its F value never rose above 1.5, none of the experiments resulted in curves which intersected.

With copper, the only experiment which resulted in curves which intersected was carried out at pH 3.94 where its F value was comparatively small (maximum value of ca. 9 compared with 40 at pH 4.57 and 400 at pH 5.04). No experiments were conducted at pH values less than 3.94 because of the increasing difficulty of measuring K_1.

In Table 16 are compared the F values of the metals at pH 5.04 and at various glutamate concentrations.

Table 16

C_g	$[Glu^{2-}]$	Cu	Ni	Co	Zn
mM	$M.10^8$	F	F	F	F
73.6	116	418	1.95	1.13	1.13
48.0	75.4	197	1.61	1.09	1.21
16.0	25.1	34.6	1.20	1.03	1.07
9.6	15.1	17.4	1.12	1.02	1.04
6.4	10.1	10.7	1.08	1.01	1.03
3.2	5.0	5.2	1.04	1.01	1.01

For the calculations it was necessary to know the pK values of glutamic acid and the stability constants of the various metal ions with glutamate. The values used are recorded in Table 17. They were taken from ref. 47.

Table 17

	K_1	K_2	K_3
H^+	9.67	4.28	2.30
Co	5.06	3.40	
Cu	7.85	6.55	
Ni	5.90	4.44	
Zn	5.45	4.01	

Discussion

The values of K_2 shown in Table 15 are calculated in terms of the total molar concentration of Schiff's base and not of a particular ionic species. The results are therefore pH dependent.

In order to express K_2 in terms of anionic Schiff's base, the dissociation constants of the uncomplexed Schiff's base are required. These values are not available for this system, but are recorded for pyridoxylidenevaline by Metzler (22).

Metzler measured the apparent equilibrium constant K_{pH} between pyridoxal, valine and the corresponding Schiff's base at numerous pH values, where

$$K_{pH} = [\text{Schiff's base}]/[\text{Pyridoxal}][\text{Valine}]$$

Defining the actual equilibrium constant K as

$$K = [SB^{2-}]/[Py^-][Val^-]$$

Metzler related K_{pH} to K by taking into account the pK values of the various dissociable groups. These were known for pyridoxal and valine but unknown for the Schiff's base. One of the three pK's attributed to the latter was given a value of 10.49 from observations of spectral changes of the Schiff's base about this pH, while another (the carboxyl group) was assumed to have the same value in the Schiff's base as in valine. The best theoretical fit of the observed K_{pH}/pH graph was obtained taking values of 1.65 for logK and 5.88 for the third pK (attributed to the ring nitrogen).

The results from the stability constant determinations on the metals Cu(11) and Ni(11) shown in Table 15 were recalculated in terms of the concentrations of penta-anionic Schiff's base and tri-anionic chelates by adding the factor ($\log[SB']/[SB'^{5-}] - \log[MSB']/[MSB'^{3-}]$). The term $[SB']/[SB'^{5-}]$ was calculated by using Metzler's values of 10.49 and 5.88 for the two unknown pK's, and assuming that the other dissociable protons in the Schiff's base have pK's comparable to those in free pyridoxal phosphate and glutamate (1.89 for primary phosphate, 6.32 for secondary phosphate and 4.28 and 2.30 for the carboxyl groups of glutamate).

Similarly $[MSB']/[MSB'^{3-}]$ was calculated by taking Davies' (36) values for the pK's of the ring nitrogen in the complexes of 5.6 and 6.7 for copper and nickel respectively. The other pK's were taken as 1.89, 2.30 and 6.32 for both copper and nickel complexes.

The recalculated results are shown in Table 18.

Table 18

pH	3.94	4.57	5.04	6.37
K' . Ni		10.01	9.76	9.45
K' Cu	14.28			

These values are comparable in magnitude to those obtained by Davies et al. (36) for the pyridoxal-valine system (14.5 and 10.8 for copper and nickel respectively).

Determination of the Equilibrium Constant for the Formation of the Schiff's Base from Pyridoxal Phosphate and Glutamate.

The formation of Schiff's bases between pyridoxal (or its phosphate) and aminp acids has already been widely reported (10,18-22). The equilibrium constants for some amino acids are recorded in several (10,19).

With pyridoxal and monobasic amino acids the problem of relating the observed apparent equilibrium constants to the ionic species actually taking part in the equilibrium is fairly easily resolvable (22). However, in the case of pyridoxal phosphate and glutamic acid an equilibrium involving the totally ionised species would result in penta-anionic Schiff's base formation. Consequently it may be expected that the observed equilibrium constant K might deviate from that defined as

$$K = [SB'^{5-}]/[PyP^{3-}][Glu^{2-}]$$

This deviation, together with the difficulty in calculating $[SB'^{5-}]$ at various pH values - because of the unknown pK values of the Schiff's base - made it necessary to evaluate K_1 at each pH required. K_1 is defined as

$$K_1 = [\text{Total Schiff's base}]/[\text{Total free PyP}][\text{Free Glu}]$$

or

$$K_1 = [SB']/[PyP]C_g \qquad (i)$$

The substitution of the total concentration of glutamate C_g for total free glutamate is valid because of the large excess of glutamate in the following experiments.

The optical density D is then given by:

$$D = [SB']E_{sb} + [PyP]E_p \qquad \text{(ii)}$$

Total pyridoxal phosphate in the reaction mixture is

$$C_p = [SB'] + [PyP] \qquad \text{(iii)}$$

Eliminating [PyP from (ii) and (iii),

$$D = [SB']E_{sb} + (C_p - [SB'])E_p$$

$$= [SB'](E_{sb} - E_p) + C_pE_p \qquad \text{(iv)}$$

Eliminating [PyP] from (i) and (iii),

$$K_1 = [SB']/(C_p - [SB'])C_g$$

or $$[SB'] = K_1C_gC_p/(1 + K_1C_g) \qquad \text{(v)}$$

Substituting (v) in (iv),

$$D = C_pE_p + C_p(E_{sb} - E_p)K_1C_g/(1 + K_1C_g)$$

If $D_o = C_pE_p$ the optical density of PyP alone

Then $D - D_o = K_1C_pC_g(E_{sb} - E_p)/(1 + K_1C_g)$

This rearranges to

$$1/C_g = K_1C_p(E_{sb} - E_p)/(D - D_o) - K_1$$

Thus plotting $1/C_g$ against $1/(D - D_o)$ should give a straight line The negative intercept on the ordinate is then K_1.

Experimental

1.5 ml of acetate buffer were pipetted into a 3 ml quartz cell thermostatted at 25.0°C, together with 0.25 ml of pyridoxal phosphate solution. The volume was made up to 3.0 ml with NaCl solution of the same ionic strength as the glutamate solution to be used. The optical density of the solution (0.2 mM in PyP)was measured at 24,500 cm^{-1}, the wavelength of an absorption peak in the absence of metal ions. The optical densities of solutions 0.2 mM in pyridoxal phosphate were then measured in the presence of various concentrations of sodium glutamate (32 to 112 mM). The ionic strength was kept constant with NaCl. The sodium glutamate solution had previously been adjusted to the pH of the buffer to prevent changes in the pH of the reaction mixture at high concentrations of glutamate.

Graphs of $1/C_g$ against $1/(D - D_o)$ were plotted at each pH (Fig 58). The 'best' straight line was drawn through the points and K_1 was measured as the negative intercept on the ordinate. The probable error was determined by drawing other 'possible' lines through the experimental points and measuring the deviations from the 'best' value.

The results are shown in Table 19.

Table 19

pH	3.95	4.57	5.04	5.86	6.37
K_1	6.4 ± 0.2	7.4 ± 0.5	7.3 ± 0.1	11.2 ± 0.5	16.1 ± 0.2

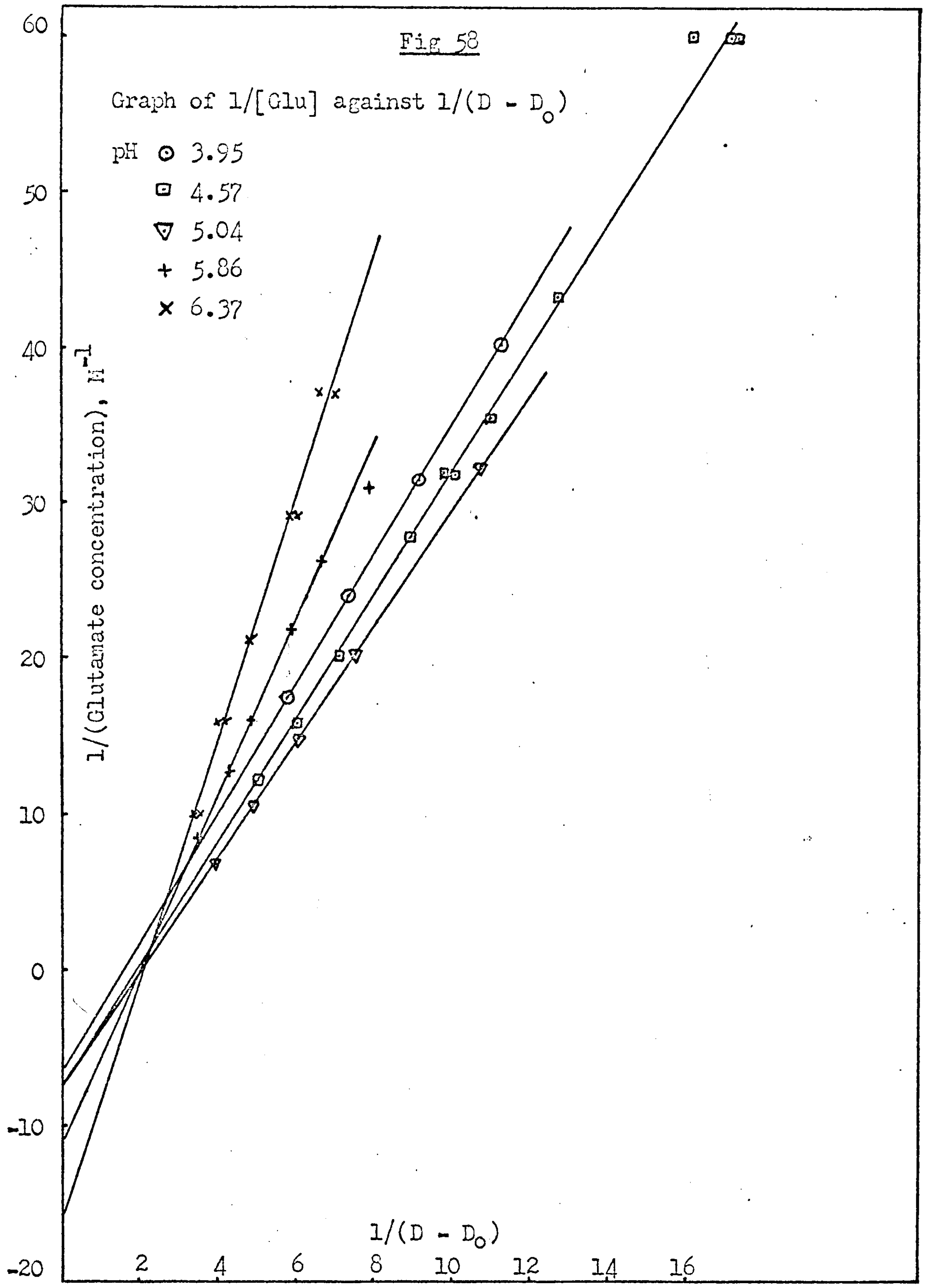


Fig 58
Graph of 1/[Glu] against 1/(D - D$_o$)
pH 3.95
4.57
5.04
5.86
6.37
1/(Glutamate concentration), M^{-1}
1/(D - D$_o$)
60
50
40
30
20
10
0
-10
-20
2
4
6
8
10
12
14
16

(b) Determination of the 'Mixed' Stability Constants of Schiff's Bases with various metals.

The results from p 136 suggest that metal ions can form 'mixed' complexes with Schiff's bases and their constituents. The evaluation of the stabilities of these complexes spectrophotometrically depends upon there being measurable spectral changes associated with each addition of ligand. This is true for the addition of Schiff's bases to the metal but may not necessarily be so for further addition of glutamate or pyridoxal phosphate. A potentiometric titration technique is therefore more suitable for this study.

Watters (48) successfully developed Bjerrum's method (43) of calculating the average number of each species associated to the metal in a system of two co-ordinating ligands (ethylenediamine and oxalic acid). The method is, however, dependent upon the basicities of the two ligands being widely different so that mixed complexes are not formed in the presence of appreciable concentrations of undissociated acid. This condition does not apply in the present although it may, as a first approximation, in the system M^{2+}-pyridoxamine phosphate-α-keto-glutarate, where the pK values of α-ketoglutaric acid are relatively low. For this reason Watters' method of calculation was tried.

If the overall stability constants for the formation of complexes of the type MP_pG_g are defined as:

$$Q_{pg} = [MP_pG_g]/[M][P]^p[G]^g \quad \text{omitting charges} \qquad (i)$$

Then the average number of pyridoxamine phosphate molecules associated to each metal ion is:

$$\bar{n}_p = \frac{[MP] + 2[MP_2] + [MPG] + 2[MP_2G] + [MPG_2] + 2[MP_2G_2]}{C_m}$$

$$= \frac{Q_{10}P + 2Q_{20}P^2 + Q_{11}P.G + 2Q_{21}P^2.G + Q_{12}P.G^2 + 2Q_{22}P^2.G^2}{1 + Q_{10}P + Q_{20}P^2 + Q_{11}P.G + Q_{21}P^2.G + Q_{12}P.G^2 + Q_{22}P^2G^2}$$

omitting square brackets for simplicity.

