PHYSICO-CHEMICAL STUDIES ON THE INTERACTION OF METAL IONS & SILICIC ACID WITH CASEIN, α - CASEIN & TRANSFUSION GELATIN

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By

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C_E_R_T_I_F_I_C_A_T_E

Certified that the thesis entitled " PHYSICO-CHEMICAL STUDIES ON THE BINDING OF METAL IONS AND SILICIC ACID SOL WITH CASEIN, α_s-CASEIN AND TRANSFUSION GELATIN" which is being submitted by Mr. Surendra Kumar Agarwal for the award of Doctor of Philosophy in Chemistry of the Roorkee University, is a record of his own work under my supervision and guidance. The matter embodied in this thesis has not been submitted for the award of any other degree of any University.

This is further to certify that he has worked for a period of two years and about two months from September 23, 1963 to December 5,1965 at this University to prepare this thesis.

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A_C_K_N_O_W_L_E_D_G_E_M_E_N_T

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C_O_N_T_E_N_T_S

PART B:

G_E_N_E_R_A_L I_N_T_R_O_D_U_C_T_I_O_N

GENERAL INTRODUCTION

Proteins are large molecules generally classed under what are named as macromolecules. The commonly known examples are gelatin, casein, myocin, egg albumin, haemoglobin etc. In the language of a chemist these may be considered to be built up from structural units consisting of amino acids in the main chain, being simple proteins. But there exist other components also, what are called prosthetic groups, resulting in the formation of complex or conjugated proteins or proteids. Amongst these may be mentioned the glucoproteins, e.g., ovalbumin, mucin; phosphoproteins, e.g., casein; having the carbohydrates, and phosphoric acid as the prosthetic groups respectively and the nucleoproteins and chromoproteins. But for these minor differences the proteins are chemically alike and their structure is characterised by the polypeptide chains present in them.

From the structural view point the proteins are of two types; the fibrous proteins, and the globular or corpuscular proteins. Of the former the simplest is the fibroin of silk, containing an elongated trans-polypeptide chain. Other examples of fibrous proteins are the keratins (proteins from hair and fur of animals, feathers, nails, horns, quills etc.) which are folded and cross-linked and when stretched are like silk. The other variety, viz.,

collagen is cis-polypeptides, permanently folded and unlike keratins cannot be easily stretched due to the presence of certain side groups. Another class comprises globular or corpuscular proteins which are completely folded into dense structural units, giving what may be termed as colloid crystals as in the case of colloidal solutions of egg albumin and haemoglobin.

Certain properties, like denaturation, swelling, gelation and amphoteric character appear to be intimately connected with the structural characteristics of the proteins. The presence of these properties may be attributed to the existence of groups or side chains other than those present in the main chain. These can be the carboxylic and the amino groups responsible for their amphoteric character(the behaviour wrongly assigned to the amino acids in the main chain) and the paraffinic, phenolic or sulphydryl groups so very essential for the menifestation of hydrophobic or hydrophilic behaviour in them. The presence of such groups implies existance of Vander Waal's forces, dipole attraction between hydrophilic groups, formation of heteropolar bonds etc.in the proteins. Their swelling property in presence of water is a direct consequence of the loosening of dipole attraction in polar solvents. In a similar fashion denaturation-a property involving decreased solubility in water and increased opalescence-

may be ascribed to the rupture of certain linkages in the native protein and involves partial combination between the ionisable groups,such as S-S,CO-S,CO-NH-CO of the protein. Moreover,the chain length plays an important role in determining the crystallising tendency of the proteins. Thus it is found that molecules with unequal chain length do not form regular patterns and there the possibility of crystallisation is very remote.

Proteins,being intrinsically unstable,are highly reactive and interact almost with all anions and cations, with lipides ,with carbohydrates and with one another. Such interactions have been of prime importance in studying the applied aspects of protein chemistry. Many of the properties of silk and wool,especially related to their tensile strength and elasticity, may be modified by interaction with other substances. Von Weimarn has shown that silk may be dissolved in concentrated solution of sodium thiocynate and precipitated again in the form of glass, or it may be spun,stretched and stroked until it is far stronger than natural silk but is more brittle.

With globular proteins reactions with detergents (soap solutions) or organic solvents have proved to be of immense intrest to the technologist. The presence of detergents unfold the native structure resulting in the formation of elastic and highly double refracting fibre[1].

This could be achieved with egg albumin,wheat glutemin, casein,zein and blood albumin(2-6). Many laboratories have used highly concentrated organic solvents in place of detergents. The problem of the structural transformation of corpuscular to fibrous proteins carries great commercial potentialities-so much so that it should be possible to convert even tobacoo mosaic virus into textiles," an overcoat from a deseage"(7).

The process and technique of leather tanning is punctuated with reactions involving the interaction with organic and inorganic compounds. This process involves the turning of hydrated animal hides susceptible to putrefaction into dehydrated material,collagen tannate, by interaction with tannin. The use of tannin as a tanning reagent has been replaced by chromium salts, which being polyvalent,like tannin link the neighbouring chains together. Infact,tanning reagent are those which precipitate the gelatin of the hides in an insoluble form. Thus formaldehyde and syntan may be employed in tanning and are found to provide strong permanent cross-linkages (8).

Proteins,although basically different from high polymers in their structure (with free terminal groups), have found some use in plastics and in the manufacture of fibres. In both the cases modified protein products are obtained by interacting the protein with formaldehyde,

the process involving the joining of the two protein chains by means of a CH_2 group. Various patents, from time to time, have appeared in the literature for preparing casein fibres(9), casein wool and casein filaments(10). In all these mixtures of casein with a soap like sodium lauryl sulphate and formaldehyde is extruded in a coagulating bath containing sodium aluminate or zinc chloride. Casein fibre has an opaque silk like lusture similar to natural protein fibres and resembles certain types of rayon. Here too, formaldehyde is used to establish methylene bond but the extra tensile strength and elasticity is realised from aluminium sulphate or sodium aluminate by forming aluminium bridges.

A critical study of metal-protein interaction is of fundamental importance in investigating the action of ions in various tissues as well as in determining the role of enzymes in metabolic transformations. The latter are defined as organic catalysts of colloidal nature produced by living organism and are associated with many metal ions like, zinc, magnesium, manganese, iron or copper, probably in the form of coordination compounds. The presence of these metals is necessary if the enzyme has to retain its catalytic activity. Amongst the simple enzymes may be mentioned: urease(11)(for hydrolysing urea to ammonia and carbondioxide) whose activity is lost on

interaction with mercury through its -SH groups: carboxypeptidase(12,13) (used for breaking down peptides with a free terminal carboxyl group) where magnesium ions are indispersible for the action; pepsin and pepsinogen(14) (the enzyme secreted by gastric mucosa); trypsin (15) (the protase from the digestive tract which acts in the alkaline medium); α-amylase(16) (obtained from pig pancrease or human saliva and can split up starch in the middle of the molecule) in which inactivation sets in by the presence of heavy metal salts.

