STUDY ON FORMATION AND STABILITY CONSTANTS OF FOLIC ACID METAL ION COMPLEXES

Drug Profile of Folic Acid

Folic acid occurs in yeast, spinach and liver. The structure of folic acid and its synthesis was reported by Angter et al (268). Much of the folic acid isolated from natural sources, such as yeast and liver is thought to be present in the natural state as citrovorum factor (269). Folic acid is present (about 5 parts per million) in the dried pollen of various species of plants.

Structure

\[
\begin{align*}
\text{Molecular formula} & \quad C_{19}H_{19}N_7O_6 \\
\text{Molecular weight} & \quad 441.4
\end{align*}
\]

Chemical nomenclature

Acidum Folicum; Folacin; Pteroylglutamic acid; Pteroylmono glutamic acid; Folinsyre; N-4-(2-Amino-4-hydroxy-pteridin-6-ylmethylamino)benzoyl-L-(+) glutamic acid.

It is usually seen as a yellow-orange, odourless, tasteless, microcrystalline powder. It chars and decomposes at about 250°C. It is soluble in 5,000 parts of boiling water; 200,000 parts in cold water at pH 4; 20,000 parts at pH 5 and 70 parts at pH 7.
It is very slightly soluble in water, methanol, ethanol, acetone, chloroform ether and benzene, relatively more soluble in acetic acid, phenol and pyridine, but soluble in dilute solutions of alkali hydroxides, carbonates, hydrochloric acid and sulfuric acid.

It usually contains two molecules of water of crystallization, one which is loosely bound and the other is lost on drying at 75° (below 5 mm Hg). The specific rotation of a 0.5% w/v solution in 0.1 N sodium hydroxide is +20°.

It is incompatible with oxidizing and reducing agents and with ions of heavy metals. Alkaline solution of folic acid is sensitive to oxidation. Acid solutions of folic acid are sensitive to heat but towards neutrality, stability progressively increases. In aqueous solution, it is most stable above pH 6. Below pH 7, nicotinamide is used to increase solubility. The sodium salt is more soluble than 1 in 70, and may be sterilized by autoclaving at 15 lb. pressure.

Folic acid is very sensitive towards sunlight. The decomposition of folic acid by light is greatly intensified by riboflavin. As little as 50 mg of riboflavin per 100 ml of solution can accelerate the destruction of 10 mg of folic acid (270).

Ionisation constant pKa of folic acid is 8.6 UV absorption spectra of folic acid shows λ max at 256, 283 and 365 nm.
Pharmacology

Folic acid is a member of Vitamin-B group. It gets reduced in the body to tetrahydrofolate. It is a coenzyme for various metabolic processes including the synthesis of purine and pyrimidine nucleotides, and hence in the synthesis of DNA. It is also involved in some amino acid conversion, and in the formation and utilization of formate.

At clinical level, folic acid rapidly cures the anaemia of pregnancy and macrocytic anaemia, sometimes arising after operations of the small intestine. In these two conditions, Vit $B_{12}$ is ineffective. On the other hand, folic acid is not so effective in pernicious anaemia as Vit $B_{12}$. Folic acid has been found to be helpful but not curative in the tropical bowel disease known as sprue.

Deficiency of folic acid leads to megaloblastic anaemia. It results from a diminished intake, from malabsorption, or from the concomitant use of anticonvulsants or dihydrofolate reductase inhibitors such as pyrimethamine, trimethoprim or methotrexate. Folic acid does not correct folate deficiency due to dihydrofolate reductase inhibitors. Calcium folinate is used for this purpose.

Treatment of megaloblastic anaemia

In the treatment of megaloblastic anaemia folic acid is given orally. Initially dosage of 10 to 20 mg daily for 14 days, or until a haemopoietic response has been obtained. The daily maintenance dose is 2.5 to 10 mg.
Folate compounds decrease formate accumulation after methanol treatment by stimulating formate oxidation or utilisation, and suggest a possible use of folates in the treatment of certain cases of human methanol poisoning (271). Administration of folate, with ethanol reversed the inhibitory action of ethanol on several human intestinal glycolytic enzymes (272).

Patients treated with pyrimethamine, chloroquine phosphate and sulfisoxazole for Plasmodium falciparum malaria, are supplemented with folic acid. A reduced incidence of anaemia and leukopenia, a more rapid reticulocytosis, and an increased platelet response are observed in folic acid supplemented patients. The antimalarial effect of pyrimethamine was not inhibited by the addition of folates to the treatment regimen (273).

Toxicity

Toxicity symptoms of folic acid in pharmaceutical doses are gastrointestinal and nervous. It includes anorexia, nausea, abdominal distension and discomfort, flatulence, sleep disturbances, vivid dreaming, irritability, excitability, rash, bronchospasm and over activity. EEG changes and convulsions are reported with intravenous therapy of folic acid.

Assay

Folic acid is commonly assayed by measuring (by diazotization) the amount of aromatic amines liberated by reduction with zinc amalgam and mineral acid. A correction is made for any free p-aminobenzoic or aminobenzoylglutamic acid present (274).
The microbiological assay using *Lactobacillus casei* provides a further check on the purity of folic acid (275). The need for thorough analysis has been demonstrated by Nichol (276) who has used paper chromatography to reveal the presence of pteroic acid in a "highly purified" specimen of folic acid. In addition, the purest obtainable specimens of aminopterin and other "folic acid inhibitors" were shown by this technique to contain sufficient folic acid or pteroic acid to account for a growth promoting action on an antagonist resistant strain of *Streptococcus faecalis*.

**Folic Acid Metal Ion Complexes**

It was first pointed out in 1949 that pteridines containing a 4-hydroxy group combine with the cations of heavy metals, to give 1:1 complexes and at higher pH value (about pH 7.00), 2:1 complexes (277).

All natural pteridines, except the citrovorum factor, combine with metals in this way. So far no metal complexes of pteridine have been isolated from nature. In fact, the customary methods of isolation make use of strongly alkaline (or acidic) condition which would break up such complexes. Nevertheless, it is likely that some of the biological actions of pteridines are effected through these metal complexes.