Similarly,

$$\bar{n}_g = \frac{Q_{01}G + 2Q_{02}G^2 + Q_{11}P.G + 2Q_{12}P.G^2 + Q_{21}P^2.G + 2Q_{22}P^2.G^2}{1 + Q_{01}G + Q_{02}G^2 + Q_{11}P.G + Q_{12}P.G^2 + Q_{21}P^2.G + Q_{22}P^2.G^2}$$

The equations containing the experimental variables are derived as follows:-

Total PyP $\quad P_t = AP + C_m\bar{n}_p \quad$ (ii)

and total αKG $\quad G_t = BP + C_m\bar{n}_g \quad$ (iii)

where A and B are functions of pH and pK values, relating the concentrations of anionic ligand to the total free ligand concentrations.

If the average number of protons associated to each unbound ligand are $\bar{n}_{ap}$ and $\bar{n}_{ag}$ then,

$$\bar{n}_{ap} = (H_t^p - p - [H^+]_p)/(P_t - \bar{n}_pC_m) \quad \text{(iv)}$$

and

$$\bar{n}_{ag} = (H_t^g - g - [H^+]_g)/(G_t - \bar{n}_gC_m) \quad \text{(v)}$$

Where:- p and g are the numbers of moles of OH^- required by each ligand to give the experimental value of $[H^+]$, $p + g = m$;

P_t and G_t are the total molar concentrations of PyP and αKG; H_t^g and H_t^p are the total molar protons contributed by each ligand.

Multiplying (iv) and (v) together and rearranging gives,

$$(\bar{n}_{ap}\bar{n}_p + \bar{n}_{ag}\bar{n}_g) = (\bar{n}_{ap}P_t + \bar{n}_{ag}G_t - H_t^p - H_t^g + m + [H^+])/C_m \qquad (v)$$

$\bar{n}_{ap}$ and $\bar{n}_{ag}$ can be calculated as before (p 91) and the factor $(\bar{n}_{ap}\bar{n}_p + \bar{n}_{ag}\bar{n}_g)$ evaluated quite easily from experimental results, but equations (ii), (iii) and (v) do not provide sufficient information to evaluate $\bar{n}_p$ and $\bar{n}_g$ separately, and the corresponding values of P and G against which $\bar{n}_p$ and $\bar{n}_g$ must be plotted.

A method similar to that employed by Leussing (49) was therefore used. He found the best theoretical fit of an experimental potentiometric titration curve by a 'least squares' calculation using an I.B.M. 7090 computer. His experimental data was obtained for a titration of Ni(11) and pyruvate with glycinate.

Theory

In a solution containing metal ions, pyridoxal phosphate and glutamate,

Total metal concentration

$$M_t = M^{2+} + MP^- + MP_2^{4-} + MG^o + MG_2^{2-} + MPG^{3-} + MPG_2^{5-} + MP_2G^{6-} + MP_2G_2^{8-} \qquad (vii)$$

where MP $\equiv$ [MP^-] etc.

Total PyP concentration

$$P_t = H_4P^+ + H_3P + H_2P^- + HP^{2-} + P^{3-} + MP^- + 2MP_2^{4-} + MPG^{3-} + MPG_2^{5-} + 2MP_2G^{6-} + 2MP_2G_2^{8-} + SB' \qquad (viii)$$

Total glutamate concentration

$$G_t = H_3G^+ + H_2G + HG^- + G^{2-} + MG + 2MG_2^{2-} + MPG^{3-} + 2MPG_2^{5-} + MP_2G^{6-} + 2MP_2G_2^{8-} + SB' \quad (ix)$$

and total replaceable hydrogen

$$H_t = 3H_3G^+ + 2H_2G + HG^- + 4H_4P^+ + 3H_3P + 2H_2P^- + HP^{2-} + H^+ + OH^- \quad (x)$$

OH^- represents the total alkali added initially or during the course of the titration. H^+ is the concentration of dissociated hydrogen ions.

Using the above definition of the overall stability constants of the system Q_{pg}, and defining the Schiff's base formation constant as

$$Q_{sb} = [\text{Total free SB'}]/P.G \quad (xi)$$

The overall stability constants are used as these are thermodynamic entities. The stepwise constants would be expected to vary depending upon the order of ligand association, and would also contain statistical discrepancies.

The stability constants defined above would not be expected to have the same values as those calculated earlier (p 129) as the latter incorporate the pK values of the free Schiff's base and are therefore pH dependent. The constants Q_{pg} are calculated in terms of $[P^{3-}]$ and $[G^{2-}]$ only and their values are independent of pH. Furthermore, the method need draw no distinction between the possibilities

(a) complexing of the already formed Schiff's base

and (b) prior complexing of PyP and Glu followed by intramolecular condensation.

Thus it is immaterial whether or not the pyridoxal phosphate and the glutamate have already condensed or will subsequently do so. The only reason for the introduction of Q_{sb} is to take into account the removal of pyridoxal phosphate and glutamate due to the formation of free Schiff's base, and not as a vital part of the calculation (as was the case in the previous method p 125). As a first approximation Q_{sb} could be ignored as it is not very great at pH values below 7.

The dissociation constants of glutamic acid are given by:-

$$k_1' = a[G^{2-}]/[HG^-]$$

$$k_2' = a[HG^-]/[H_2G] \qquad \text{(xii)}$$

and $$k_3' = a[H_2G]/[H_3G^+]$$

and those of pyridoxal phosphate by:-

$$k_1 = a[P^{3-}]/[HP^{2-}]$$

$$k_2 = a[HP^{2-}]/[H_2P^-]$$

$$k_3 = a[H_2P^-]/[H_3P] \qquad \text{(xiii)}$$

and $$k_4 = a[H_3P]/[H_4P^+]$$

Substituting equations (i), (xi), (xii) and (xiii) into equations (vii)-(x) gives:

$$M_t = M(1 + Q_{10}P + Q_{20}P^2 + Q_{01}G + Q_{02}G^2 + Q_{11}P.G + Q_{12}P.G^2 + Q_{21}P^2G + Q_{22}P^2G^2) \qquad \text{(xiv)}$$

omitting concentration brackets and charges for simplicity.

$$P_t = P(1 + a/k_1 + a^2/k_1k_2 + a^3/k_1k_2k_3 + a^4/k_1k_2k_3k_4) + Q_{sb}P.G + M.P(Q_{10} + 2Q_{20}P + Q_{11}G + Q_{12}G^2 + 2Q_{21}P.G + 2Q_{22}P.G^2) \qquad \text{(xv)}$$

$$G_t = G(1 + a/k_1' + a^2/k_1'k_2' + a^3/k_1'k_2'k_3') + Q_{sb}P.G + M.G(Q_{01} + 2Q_{02}G + Q_{11}P + 2Q_{12}P.G + Q_{21}P^2 + 2Q_{22}P^2G) \quad \text{(xvi)}$$

$$H_t = G(a/k_1' + 2a^2/k_1'k_2' + 3a^3/k_1'k_2'k_3') + H^+ + OH^- + P(a/k_1 + 2a^2/k_1k_2 + 3a^3/k_1k_2k_3 + 4a^4/k_1k_2k_3k_4) \quad \text{(xvii)}$$

When account has been taken of the dilution caused by adding v ml of titrant to 100 ml of solution, the terms in P_t, G_t, H_t and M_t decome $100P_t/(100 + v)$ etc.

The procedure was to assume values of Q_{11}, Q_{12}, Q_{21} and Q_{22} and to calculate a theoretical titration curve. The constants were then systematically altered to give a 'least squares' fit between experimental and calculated curves. Several methods were attempted which differed in the calculated variable used in the least squares operation. These were:

(a) Residuals in pH.

This method is basically the one employed by Leussing (49). A solution of pyridoxal phosphate and metal salt was titrated with 0.05 M disodium glutamate. OH^- in the above equations was zero throughout.

The theoretical pH was calculated as follows:-

When equations (xv) and (xvi) are rearranged in terms of a^3 and a^4 this gives,

$$a^4/k_1k_2k_3k_4 = 100P_t/(100 + v)P - B.M - Q_{sb}G - (1 + a/k_1 + a^2/k_1k_2 + a^3/k_1k_2k_3) \quad \text{(xviii)}$$

and

$$a^3/k_1'k_2'k_3' = 100\,G_t/(100 + v)G - A.M \div Q_{sb}P - (1 + a/k_1' + a^2/k_1'k_2') \quad \text{(xix)}$$

where

$$A = Q_{01} + 2Q_{02}G + Q_{11}P + 2Q_{12}P.G + Q_{21}P^2 + 2Q_{22}P^2G$$

and $$B = Q_{10} + 2Q_{20}P + Q_{11}G + 2Q_{21}P.G + Q_{12}G^2 + 2Q_{22}P.G^2$$

Substituting a^3 from equation (xix) into (xviii); a^4 and a^3 from equations (xviii) and (xix) into (xvii) and rearranging gave,

$$A'a^2 + B'a + C' = 0 \qquad \text{(xx)}$$

where $$A' = (2G/k_1'k_2' - k_3'[3G/k_1'k_2'k_3' - P/k_1k_2k_3] - 2P/k_1k_2)$$

$$B' = (G/k_1' - k_2'k_3'[3G/k_1'k_2'k_3' - P/k_1k_2k_3] - 3P/k_1)$$

and $$C' = k_1'k_2'k_3'(3G/k_1'k_2'k_3' - P/k_1k_2k_3)(100G_t/[100 + v]G - AM - Q_{sb}P - 1)$$
$$+ 4P(100P_t/[100 + v]P - B.M - Q_{sb}G - 1)$$
$$+ OH^- - 100H_t/(100 + v) + H^+$$

Thus for any given values of A', B' and C' the corresponding value of a can be calculated from equation (xx) by

$$a = \frac{-B' - (B'^2 - 4A'C')^{\frac{1}{2}}}{2A'} \qquad \text{(xxi)}$$

(The minus sign is used in equation (xxi) as this gives the positive root - i.e. A' turns out to be negative).

The constant term C' includes a term H^+ which is in fact an experimental variable. In practice H^+ was included in the B' term by using the relationship:

$$H^+ = a^{0.98} \tag{xxii}$$

As it is difficult to incorporate an evaluation of this in the program, eqation (xxii) was rewritten as,

$$H^+ = Fa$$

where F is a factor greater than unity which depends upon the pH. F was evaluated at 0.5 pH unit intervals from 2.0 to 7.5 and stored in the computer. The program was designed to linearly interpolate the appropriate value of F each time the pH was calculated from equation (xxi).

Equations (xiv), (xv) and (xvi) had to be solved for M, P and G so that corresponding values of these could be substituted into equation (xxi) to give the calculated pH. As each solution required all the values of M, P, G, v and a, initial guesses had to be made for those which were not available until some later stage in the program, and the set of solutions re-iterated until consecutive solutions did not differ by more than specified amounts.

These solutions were carried out as follows:-

Rearranging equation (xv) gave,

$$f(P) = P(1 + a/k_1 + a^2/k_1k_2 + a^3/k_1k_2k_3 + a^4/k_1k_2k_3k_4) + B.M.P + Q_{sb}P.G - 100P_t/(100 + v) \tag{xxiii}$$

f(P) is zero only at 'correct' values of P and G. These values were found by assuming a value for G (as equation (xvi) is explicit in G_t it was simpler to guess values of G and calculate G_t after solution of the equations for P, than vice versa) and increasing P by finite steps (usually by doubling its previous value) until $f(P)$ changed sign. The 'correct' value of P then lay between the last and penultimate values of P. The root was then found to within the required tolerance (0.2% of P was taken for this) by means of a Newton-Raphson refinement (50). This refinement (based on Taylor's theorem) involved the re-iteration of the calculation

$$P_{n+1} = P_n - f(P)/f'(P) \qquad \text{(xxiv)}$$

where P_{n+1} is a better approximation to the true root than P_n, and $f'(P)$ is the derivative of $f(P)$ with respect to P.

i.e.
$$\begin{aligned} f'(P) = {} & (1 + a/k_1 + a^2/k_1k_2 + a^3/k_1k_2k_3 + a^4/k_1k_2k_3k_4) \\ & + M(Q_{10} + 4Q_{20}P + Q_{11}G + Q_{12}G^2 + 4Q_{21}P.G + 4Q_{22}P.G^2) \\ & - P.B^2.M^2/M_t + Q_{sb}G \end{aligned}$$

The procedure was, then, to select a value of G and to find the appropriate value of P which made $f(P) = 0$. The theoretical value of a was then calculated from equation (xxi) and tested to see if it was positive. If it was not, P was further incremented to find the second root until such a time as a was positive. (In actual fact, Descartes rule of signs* shows that there is only one positive root for $f(P)$ when all the stability constants are positive. However, the program had to be written so other roots could be found if the constants were not positive because the method of curve fitting involved changing the signs of the constants to observe the effect on the residuals). The total concentration of glutamate G_t was then evaluated from equation (xvi), and the volume of titrant (v of 0.05 M disodium glutamate) required to give that value of G_t was calculated.