Besides the enzymes described above there exists metal proteides with enzymatic properties, like the copper proteides; polyphenoloxidase(17,18) containing 0.2 to 0.3% copper; monophenoloxidase(19) which contains 0.23% of copper and is able to oxidise p-cresol ten times faster than catechol and ascorbic acid oxidase(20) having 0.25% of copper bound in the complex. There are zinc and magnesium proteids also, e.g., carbonic anhydrase containing 0.2% zinc (21) which is extra-ordinarily sensitive to sulphanilimide which has an inhibiting effect even at a concentration as low as 2×10^{-6} molar; enolase (22,23), the effective catalyst for the removal of water from 2-phosphoglyceric acid is a magnesium compound; hexokinase(24), which catalyses the biochemically vital reaction:

glucose + ATP $\longrightarrow$ glucose 6 phosphate + ADP,
may contain both magnesium and manganese ions. phosphotases, which bring about the fission of phosphates are mostly activated by magnesium ions but some of them also get activated by manganese, iron(ii), cobalt(ii), nickel(ii), calcium, barium and cadmium ions(25). Similarly arginase, responsible for the breakdown of arginine to ornithine and urea, though contains complex bound manganese, is activated by cobalt, nickel, iron and vanadium(26). Other processes related to animal metabolism involving metal ions are: the phosphorolytic degradation of organic molecules by phosphotases(27,28) which have magnesium as the metal ion constituent; carbohydrate phosphorylation reaction with zinc protein, insulin(29).

The metal complexes so far considered are labile in character with the result that investigations with them have not been conclusive. Fortunately, however, we have a group of molecules, known as porphyrins, in which the metal ion is firmly bound and, therefore, can be usefully employed for getting a better insight in the mechanism of biological processes. Porphyrin complexes contain iron as the metallic constituent, e.g., haeme-iron(ii) protoporphyrin, its oxidised form haemin-iron(iii) protoporphyrin(30) and the binuclear complex-haematin. More information on this subject is available from the

recently published reviews(31 - 38).

CHARACTERISATION OF PROTEINS.

Many complexities arise during the investigations on proteins which are attributed to factors like, uncertainty in molecular weight,variation in chemical behaviour depending on the nature of origin,purity and their great sensitivity to denaturation. A large number of physico-chemical methods,viz.,ultracentrifugation(39-40),ultrafiltration(41-45), magnetic suceptibility(46-48),light scattering (49-51),solubility (52),viscosity(53),precipitation(54-56),migration in electric field (57-59),equilibrium dialysis(60-69), potentiometry and pH metry (70-75),polarography(76-84), spectrophotometry(85-93) and electrophoretic mobility (94-95) have provided means of ascertaining the mode of binding of the metals to the protein and in achieving greater success in the solution of the quantitative aspect of the problem.

The first important factor in the study of metal-protein interaction is the hydrogen ion equilibria.These studies are indispensible for the determination and characterisation of different ionisable sites acting as donor groups for the coordination of metal ions in the proteins. First theoretical interpretation of the hydrogen ion equilibria was given by Linderstrom-Lang (96-97) on the basis of Debye-Huckel theory. This was

further extended by Cannan et al(98,99), Kirkwood(100), and Scatchard(101). By the extensive studies Tanford (102-112) was able to determine the number of different reactive sites as well as their pK values, using the equation(113),

$$\log \frac{r}{n-r} = (pK_{int}) \pm 0.868\, ZW$$

where n is the number of ionisable groups with pK equal to (pK_{int}), r the number of groups ionised, Z the net charge on the protein molecule and $W = \frac{N e^2}{2DRT}\left(\frac{1}{b} - \frac{k}{ka}\right)$

where b is the radius of the protein, a the radius of exclusion and N, e, D, T and R have the usual significance as in the Debye-Huckel theory. If the Linderstrom-Lang's model of protein is adequate, a plot of pH-log $\frac{r}{n-r}$ against Z or more conveniently (114) h, the number of protons bound per protein molecule, would be linear with the intercept at h = 0 equal to pK_{int}. The value of the electrostatic factor W can be computed from the slope of the straight line. Hence it is found that hydrogen ion titration curves are enough to provide the necessary information regarding the electro-chemical nature of the protein molecule with an additional knowledge of number of ionisable groups.

THEORY OF MULTIPLE EQULIBRIA (115).

When proteins combine reversibly with small molecules or ions the usual laws of equilibrium govern the reaction. Since proteins in general offer a large number of reactive sites for metal ion binding, therefore,the successive reactions in the multiple equilibria of a metal ion M,with a protein molecule P,having n sites for combination may be represented by the equations.

$$P - M \quad PM$$
$$PM - M \quad PM_2$$
$$PM_2 - M \quad PM_3$$

$$PM_{n-1} - M \quad PM_n$$

The individual formation constants may be given according to law of mass action

$$K_1 = \frac{(PM)}{(P)\,(M)}$$

$$K_2 = \frac{(PM_2)}{(PM)\,(M)}$$

$$K_3 = \frac{(PM_3)}{(PM_2)\,(M)}$$

$$K_n = \frac{(PM_n)}{(PM_n-1)(M)}$$

Now if $V = \frac{\text{Moles of bound M}}{\text{Moles of total protein}}$ it can be shown that

$$V = \frac{(PM)-2(PM_2)\ \text{----}\ n(PM_n)}{(P)-(PM)-\ \text{.....}\ (PM_n)} \quad \text{...(i)}$$

$$\text{Or} \qquad V = \frac{K_1(M) - 2K_1K_2(M)^2 - \ldots\ldots n\,K_1K_2\ldots K_n(M)^n}{1-K_1(M)-K_1K_2(M)^2 \ldots\ldots K_1K_2\ldots K(M)^n} \ldots (ii)$$

If each site, uninfluenced by its neighbouring sites has the same affinity for M, then $K_1 \ldots K_n$ are not independent but bear a relationship with each other and to a single intrinsic constant K.

$$K_2 = \frac{n-i-1}{i} K \text{ where } i = 1, 2 \ldots n$$

equation 2 therefore reduces to

$$V = \frac{nK(M)}{1-K(M)} \ldots\ldots\ldots\ldots \quad (iii)$$

But when an ion is bound to protein, it tends to reduce the affinity of the protein for the second on-coming ion due to electrostatic repulsion and hence equation 2 would no longer be valid. This electrostatic factor may be introduced into equation(3) by an approximation (96), fairly accurate for the magnitudes of the electrostatic effects commonly encountered in ion-protein interaction and thus the equation(3) becomes

$$V = \frac{nKe^{-2z^2W}(M)}{1 - Ke^{2Z2W}(M)} \ldots\ldots\ldots (iv)$$

where Z is the charge of the metal ion and W a quantity related to the electrostatic work required to bring the ion M to the surface of the protein molecule, and for a shperical model it is given by the expression

$$W = \frac{Ne^2}{2DRT} \left(\frac{1}{b} \right) - \frac{K}{1-K_a}$$

where b is the radius of protein,a the radius of exclusion and N,e,D,R and T have the usual significance in the Debye-Huckel theory. In a competitive interaction,the two cations,say M and N may compete for the same site. Neglecting electrostatic interaction factor,the equation for a single set of sites reduces to,

$$V_M = \frac{n K_M (M)}{1 - K_M (M) - K_N (N)}$$

METAL-PROTEIN SYSTEMS

In the foregoing review we laid emphasis on the qualitative study of the metal-protein interactions. The extremely important but less familiar aspect of the problem is the quantitative studies of the metal-protein interactions. The last fifteen or twenty years have witnessed great theoretical and experimental advances in this direction to which many brilliant workers have made extremely useful contributions. Amongst them may be named Klotz(116-121),Albert(122-124),Monk(125),Li and Coworkers(126-129),Gurd(130,131), Scatchard(132),Tanford(133-136),Gustaveason(137-139), Schulman(140) etc.. A review of their work has revealed that the theoretical and practical advances made so far have been due to the selective application of modern physico-chemical techniques. The studies carried out so far,may be divided under the following heads