Silver and mercuric ions are known to form complexes with folic acid (278). The stability constant for mercuric : folic acid (1:2) and silver : folic acid (1:1) complexes are $0.3 \times 10^3$ and $0.8 \times 10^5$ respectively. It was concluded from IR studies
of the complexes that coordination take place at 4-OH and 5-nitrogen in 4-hydroxy-2-aminopteridine fragment (278).

**Formation of metal ion chelates in aqueous solutions**

In aqueous solution, metal ions by themselves are always highly solvated. Hydrolysis occurs when the coordinated water molecules successively lose protons by dissociation.

The ability of chelating agents to compete for aquated metal ions may be expressed quantitatively as the equilibrium constant for the reaction of the metal ion $M^{m+}$ and $L^{n-}$ to form the complex, $ML^{(m-n)+}$. In general, the true thermodynamic equilibrium constant (applicable at infinite dilution of the reacting species) is not easily determined and most investigators report a stoichiometric or concentration constant $K$, where

$$K = \frac{[ML^{(m-n)+}]}{[M^{m+}][L^{n-}]}$$

These practical constants incorporate activity coefficients which are less than unity and are held invariant during experiments by the addition of high, constant concentration of background electrolyte(s). Sodium perchlorate and potassium nitrate are commonly employed as background electrolytes. The inertness of background electrolytes facilitates thermodynamic interpretation of the equilibrium constant, but is of less value in calculation of concentration of complexes in biological fluids. Therefore, sodium chloride is employed as the background electrolyte for the determination of formation constant.
Whenever, the coordination of a metal ion is at least twice the effective denticity of the ligand, an $ML_2$ type complex may be formed. Such stepwise coordination often extends to other stoichiometries such as $ML_3$ and $ML_4$. In these cases, it is convenient to define the equilibria in terms of overall formation constant, $\beta_1$ which relates the concentration of each species to the free concentration of its component. Omitting charges for the sake of clarity, this can be written as:

$$[ML] = \beta_1 [M][L]$$
$$[ML_2] = \beta_2 [M][L]^2$$
$$[ML_3] = \beta_3 [M][L]^3$$ etc...

Note that $\beta_1 = K$ (the first stepwise equilibrium constant).

In fact, relatively few metal-ligand systems confine themselves solely to the formation of these $ML_1$, $ML_2$ and $ML_3$ complexes. Most of them include other species such as the protonated complex $MLH$ and the hydroxy complex $ML(OH)$. In addition, the formation of polynuclear complexes of the type $M_2L_2$ or $M_2L_3$ takes place. The recognition of complexes other than the single, stepwise species is fundamental to modern approaches in aqueous inorganic chemistry. Accordingly, it is necessary to formulate the equilibrium concentration of various complex species as a general expression such as

$$S_j = \beta_j \prod_i x_i^{k(i,j)}$$

where $S_j$ is the concentration of complex $j$, $\beta_j$ is the corresponding formation constant, $x_i$ is the free concentration of the $i^{th}$ component, $k(i,j)$ is the stoichiometric coefficient of component $i$ and $j$. 


While the formation constants do reflect in intrinsic strength of metal-ligand binding, many other factors contribute towards the final distribution of complexes e.g. (i) in aqueous solution, the equilibria always involve some competition with hydroxide ions and (ii) most complexing reactions involve the displacement of protons from the ligand by the metal ion. Both of these phenomena make complex distributions generally dependent on the pH of the solution.

In general, the overall outcome of equilibrium in such multicomponent system must be determined mathematically. This is done by solving the set of mass balance equations in which the total concentration of each component is made up of the sum of contribution from each of the constituent species (279). Thus, $T_i = x_i + \sum_j S_j K(i,j)$ where $T_i$ is the total concentration of the $i^{th}$ component.

By substituting for the concentration of each complex species, $S_j$, the mass balance relationship can be expanded into a set of non-linear equations expressing each total concentration in terms of the free concentrations of each component. Hence, one obtains a set of $n$ equations in $n$ unknown, where $n$ is the number of components.

2.1 Stability of Metal Chelates

In-order to know the absolute co-ordinate bond energy, we must have thermodynamic data on gas phase reactions such as

$$ML(n-1)(g) + L(g) \rightleftharpoons ML_n(g)$$
where \( n \) is the co-ordination number of the metal \( M \), \( L \) is the monodentate ligand. Actually, very little direct data of this kind (\( \Delta H_{n} \)) are available. Stability constants of complexes in solution provide an alternative measurable path to the desired information to a good approximation.

Metal ions in aqueous solution interact strongly with water molecules to form strong co-ordinate linkage by virtue of the lone electron pairs. The actual state of metal ions in solution is that of an aquo complex in which water molecules are to be regarded as ligands. The formation of metal complexes in solution with particular ligand must occur via the successive replacement of hydration molecules e.g.

\[
\begin{align*}
[M(H_2O)](aq) + L(aq) & \rightleftharpoons [M(H_2O)_{N-1}L](aq) + H_2O(aq) \\
[M(H_2O)_{N-1}L](aq) + L(aq) & \rightleftharpoons [M(H_2O)_{N-2}L_2](aq) + H_2O(aq)
\end{align*}
\]

For each of the above equilibria, we can write an expression according to the law of mass action as follows:

\[
\begin{align*}
M + L & \rightleftharpoons ML \quad K_1 = [ML]/[M][L] \\
ML_{N-1} + L & \rightleftharpoons ML_N \quad K_N = [ML_N]/[ML_{N-1}][L]
\end{align*}
\]

The constants \( K_1, K_2, K_3, \ldots, K_N \) are called the stepwise formation constants or stability constants.
2.2 Importance of stability constants

(i) Stability constant of a complex is an important measurable quantity to explain and to correlate the phenomena in complex formation.

(ii) It is useful in problems in theoretical chemistry because of its relation to the M-L bond strength.