The experimental data stored in the computer consisted of the set of readings of volume of titrant against pH. From the value of v, calculated as above, the appropriate experimental pH was interpolated (by a Lagrange interpolation (51) using the calculated v and the nearest experimental v bracketed by two others) and the value of $(pH_{calc} - pH_{expt})$ found. The procedure was repeated for about 20 values of G and the pH residual calculated. The residual U is defined as:-

$$U = \sum (pH_{calc} - pH_{expt})^2$$

The summation is carried out over all the trial G values.

The method was to minimise U by means of a library 'routine' (52) which systematically altered the guessed values of Q_{11}, Q_{12} etc. Computation was designed to cease when successive calculations of U did not cause any of the constants to be altered by more than 2% of its value at that time.

The value of Q_{sb} required during execution of the program was found by interpolation of experimental values of Q_{sb}/pH. These values were obtained as follows. A graph of K_1 (from p 143, and

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* This states that the number of positive roots of a polynomial with real coefficients is equal to the number of sign changes of that polynomial.

including a value of $K_1 = 80$ at pH 7.5 (53)) against pH was plotted and values of K_1 were interpolated at 0.2 pH unit intervals from pH 3.5 to 7.6. These K_1 values were expressed in terms of total pyridoxal phosphate and total glutamate concentrations whereas Q_{sb} was required in terms of P^{3-} and G^{2-}. When the necessary conversion had been carried out (by using the known pK values of PyP and Glu) it was found that Q_{sb} changed by powers of ten for each pH increment of 0.2. As interpolation of such data might have produced spurious results, Q_{sb} was divided by a^3. Q_{sb}/a^3 was reasonably constant over the pH range of interest and so this was the form in which the data was fed into the computer. A Lagrange interpolation (using the calculated pH and the three nearest stored pH values) was carried out at each iterative step of the solutions described above, and the interpolated value of Q_{sb}/a^3 multiplied by a^3.

As the values of G increased uniformly the program was designed to use the 'correct' values of P, a, v and Q_{sb} obtained at one G value as initial guesses for the next, and to return to the values set at the start of the program only after the last G value had been used. The computer was then ready to take the first G value again when U was next calculated.

It was found that, during execution of the program, the iterations did not produced the expected convergence to give the required results, but diverged with increasing rapidity. The program usually faulted when erroneous values made certain instructions ridiculous (e.g. instructions to find the square root of a number which turned out to be negative).

Possible reasons for the divergence are:

(i) a, as calculated from equation (xxi), is sensitive to only the first power of G_t, whereas G_t, as calculated from equation (xvi), is sensitive to powers of a up to 3. Thus any error in a produces a larger error in G_t which in turn produces an even larger one in a.

(ii) The region of the experimental curve necessarily used in the determination of the constants occurred in the pH range where the terms $a^2/k_1'k_2'$ and $a^3/k_1'k_2'k_3'$ in equation (xvi) are more important than the terms 1 and a/k_1'. This accentuated the dependence of G_t on a^3.

Leussing (49) worked with a system in which the highest powers of a encountered were squared terms. He also worked in a pH range where, presumably, these squared terms were of little importance and thus avoided the difficulties experienced here.

(b) Residuals in volume of titrant.

Obviously any method which depends upon the evaluation of pH will not converge. In this method the pH was fixed at an experimental value and the theoretical volume of 0.05 M disodium glutamate required to give this pH was calculated.

The method of finding the required value of P was the same as in (a) above. G, however, was evaluated explicitly for each trial P by rearranging equation (xvii):

$$G = \frac{100H_t/(100 + v) - H^+ - P(a/k_1 + 2a^2/k_1k_2 \ldots\ldots\ 4a^4/k_1k_2k_3k_4)}{a/k'_1 + 2a^2/k'_1k'_2 + 3a^3/k'_1k'_2k'_3} \qquad \text{(xxv)}$$

M was evaluated from equation (xiv) and the resulting values substituted into equation (xvi). The volume v was then calculated as

$$v = 100G_t/(0.05 - G_t)$$

The whole operation was repeated until successive iterations produced changes in v of no greater than 0.01 ml. P was incremented as before and the whole operation repeated until f(P) became zero. (In the final stages P was altered by the Newton-Raphson method described above).

The summation

$$U = \sum_{\substack{\text{all expt} \\ \text{pH values}}} (v_{calc} - v_{expt})^2$$

was carried out and U minimised by means of the library routine.

It was found in practice that the evaluation of U took so long that it could not be performed the number of times necessary (12 times in this case) for one complete cycle of the library routine in the time requested (about 50 seconds). As only one iteration of the stability constants takes place per cycle of the library routine, it would obviously be very expensive in computer time to carry out the estimated number of cycles necessary (about 10). However, the program was of use, in the absence of the library routine, to study the effect of variation of the values of the stability constants on the computed volume of titrant.

Seven experimental points were taken from the titration of Cu(11) and pyridoxal phosphate with disodium glutamate, and the volume of titrant was calculated at each for several values of the four constants Q_{pg}. The calculated and experimental volumes are compared in Table 20. The constants Q_{pg} were increased in turn by a factor of 10^2 within the limits of 10^{17} to 10^{27} for Q_{11} and Q_{12}, and 10^{20} to 10^{30} for Q_{21} and Q_{22}.

The time taken to evaluate these results was reduced to a fifth by rearranging equation (xv) explicitly in terms of P:-

$$P = \frac{100P_t/(100 + v) - Q_{sb} - B.M}{1 + a/k_1 + a^2/k_1k_2 + a^3/k_1k_2k_3 + a^4/k_1k_2k_3k_4} \qquad \text{(xxvii)}$$

This meant that the incremental increase of P and the Newton-Raphson refinement could be omitted. G was calculated from equatión (xxv); G_t from (xvi); v from (xxvi) and P from (xxvii). The procedure was repeated until v changed by less than 0.01 ml between successive iterations.

Results calculated in this way agreed exactly with those shown in Table 20.

Table 20

	v Expt	Calculated v ml 10^{17}	10^{19}	10^{21}	10^{23}	10^{25}	10^{27}
Q_{11}							
	2.91	1.43	1.39	7.63	13.57	13.69	13.69
	6.78	3.43	8.08	15.30	15.30	15.31	15.31
	9.79	16.38	19.99	20.07	20.07	20.07	20.07
	12.12	22.39	23.05	23.06	23.07	23.07	23.07
Q_{12}							
	6.78	3.43	3.43	3.43	3.43	3.43	3.83
	9.79	16.37	16.37	16.38	16.46	20.71	24.92
	12.12	22.39	22.39	22.39	22.78	27.32	27.98
	15.85	26.71	26.71	26.73	28.37	31.58	31.68
	19.90	29.43	29.43	29.53	32.80	34.31	34.33
	26.10	34.17	34.18	35.02	38.81	39.04	39.04
Q_{21}		10^{20}	10^{22}	10^{24}	10^{26}	10^{28}	10^{30}
	6.78	3.43	3.43	3.43	3.43	4.37	15.02
	9.79	16.38	16.38	16.38	19.91	24.65	25.82
	12.12	22.44	22.44	22.93	27.41	29.28	29.26
	15.85	26.71	26.71	29.67	32.73	33.01	33.04
	19.90	29.42	29.42	33.35	34.23	34.25	34.26
	26.10	34.17	34.17	34.91	34.92	34.93	34.94
Q_{22}							
	15.85	26.71	26.71	26.71	26.71	26.79	30.65
	26.10	34.18	34.18	34.19	35.50	39.35	39.89

The results in Table 20 show that, for experimental volumes greater than 6.78 ml, the smallest calculated volumes are

(i) independent of the values of any of the constants;

and (ii) consistently much larger than the experimental values.

The independence of the minimum values of v on the values of the constants means that v_{min} is a function only of the stability constants of the simple complexes of pyridoxal phosphate and glutamate with Cu(11). The fact that v_{min} is still too large, even without mixed complex formation, indicates that the original equations cannot fully describe the system.

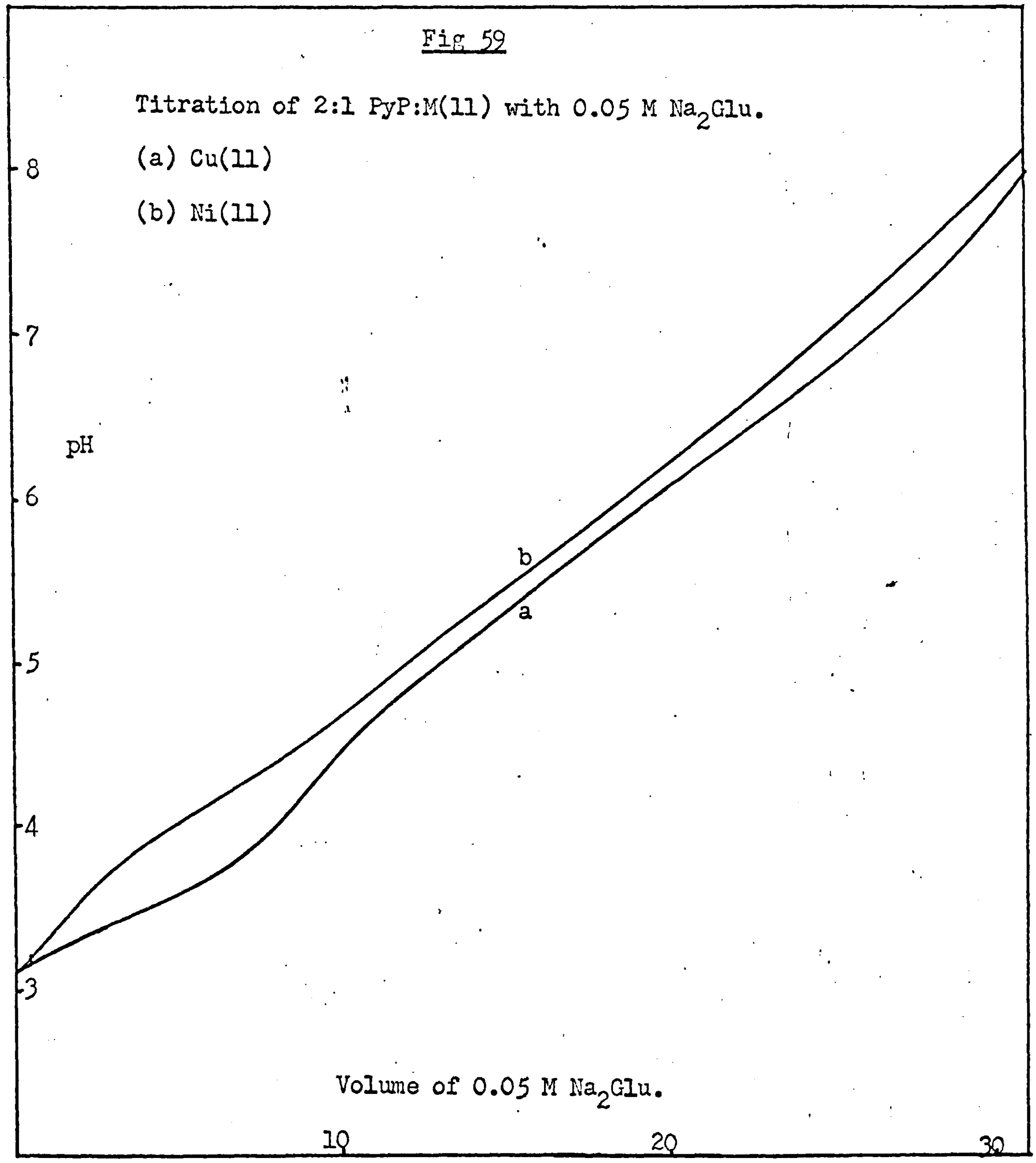
The experimental titration curves for Cu(11) and Ni(11) with pyridoxal phosphate are shown in Fig 59.

As the pH of these solutions only rose to about 7.5 after ca. 30 ml of titrant (0.05 M Na_2Glu) had been added, the buffer action of the excess glutamate (present in excess of a 1:2 metal:glutamate ratio after only 10 ml of titrant had been added) influenced the pH of the solution more than was desirable in the range where higher complexes would be formed. This led to the third method attempted.

(c) Titration of metal, pyridoxal phosphate and glutamate with standard alkali.

50 ml of 0.01 M pyridoxal phosphate, 10 ml of M KCl, 5.0 ml of 0.05 M $CuSO_4$, 10 ml of 0.05 M monosodium glutamate and 10 ml of 0.100 N HCl were pipetted into the titration apparatus described earlier (p 99) and the volume made up to 100 ml with CO_2- free distilled water. The solution was titrated with 0.100 N sodium hydroxide. The

titration was repeated with 5.102 x10^{-2} M $NiSO_4$. These titration curves are shown in Fig 60 along with the curve produced in the absence of any metal.