(i) the evaluation or determination of the intrinsic association constant,(ii) a comparision of these constants with the formation constants of simple ligands having the same donor groups as the proteins, (iii) experiments performed to show the absence or presence of the phenomenon of chelation and (iv) the study of systems involving 'sluggish equilibrium'e.g., formation of poly nuclear complexes(141) of iron, chromium and aluminium. The results reveal that the interaction of mercury,copper,silver,zinc,lead and cadmium with proteins have been the most extensively investigated. These metals are strongly bound to different reactive sites on the protein molecule,being preferentially bound to - SH group at lower concentration. Moreover,they generally form stronger bonds with nitrogen than with oxygen atom,and hence their interaction with imidazole, ϵ -amino and guanidino groups are more significant.

The interaction of copper with serum albumin and also the methylated proteins(142) have been investigated by Klotz(143-147). These studies indicate the binding of copper with carboxyl,imidazole and the amino groups of the proteins. Klotz(148-167) and coworkers also studied the combination of copper with α-casein β-lectoglobin, γ-globin and lysozyme.

The combination of mercury or its mono-alkyl

derivatives(168) with the -SH group of the protein have been carried out (169-171). The results with serum albumin indicate,that one metal ion is bound with two protein molecules forming a dimer. Higher concentration of the metal ion is found to oppose the formation of dimer and so also the excess of halide ions. Methyl mercury salts react quantitatively with sulphydryl groups with pK = 4.5.

Interaction of zinc and cadmium have been studied in detail by Gurd and Goodman(171,172).They concluded that zinc combines with the imidazole groups of protein in the pH range 5-6.8 reversibly at 0°C. Tanford(173) also derived the same conclusions employing polarographic technique. Gurd(174)studied the interaction by equilibrium dialysis. The nature of zinc -insulin complex has been investigated by Tanford and Epstein(175) by pH metric and polarographic methods. They concluded that each zinc was bound to two imidazole groups below pH 6.5.

In contrast to transition metals,alkaline earth metals ions form complexes with weaker binding and combination usually takes place at the charged sites. These results point to the fact that in protein complexes of alkaline earth cations the affinity parallels that of small ligands.

From the foregoing review it is clear that very

little attention has been paid for the quantitative elucidation of the binding of metal ions with proteins like,gelatin,casein etc.. Extensive studies in this direction were taken out a few years ago by Malik and Coworkers(180-185) employing transfusion gelatin (a protein of the collagen type, important from pharmacological point of view(186) and has a simpler configuration and known molecular weight).Some of their conclusions are listed below:

1. Hydrogen ion equilibria studies on transfusion gelatin carried out at different temperatures revealed the presence of 84 carboxyl,23 amino,3.8 imidazole and 43 guanidinium groups per 75 x 10^3 gms. of protein. The results confirmed the values of amino acid analysis and also those obtained from the observations of apparent heats of ionisation. Tanford's equation was found to be applicable only in the carboxyl region(W=0.027).

2. The influence of the protein concentration,metal ion concentration and pH on the i_d/i_{d0} values was studied by carrying out polarographic measurement in mixtures of cupric sulphate and transfusion gelatin.It was found that a decrease in value took place with increase in protein concentration and decrease in the metal concentration. The latter effect has been attributed to the binding of the copper with transfusion gelatin. Evoluation of log K showed that the copper is more strongly bound to

the amino than the carboxyl group. Increase in pH resulted in an increase in the $i_{d/i_{dO}}$ value also.

Polarographic and pH metric studies of mixtures containing lead nitrate and transfusion gelatin carried out with varying concentration of the reactants and under varying pH conditions have shown the existence of a metal-protein complex in the pH range 3.7-5.6. Experiments carried out at higher pH range(5.9-6.8)gave little or no evidence for the binding of lead to the imidazole groups. An alternative method,identical in principle with Bjerrum's method,was introduced to calculate V_M (active sites covered by the metal) from the difference in the hydrogen ion data in the presence and absence of metal.Evidence for one to one binding of plumbous ion to the carboxylate ~~ion~~ groups of transfusion gelatin,with values of 1.87 and 2.593 K cals for the intrinsic association constant and free energy respectively,were obtained.

Polarographic studies with zinc and cadmium (using Tanford's method) showed that two atoms of each cadmium and zinc were found to be bound per protein molecule in the pH range 9.0 to 11.0 with association constants 2.778 and 2.380 respectively. In the lower pH range one cadmium ion combined with the carboxyl groups while no such tendency could be seen with the amino group of the protein . The order is copper, > cadmium > zinc. It would be interesting to show that

it was for the first ~~the~~ time that the evidence of the binding of zinc to amino group of a protein was available on the basis of these studies.

Recently the interaction of metal hydrous oxide sols with proteins have been studied by Malik and Coworkers(176-179). This aspect of study is likely to give an answer to the unsolved problem of the role of "Kushtas" and "Bhasams" in the indigineous system of medicine (Unani or Ayurvedic). This is yet to be extensively studied in order to give a clear picture of the influence of colloidally dispersed metal ions,when taken orally, on the metabolic activity of human beings. Preliminary studies carried out by them during the last few years have provided the following useful informations:

1. pH metric titrations and viscometric studies on the interaction of gelatin with aluminium,iron,cobalt, nickel and silver show that the aluminium ions from its colloidal solutions combine with free carboxyl groups of the protein in the pH range 3.0-5.0. No evidence of such a binding in available with iron.On the other hand cobalt,nickel and silver hydrous oxide sols unlike alumina,do not give evidence of the availability of these metals for interaction with the protein to give definite complexes. Viscosity-concentration curves,however,give indication of the formation of adsorption complexes with the colloidal solutions.

2. pH metric titrations of anionic haemoglobin with aluminium, chromium and beryllium reveal that these metals make themselves available from their respective hydrous oxide sols for interaction with the protein, the combining power being of the order Al(III) > Cr(III) > Be(II). Here too carboxyl groups are involved.

3. With another important protein, casein, evidence is forthcoming for the availability of cobalt, nickel and silver from their hydrous oxides for interaction with this protein. Unlike the previous studies, here ~~with~~ the possibility of the metal ions combining with the imidazole group of the casein is indicated.