(iii) The conditions required for maximum formation of a complex can be predicted on the basis of its stability constant.

(iv) If we know all the stability constants for a given system, it is possible to calculate the equilibrium concentrations of different species of a metal complex.

(v) Stability constants can be used to devise analytical and separation procedures.

(vi) Stability constants are useful to control the metal ion concentrations in solution.

2.3 Factor influencing stability

Interpretation of stability of a complex implies following considerations: (i) the metal-ligand bond energy, (ii) the heats of solvation of the various species, and (iii) the entropy changes involved. These, in turn, would depend on (i) temperature, (ii) nature of solvent, (iii) nature of metal, and (iv) nature and number of ligands. Some of the generalizations relating to the nature of the metal and of the ligand are discussed briefly.

2.3.1 Nature of Metal

(i) Charge density

The stability of ionic complexes increases with the ionic charge and decreases with the ionic radius of the metal ion.
The term "ionic potential" is sometimes used to express the combined effect. The metal ions with large ionic potentials are more suitable for complexation. In general, such a relation is observed for ionic chelates, e.g. alkali and alkaline earth metals. The adherence to the relationship is sometimes used as a criterion for ionic bonding. The outer electron configuration of the metal ion forms an important consideration in complex stability. Martell and Calvin (280) on the basis of Born equation (281), correlated the stability constant with the $Z^2/r$ value for the metal ion. Davies and Williams (282) have suggested the use of the reciprocal of the radius of the hydrated metal ion in the case of outer sphere complexes.

(ii) Available orbitals

Since electron pairs from ligand atoms occupy vacant orbitals on the central metal ion, the co-ordinate covalent bond strength depends on the ionization potential of the metal atom. In comparing the stabilities of chelates of a given ligand with various ions, usually there is a good linear relationship between stability constants and the ionization potentials of the central ions (280).

Fernelius et al (283) have pointed out linearity between stability constants and electronegativity of the metal ions. Calvin (280) has demonstrated the equivalence of ionization potential and electronegativity-hybrid bond strength product as measures of chelating tendencies.
Irving and Williams (284) have shown that for a number of ligands, the stability sequence for the first row bivalent transition metals is $\text{Mn} < \text{Fe} < \text{Co} < \text{Ni} < \text{Cu} < \text{Zn}$.

2.3.2 (i) Nature of ligand

The ligand characteristics influencing stability of the metal chelate are (i) the size of chelate ring, (ii) the number of chelate rings, (iii) the basic strength of the ligand, (iv) substitution in the chelating molecule which may modify the basic strength or bonding or may introduce steric effects and (v) the nature of donor atoms.

Although metal chelates have ring size ranging from four to nine members, those with five and six membered rings are more stable. Pfeiffer (285) observed that the five membered ring, in general, is more stable when it is saturated, but six-membered rings are favored when they are unsaturated.

The metal chelate stability increases with the number of rings formed, i.e. with the effective dentate nature of the chelating molecule. Some excellent quantitative studies allowing comparison between stability and the tying together of donors to form additional rings have been reported (286).

Co-ordination is equivalent to neutralization in the Lewis sense, and hence it seems logical to expect some correlation between basic strength of a ligand and the stability of its metal
complexes. The linear relationship between the basic strength of a ligand and the stability of its metal ion complexes has been observed (287). For ligands with different types of basic centre, correlations have been made with $pK_a$ of the more basic centre, although some authors prefer to use a sum.

Irving and Rossotti (283) have discussed the thermodynamic relationship derived by Duncan (288).

$$\log K = pK - \left( \frac{G^o_{ML} - G^o_{HL}}{RT} \right) + B$$

where $G^o_{ML}$ and $G^o_{HL}$ are the partial molar free energies of ML and HL; $B$ is constant for all complexes of the given metal ion, independent of the nature of L.

The presence of non-chelating substituents in a chelating molecule may cause steric effects besides altering the basicity of the ligand. These might exert an adverse effect on stability (289).

A different type of steric inhibition in which the substituent interferes with the planar configuration, and hence with the possible resonance stabilization, has also been reported (290). Another type of steric effect emphasizes the need for mutual compatibility between the special requirements of metal ion and ligand.

2.4 Determination of stability constants by pH metry

Theoretically, a metal complex or a metal chelate, $ML_n$, in solution, can be regarded as formed by a succession of reversible steps.
where \( M \) is the uncomplexed metal ion and \( L \) is the free ligand (charges on \( M \) and \( L \) are omitted for clarity and generality).

Each step is governed by the corresponding equilibrium constant defined as:

\[
T_{k_n} = \frac{[ML_n]}{[ML_{n-1}][L]} \quad \ldots \ldots \quad (2)
\]

\( T_{k_n} \) is the activity quotient for the reaction step. More strictly, these are stages of substitution in the solvated ion, viz.,

\[
M(H_2O)_x + L \rightleftharpoons M(H_2O)_{x-1}L + H_2O
\]

The omission of solvent from equation (1) is justified, provided that the reactants are in such low concentrations that activity of solvent remains effectively constant (291).

\[
ML_{n-1} + L \rightleftharpoons ML_n \quad \ldots \ldots \quad (3)
\]

\( T_{k_n} \) is called the \( n \)th thermodynamic metal-ligand stability or formation constant. The overall thermodynamic metal-ligand stability constant \( T_{B_N} \), will be activity quotient corresponding to the overall reaction.

\[
M + NL \rightleftharpoons ML_N \quad \ldots \ldots \quad (4)
\]

and will be given by

\[
T_{B_N} = \frac{[ML_N]}{[M][L_N]} \quad \ldots \ldots \quad (5)
\]

Obviously, the overall and stepwise stability constants are related

\[
T_{B_N} = T_{k_1} T_{k_2} \ldots \ldots T_{k_N} = \frac{N}{n=1} T_{k_N} \quad \ldots \ldots \quad (6)
\]
On the other hand, usually the ligand is a conjugate base of a weak acid. If we assume that ligand takes up $j$ protons, we have another set of equilibria.