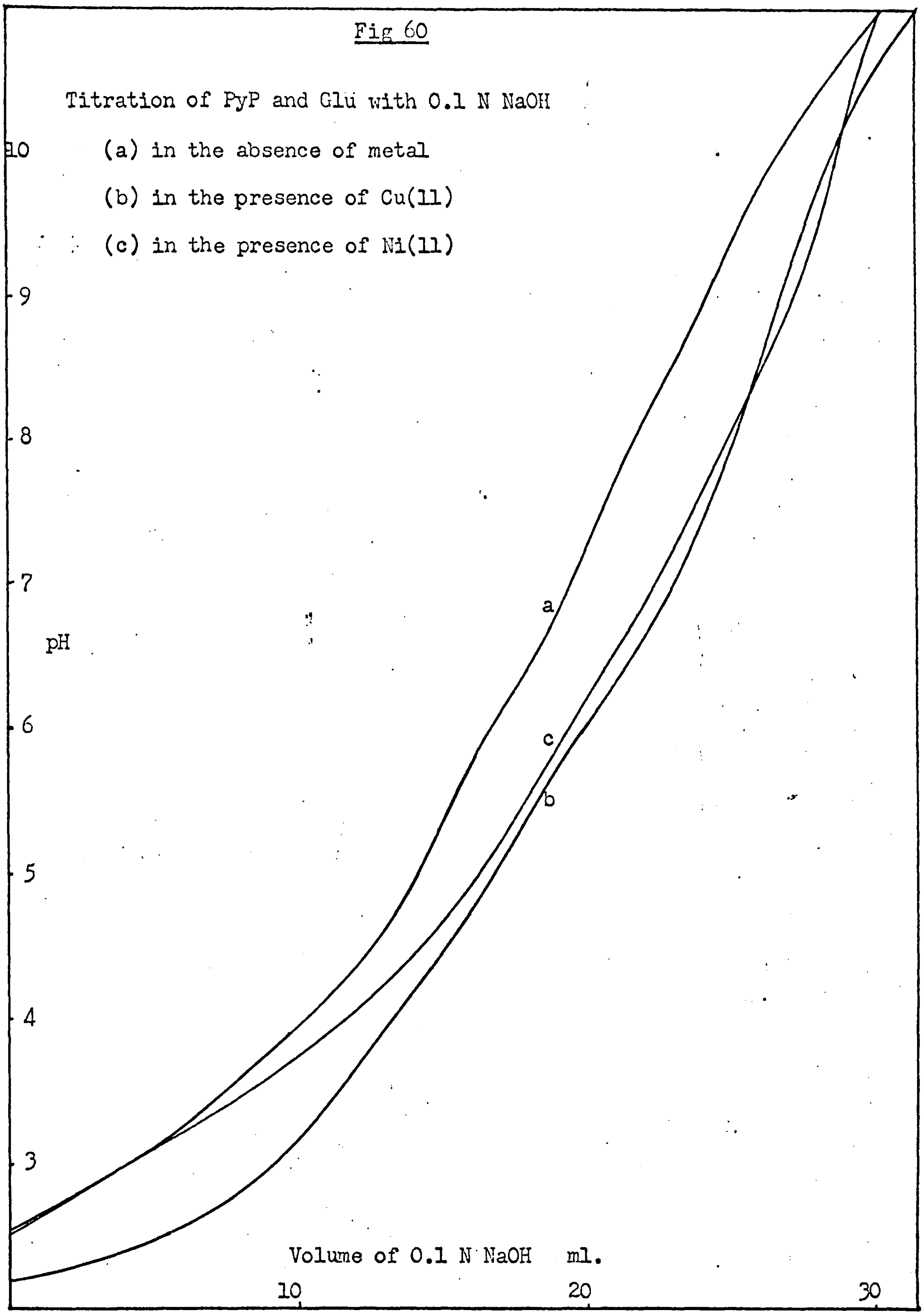


Fig 60
Titration of PyP and Glu with 0.1 N NaOH
(a) in the absence of metal
(b) in the presence of Cu(11)
(c) in the presence of Ni(11)
10
9
8
7
6
5
4
3
pH
a
c
b
Volume of 0.1 N NaOH ml.
10
20
30

The program for the treatment of this experimental data was initially designed to calculate the volume of titrant required to give the experimental pH. However, iterative solutions of the explicit equations in P, G and v (i.e. a rapid method of computation similar to that discussed above) did not converge. The problem was overcome by keeping both the experimental pH and the volume fixed and calculating the theoretical volume of 0.05 M glutamate (calculated as Na_2Glu) required to give those conditions. This volume was compared with the actual of glutamate used (10 ml) and the difference taken as a measure of the accuracy of the estimated stability constants. The process was repeated for several experimental pH/volume readings.

This method is then identical to the second method of section (b) above except that the total concentration of hydrogen ions H_t has different values at each experimental pH. G is then evaluated from a modification of equation (xxv):-

$$G = \frac{(100H_t - 0.1V)/(100 + V) - H^+ - P(a/k_1 + 2a^2/k_1k_2 \ldots\ldots 4a^4/k_1k_2k_3k_4)}{a/k_1' + 2a^2/k_1'k_2' + 3a^3/k_1'k_2'k_3'}$$

Here V is the experimental volume of OH^-.

The theoretical volume v of glutamate is calculated from equation (xxvi), P from equation (xxvii) and the concentration of free metal ions M from equation (xiv).

The results of these calculations for the Cu(11) system are shown in Table 21. The stability constants Q_{pg} were varied as follows:-
Q_{11} in steps of 10 from 10^{17} to 10^{20}; Q_{12} in steps of 10 from 10^{22} to 10^{25};

Table 21

V	Stability Constants (expressed as logs)				v
	Q_{11}	Q_{12}	Q_{21}	Q_{22}	
2.10	17	22	25	25	5.47
7.50	17	22	25	25	5.39
12.28	17	22	25	25	7.38
	18				8.37
	19				13.71
	20				19.12
	17	22	27	25	7.55
			29		14.72
			31		24.68
13.90	17	22	25	25	12.00
	18				17.90
	19				20.70
	20				21.15
	17	22	27	25	19.10
			29		26.54
			31		28.45
19.91	17	22	25	25	25.72
	18				24.54
	19				22.08
	20				19.44
	19	24	27	25	25.53
			29		25.79
			31		25.81

V	Q_{11}	Q_{12}	Q_{21}	Q_{22}	v
19.91	20	22	27	25	19.45
		23			24.45
		24			24.47
		25			24.58
27.12	17	22	25	25	12.22
	18				11.89
	17	23	25	25	12.31
		24			12.82
		25			14.03

Q_{21} in steps of 10^2 from 10^{25} to 10^{31} and Q_{22} in steps of 10^4 from 10^{25} to 10^{29}. Only those variations of the stability constants which caused a change in v are shown in the above table.

Apart from those results corresponding to low experimental pH values, the calculated volumes of glutamate are well above the required value of 10 ml. The discrepancy is greatest in the middle of the pH range. Obviously no single set of values for the stability constants Q_{sb} will fit all the experimental points equally well. This supports the suggestion made earlier that other equilibria that are not accounted for in the theory so far must be present in the system.

An indication of the nature of these other equilibria is given on p 116-122. The values of the stability constants of pyridoxamine phosphate and metal ions were found to be affected by the degree of protonation of the complexes formed. As the complexes of Schiff's bases form at a much lower pH than the complexes of their constituents (Fig 60), it may be expected that the effects of such a protonation would be much more important.

In order to compensate for these effects, the dissociation constants of the various p[illegible] complexes are required. As a first approximation the pK values of the complexes were assumed to be the same as the unco-ordinating groups in the free ligand. (It was also necessary to assume that the metal co-ordinated to the imine notrogen and to the phenolic oxygen, - see Introduction). It has been shown by several authors (see Introduction) that the pK of the ring nitrogen does not remain unaltered in the complexes of Schiff's bases, but is decreased by amounts depending upon the metal involved. The program was written to allow for variation of this pK to study the effect on the 'best' set of constants. Protonation of the simple complexes was ignored.

The correction for protonation of the various complexes was carried out by multiplying each term in equations (vii) to (ix) that refers to a mixed complex, by a factor which depends upon the the number of dissociable groups of that complex and the pK values of these groups. These factors were designated the symbols W(10) to W(12) for the terms MPG^{3-}, MPG_2^{5-}, MP_2G^{6-} and $MP_2G_2^{8-}$ respectively. Each factor is then the ratio between the total concentration of a mixed species and the concentration of its anion.

Equation (x) had also to be altered to take into account the number of protons associated to each mixed species. The symbols W(16) to W(19) were used for this purpose. Then:-

$$W(10) = 1 + a/k_5 + a^2/k_5k_2 + a^3/k_5k_2k'_2 + a^4/k_5k_2k'_2k'_3$$

$$W(11) = 1 + a/k_5 + a^2/k_5k_2 + a^3/k_5k_2k'_2 + a^4/k_5k_2k'^2_2 + a^5/k_5k_2k'^2_2k'_3 + a^6/k_5k_2k'^2_2k'^2_3$$

$$W(12) = 1 + a/k_5 + a^2/k_5^2 + a^3/k_5^2k_2 + a^4/k_5^2k_2^2 + a^5/k_5^2k_2^2k'_2 + a^6/k_5^2k_2^2k'_2k'_3$$

$$W(13) = 1 + a/k_5 + a^2/k_5^2 + a^3/k_5^2k_2 + a^4/k_5^2k_2^2 + a^5/k_5^2k_2^2k'_2 + a^6/k_5^2k_2^2k'^2_2 + a^7/k_5^2k_2^2k'^2_2k'_3 + a^8/k_5^2k_2^2k'^2_2k'^2_3$$

and

$$W(16) = a/k_5 + 2a^2/k_5k_2 + 3a^3/k_5k_2k'_2 + 4a^4/k_5k_2k'_2k'_3$$

$$W(17) = a/k_5 + 2a^2/k_5k_2 + 3a^3/k_5k_2k'_2 + 4a^4/k_5k_2k'^2_2 + 5a^5/k_5k_2k'^2_2k'_3 + 6a^6/k_5k_2k'^2_2k'^2_3$$

$$W(18) = a/k_5 + 2a^2/k_5^2 + 3a^3/k_5^2k_2 + 4a^4/k_5^2k_2^2 + 5a^5/k_5^2k_2^2k'_2 + 6a^6/k_5^2k_2^2k'_2k'_3$$

$$W(19) = a/k_5 + 2a^2/k_5^2 + 3a^3/k_5^2k_2 + 4a^4/k_5^2k_2^2 + 5a^5/k_5^2k_2^2k'_2 + 6a^6/k_5^2k_2^2k'^2_2 + 7a^7/k_5^2k_2^2k'^2_2k'_3 + 8a^8/k_5^2k_2^2k'^2_2k'^2_3$$

In the above equations k_5 has been substituted for the dissociation constant of the ring nitrogen (previously k_2 - see p 149). Terms which include the dissociation constant of the primary phosphate, k_4, have been omitted as these should be small compared eith the other terms in the pH range where protonation of the complexes is important. Furthermore, the inclusion of k_4 would have caused terms in a^{10} to appear in the calculation. This means that at a pH value of 10 the magnitude of these terms would have approached the 'overflow' value of the computer cells of about 10^{-120}.

Including the factors W(16) to W(19) in the equation for H_t (x) gives:

$$H_t = G(a/k_1' + 2a^2/k_1'k_2' + 3a^3/k_1'k_2'k_3') + H^+ + OH^- + Q_{11}W(16)P.G$$
$$+ Q_{12}W(17)P.G^2 + Q_{21}W(18)P^2G + Q_{22}W(19)P^2G^2$$
$$+P(a/k_1 + 2a^2/k_1k_2 + 3a^3/k_1k_2k_3 + 4a^4/k_1k_2k_3k_4)$$

The value of k_5 was initially taken as that of the dissociation constant of the ring nitrogen in pyridoxal phosphate (pK = 8.69 - see p 106). The values of the stability constants Q_{pg} were varied between wide limits and the effect on the calculated volume of glutamate observed. A selection of these results is shown in Table 22 for the system Cu(11)/pyridoxal phosphate/glutamate, and in Table 23 for the system Ni(11)/pyridoxal phosphate/glutamate. Q_{22} has been omitted as it was found to have no effect on the computed volume at any experimental point. Q_{12} is included although it had only a limited effect on v.