The work described in this thesis deals with the extensive studies and advancement made on the binding of metal ions with transfusion gelatin, casein and α_s-casein.

Statement of the problem

The information contained in the following pages have been divided into two parts, A and B.

Part A.

Quantitative studies on the interaction of metals with proteins.

Chapter 1: Potentiometric studies on the interaction of magnesium, manganese and strontium with transfusion gelatin.

Chapter 2: Equilibrium dialysis studies on the binding of magnesium nickel and cobalt with transfusion gelatin.

Chapter 3: pH-metric studies on the interaction of copper, zinc, magnesium, manganese and strontium with casein and α_s-casein.

Chapter 4: Polarographic studies on the binding of copper to α_s-casein.

Part B.

Studies on the mutual interaction of hydrophilic and hydrophobic sols.

Chapter 5: pH-metric studies on the interaction between silicic acid sol or Tabaschir sol and Transfusion gelatin.

Chapter 6: This deals with the variation in viscosity during the sol-gel transformation of some, casein, α-casein, sodium metal silicate, and formaldehyde, mixtures.

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C_H_A_P_T_E_R I

Potentiometric studies on the binding of magnesium, manganese and strontium with transfusion gelatin.

I_N_T_R_O_D_U_C_T_I_O_N

pH-metry as an indirect method for indicating metal-protein interactions has been successfully employed by several workers(96,98-101,185,187-189) for estimating bound hydrogen ions in proteins. The hydrogen ion equilibria gives invaluable information(102,190,191) about the intrinsically identical groups of particular type and their pK values,configurational changes occuring during the titration of protein with acid or alkali; and the average net charge on a protein molecule. Besides this, technique has been utilized to study the binding of simple monovalent anions or cations,where the interaction is purely electrostatic in nature. It has been argued that the change in PH by the addition of alkali metal salt to a protein solution of isoionic pH,towards the alkaline side is indicated in the binding of anions and the reverse effect is shown in the binding of cations( ).This method has, however,got its limitations in determining the binding of metal cations to proteins(in conventional type of reversible binding) in view of the difficulties encountered in the choice of suitable electrode in realising the condition where accurate measurements may be made. It is,therefore, of great interest to develop an indirect method based solely on pH measurement which can be used successfully to estimate the bound metal ions to protein. Earlier

communications from Malik and Coworkers(192,193) gave enough evidence that hydrogen ion titration curves of proteins in presence and absence of metal ions may be used to calculate the binding data and relevant intrinsic association constant of metal protein interactions.

The work,described in the present chapter,deals with the studies on the binding of some physiologically important metal ions,like magnesium,manganese and strontium with transfusion gelatin by pH metric method. The results have given evidence for the binding of these metal ions at different sites of transfusion gelatin- a study which has not hither to been made with fibrillar proteins.

A knowledge of the development made in electrochemistry of proteins becomes of primary importance for workers in the field of metal-protein complexes;since the studies on the binding remain purely of qualitative nature unless data on acid-base binding capacities of proteins are made available. A metal ion has to compete with hydrogen ions(which surround the protein molecule) for a common site in the process. Such competitive process necessiates exact knowledge of hydrogen ion binding capacities and their pK values. A short summary of the work done on hydrogen ion binding to different proteins is therefore worth describing.

Early attention was focused on the maximum amount

of acid and base with which proteins can combine as indicative of the number of dissociable cationic or anionic groups which they possess. This was later developed to give the equilibrium constants of various types of dissociable groups. Burgarozky and Liberman (193) were the first to report the results on the electromotive force determination of the acid-or base-binding. Since then a number of workers,notably, Robertson(194),Rohonyi(195),Manabe and Matulla(196), Basel and Matulla(197),Pauli(198),Llyod and Mayer(199) have worked in this direction. Conductometric (200-201) and Cryoscopic methods(202) were met with little success. Van Slyke(203),orying and Pauling(205), Bracewell(206) and Izaquirre(207) explained the phenomena of hydrogen ion binding on the basis of physical adsorption. The first explaination based on purely chemical view point was put forward by Loeb in 1922 and further developed by Linderstron-Lang(96),who gave the adequate theoretical interpretation of the phenomena on the basis of Debye-Huckel theory. Since then many modifications have been put forward by different workers namely Cannan,Kibrick and Palmer (98,99),Kirkwood(100), Scatchard(101) and Tanford(102-112).

The titration curves in these cases may be divided into a number of regions in which the different groups ionise. The titrable groups which occur in

greatest abundance are the side chain carboxyl groups of glutamic acid and aspartic acid;the amino groups of the lysine residue;the guanidyl groups of arginine residue;the imidazole groups of histidine residue;and the phenolic groups of the tyrosine residue. Less frequent are the thiol groups of cysteine;the phosphoric acid groups of phosphoseriene and phosphothreonine. The number of these ionisable groups of various proteins such as Haemoglobin(208-210),Serum albumin(105),Eggalbumin(98), β-Lactoglobulin(4,211),Lysozyme(107),Insulin(72,104), Conalbumin(114),Ribonuclease(103),Wool keratin(212), Myosin(213),Casein, α-, β- and γ-caseins(214), Collagen(215),Gelatin(216-227),Metmyoglobin(228) and Bovine Thrombin(229) have been determined by the pH-metric method.

Metal-protein binding

The more important study, the study of metal protein system has elucidated a number of reviews.These include that of Greenberg (230); on the early studies on the binding of alkaline earth metals and that of Klotz(231) who gave theories and experimental methods for the study of binding. Lehninger(232) and Williams (233) considered the enzymatic activity of metal-protein complexes in the light of the reaction involving the formation of metal complexes with small organic ligand.

Some authors have devoted themselves exclusively to the study of metal protein complexes(234-242).

Knowledge about the metal complexes of simple organic molecule was first given by Bjerrum's work on 'Metal amine formation in aqueous solution'(243).His method was further extended to metal complexes of amino acids, peptides and finally to metal-protein complexes. The basic concept on which hydrogen ion titration data may be utilized to elucidate the nature of the binding of ions other then hydrogen ions was,however,put forward by Tanford((73).

Tanford's method for studying binding of metals.

Generally the metal ions form complexes with the substances which combine with the hydrogen ions. So there is a competition between the metal ions and hydrogen ions for the common site. This may be represented as,

$$M^{-z} + NH_3 \quad M(NH_3)^{-z}, M(NH_3)_2^{-z} \quad \text{.....(1)}$$

the equilibrium in such a reaction,may be measured indirectly by observing the displacement of corresponding hydrogen ion equilibrium.

$$NH_4^+ \quad NH_3 + H^+ \quad \text{.....(2)}$$

The hydrogen ion equilibria is expressed interms of dissociation constants. It is assumed that all ionisable groups of a particular type are intrinsically

identical,the degree of dissociation of such groups at one particular pH is given by:

$$\log \frac{x_i}{1-x_i} = pH - (pK_{int}) - 0.868\, ZW.$$

here x_i represents the degree of dissociation of groups of the ith kind,Z is the net charge on the protein molecule at a given pH and W is an emperical parameter which depends (for a particular temperature and a given ionic strength) on the size and shape of the protein molecule. If n_i is the total number of intrinsically identical ionisable groups of the i th type and r_i is the number of such groups dissociated at a given pH then the above equation becomes:

$$\log \frac{r_i}{n_i-r_i} = pH - (pK_{int}) \pm 0.868\, ZW.$$

This equation is generally applied to determine the pK_{int} values of different ionising groups in the protein molecule. Since in presence of metal ion a fraction of a particular type of groups will be removed from participation in hydrogen ion equilibria due to metal ion binding;a shift in pH would therefore,be observed. In other words,the hydrogen ion equilibria will be shifted towards the basic side of the functional groups. It is thus possible to obtain the binding data directly from the difference in hydrogen ion titration curves of protein in presence and absence of metal ions. Incidentally the treatment is

identical with Bjerrum's method.