\[
\begin{align*}
L + H & \rightleftharpoons LH \\
LH + H & \rightleftharpoons LH_2 \\
LH_{j-1} + H & \rightleftharpoons LH_j \\
\end{align*}
\] ........ (7)

Each of the above equilibria is governed by its equilibrium constant

\[
K_{H_j}^k = \frac{[LH_j]}{[LH_{j-1}][H]} ........ (8)
\]

$K_{H_j}^k$ is called the $j^{th}$ thermodynamic proton-ligand stability constant and is the reciprocal of the thermodynamic dissociation constant of the acid $LH_j$ which dissociates as

\[
LH_j \rightleftharpoons LH_{j-1} + H ........ (9)
\]

$T_{B}H_j$, the overall thermodynamic proton-ligand stability constant, is given by

\[
T_{B}H_j = \frac{[LH_j]}{[L][H]^j} ........ (10)
\]

and it refers to the reaction $L + JH \rightleftharpoons LH_j$ .... (11)

Again $T_{B}H_j = T_{k_1}^H, T_{k_2}^H, ... T_{k_j}^H = \prod_{j=1}^{\pi} T_{k_j}^H$ .... (12)

It is now assumed that ionic or molecular species involving $M, L$ and $H$, other than those shown in the above equilibria, do not exist in solution.

The thermodynamic constants are difficult to determine. Therefore, it is customary to control activity coefficients by using an inert supporting electrolyte in determination of
stability constants. These constants are called the stoichiometric, or apparent, or concentration constants. All the terms of their equilibrium expressions are given as concentration in moles per litre.

Thus,
\[ k_n = \frac{ML_n}{ML_{n-1}L} = \text{n}^{\text{th}} \text{ stoichiometric metal-ligand stability or } \text{formation constant} \quad \ldots \ldots \quad (13) \]

and
\[ \beta_n = \text{n}^{\text{th}} \text{ overall stoichiometric metal-ligand stability constant.} \]
\[ \beta_n = \frac{ML_n}{[M][L]^n} = \sum_{n=1}^{n} k_n \quad \ldots \ldots \quad (14) \]

Similarly, stoichiometric proton-ligand stability constants are given by
\[ k_H^j = \frac{LH_j}{LH_{j-1}H} \]
\[ \beta_H^j = \frac{LH_j}{[L][H]^j} = \sum_{j=1}^{j} k_H^j \]

Rossotti (291) has discussed the choice of a suitable salt medium. Potassium nitrate is the most frequently used supporting electrolyte because of very slight complexing tendency of nitrate ion. Molar concentrations are used in place of activities throughout the subsequent treatment.

2.5 Experimental Methods

Various experimental procedures have been proposed for the determination of stability constants from the above equations. The methods applicable to stepwise complex formation in an ionic medium are divided into two groups (292).
(i) Methods involving direct measurement of the equilibrium concentration of an individual species participating in the complex formation, viz., electrometric methods, solubility determinations, solvent extraction and ion exchange.

(ii) Methods based on measurement of some property that depends on the concentrations of several species in solution. Among these, the spectrophotometric and the cation exchange method are important.

pH metric method is the most accurate, reliable, and widely used procedure. The pH of the solution is sensitive to complex formation. The chelation results in release of hydrogen ions from the acid or lowers the concentration of the base. This method was introduced by Bjerrum (293).

Several authors (291) have reviewed the experimental techniques.

Usually, the computation of stability constants from experimental data comprises the following three steps:

(i) Evaluation of the formation curve of the system.

(ii) Calculation of stoichiometric $K_n$'s of the system by direct solution of the formation function or

(iii) Conversion of stoichiometric constants into thermodynamic constants.

2.6 Evaluation of the formation curve

For systems involving a ligand which is a conjugate base of the weak acid, the pH-metric method introduced by Bjerrum (293) has been widely employed.
The method, as adopted by Irving and Rossotti (294), has several advantages. Here, it is neither necessary to convert the pH meter readings to stoichiometric hydrogen ion concentrations nor to know the stoichiometric concentration of the neutral salt added to maintain the ionic strength constant. The method is valid both in water and in mixed solvents. Moreover, the dissociation constants of the chelating agents are obtained under the same experimental conditions as the metal ligand formation constants. Therefore, this method is employed in the present work.

EXPERIMENTAL

Apparatus

Melting points were determined in open capillaries and are uncorrected.

(i) Spectrophotometer

All absorption spectral measurements were taken on Beckmann 25 model spectrophotometer equipped with two matched cuvettes having 1 cm light path.

(ii) IR Spectrophotometer

IR Spectra were recorded on a Perkin-Elmer 337 Grating spectrophotometer in KBr disc.

(iii) Atomic absorption spectrophotometer

A GBC Model 901 atomic absorption spectrophotometer had been used.

(iv) pH meter

The pH measurements were carried out on spectronic pH meter 324.
The pH meter has accuracy of $\pm 0.01$ pH and reproducibility of $\pm 0.02$ pH in standard operation. A glass electrode recommended for over pH range 0-14 and temperature range 15° to 80°, was used in combination with standard calomel reference electrode.

When the apparatus was not in use, glass electrode was immersed in distilled water. After dipping into strongly alkaline solution during pH titration, it was momentarily immersed in 0.1 M HCl and then rinsed thoroughly with distilled water. Occasionally, it was left over night in 0.1 M HCl.

**Calibration of pH meter**

The pH meter was standardised at the desired temperature with buffer solutions such as 0.05 M potassium hydrogen phthalate (pH 4.00 at 25°C and 4.01 at 35°C), 0.01 M borax (pH 9.17 at 25°C and 9.10 at 35°C) National Bureau of Standard buffers. Standardisation was checked by at least two buffers before and after each titration.