The results for the Cu(11) system are consistently low and this made it difficult to pick out the 'best' set of constants. The results do, however, show that the most important species in solution are those of CuPG and CuP_2G. The results for the Ni(11) system show that there exist values of the constants for which the calculated volumes are greater than 10 ml (as would be expected if the guessed values of the constants were too small). By picking out all the values of the constants which gave values of v as near 10 ml as possible, it was found that the most consistent set of results for all the experimental

Table 22

V	Q_{11}	Log Q_{pg} Q_{12}	Q_{21}	pk_5 = 8.69 v	5.6 v
2.10	6	6	6	5.47	5.47
	8			5.47	5.47
	10			5.00	5.47
	12			0.53	5.43
	6	6	9	5.47	5.47
			12	5.46	5.47
			15	1.79	5.47
7.50	6	6	6	5.39	5.39
	8			5.34	5.39
	10			2.88	5.38
	12			0.07	5.02
	6	6	9	5.39	5.39
			12	5.10	5.39
			15	0.09	5.39
12.28	6	6	6	7.22	7.26
	8			4.53	7.26
	10			0.12	6.91
	12			0.01	1.17
	6	6	9	7.06	7.26
			12	0.27	7.26
			15	0.00	7.14

			pk_5 =	8.69	5.6
V	Q_{11}	Q_{12}	Q_{21}	v	v̇
13.90	6	6	6	8.52	8.70
	8			2.75	8.68
	10			0.05	7.39
	12			0.00	0.45
	6	6	9	7.66	8.70
			12	0.07	8.70
			15	0.00	8.05
19.91	6	6	6	7.40	12.65
	8			0.58	11.70
	10			0.01	3.03
	6	10	6	7.40	12.65
		12		6.97	12.65
	6	6	9	3.54	12.65
			12	0.01	12.59
			15	0.00	4.21
27.12	6	6	6	2.92	13.27
	8			0.03	
	6	8	6	2.84	13.21
		10		2.84	11.24
		12		1.50	
	6	6	9	1.95	11.29
			12	0.00	2.87

Table 23

V	Q_{11}	Q_{12}	Q_{21}	v
5.42	4	4	4	11.91
	6			11.90
	8			11.25
	10			1.66
	6	6	7	11.91
			10	11.80
			13	1.04
10.88	4	4	4	10.12
	6			10.03
	8			5.16
	10			0.11
	6	6	7	10.02
			10	7.03
			13	0.03
16.82	4	4	4	7.46
	6			5.68
	8			0.22
	6	6	7	5.59
			10	0.28
23.97	4	4	4	11.56
	6			1.03
	4	6	4	11.55
		8		11.11
		10		5.46

V	Q_{11}	Q_{12}	Q_{21}	v
23.97	4	4	7	5.27
			10	0.03
28.50	4	4	4	9.96
		6		9.89
		8		8.43
	4	4	7	7.61
			10	0.19

points was $Q_{11} = 10^5 \pm 10$ and $Q_{21} = 10^6 \pm 10$. The only value that could be assigned to Q_{12} was $< 10^8$. This was because the insensitivity to Q_{12} of the computed volume prevented the comparison of the effects of varying Q_{12} at several experimental points.

In order to see if the low results for the Cu(11) system were caused by the assumption that the pK of the ring nitrogen remained constant during chelation, the effect of putting $pk_5 = 5.6$ was observed. (This is the value obtained by Davies (36) for the copper pyridoxylidene-valine system). The results are shown in the last column of Table 22. They indicate that the most suitable constants have a much higher average value than when k_5 is assumed to be unaffected by chelation. The results at higher pH values (i.e. higher values of V in Table 22) are also more satisfactory in that v can have values greater than 10 ml (e.g. when the constants are too small). However, the results are not consistent enough throughout the range for an estimate of the 'best' set of constants to be made.

This method of determining mixed stability constants was adapted for the system M(11)/pyridoxamine phosphate/αketoglutarate. The adaptation consisted simply of altering the pK values of the respective ligands and the order in which they appeared in various parts of the program. Although the adaptation was quite simple, it was found that computation time was increased about 10-fold and that the computed volumes became negative at high pH values.

A selection of these results are shown in Table 24 for the Cu(11) and Ni(11) systems. The titration curves are shown in Fig 61.

Table 24

	V	Q_{11}	Q_{12}	Q_{21}	v
Cu(11)					
	7.23	6	6	6	9.37
		8			9.12
		10			2.47
		8	8	6	9.12
			10		9.00
			12		5.15
	12.29	6	6	6	6.28
		8			4.61
		10			0.17
		6	8	6	6.28
		8			4.61
		10			0.17
		6	8	6	6.28

V	Q_{11}	Q_{12}	Q_{21}	v
12.29	6	10	6	5.77
		12		1.75
18.27	6	6	6	-10.53
<u>Ni(11)</u>				
7.51	6	6	6	11.12
	8			10.46
	10			1.52
	6	8	6	11.11
		10		10.72
		12		4.31
13.95	6	6	6	10.69
	8			1.92
	6	8	6	10.51
		10		5.62
		12		0.79
	6	6	9	10.69
			12	10.68
			15	6.69
	6	6	6	-1.76

These results show that the constant Q_{12} is more important in the PamP-αKG than in the PyP-Glu system.

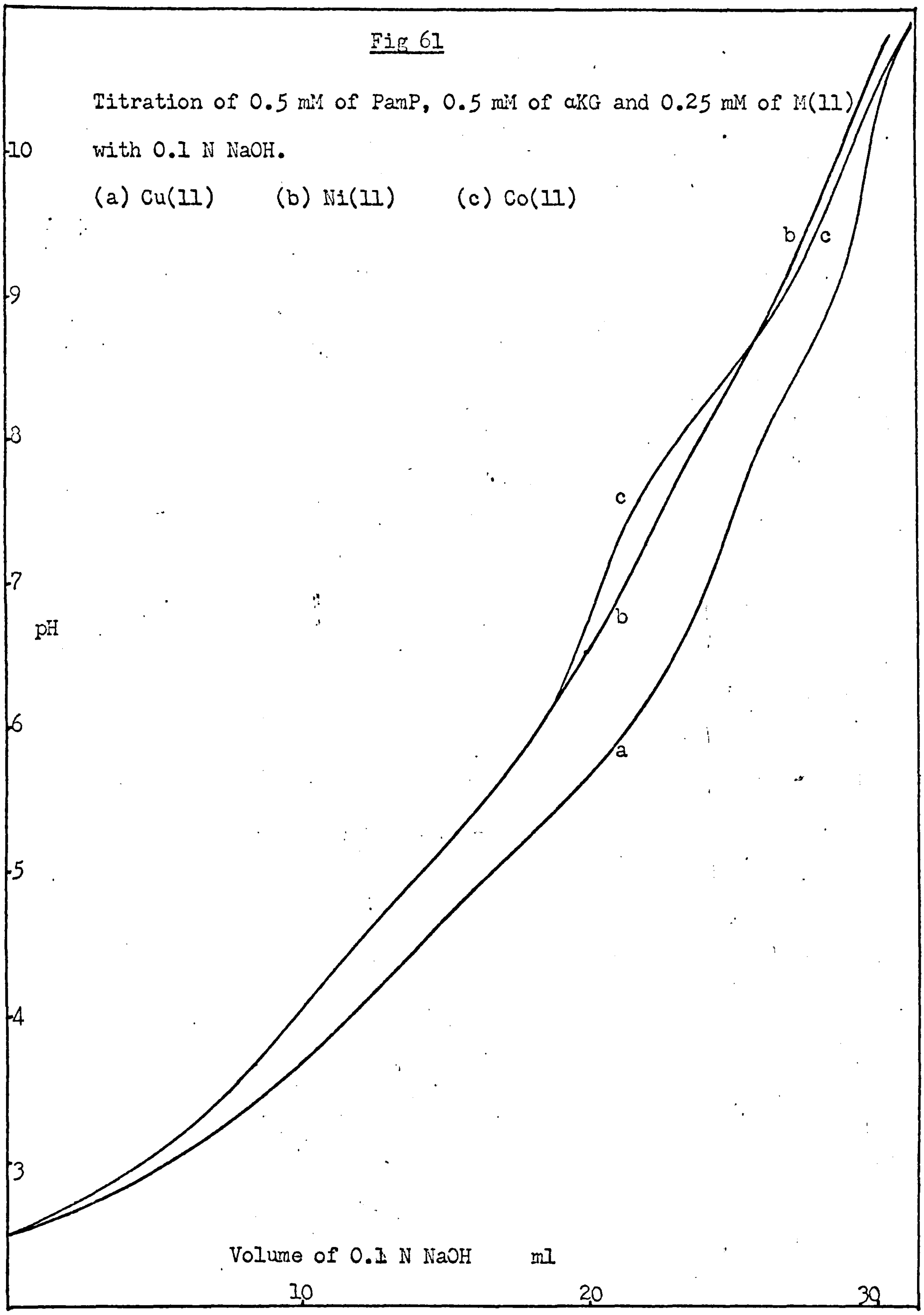


Fig 61

Titration of 0.5 mM of PamP, 0.5 mM of αKG and 0.25 mM of M(11) with 0.1 N NaOH.

(a) Cu(11) (b) Ni(11) (c) Co(11)

Discussion

The complexity of the equations describing co-ordinating systems which contain more than one ligand, makes it unlikely that evaluation of the stability constants could be carried out by conventional means. As no rigorous method exists for the solution of equations of higher order than 4 (i.e. a quartic equation), iterative procedures, similar to those developed here, must be employed. These methods require so much calculation that their application would be unthinkable without the use of a high speed computer. Even so, little is known about the behaviour of non-linear equations when subjected to re-iterative solution. The solutions can either

(i) converge on the 'correct' or 'incorrect' root;

(ii) diverge;

or (iii) oscillate around a continuous path.

As a chemical system is reproduceable merely by mixing the same reactants in the same concentrations, it is expected that a unique solution exists for the equations describing that system. Consequently, if the iterations converge, as in (i) above, the resulting solution must be the correct one.

In order to find the best set of constants to fit this solution at all experimental points an optimisation method is desirable. The trial and error method used to evaluate the results in Tables 20-24 can only give an indication of the order of magnitude of the required constants if, as occurs here, there is some variation in the calculated volumes over the experimental range. However, the optimisation method discussed earlier (p 154) changed the signs of the constants in order to observe the effect on the 'least squares' fit. This technique enabled larger steps in the right direction to be taken than would otherwise have been possible. However, by changing the signs, the equations no longer represent a physical system, and, as Descartes rule (p 154) states, more than one suitable solution is possible.

The first method to be developed in (b) above (p 157) was designed to test each root to see if it was acceptable (i.e. to see if it was positive and resulted in all the calculated experimental parameters being positive). If it was not, the variable P was incremented until the correct root was found. Consequently, an optimisation based on this method would be expected to work because incorrect solutions are ignored. The disadvantage of the method is that a great deal of computer time is required.

The second method, developed in (b) and (c) above, relied upon the fact that there exists only one solution of the explicitly stated equations which describe the system. This method worked adequately on its own and was rapid enough to be used with the optimisation 'routine', but as the routine changed the signs of the constants and thereby produced more roots, the actual solutions found are those with values nearest to the numbers already contained in the computer cells from previous calculations. Tests could be devised to see if the solutions were valid, but these tests would be useless on their own. Information is also required on where to look for the correct root should the calculated root be incorrect. This information can be provided by the derivatives of the parameter being calculated (in this case v) with respect to each of the constants required. A library routine is available (52) for such an optimisation which utilises these derivatives.

Attempts to differentiate v were unsuccessful because of the mathematical complications involved.

Conclusions

The results shown in Tables 31-23, although inconclusive, do indicate that the presence of significant concentrations of the species MP_2G_2 is unlikely. They also indicate that the species MPG_2 exists but that its presence is not vey important in solutions of 1:1 glutamate:pyridoxal phosphate ratio (the ratio in the solutions titrated). The presence of the latter species was postulated earlier (p 136-7) when solutions containing an excess of glutamate were being investigated.

It would be expected that the most important species in solution are those whose stability constants cause the greatest variation in v (Tables 20-4) near the value of v = 10. These are MPG and, more surprisingly, MP_2G.

The programs used in this work appear in Appendix V.

General Discussion and Conclusion.

Cu(11) ions have been found to catalyse strongly the transamination of pyridoxamine phosphate and α-ketoglutaric acid. With reactant concentrations of 0.3 mM pyridoxamine phosphate, 16 mM α-ketoglutaric acid and 0.6 mM Cu(11) the equilibrium was in favour of almost complete conversion to pyridoxal phosphate and glutamate. Transamination of pyridoxamine phosphate in the presence of other metal ions (Ni(11), Co(11) ans Zn(11)) was found to take place too slowly to be measured accurately by the methods employed (p 62) and the equilibria were very unfavourable to reaction products. As the rate of the reverse reactions (i.e. PyP + Glu $\rightleftharpoons$ PamP + αKG) was almost constant (to within a factor of 3 - see Table 3 p 47) at a given pH, the position of the equilibrium must be decided by the factors influencing the rate of PamP-αKG transamination. According to the principle of microscopic reversibility, kinetic factors would be expected to have similar influences on both the forward and reverse reactions. Consequently, the fluctuation of the equilibrium point must be caused by thermodynamic effects. These include the stabilities of the initially formed complexes; the pK values of the ligands; and the equilibrium constants of Schiff's base formation.

Because of the structural similarities of aldimine and ketimine Schiff's bases, it is unlikely that the stability constants of MSB' and MSB" are significantly different. However, examination of the titration curves on p 162 shows that even at pH 2.2 there is appreciable formation of CuSB'; much more so than with CuSB". The difference is even more noticeable with Ni(11). The titration curve of Cu(11)/PamP/αKG (Fig 61) deviates appreciably from that of PamP/αKG alone at about pH 3.8, whereas with Ni(11)/PamP/αKG the pH is in the region of 6. This indicates that the main influence on the concentration of the reactive complex is the very high pK value for the amine nitrogen in pyridoxamine phosphate (p 106). Metals other than copper cannot effectively compete with the hydrogen ion for the ligand anion.

If it is assumed that the pH/rate profiles of both transamination reactions in the presence of any metal are similar in character to those for Cu(11) (see Figs 2 and 23), then significant concentrations of MSB' for Cu(11), Ni(11), Co(11) and Zn(11); and of MSB" for Cu(11), can be formed at pH values at which an appreciable rate of transamination is possible. However, for the complexes MSB" for Ni(11) and Co(11), the pH values above which significant concentrations of complex are formed, are above the rate maximum in Fig. 23. Consequently, the rates of transamination of pyridoxamine phosphate and α-ketoglutarate in the presence of Ni(11) and Co(11) will always be low.