E_X_P_E_R_I_M_E_N_T_A_L

Transfusion gelatin (Conc.6.0%, mol.wt.75,000) as supplied by the Director,N.C.L.,Poona, was used throughout these investigations.

Stock solutions of metal salts,namely,magnesium chloride,manganese chloride, and strontium nitrate (all A.R.products) were dissolved in double distilled water and their strength determined gravimetrically. The solutions of requisite strength were made by subsequent dilution. Potassium chloride and potassium nitrate solutions of one molar concentration were also prepared to maintain the ionic strength to a constant value.

Hydrochloric acid and potassium hydroxide solutions were prepared by diluting the stock solutions, whose strength was checked gravemetrically. Carbonate free potassium hydroxide was prepared as recommended by Kolthoff(244).

Procedure: Varying volumes of hydrochloric acid (0.0861 M) viz.,1.6,1.0,0.8,0.7,0.5,0.3,0.1,0.02 and 0.0 mls.and 0.2,0.3,0.4,0.6,1.0,1.1 and 1.5 mls.of potassium hydroxide (0.0605 M) were taken in different pyrex tubes. Two such sets were prepared. In one set only the metal salt solutions(one ml.in each case)was

added,while in the other set transfusion gelatin(one ml.) was also added alongwith the metal salt solutions. The total volume was made upto 10 mls.in each case,first adding the requisite amount of potassium chloride or potassium nitrate,as is the case, to maintain the ionic strength constant at 0.15, and then adding water.The following sets were arranged and followed up by pH-metric studies:

1. Acid or base only.
2. Acid or base + one ml.transfusion gelatin.
3. Acid or base + one ml.magnesium chloride.
4. Acid or base + one ml.magnesium chloride.+one ml. transfusion gelatin.
5. Acid or base + one ml.manganese chloride.
6. Acid or base + one ml.manganese chloride+one ml. transfusion gelatin.
7. Acid or base + one ml.strontium nitrate.
8. Acid or base + one ml.strontium nitrate+ one ml. transfusion gelatin.

The studies were made in the above order.The concentration of the metal salts solution added was noted in each case.

pH-measurements: pH measurements were made on a Cambridge Bench Type pH-meter,with the glass electrodes. Each division on the scale could read upto 0.02 pH units. Special blue high 'Alki' glass electrode was used to measure the pH of highly alkaline solutions(pH above 9.00). Before carrying out measurements pure nitrogen gas was

passed for 10 mts.to remove any dissolved carbon-dioxide and to make the atmosphere,in the beaker,inert. The buffers used for the standardisation of pH meter were made by dissolving potassium hydrogen phthalate (0.05M solution) and Borax(0.05M) for the two acid and basic ranges of the pH.

The pH was measured within one hour of mixing the reagents and also after 24 hours. The pH remained the same in both the cases.

Calculation: The calculations were made using the equation of Cohn and Edsall(245).

If we consider a solution of hydrochloric acid of C_1 M/litre and the concentration of hydrogen ions $[H^+]$ as C_1M/litre,then the pH of the solution is given by $pH_1 = -\log f_1C_1$ where f_1 is the hydrogen ion activity coefficient in this solution. If the same solution contains g gm/litre of isoionic protein, the hydrogen ion concentration will be C_2,since (C_1-C_2)/g gm.mole of hydrogen ions will have combined with each gram of protein, and the pH will be $pH_2 = -\log f_2C_2$.

Then $\log C_2/C_1 = pH_1 - pH_2 + \log f_1/f_2$. The assumption of Cohn and Bergmann,discussed by Cohn and Edsall, was that $f_1 = f_2$, so that the last term disappeared. For this assumption to be true,the ionic strength of the two solutions should be the same.This is equivalent to the assumption that the isoionic

protein molecule,which bears equal number of opposite charges,makes no contribution to the ionic strength, while the additional charges borne by the protein molecule as a result of ion binding contribute as if they were separated small ions. The equations can be manipulated to give,

$$\frac{C_1 - C_2}{g} = \frac{C_1}{g} \left\{1\text{-antilog } (pH_1 - pH_2)\right\}$$

for bound hydrogen ion and

$$\frac{C_1 - C_2}{g} = \frac{C_1}{g} \left\{1\text{- antilog } (pH_2 - pH_1).\right\}$$

for the bound hydroxyl ions.

Experiments were also performed to show the reversible nature of hydrogen ion equilibria in presence of metal ions(105).

The results are summarised in the following tables.

T_A_B_L_E NO. 1

Conc. of transfusion gelatin = 0.6%

Total volume = 10 mls. Ionic strength =0.15

Temperature 25°C .

HCl(0.0861 M) added mls.	pH - values			
	With acid or alkali	With protein	2.0×10^{-3} M Mg^{2+}	2.0×10^{-3} M Mg^{2+} + protein
1.6	1.96	2.18	1.96	2.17
1.0	2.12	2.58	2.10	2.56
0.8	2.22	2.94	2.22	2.94
0.7	2.26	3.30	2.28	3.30
0.5	2.44	3.92	2.40	3.96
0.3	2.66	4.42	2.66	4.44
0.1	3.14	5.46	3.10	5.50
0.02	3.80	6.68	3.78	6.50
0.0	5.64	7.08	5.74	6.98
K OH mls.				
0.2	10.92	8.52	10.50	8.68
0.3	11.14	9.45	10.38	9.24
0.4	11.28	9.66	10.40	9.68
0.6	11.38	10.12	10.58	10.12
1.0	11.66	11.10	11.18	10.85
1.1	11.76	11.18	11.26	10.82
1.5	11.86	11.54	11.56	11.13