**Chemicals and Reagents**

Folic acid (USP), Potassium hydrogen phthalate (Glaxo), Borax (Glaxo), Sodium hydroxide (Glaxo), Perchloric acid (E.Merck), Calcium carbonate (Glaxo), Magnesium carbonate (Glaxo), Barium carbonate (Glaxo), Strontium carbonate (Glaxo), Copper carbonate (Glaxo), Cobalt carbonate (SD's), Manganese carbonate (S.M.), Zinc oxide (E.Merck), Cadmium oxide (SD's), Reduced iron (S.M.), Ammonia solution (E.Merck), Disodium edetate (BDH), Ammonium
chloride (BDH), Eriochrome Black T (E. Merck), Xylenol orange (BDH), Sulfuric acid (E. Merck), Hexamine (SD's), Hydroxylamine hydrochloride (E. Merck), Triethanolamine phosphorus pentoxide (E. Merck), Nitric acid (E. Merck), Hydrochloric acid (E. Merck), Perchloric acid (E. Merck).

**Water**

The distilled water was twice redistilled over alkaline potassium permanganate from an all glass (pyrex) unit. The final distillate was stored in a well stoppered pyrex container. The pH of this water was about 6.8.

**Sodium hydroxide solution (0.1 M)**

Sodium hydroxide pellets (4.2 g) were dissolved in and diluted with water to 1000 ml.

The solution was standardised as follows: potassium hydrogen phthalate (100 mg) was dissolved in water (20 ml) and titrated with 0.1 M NaOH solution potentiometrically.

**Perchloric acid solution (0.1 M)**

Perchloric acid (8.5 ml; 70% W/V) was mixed with water and diluted to 1000 ml with the same solvent.

The solution was standardised by titrating 10 ml of 0.1 M sodium hydroxide solution against the prepared perchloric acid solution. The end point was detected potentiometrically.
Metal perchlorate solutions (0.05 M)

The excess of calcium carbonate, magnesium carbonate, barium carbonate, strontium carbonate, copper carbonate, cobalt carbonate, manganese carbonate, zinc oxide, cadmium oxide and reduced iron were added in excess to perchloric acid solution (100 ml; 0.1 M). They were allowed to react at room temperature for 3-4 hours with occasional shaking. The suspensions were filtered through Whatman 42 filter paper. The pH of the filtrate was found to be between 6 and 7.

Preparation of Folic acid solution (0.05 M)

Folic acid (550 mg) was dissolved in ammonia solution (0.625 ml; 25% w/v) and diluted to 25 ml with water.

Disodium edetate solution (0.05 M)

Disodium edetate (18.617 g) was weighed accurately and dissolved in warm water (200 ml). It was then diluted to 1000 ml with water.

Ammonia-Ammonium chloride buffer solution (pH 10)

Ammonium chloride (17.5 g) was added to ammonia solution (142 ml; 25% w/v) and diluted to 250 ml with water. The pH of the solution was adjusted to 10.

Eriochrome Black T Solution

Eriochrome Black (0.2 g) was dissolved in a mixture of triethanolamine (15 ml) and absolute ethanol (5 ml).

Xylenol Orange solution

Xylenol orange (50 mg) was dissolved in water (10 ml).
General Procedure for Standardisation of Metal Perchlorate Solutions

(a) Using Eriochrome Black T Indicator

Metal perchlorate solution (5 ml) was mixed with ammonia-ammonium chloride buffer solution (10 ml; pH 10). Eriochrome Black solution (3-4 drops) was added to it and titrated against standard disodium edetate solution (0.05 M) till the color was changed from pink to pure blue.

The perchlorate solutions of calcium, magnesium, zinc, barium, and strontium were standardized by the above method.

(b) Using Xylenol Orange Indicator

To metal perchlorate solution (5 ml), Xylenol orange solution (2-3 drops) was added and then diluted with sulfuric acid until the color changed from red to yellow. Powdered hexamine was added to it to get deep red color. The solution was warmed to 40°C and titrated against standard disodium edetate (0.05 M), till the color changed from red to yellow orange.

The perchlorate solutions of cobalt and cadmium were standardized by the above method.

(c) Standardization of Manganese Perchlorate Solution

To metal perchlorate solution (1 ml), a few crystals of hydroxylamine hydrochloride and water (10 ml) were added. The solution was warmed and neutralized with dilute sodium hydroxide, if necessary. Triethanolamine (3 ml), ammonia-ammonium chloride buffer solution (2 ml; pH 10) and Eriochrome T indicator solution
(2 drops) were added to it. The reaction mixture was titrated against standard disodium edetate (0.05 M) till the color changed from pink to blue.

\[
\text{Molarity of metal perchlorate} = \frac{\text{Vol. of EDTA} \times \text{Molarity of EDTA}}{\text{Vol. of metal perchlorate solution}}
\]

(d) Using Pyrocatechol Violet Indicator

To metal perchlorate solution (5 ml), sufficient pyridine was added to produce intense blue color. Pyrocatechol violet solution (5-6 drops) was added to it and titrated against standard disodium edetate solution (0.05M) till the color changed from blue to yellowish green.

Copper perchlorate solution was standardised by this method.

(e) Colorimetry

Ferrous sulfate (0.35g) was dissolved in water (25 ml). 1 ml of this sample (0.05M) was mixed with sodium acetate acetic acid buffer (2 ml) and 1 ml of 1,10-phenanthrolinium chloride solution. The solution was diluted to 25ml with water and absorbance was measured at 510 nm on colorimeter.

The metal perchlorate solution (1 ml) was treated similarly and absorbance was compared with that of standard Fe\(^{2+}\) solution.
Calvin-Bjerrum pH Titrations

The titrations were carried out in 100ml beaker. The pH meter was calibrated as described earlier.

(A) pH metric titrations of a mixture of folic acid and metal perchlorate (Ca$^{2+}$, Mg$^{2+}$, Ba$^{2+}$, Sr$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Fe$^{2+}$) were carried out at 37 ± 0.1°.

(B) The total volume $V^o$ in each set was 50 ml, keeping constant ionic strength $i = 0$.

The following sets of mixtures were prepared for titration:

(1) 18 ml of HCIO$_4$ (0.1 M) + 32 ml of water.