It was found that transamination of pyridoxal phosphate and glutamate was slower in the presence of metal ions if account is taken of the low concentration of free SB' in aqueous solution. No similar treatment can be applied to the reaction of pyridoxamine phosphate and αketoglutarate as the equilibrium constant and reactivity of the free Schiff's base (SB") are unknown. On this basis it is difficult to say whether metal ions catalyse the actual protropic shift in ketimine Schiff's bases (SB") or whether, as seems to be the case with aldimine Schiff's bases (SB'), the free Schiff's base would be more reactive if it were formed in sufficient concentration.

Appendix I

Preparation of the Complexes from Cu(11), Pyridoxal Phosphate and Sodium Glutamate; and from Cu(11), Pyridoxamine Phosphate and α-Ketoglutarate.

1.235 g of pyridoxal phosphate and 0.850 g of sodium glutamate (equimolar amounts) were dissolved in the minimum volume of water. Solid sodium bicarbonate was added until no further effervescence took place. The mixture was then added to an equilivalent weight (0.998 g) of copper acetate dissolved in a minimum volume of water. The resulting mixture was cooled in ice. The green precipitate which formed almost was filtered off at the pump. Further yields were obtained by treating the filtrate with methyl alcohol, and subsequently a third yield was obtained with ether. (However, this third yield tended to become tarry when separated from the mother liquor and was discarded.)

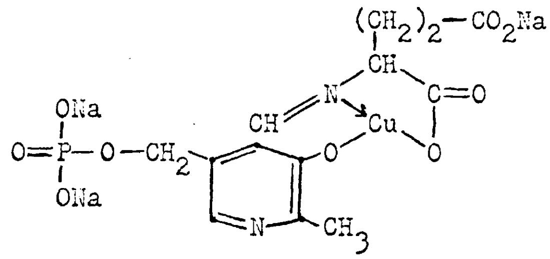
The precipitates were washed with methyl alcohol and dried in vaccuo. Recrystallisation from water was not possible because of the solubility of the complex. The total yield was 0.67 g.

Analysis of the dehydrated complex (by Bernhardt (54)) gave the results shown in Table 25.

Table 25

	C	H	N
Bernhardt	32.24	3.10	5.27%
Theoretical for Diag. 1	31.34	2.61	6.09%

The data from Bernhardt was used to calculate the percentage of water in the hydrated complex. The experimental value is 8.16% compared with the theoretical value for 2 moles of water of 7.42%.



(1)

The complex of Cu(11), pyridoxamine phosphate and α-ketoglutarate was prepared in a similar manner. 5 mM of copper acetate were dissolved in a minimum volume of warm water. To this solution was added 5 mM of pyridoxamine phosphate hydrochloride and 5 mM of α-ketoglutarate followed by sufficient sodium bicarbonate to neutralise all the acidic protons. The solution was then left at 0°C for about 3 hours after which time a slight gelatinous precipitate had appeared. The jelly was filtered off and discarded. Methyl alcohol was added to the filtrate whereupon a flocculent green precipitate was formed. This was filtered of at the pump, washed with methyl alcohol and dried in vaccuo. The yield was about 2 g.

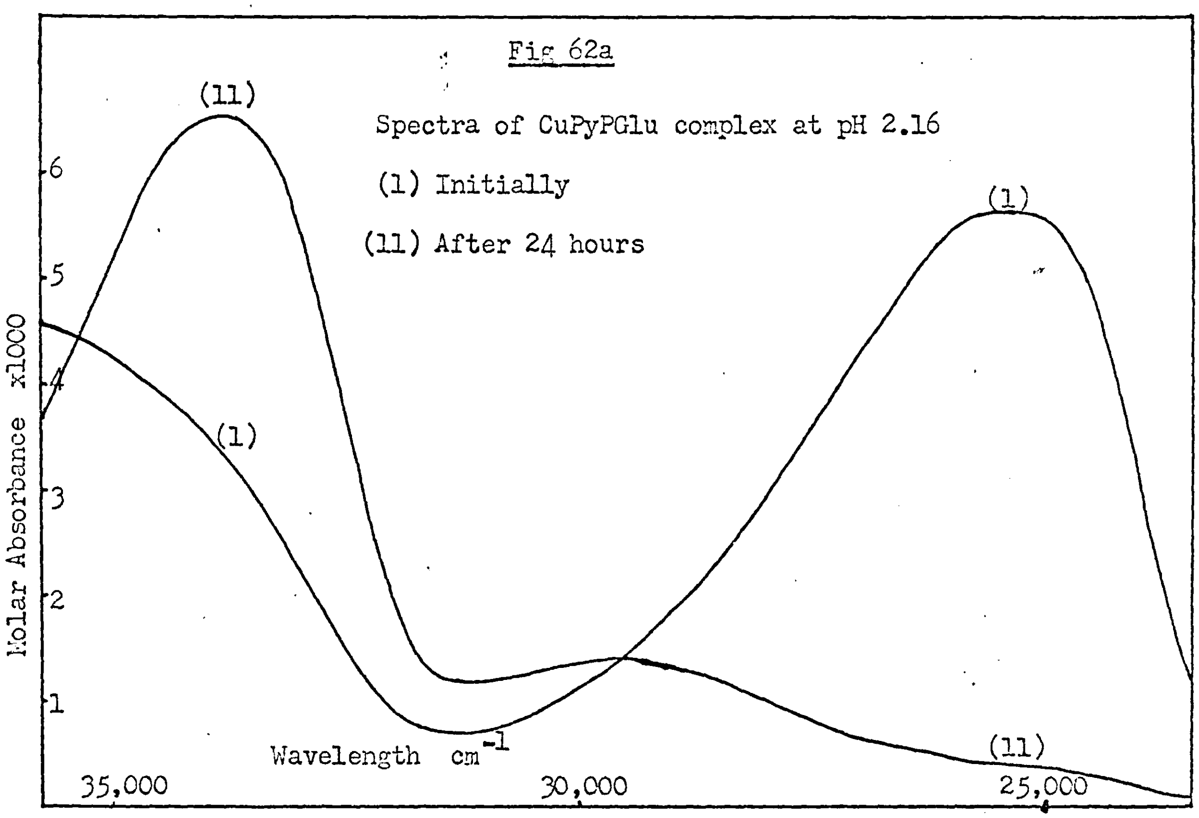
Analysis gave the results shown in Table 26.

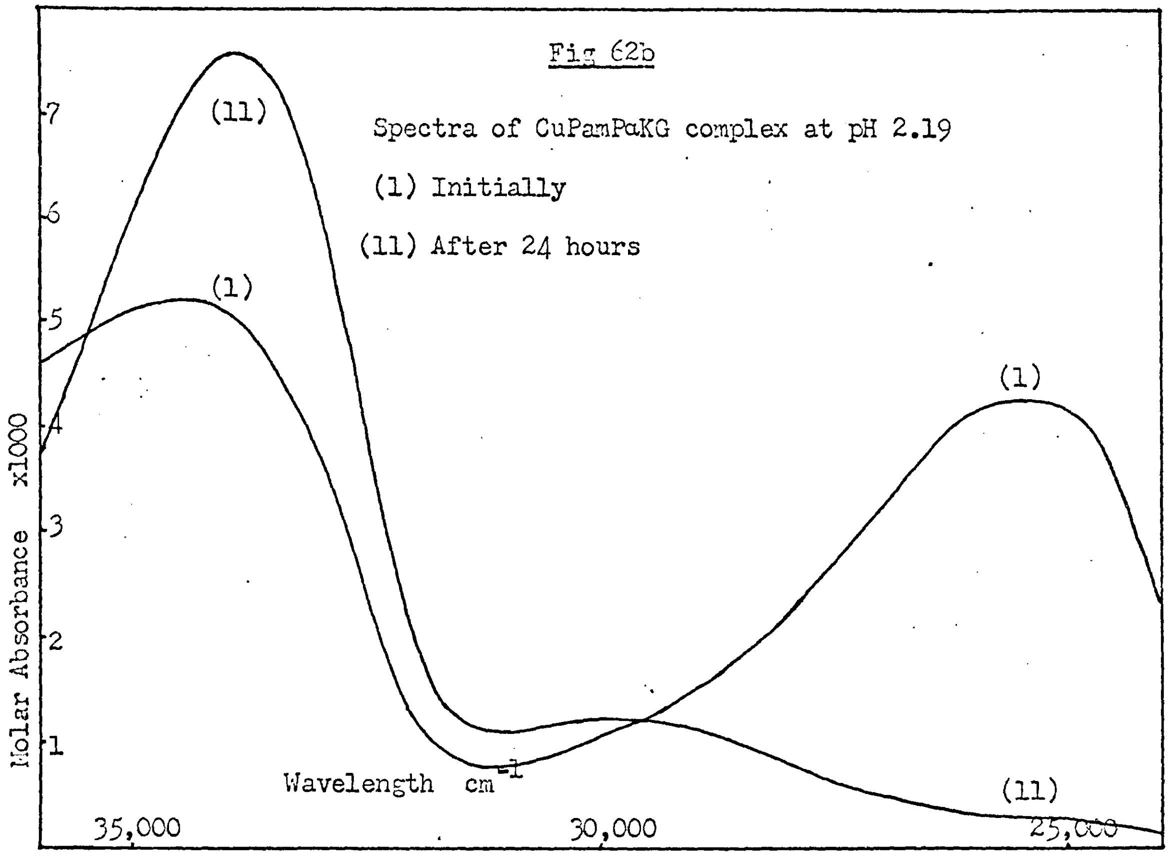
Table 26

	C	H	N
Bernhardt	32.82	3.56	6.12%
Theoretical for Diag 1	31.34	2.61	6.09%

The theoretical results are based on the same empirical formula as Diag. 1 above. The percentage of water in the hydrated complex was 7.27% compared with a theoretical value of 7.42% for 2 moles of water.

2.48 mg of each of the above complexes was weighed out and dissolved in 25 ml of acetate buffer. The spectrum of the resulting solution (0.2 mM - based on a molecular weight of 496.5 from Diag 1) was recorded immediately and at approximately 8 hour intervals for two days. These spectra were compared with spectra of solutions which were 0.2 mM in each of the reactants Cu(11), pyridoxal phosphate and glutamate; and Cu(11), pyridoxamine phosphate and α-ketoglutarate. The spectra of the reaction mixtures are shown in Fig 62.





It was found that the final spectra from the complex of CuPyPGlu; the complex of CuPamPαKG; and the solution containing Cu(11), pyridoxal phosphate and glutamate, were almost identical. At low pH values these spectra were totally different from the final spectrum of a solution containing Cu(11), pyridoxamine phosphate and α-ketoglutarate. (Figs 63 and 64).

The above observations indicate that the complex prepared from pyridoxamine phosphate and α-ketoglutarate has already undergone transamination during the time taken for its preparation.

The reaction which takes place on dissolving the complexes in buffer is probably the hydrolysis of the complex. This proceeds until an equilibrium is reached. At pH values as low as 2.4 and in the absence of an excess of glutamate, this equilibrium is expected to favour the

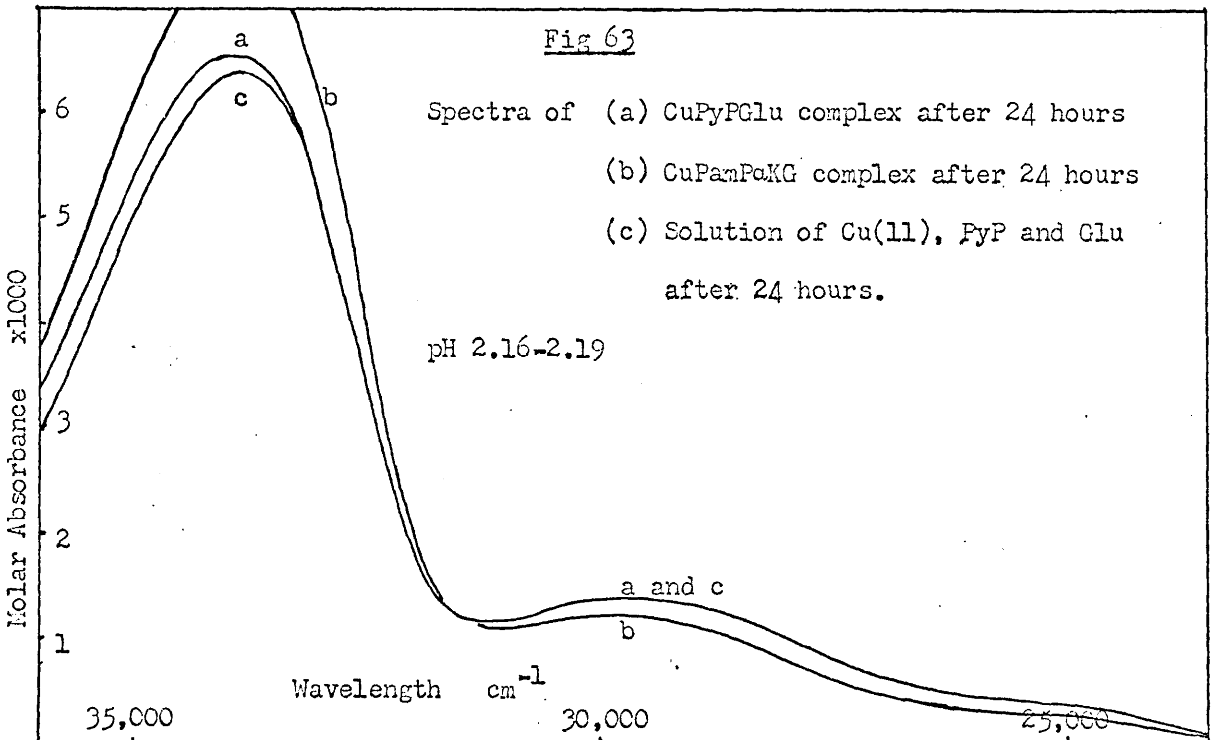


Fig 63

Spectra of (a) CuPyPGlu complex after 24 hours
(b) CuPamPαKG complex after 24 hours
(c) Solution of Cu(11), PyP and Glu after 24 hours.