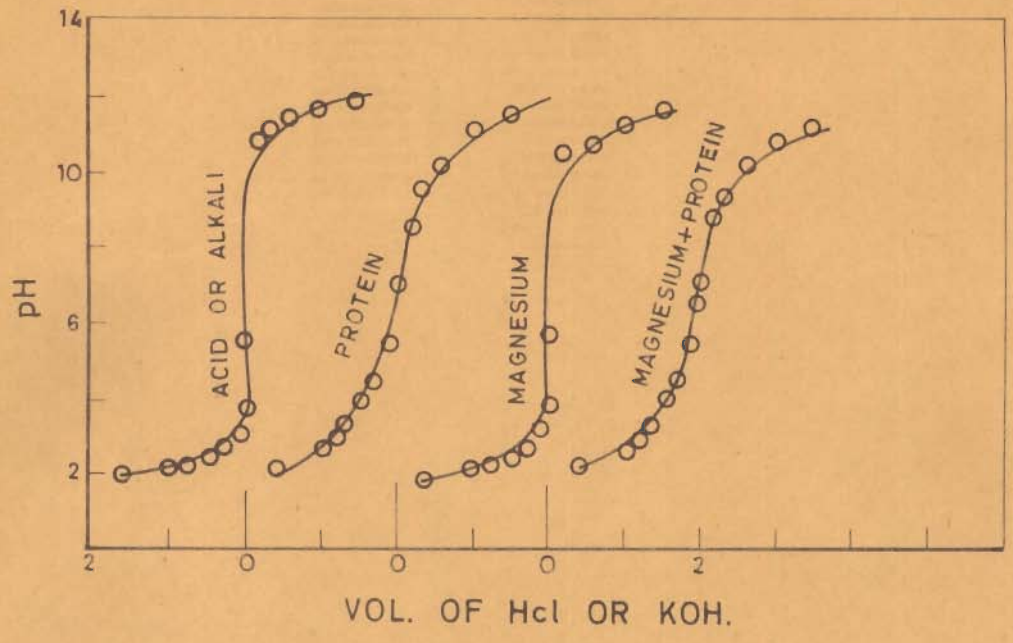

FIGURE 1

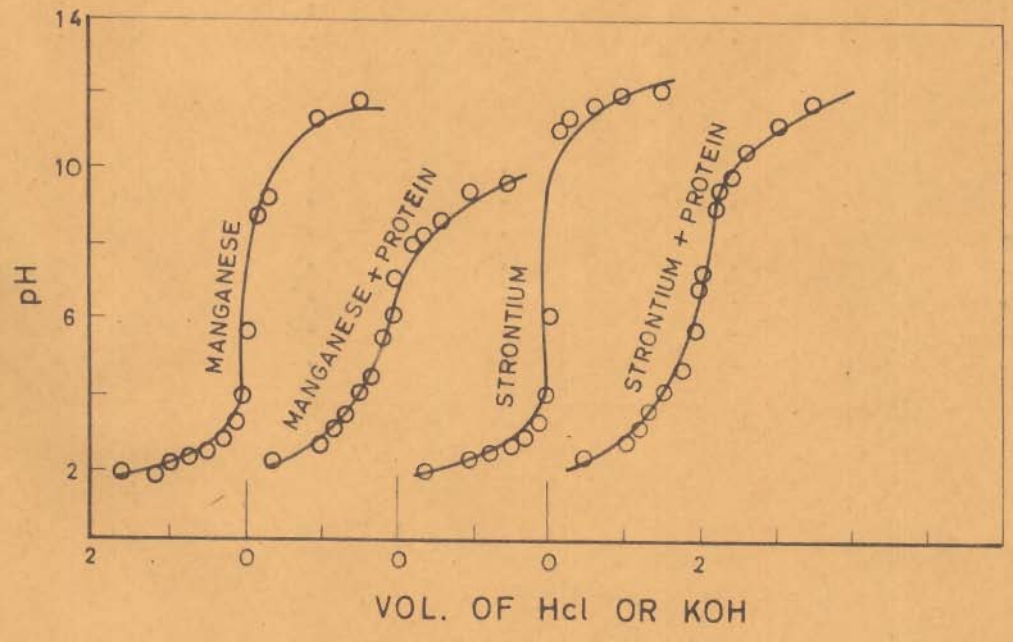

FIGURE 2

T_A_B_L_E 2

Conc.of transfusion gelatin = 0.6%

Total volume = 10 mls. Ionic strength =0.15

Temperature 25°C.

HCl(0.0861 M) added mls.	pH -values			
	$1.0x10^{-3}$M Mn^{2+}	$1.0x10^{-3}$M Mn^{2+} + protein	$1.0x10^{-3}$M $Sr.^{2+}$	$1.0x10^{-3}$M Sr^{2+}+ protein
1.6	1.95	2.17	1.96	2.18
1.0	2.15	2.61	2.22	2.58
0.8	2.26	2.98	2.31	3.04
0.7	2.29	3.44	2.34	3.42
0.5	2.44	3.97	2.47	3.94
0.3	2.66	4.38	2.70	4.52
0.1	3.16	5.38	3.14	5.51
0.02	3.76	6.76	3.82	6.70
0.0	5.56	7.04	5.86	7.10
K OH mls.				
0.2	8.65	7.80	10.90	8.82
0.3	9.10	7.98	11.14	9.28
0.4	8.40	8.10	11.30	9.62
0.6	8.76	8.40	11.47	10.20
1.0	11.20	9.34	11.72	11.05
1.1	11.30	9.42	11.80	11.12
1.5	11.72	9.54	11.94	11.58

T_A_B_L_E 3

Conc.transfusion gelatin =0.6% (0.8×10^{-4} M)

Ionic strength= 0.15 Temperature 25°C

H^+ added mols/Lx10^3	pH	Bound H^+ moles/mole protein	Mole of H^+ dissociated per mole of protein
13.776	2.18	70	-
8.61	2.58	70	-
6.888	2.94	70	-
6.027	3.30	68	2
4.305	3.92	52	18
2.583	4.42	32	38
0.861	5.46	11	59
0.172	6.68	2	68
-	7.08	-	70

Base (OH^-) added moles/L.10^3	pH	Bound OH^- moles/mole protein.	Moles of H^+ dissociated per mole of protein
1.210	8.52	15	85
1.815	9.45	22	92
2.420	9.66	30	100
3.630	10.12	43	113
6.050	11.10	55	125
6.655	11.18	61	131
9.075	11.54	63	133

T_A_B_L_E 4

Conc.transfusion gelatin = 0.6%(0.8×10^{-4} M)

Ionic strength = 0.15 Temperature 25°C.