(2) 5 ml of Folic acid solution (0.1 M) + 18 ml of HCIO$_4$ (0.1 M) + 27 ml of water.

(3) 5 ml of Folic acid solution (0.1 M) + 2 ml of metal perchlorate solution (0.05 M) + 18 ml of HCIO$_4$ (0.1 M) + 25 ml of water.

(a) Titration of the Perchloric Acid mixture No.(1)

Mixture No.1 was taken in the titrating vessel and titrated against standard sodium hydroxide (0.1M). The change in the pH of the solutions with each addition of sodium hydroxide solution was recorded (Table 12).

(b) Titration of the Mixture No.(2) containing Perchloric acid and Ligand

Mixture No.2 was taken in a clean titrating beaker and titrated as above (Table 12).
Metal Conc. = 0.05M (Ca, Mg, Ba, Sr, Cd, Zn, Cu, Co, Mn, Fe)  
Folic acid conc. = 0.1M; Free acid = 0.1M HClO₄; Titrant = 0.095N NaOH.

Table-12: Titration Data on Metal-Ligand Stability Constants of Folic Acid with Some Divalent Metal Ions

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<th>Volume of alkali added</th>
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<th>Ligand</th>
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<th>Mg²⁺</th>
<th>Ba²⁺</th>
<th>Sr²⁺</th>
<th>Cd²⁺</th>
<th>Zn²⁺</th>
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</table>

*Average of 5 determinations
(c) Titration of the Mixture No.(3) containing Perchloric acid, Folic acid and Metal perchlorate

Mixture No.3 was taken in a beaker and titrated as above (Table 12). The change in color of the reaction mixture and appearance of turbidity were observed during the titration.

Preparation of Folic Acid-Metal Ion Complexes

Folic acid (2.2 g) was dissolved in sodium hydroxide solution (100 ml; 0.1 N). The metal perchlorate solution (40 ml; 0.05M) was added to it and mixed thoroughly. The pH of the reaction mixture for precipitation of the complexes (as given in Table 13) was adjusted by adding dropwise the solution of perchloric acid (0.1M) with constant stirring. The reaction mixture was digested in a water bath (approx. 60°) for about half an hour to coagulate the precipitates. It was then cooled to room temperature and filtered with suction. The residue was washed thoroughly with water and dried at 80° in an oven, followed by drying in a desiccator over P₂O₅.

The melting points of the complexes are recorded in Table 13.

Metal-ion content determination

Chemical Method

Metal complex (500 mg) was weighed accurately and then decomposed with a mixture of conc. perchloric acid (25 ml) and nitric acid (25 ml). The residue was dissolved in hydrochloric acid (25ml; 0.1N) and diluted to 250 ml with water. An aliquot of the solution was used for determination of the metal content.
<table>
<thead>
<tr>
<th>No.</th>
<th>Complex</th>
<th>Precipitation pH $\pm 0.3$</th>
<th>M. P. $[^\circ\text{D}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CF$_2$ Ca</td>
<td>4.3</td>
<td>207</td>
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<tr>
<td>2.</td>
<td>CF$_2$ Mg</td>
<td>4.2</td>
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<tr>
<td>3.</td>
<td>CF$_2$ Ba</td>
<td>5.1</td>
<td>200</td>
</tr>
<tr>
<td>4.</td>
<td>CF$_2$ Sr</td>
<td>4.8</td>
<td>210</td>
</tr>
<tr>
<td>5.</td>
<td>CF$_2$ Zn</td>
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<td>CF$_2$ Cu</td>
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<td>CF$_2$ Co</td>
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<tr>
<td>8.</td>
<td>CF$_2$ Mn</td>
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<td>9.</td>
<td>CF$_2$ Cd</td>
<td>4.4</td>
<td>220</td>
</tr>
<tr>
<td>10.</td>
<td>CF$_2$ Fe</td>
<td>5.3</td>
<td>220</td>
</tr>
</tbody>
</table>
The metal-ions were determined volumetrically by titrating with disodium edetate solution as described under standardisation of metal perchlorates. The results were compared with those obtained by atomic absorption spectrophotometric analysis (Table 17).

RESULTS AND DISCUSSION

(A) Complex formation

Folic acid is sparingly soluble in water so it was dissolved in minimum quantity of sodium hydroxide solution (about 2.0 to 2.4 equivalent quantities) to form sodium folate which in presence of metal perchlorate forms 2:1 sodium folate : metal ion complex. In presence of excess of perchloric acid it is converted into folic acid metal ion complex having free carboxylic acid groups. Perchloric acid was used because it forms sodium perchlorate with sodium hydroxide which serves as a supporting electrolyte.

Complex formation at a particular pH is determined by Calvin-Bjerrum pH titrations. Titration curves are obtained by adding sodium hydroxide solution to the mixture containing (i) folic acid and perchloric acid; and (ii) folic acid, metal perchlorate and perchloric acid (Table 12). The pH obtained was plotted against volume of sodium hydroxide solution. From these potentiometric titration curves, it is seen that initially both titration curves are superimposing. The distance between the two curves increases gradually to a maximum at around pH 4.5 showing the formation of maximum amount of the complex. As titration proceeds beyond this pH, it decreases gradually. Generally, the complex formation takes place between pH 3.5 and 5.5.
(B) Confirmation of complex formation

In the preliminary experiments, folic acid metal ion complex formation is indicated from the following evidences.

(i) The distance between the titration curves of folic acid-metal ion perchlorate mixture and that of folic acid alone shows the formation of complex. (Table 12)

(ii) Metal ion content determination by chemical method. (Table 17).

(iii) Bathochromic shift observed in UV-spectrum of folic acid in the presence of metal. (Table 18)

(iv) Absence of absorption peak around 3300 cm\(^{-1}\) in IR of folic acid metal ion complex. A sharp peak at 825 cm\(^{-1}\), 525 cm\(^{-1}\) and 450 cm\(^{-1}\) shows the presence of metal.