pH 2.16-2.19

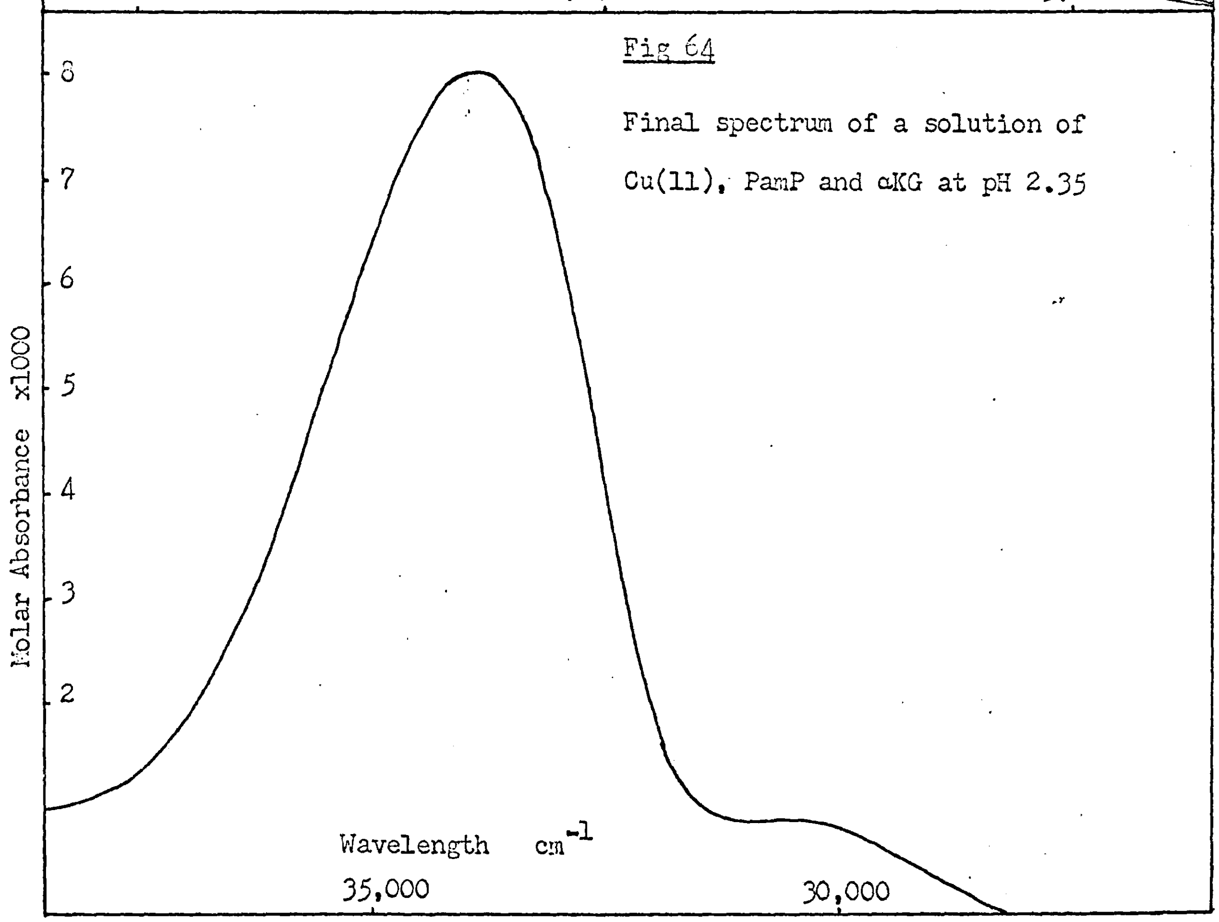


Fig 64

Final spectrum of a solution of Cu(11), PamP and αKG at pH 2.35

products of hydrolysis, as is shown by Fig 63.

Kinetic studies on the disappearance of the peak at 25,500 cm^{-1} showed that the reaction was neither first nor second order over a range of about 50% reaction. The hydrolysis of the complexes was not studied further.

Appendix II

The following abbreviations and symbols have been used throughout this thesis.

PyP	Pyridoxal phosphate (P also used for [PyP])
PamP	Pyridoxamine phosphate (P also used for [PamP])
Glu	The glutamate anion (G also used for [Glu])
αKG	The α-ketoglutarate anion (G also used for [αKG])
SB'	The Schiff's base derived from PyP and Glu
SB"	The Schiff's base derived from PamP and αKG
SB	Any Schiff's base in general
SBOH	Any carbinolamine
D	Optical density
D_o	Initial optical density
E	Extinction coefficient
[ ]	Concentration of species in brackets. These brackets have been omitted in certain parts for convenience. Where this is so the character '.' distinguishes between [CuP] ≡ CuP and [Cu][P] ≡ Cu.P etc.
C_p	Total concentration of PyP or PamP
C_m	Total concentration of metal ion
$\bar{n}_a$ or $\bar{n}'_a$	Average number of protons associated to ligand
$\bar{n}$ or $\bar{n}'$	Average number of ligand molecules associated to metal
[A]	Concentration of free ligand anion
(A)	Total concentration of free ligand
C_a	Total concentration of ligand

C_h	Total concentration of replaceable protons
C_{oh}	Concentration of OH^- in titrant
m	Total moles of titrant added
v	Volume of m moles of titrant
v'	Volume of titrant required during blank titration
p[ ]	Potential of species in brackets (- log[ ])
C_g	Total concentration of glutamate

Appendix III

Buffers Used in the Present Work

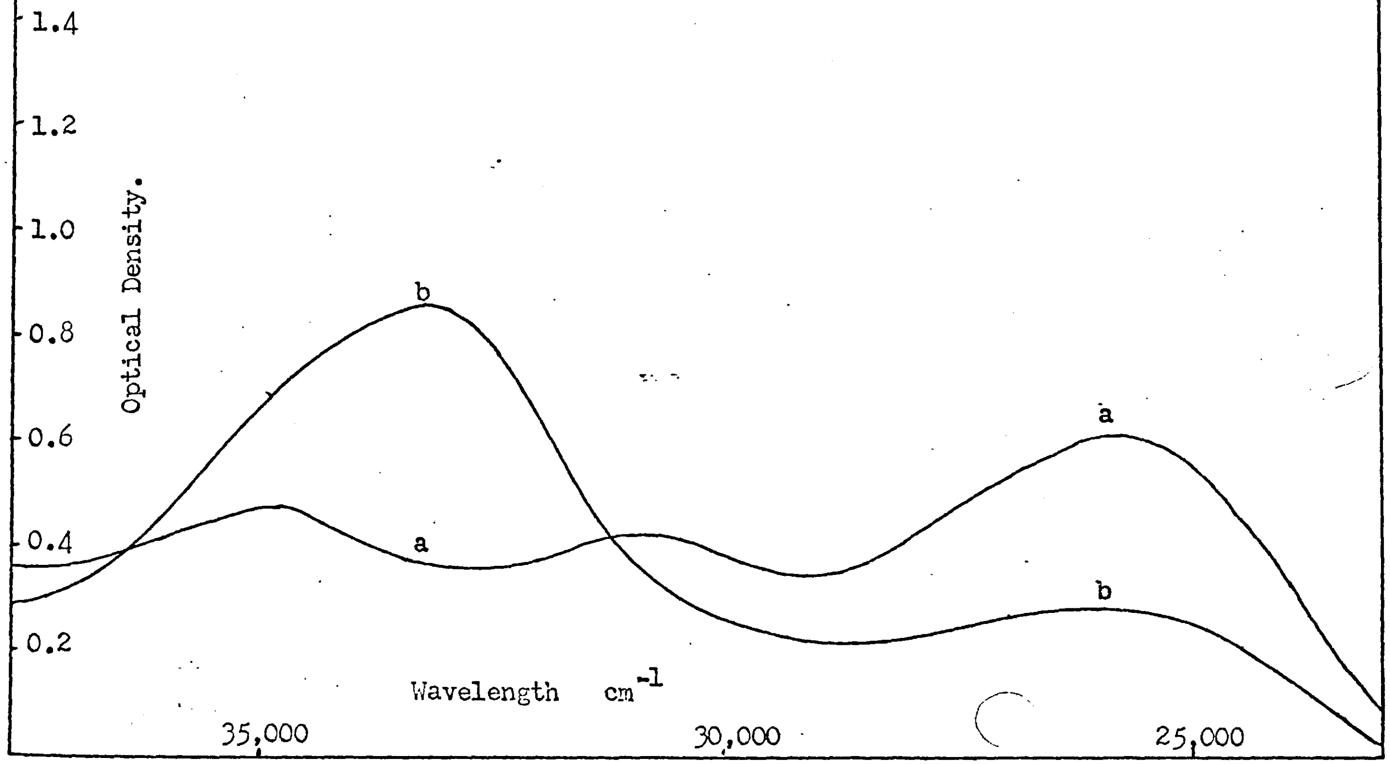
Acetate was found to be the only buffer suitable for systems containing strongly co-ordinating ligands. Sodium hydroxide was used in one instance (p 66) for pH values above 7, but it was difficult to control, and the resulting pH depended very much upon the pK values of the reactants.

The acetate buffer was made up by pipetting 20 ml of molar sodium acetate into a 100 ml standard flask. V ml of N HCl were then added together with (30 - V) ml of molar NaCl. The volume was made up to 100 ml with distilled water.

Borate was examined as a possible buffer for the higher pH range but it was found that the spectra of pyridoxal phosphate in borate and acetate buffers at the same pH were different. This suggests that some reaction takes place between pyridoxal phosphate and borate making the latter unsuitable as a buffer. The spectra of pyridoxal phosphate in borate and acetate buffers are shown in Fig 65. The spectra of pyridoxal phosphate in the presence of, and in the absence of acetate buffer are identical at the same pH.

Fig 65

Spectra of pyridoxal phosphate at pH 7 in (a) Acetate buffer
(b) Borate buffer



Appendix IV

Reagents Used in the Present Work

Pyridoxal phosphate ('purum' grade) was obtained from 'Fluka' and used without further purification. Solutions were made up in blackened flasks and were kept for a maximum of 3 days in a refrigerator. During the stability constant determinations of p 123 et seq., solutions were made up afresh each day.

Pyridoxamine phosphate hydrochloride (purissimum grade) was also obtained from 'Fluka' and used without further purification. Solutions were stored in darkened flasks in a refrigerator and kept for a maximum of 3 days.

Monosodium glutamate (reagent grade) was obtained from B.D.H. and used without further purification. Solutions were stored in a refrigerator and kept for a maximum of 2 days.

Reagent grade α-ketoglutaric acid was obtained from B.D.H. and also used without further purification. Solutions which had been partially neutralised were frozen solid immediately after use in order to retard the rapid growth of bacteria. No solution was kept for more than 1 day.

Appendix V

The Computer Programs Used in this Work

On the following pages is shown the latest program used to determine the mixed stability constants of Cu(11), PyP and Glu, together with some typical data. The location of the variables is shown below.

C	The concentration of PyP^{3-}
G(0) or G(P-1)	The concentration of Glu^{2-}
D(I+10), E(I+10)	Corresponding values of pH and $[H^+]/a$ correction factor F
U(I-1), V(I-1)	Corresponding values of pH and K_1/a^3
F(I-1)	Dissociation constants of the ligands
A(I-1), B(I-1)	Corresponding values of experimental v/a readings
C(I-1)	Values of known simple stability constants
V, W	Current values of v and a
X(0)-X(3)	Values of the mixed stability constants
Z	Interpolated value of Q_{sb}
H(0)	Interpolated value of $[H^+]/a$ correction factor F
W'	Calculated value of titrant volume v

The function of the subroutines are:

91)	Interpolation of Q_{sb}, interpolation of F
10)	Iterative evaluation of the experimental parameters.

The programming language is EXCHLF Autocode (1966).