H^+ added mols/L x 10^3	pH	Bound H^+ Moles/Mole protein	Mole of H^+ dissociated per mole of protein
13.776	2.17	70	-
8.61	2.56	70	-
6.888	2.94	70	-
6.027	3.30	68	2
4.305	3.96	52	18
2.583	4.44	32	38
0.861	5.50	11	59
0.1722	6.50	2	68
-	6.98	0	70

Base (OH^-) added moles/L.10^3	pH	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole of protein
1.210	8.68	15	85
1.815	9.24	21	91
2.420	9.68	24	94
3.630	10.12	30	100
6.05	10.85	40	110
6.655	10.82	53	123
9.075	11.13	71	141

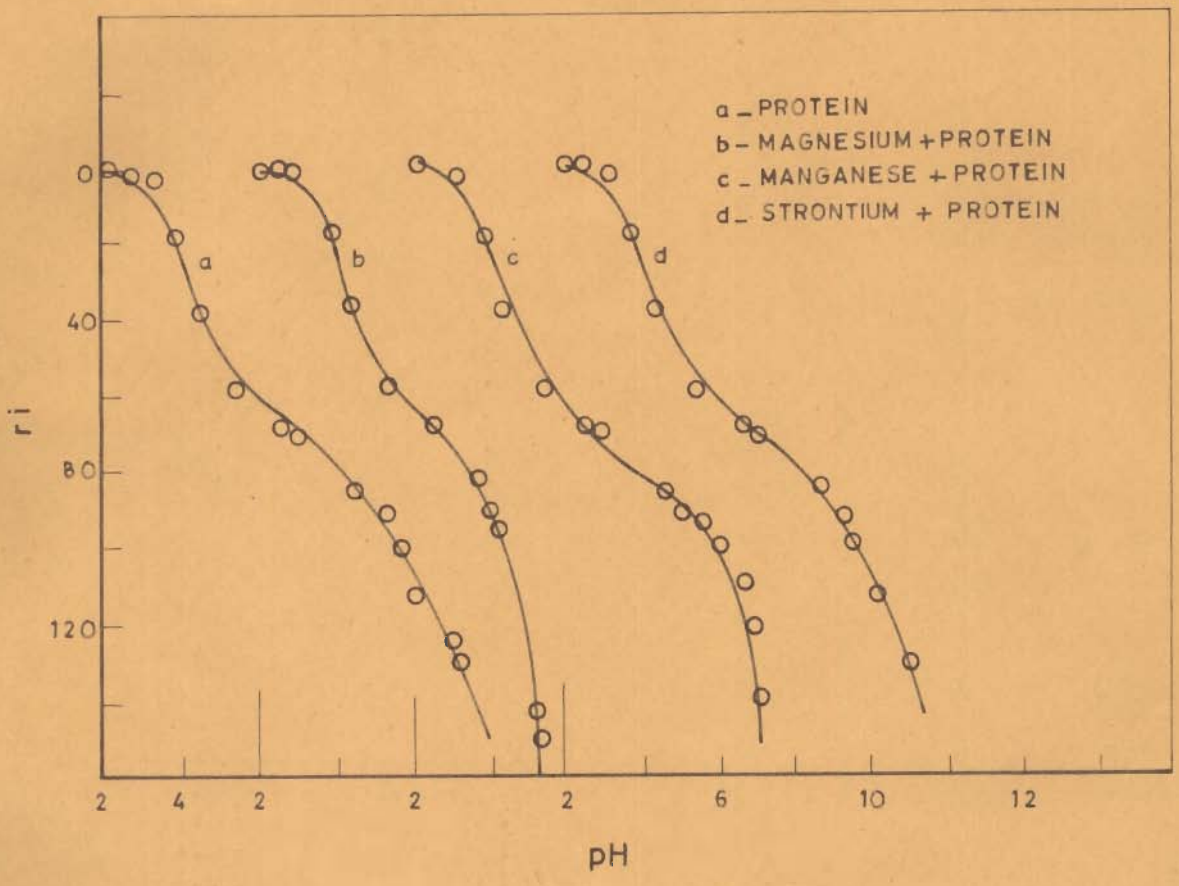
a _ PROTEIN
b – MAGNESIUM + PROTEIN
c _ MANGANESE + PROTEIN
d _ STRONTIUM + PROTEIN
a
b
c
d
0
40
80
120
ri
2
4
2
2
2
6
10
12
pH

FIGURE 3

T_A_B_L_E 5

Conc.transfusion gelatin =0.6% (0.8×10^{-4} M)

Ionic strength = 0.15 Temperature 25°C

H^+ added mols/L $\times 10^3$	pH	Bound H^+ moles/mole protein	Mole of H^+ dissociated per mole of protein
13.776	2.17	70	-
8.61	2.61	70	-
6.888	2.98	70	-
6.027	3.44	70	-
4.307	3.97	52	18
2.583	4.38	32	38
0.861	5.38	11	59
0.172	6.76	2	68
-	7.04	-	70

Base(OH^-) added moles/L.10^3	pH	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole of protein
1.210	7.80	13	83
1.815	7.98	21	91
2.420	8.10	15	85
3.630	8.40	25	95
6.05	9.34	75	145
6.655	9.42	82	152
9.075	9.54	112	182

T_A_B_L_E 6

Conc.transfusion gelatin =0.6% (0.8×10^{-4} M)

Ionic strength =0.15 Temperature 25°C

H^+ added mols/L x 10^3	pH	Bound H^+ moles/mole protein	Mole of H^+ dissociated per mole of protein
13.776	2.18	70	-
8.61	2.58	70	-
6.888	3.04	70	-
6.027	3.42	69	1
4.305	3.94	52	18
2.583	4.52	32	38
0.861	5.51	11	59
0.172	6.70	2	68
-	7.10	-	70

Base(OH^-) added moles/L.10^3	pH	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole of protein
1.210	8.82	15	85
1.815	9.28	22	92
2.420	9.62	30	100
3.630	10.20	43	113
6.05	11.05	60	130
6.655	11.12	66	136
9.075	11.58	63	133

RESULTS AND DISCUSSION

The pH values recorded in tablesland 2 were used to calculate the extent of binding by the help of the equations earlier cited. Actually the difference between the added and the free hydrogen ions gives the number of hydrogen ions bound to the protein. From this r, the number of hydrogen ions dissociated per protein molecule, is evaluated both in presence and absence of metal ions. Thus,titration curves for the systems magnesium-transfusion gelatin,manganese-transfusion gelatin and strontium-transfusion gelatin and transfusion gelatin alone are obtained by Plotting 'r 'against pH.

The titration curves of metal-transfusion gelatin system are strikingly different from the titration curve of transfusion gelatin(Fig.3).It has been observed that the activity of hydrogen ions is greatly increased when metal ions are added to the protein and the hydrogen ion titration curves show a shift towards the basic side of the functional groups. This would be the case if hydrogen ions are assumed to be replaced by the metal ions. Such observations point towards the fact that metal ions compete very well with the hydrogen ions in the interaction process. Thus the excess of hydrogen ions available in presence of metal would indirectly give the extent

of metal ion binding. Tables 3-6 show that a limited number of hydrogen ions are displaced at different pH values when magnesium, manganese and strontium are added to the titration mixture. If we assume that a 'one to one binding' is favoured as suggested by Gurd and Murray then the number of hydrogen ions displaced by the metal directly gives the number of metal ions bound per protein molecule(V_M).

The method of dividing a titration curve into different ranges as set out by Cannan is still satisfactory.

In the solution of high ionic strength the maximum binding is attained between pH 2.0 to 5.5. Between this pH the binding is attributed to those side chain carboxyl groups not present as amides; the portion between pH 6.0-8.5 is attributed to imidazole and α-amino groups present in small quantities. The titration from pH 8.5 to the highest pH is not always well defined. At higher pH range (between pH 11.0 to 12.0) it is attributed to ϵ-amino groups of lysine, phenolic hydroxyl groups of tyrosine and sulphydryl groups of cysteine.