(v) Molecular weight determination. (Table 19)

(vi) X-Ray diffraction shows the presence of metal in stable position. (Table 20).

In order to determine the moiety of folic acid involved in metal ion complex formation, the molecule of folic acid may be divided into two parts, (i) Benzoylglutamic acid and (ii) Pteridine portion.

The complex formation in benzoylglutamic acid may involve \(\text{HOOC-CH-CH}_2\text{-CH}_2\text{-COOH}, -\text{NH-CH-COOH or -NH-CH-CH}_2\text{-CH}_2\text{-COOH moiety.}

If the complexation occurs with these moieties, the formation of 5-, 7- and 8-membered chelates are possible. However, 7- and 8-membered rings are reported to be unstable (295).
Since, the complexes give positive tests for free carboxylic acid and are soluble in sodium bicarbonate or sodium hydroxide solution, the possibility of involvement of carboxylic group in complex formation is less. Further, it is reported that stability of metal ion complexes with benzoylglutamic acid is very poor (295).

Therefore, it seems that the complex formation takes place in pteridine moiety.

One of possible sites for complex formation in pteridine fragment is at N-1 and -NH₂ in position 2. However, the chelate thus formed may have 4-membered ring, which are reported to be unstable (296). Further, IR spectra of complexes indicates the presence of primary amino group.

The other two possibilities are the involvement of 4-OH and 5-N or 5-N and 10-N. In the formation of latter complex, there is no possibility of hydrogen ion production. While in the present case, the titrable hydrogen ion is formed. Hence, the 4-OH and 5-N take part in metal ion complex formation.

It is also noteworthy that the physiological activity of folic acid in its metal ion complexes remains intact because the active centres 8-N and 10-N in folic acid molecule do not take part in metal ion complexation (297). We support, therefore, the structure of folic acid metal ion complexes as proposed by Albert et al. (295).
(c) Study of Stability Constants

Calculations of stability constants of folic acid metal ion complexes:

The reaction between folic acid and metal perchlorate can be written as

\[ 2 \text{Folic acid} + M(C104)_2 \longrightarrow (\text{Folic acid})_2M + 2\text{HClO}_4 \]  

(I) Folic acid \((L)\) reacts with metal ion \((M)\) to give complex \((ML)\).

\[ M + L \rightleftharpoons ML \]  

The equilibrium constant \((K_1)\) is given by expression

\[ K_1 = \frac{[ML]}{[M][L]} \]  

(ii)

Since folic acid forms stepwise complex with another ligand,

\[ ML + L \rightleftharpoons ML_2 \]  

(iii) The complex formation constant \((K_2)\) is given by expression

\[ K_2 = \frac{[ML_2]}{[ML][L]} \]  

(iv)

The overall formation constant \((\beta)\) of the system is calculated from the following equation

\[ \beta = K_1K_2 = \frac{[ML_2]}{[M][L]^2} \]  

(v)

where \(K_1\) and \(K_2\) = formation constants

\[ \beta = \text{stability constant} \]

(II) \(\bar{n} = \frac{\text{Total concentration of ligand bound}}{\text{Total concentration of metal ion}}\)

\[ = \frac{[ML] + [ML_2]}{[M] + [ML] + [ML_2]} \]
The \( \bar{n} \) value can be calculated from the above equation (298),
where \( \bar{n} \) = average number of ligand groups bound per metal ion.

\[
\bar{n} = \frac{d \times N_{\text{NaOH}}}{\text{Total mmol of metal}}
\]

where \( d \) = horizontal distance between the two curves at a given pH.

'd' represents the amount of NaOH required to titrate the H\(^+\) ions
liberated by the formation of a complex.

\[ N = \text{normality of NaOH solution.} \]

(III) \[ [L] = \frac{K_a \{[HL]_{\text{initial}} - [\text{NaOH}]\}}{[H_2O^+]} \]

\[
\therefore p[L] = pK_a - \text{pH} - \log \left\{ [HL]_{\text{initial}} - [\text{NaOH}] \right\}
\]

where \([L]\) = concentration of free ligand

\([\text{NaOH}]\) = concentration of alkali added

\([\text{ML}]_{\text{initial}}\) = initial concentration of ligand

The \( \bar{n} \) and corresponding \( p[L] \) values for folic acid metal
ion complexes were calculated (Table 14). The maximum \( \bar{n} \) values
for Ca\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Mn\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\) and Fe\(^{2+}\)
metal ion complexes of folic acid obtained were 2, which confirms
that the metal to folic acid complex ratio is 1:2 under the
experimental conditions.

From Table 14 log \( K_1 \) and log \( K_2 \) can be determined (298).

\[
\log K_1 = p[L] \text{ at } \bar{n} = 0.5 \\
\log K_2 = p[L] \text{ at } \bar{n} = 1.5
\]

when \( \bar{n} \) is less than 1.5, log \( K_2 \) can be calculated from the
following equation.
Table 14: n and corresponding p(L) values for folic acid metal ion complexes

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</tbody>
</table>

*CF2Ca, CF2Mg, CF2Ba, CF2Sr, CF2Co, CF2Zn, CF2Cu, CF2Cd, CF2Mn, CF2Fe are complexes of 2 moles of folic acid with 1 mole of metals viz. Calcium, Magnesium, Barium, Strontium, Cobalt, Zinc, Copper, Cadmium, Manganese and Iron.
2 \log K_1 K_2 = p[L] \text{ at } \bar{n} = 1.0

\therefore \log K_2 = 2 \log K_1 K_2 - \log K_1 \text{ and }

\log \beta = \log K_1 + \log K_2

The data of stability constants of folic acid metal ion complexes are given in Table 15.

**Stability constants of Folic acid with divalent metals**

The stability constants of folic acid with the divalent metal ions are studied under two categories:

**Category-I:** Transition metals viz. Cu$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Fe$^{2+}$

**Category-II:** Alkali earth metals viz. Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$

The stability constants of folic acid with these metal ions are discussed on the basis of:

(i) Variation with the atomic number.