CHAPTER 0

```
A->10
B->10
C->10
D->25
E->25
H->5
F->10
G->25
U->20
V->20
W->25
X->5
Y->5
Z->5

W(8)=20
W(4)=0

READ (C)
READ (G(0))
READ (N')
I=1(1)N'
READ (D(I+10))
READ (E(I+10))
REPEAT
READ (M')
I=1(1)M'
READ (U(I-1))
READ (V(I-1))
REPEAT
N=4
I=1(1)N
Y(I-1)=1
REPEAT
I=1(1)7
READ(F(I-1))
REPEAT

\\46)READ (M)
JUMP 47,M=0
I=1(1)M
READ (A(I-1))
READ (B(I-1))
REPEAT
I=1(1)4
READ(C(I-1))
REPEAT
READ (H)
READ(F(7))
READ(B')
```

```
O=1(1)M
NEWLINE 2
CAPTION
V(EXPT)=
V=A(O-1)
PRINT (V,2,2)
W=B(O-1)
P=1
JUMPDOWN 91
C=0.000 000 000 001
G(P-1)=0.000 000 000 1

X(0)=1000 0B'
I=1(1)4
X(0)=100X(0)
NEWLINE
CAPTION
X(0)=
PRINT (X(0),0,1)

X(1)=1000 0B'
J=1(1)4
X(1)=100X(1)
CAPTION
X(1)=
PRINT (X(1),0,1)

X(2)=1000B'
K=1(1)4
X(2)=1000X(2)
CAPTION
X(2)=
PRINT (X(2),0,1)

JUMPDOWN 10
CAPTION
V(CALC)=
PRINT (W',0,6)
NEWLINE

REPEAT
REPEAT
REPEAT
REPEAT
JUMP 46
47)END
```

```
91)I=1(1)M'
W(7)=*MOD(U(I-1)+0.43429*LOG(W))
JUMP 92,W(7)>W(8)
W(8)=W(7)
REPEAT

92)W(11)=-0.43429*LOG(W)
W(8)=20
Z=V(I-3)*(W(11)-U(I-2))*(W(11)-U(I-1))/*(*(U(I-3)-U(I-2))*(U(I-3)-U(I-1)))
Z=Z+V(I-2)*(W(11)-U(I-3))*(W(11)-U(I-1))/*(*(U(I-2)-U(I-3))*(U(I-2)-U(I-1)))
Z=Z+V(I-1)*(W(11)-U(I-3))*(W(11)-U(I-2))/*(*(U(I-1)-U(I-3))*(U(I-1)-U(I-2)))
Z=ZWWW

I=1(1)N'
H(1)=0.43429*LOG(W)+D(I+10)
H(1)=-H(1)
JUMP 109,0>H(1)
REPEAT
109)H(0)=E(I+10)+H(1)*(E(I+10)-E(I+9))/*(D(I+10)-D(I+9))

W(10)=1+W/F(7)+WW/*(F(7)F(4))+WWW/*(F(7)F(4)F(1))+WWWW/*(F(7)F(4)F(1)F(2))
W(11)=1+W/F(7)+WW/*(F(7)F(4))+WWW/*(F(7)F(4)F(1))+WWWW/*(F(7)F(4)F(1)F(1))
W(11)=W(11)+WWWWW/*(F(7)F(4)F(1)F(1)F(2))+WWWWWW/*(F(7)F(4)F(1)F(1)F(2)F(2))
W(12)=1+W/F(7)+WW/*(F(7)F(7))+WWW/*(F(7)F(7)F(4))+WWWW/*(F(7)F(7)F(4)F(4))
W(12)=W(12)+WWWWW/*(F(7)F(7)F(4)F(4)F(1))+WWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(2))
W(13)=1+W/F(7)+WW/*(F(7)F(7))+WWW/*(F(7)F(7)F(4))+WWWW/*(F(7)F(7)F(4)F(4))
W(13)=W(13)+WWWWW/*(F(7)F(7)F(4)F(4)F(1))+WWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1))
W(13)=W(13)+WWWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1)F(2))
W(13)=W(13)+WWWWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1)F(2)F(2))
W(16)=W/F(7)+2WW/*(F(7)F(4))+3WWW/*(F(7)F(4)F(1))+4WWWW/*(F(7)F(4)F(1)F(2))
W(17)=W/F(7)+2WW/*(F(7)F(4))+3WWW/*(F(7)F(4)F(1))+4WWWW/*(F(7)F(4)F(1)F(1))
W(17)=W(17)+5WWWWW/*(F(7)F(4)F(1)F(1)F(2))
W(17)=W(17)+6WWWWWW/*(F(7)F(4)F(1)F(1)F(2)F(2))
W(18)=W/F(7)+2WW/*(F(7)F(7))+3WWW/*(F(7)F(7)F(4))+4WWWW/*(F(7)F(7)F(4)F(4))
W(18)=W(18)+5WWWWW/*(F(7)F(7)F(4)F(4)F(1))
W(18)=W(18)+6WWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(2))
W(19)=W/F(7)+2WW/*(F(7)F(7))+3WWW/*(F(7)F(7)F(4))+4WWWW/*(F(7)F(7)F(4)F(4))
W(19)=W(19)+5WWWWW/*(F(7)F(7)F(4)F(4)F(1))
W(19)=W(19)+6WWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1))
W(19)=W(19)+7WWWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1)F(2))
W(19)=W(19)+8WWWWWWWW/*(F(7)F(7)F(4)F(4)F(1)F(1)F(2)F(2))
RETURN
```

```
10)A'=3/*(100+V)-H(0)W+0.000 000 000 000 0322/W-0.1V/*(100+V)
A'=A'-C*(W/F(3)+2WW/*(F(3)F(4))+3WWW/*(F(3)F(4)F(5)))
A'=A'-4CWWWW/*(F(3)F(4)F(5)F(6))
W(20)=W/F(0)+2WW/*(F(0)F(1))+3WWW/*(F(0)F(1)F(2))+X(0)Y(0)W(16)C
W(20)=W(20)+X(1)Y(1)W(17)CG(P-1)+X(2)Y(2)W(18)CC+X(3)Y(3)W(19)CCG(P-1)
G(P-1)=A'/W(20)
A=C(2)+2C(3)G(P-1)+X(0)Y(0)W(10)C+2X(1)Y(1)W(11)CG(P-1)
A=A+X(2)Y(2)W(12)CC+2X(3)Y(3)W(13)CCG(P-1)
B=C(0)+2C(1)C+X(0)Y(0)W(10)G(P-1)+X(1)Y(1)W(11)G(P-1)G(P-1)
B=B+2X(2)Y(2)W(12)CG(P-1)+2X(3)Y(3)W(13)CG(P-1)G(P-1)

W(15)=1+C(0)C+C(1)CC+C(2)G(P-1)+C(3)G(P-1)G(P-1)+X(0)Y(0)W(10)CG(P-1)
W(14)=X(1)Y(1)CG(P-1)W(11)G(P-1)+X(2)Y(2)W(12)CCG(P-1)
W(14)=W(14)+X(3)Y(3)W(13)CCG(P-1)G(P-1)
E=100H/*(*(100+V)*(W(15)+W(14)))
G=G(P-1)*(1+W/F(0)+WW/*(F(0)F(1))+WWW/*(F(0)F(1)F(2))+AE+ZC)
W'=100G/*(0.05-G)
W(5)=*MOD(W'-W(4))
D=1+W/F(3)+WW/*(F(3)F(4))+WWW/*(F(3)F(4)F(5))+WWWW/*(F(3)F(4)F(5)F(6))
C=0.5/*(100+V)
C=C/*(D+BE+ZG(P-1))
JUMP 103,W(5)>0.01
RETURN

103)W(4)=W'
JUMP 10

CLOSE

1,-12     1,-10

13
2.0   1.096   2.5   1.122   3   1.148   3.5   1.175   4   1.202   4.5   1.230
5   1.259   5.5   1.288   6   1.318   6.5   1.350   7   1.380
7.5   1.412   8   1.445

14
2.8   2.37,28   3   1.02,28   3.2   4.74,27   3.6   1.21,27
4   4.25,26   4.4   2.10,26   5   1.21,26   5.6   1.51,26
6   2.13,26   6.4   4.18,26   7   2.29,27   7.4   8.66,27
7.5   1.22,28   7.6   1.88,28

2.138,-10   5.249,-5   5.013,-3
2.065,-9    4.842,-7   1.862,-4   1.288,-2
```

6
2.10 4.677,-3 7.50 1.660,-3 12.28 1.549,-4
13.90 6.166,-5 19.91 8.610,-7 27.12 4.898,-10

1.714,6 1.20,10 7.08,7 2.51,14

2.5,-3

2.455,-6

1

5
5.42 6.383,-4 10.88 1.148,-4 16.82 6.026,-6 23.97 1.995,-8
28.50 1.191,-10

3.24,3 0 7.94,5 2.19,10

2.551,-3

3.846,-7

0.01

0

***Z

References

(1) E.E.Snell, Physiol. Revs. 33 509 (1953)

(2) A.E.Braunstein, 'The Enzymes' Ed. P.D.Boyer, H.Lardy & K.Myrback Vol. 2 p. 140

(3) D.E.Metzler, J.B.Longenecker and E.E.Snell, J.A.C.S. 76 639 (1954)

(4) D.E.Metzler, M.Ikawa and E.E.Snell, J.A.C.S. 76 648 (1954)

(5) J.B.Longenecker and E.E.Snell, J.A.C.S. 79 142 (1957)

(6) D.E.Metzler and E.E.Snell, J.A.C.S. 74 979 (1952)

(7) T.C.Bruice and R.M.Topping, J.A.C.S. 84 2448 (1962); 85 1480, 1488 (1963)

(8) L.J.Nunez and G.L.Eichhorn, J.A.C.S. 84 901 (1962)

(9) J.Olivard, D.E.Metzler and E.E.Snell, J.Biol.Chem. 199 669 (1952)

(10) Y.Matsuo, J.A.C.S. 79 2011, 2016 (1957)

(11) P.Fasella, H.Lis, C.Baglioni and N.Siliprandi, J.Inorg.Nuc.Chem. 8 620 (1958)

(12) P.Fasella, H.Lis, C.Baglioni and N.Siliprandi, Biochem.Biophys.Acta. 23 417 (1957)

(13) C.Cennammo, Boll.Soc.Ital.Biol.Sper. 37 183 (1961); 38 1037 (1962)

(14) H.Wada, E.E.Snell, J.Biol.Chem. 237 127, 133 (1962)

(15) A.E.Braunstein, Enzymol. 7 25 (1939)

(16) P.P.Cohen, Biochem.J. 33 1478 (1939)

(17) M.Dupont, J.de Chim.Phys. 1965 p.701

(18) B.E.C.Banks, A.A.Diamantis and C.A.Vernon, J.C.S. 4235 (1961)

(20) D.Heyl, S.A.Harris and K.Folkers, J.A.C.S. 70 3429 (1948)

(19) H.N.Christensen, J.A.C.S. 80 99 (1958)

(21) N.Lucas, H.K.King and S.J.Brown, Biochem.J 84 118 (1962)

(22) D.E.Metzler, J.A.C.S. 79 485 (1957)

(23) E.E.Snell, J.A.C.S. 67 194 (1945)

(24) J.Baddiley, Nature, 170 711 (1952)

(25) H.N.Christensen, J.A.C.S. 79 4073 (1957)

(26) G.L.Eichhorn and J.W.Dawes, J.A.C.S. 76 5663 (1954)

(27) J.Cattaneo, J.C.Senez and P.Beaumont, Biochem.Biophys.Acta. 44 543 (1960)

(28) H.N.Christensen and S.Collins, J.Biol.Chem. 220 279 (1956)

(29) A.E.Braunstein and M.M.Shemyakin, Biochimia, 18 393 (1953)

(30) A.M.Perault, B.Pullman and C.Valderoro, Biochem.Biophys.Acta. 46 555 (1961)

(31) G.L.Eichhorn and I.M.Trachtenberg, J.A.C.S. 76 5183 (1954)

(32) G.L.Eichhorn and N.D.Marchand, J.A.C.S. 78 2688 (1956)

(33) H.N.Christensen, J.A.C.S. 80 2305 (1958)

(34) J.B.Longenecker and E.E.Snell, Proc.Nat.Ac.Sc.U.S. Vol.42 No.5 221 (1956)

(35) D.E.Metzler, J.Olivard and E.E.Snell, J.A.C.S. 76 644 (1954)

(36) L.Davies, F.Roddy and D.E.Metzler, J.A.C.S. 83 127 (1961)

(37) R.L.Gustafson and A.E.Martell, Arch.Chem.Phys. 68 485 (1957)

(38) T.C.French and T.C.Bruice, Biochem. Vol.3 No.10 1589 (1964).

(39) E.H.Cordes and W.P.Jencks, J.A.C.S. 84 832 (1962); 85 2843 (1963)

(40) C.K.Ingold 'Structure and Mechanism in Organic Chemistry' p.466

(41) Ref 40 p 572

(42) A.A.Frost and R.G.Pearson, 'Kinetics and Mechanism' 2nd Ed. p.100

(43) J.Bjerrum, 'Metal Ammine Formation in Aqueous Solution'

(44) V.R.Williams and J.B.Neilands, Arch.Biochem. and Biophys. 53 56 (1954)

(45) H.S.Harned and B.B.Owen, 'The Physical Chemistry of Electrolyte Solutions' p.676

(46) G.Schwarzenbach,'Complexometric Titrations' pp 78-9

(47) 'Stability Constants' Chem.Soc. Special Publication No.17 (1964)

(48) J.I.Watters, J.A.C.S. 81 1560 (1959)

(49) D.Leussing, Talanta, Vol.11 189 (1964)

(50) A.D.Booth, 'Numerical Methods' p 150

(51) Ref. 50, p.25

(52) Supplied by the Institute of Computer Science, Gordon Sq. W.C.1

(53) Private communication from Miss Mary Bell, University College, London.

(54) A.Bernhardt, Mulheim Ruhr, Germany.