(ii) Order of stability constant.

(iii) Variation with the ionic radii.

**(i) Atomic number**

It has been seen from (Fig.1-3) the plot of \log K_1, \log K_2 or \log \beta against atomic number, that the stability constant increases with the increase in atomic number in case of transition metals. Exception of Cu$^{2+}$ is due to John Teller effect (299), which states that any nonlinear molecule which has unequally filled set of orbitals, will be distorted and remove the degeneracy.

In case of alkali earth metals, the stability constant decreases with the increase in atomic number. The decrease is due to ionic radii of alkali earth metals.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Log $K_1$</th>
<th>Log $K_2$</th>
<th>Log $\beta$</th>
<th>Log $K_1/K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF$_2$Mg</td>
<td>8.40</td>
<td>7.05</td>
<td>15.45</td>
<td>1.35</td>
</tr>
<tr>
<td>CF$_2$Ca</td>
<td>8.02</td>
<td>6.80</td>
<td>14.82</td>
<td>1.22</td>
</tr>
<tr>
<td>CF$_2$Sr</td>
<td>7.10</td>
<td>5.62</td>
<td>12.72</td>
<td>1.48</td>
</tr>
<tr>
<td>CF$_2$Ba</td>
<td>6.85</td>
<td>5.35</td>
<td>12.20</td>
<td>1.50</td>
</tr>
<tr>
<td>CF$_2$Cu</td>
<td>8.30</td>
<td>6.98</td>
<td>15.28</td>
<td>1.32</td>
</tr>
<tr>
<td>CF$_2$Cd</td>
<td>7.94</td>
<td>6.74</td>
<td>14.68</td>
<td>1.20</td>
</tr>
<tr>
<td>CF$_2$Zn</td>
<td>7.54</td>
<td>6.24</td>
<td>13.78</td>
<td>1.30</td>
</tr>
<tr>
<td>CF$_2$Co</td>
<td>7.26</td>
<td>6.00</td>
<td>13.26</td>
<td>1.26</td>
</tr>
<tr>
<td>CF$_2$Fe</td>
<td>7.04</td>
<td>5.70</td>
<td>12.74</td>
<td>1.34</td>
</tr>
<tr>
<td>CF$_2$Mn</td>
<td>6.37</td>
<td>5.00</td>
<td>11.37</td>
<td>1.37</td>
</tr>
</tbody>
</table>

The values of log $K_1$ and log $K_2$ are accurate to ± 0.1
Fig. 1: Effect of atomic number of metals on equilibrium constant of folic acid metal complexes.
Fig. 2: Effect of atomic number of metals on the complex formation constant $K_2$. 
Fig. 3: Effect of atomic number of metals on overall formation constant.
(ii) Order of stability constants

The comparison of log $K_1$ and log $K_2$ values (Table 15) for folic acid metal ion complexes show the following order of stability:

**Alkali earth metals**

$\text{Mg}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$

**Transition metals**

$\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$

This finding is in agreement with those of Irving and Williams (300). In all complexes, the value of log $K_1 >$ log $K_2$, and so the ratio of successive stability constants ($\log K_1/K_2$ or $\log K_1-\log K_2$) is positive.

(iii) Ionic charge and radius

The stability of a complex increases with the increase in ionic charge ($e$) of both metal ion and donor. But the stability decreases with the increase in ionic radius ($r$) of the metal ion.

It is reported that the stability constant of alkaline earth metals increases with the increase in the value of $e^2/r$. Born (281) has mathematically expressed the above relationship by the following equation.

$$E = \frac{e^2}{2r} (1-1/D)$$

where $D =$ dielectric constant of solvent and $E =$ energy change.
Since $\log \beta$ is directly related to energy, it is also proportional to ratio of square of charge of metal ion to its radius.

A linear relationship is observed between stability constants of complexes and $e^2/r$ or $1/r$ (Fig.4,5) (Table 16).

Folic acid metal ion complex (2:1)
<table>
<thead>
<tr>
<th>Metal</th>
<th>Atomic number</th>
<th>Ionic radius</th>
<th>1/r</th>
<th>$e^2/r$</th>
<th>log β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>12</td>
<td>0.61</td>
<td>1.64</td>
<td>6.56</td>
<td>15.45</td>
</tr>
<tr>
<td>Ca</td>
<td>20</td>
<td>0.93</td>
<td>1.08</td>
<td>4.30</td>
<td>14.82</td>
</tr>
<tr>
<td>Sr</td>
<td>38</td>
<td>1.06</td>
<td>0.94</td>
<td>3.77</td>
<td>12.72</td>
</tr>
<tr>
<td>Ba</td>
<td>56</td>
<td>1.27</td>
<td>0.78</td>
<td>3.15</td>
<td>12.20</td>
</tr>
<tr>
<td>Cu</td>
<td>29</td>
<td>0.68</td>
<td>1.47</td>
<td>5.88</td>
<td>15.28</td>
</tr>
<tr>
<td>Cd</td>
<td>48</td>
<td>0.91</td>
<td>1.10</td>
<td>4.40</td>
<td>14.68</td>
</tr>
<tr>
<td>Zn</td>
<td>30</td>
<td>0.70</td>
<td>1.43</td>
<td>5.71</td>
<td>13.78</td>
</tr>
<tr>
<td>Co</td>
<td>27</td>
<td>0.70</td>
<td>1.43</td>
<td>5.71</td>
<td>13.26</td>
</tr>
<tr>
<td>Fe</td>
<td>26</td>
<td>0.71</td>
<td>1.40</td>
<td>5.63</td>
<td>12.74</td>
</tr>
<tr>
<td>Mn</td>
<td>25</td>
<td>0.75</td>
<td>1.33</td>
<td>5.33</td>
<td>11.37</td>
</tr>
</tbody>
</table>
Fig. 4: Effect of ionic charge and radii of metals on stability constant.
Fig. 5: Effect of ionic radii of metals on stability constant.