However, there is no way in which those side-chain carboxyl groups present as amides may be estimated from the titration curve. The difference between the total content of dicarboxylic acids as found by amino

acid analysis and determined by titration curve normally gives the amide nitrogen content as accurately as the chemical estimation.

By the available details on the hydrogen ion equilibria of transfusion gelatin and the titration curves of the protein in presence of metal ions it is evident that the hydrogen ions are displaced by the metal in a pH range characteristic of carboxylic groups deprotonation. The results in a higher pH range(beyond pH 6.5) would indicate the binding of metal ions to imidazole, α -amino or ϵ -amino groups. One limitation of the present method,that has to be kept in view is that this method may not be reliable beyond pH 6.5. Since the calculation of binding data from pH-metric method are based on the assumption that metal ions compete with hydrogen ions in the interaction process. Actually counting of those hydrogen ions has been made which are removed from protein molecule during competition. Thus at pH 6.5 or beyond where the carboxylic groups are fully deprotonated,even if the metal binds to the protein,no displacement of hydrogen ions is possible. Therefore attempts have not been made to interpret the results above pH 6.5.

T_A_B_L_E 7

Concentration of magnesium chloride =2.0 x 10^{-3}M

Concentration of transfusion gelatin =0.8 x 10^{-4}M

pH	H^+dissociated in presence of metal ions	H^+dissociated in absence of metal ions	V_M	Free metal at equilibrium x 10^{-3}M	log K
3.0	4	2	2	-	-
4.0	24	22	2	-	-
5.0	52	48	4	-	-
5.5	61	54	7	1.44	1.812
6.0	66	59	7	-	-
6.5	72	64	8	-	-
7.0	76	67	9	1.28	3.194
8.0	89	77	12	-	-
8.5	100	85	15	-	-

T_A_B_L_E 8

Concentration of manganese chloride =2.0 x 10^{-3}M

Concentration of transfusion gelatin=0.8 x 10^{-4}M

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	V_M	Free metal at equilibrium x10^{-3}M	log K
3.0	3	2	1	-	-
4.0	24	22	2	-	-
5.0	51	48	3	-	-
5.5	58	54	4	1.68	1.485
6.0	64	59	5	-	-
7.0	73	67	6	1.52	3.119
8.0	84	77	7	-	-
9.0	97	90	7	-	-

T_A_B_L_E 9

Concentration of strontium nitrate = 2.0×10^{-3} M

Concentration of transfusion gelatin= 0.8×10^{-4} M

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	V_M	Free metal at equilibrium $\times 10^{-3}$ M	log K
3.0	4	2	2	-	-
4.0	24	22	2	-	-
5.0	50	48	2	-	-
5.5	57	54	3	1.76	1.334
6.0	62	59	3	-	-
7.0	71	67	4	1.68	2.77
8.0	81	77	4	-	-
9.0	94	90	4	-	-

T_A_B_L_E 10

Binding data calculated from titration curves.

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M	Free metal at equilibrium x 10^{-3}M	log K
		Mg^{2+} - transfusion gelatin system			
5.50	61	54	7	1.44	1.812
7.00	76	67	9	1.28	3.194
8.50	100	85	15	0.80	-
		Mn^{2+}-transfusion gelatin system			
5.50	58	54	4	1.68	1.485
7.00	73	67	6	1.52	3.119
9.00	97	90	7	1.44	-
		Sr^{2+}- transfusion gelatin system			
5.50	57	54	3	1.76	1.334
7.00	71	67	4	1.68	2.77
9.00	94	90	4	1.68	-

T_A_B_L_E 11

Intrinsic association constants of combination.

Ligand	Method	log K(Carboxyl group)			log K(imidazole group)		
		Mg^{2+}	Mn^{2+}	Sr^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}
T.Gelatin	pH-metric	1.812	1.485	1.334	3.194	3.119	2.77
Acetate		1.05[a]	-	0.97[b]	-	-	-
Imidazole		-	-	-	-	4.23[c]	-

a. Reference No.246.

b. " 247.

c. " 248.

Magnesium-transfusion gelatin system

The titration curve of magnesium-transfusion gelatin differs markedly from that of transfusion gelatin alone. The hydrogen ion equilibria has been shifted towards the basic side of the functional groups(towards lower pH) indicating thereby that the magnesium ions are themselves bound to the protein replacing the hydrogen ions. The displacement of hydrogen ions in the pH range 2.10 to 5.50 indicates that the carboxyl groups are the principal sites for the binding of magnesium ions. The number of protons displaced in this pH range are equal to 7.0. It may thus be assumed that nearly seven magnesium ions are bound to the carboxyl groups of transfusion gelatin. The logarithm of the intrinsic association constant for Mg-carboxyl groups interaction is calculated by/ inserting $V_M = 7$ in the scatchard equation (258),

$$K = \frac{V_M}{(n-V_H-V_M)C_F}$$

where V_H is the number of active sites covered by the hydrogen ions, n is the total number of such intrinsically identical sites and C_F is the metal concentration at equilibrium(free metal ions). The log K as calculated comes out to be 1.812. It is interesting to note that log K value obtained by this method compares favourably with the logarithm of the first association constant between magnesium and acetate nucleus (1.05) and magnesium and propionate nucleus (1.08). The agreement between

these values provides strong evidence for a 1:1 stoichiometry of the reaction.

In the higher pH range though the results are not considered reliable but it is clear that magnesium shows a tendency to combine with the imidazole groups and still at higher pH with the amino groups. The logarithm of the first association constant between magnesium and free imidazole nucleus is too low to be expected for a combination between magnesium and imidazole groups of transfusion gelatin.

Manganese and strontium-transfusion gelatin interaction.

Manganese and strontium both exhibit characteristics similar to magnesium. From the titration curves and tables (9 and 10) , It is evident that both the metal ions combine with the carboxyl groups. The maximum number of protons liberated in the pH range 2.0 to 5.5 are 4 and 3 for manganese and strontium respectively. The intrinsic association constants are calculated by the help of Scatchard equation. The values come out to be 1.485 and 1.334 for manganese-carboxyl groups and strontium carboxyl groups interaction respectively.

These data lead to the conclusion that amongst the three metal ions magnesium shows strong binding tendency for carboxyl groups of transfusion gelatin followed by manganese and strontium. It is interesting to note that the binding capacity follows the order of the stability constants

of the respective acetates of magnesium,manganese and strontium. Among the complexes of alkaline earth metals it has been found that the stability constant is exactly the inverse order of the radii of the cations,i.e.,the radii increase in the order,

$$Mg < Sr < Ca < Ba$$

where as the stability constant of complexes of these metal ions with different simple organic ligands decrease in the order Mg > Sr > Ca > Ba .

This tendency has been observed in acetate,propionate as well as with the amino acids. Present study shows that the same general rule is also applicable for alkaline earth cation complexes with a ligand of high complexity like transfusion gelatin. Such observations indicate that more attention should be paid to study the model compound like amino acids and simple peptides,so that a comparision can be made.

Explaination similar to that of magnesium may be offered for these systems in the neutral and the more alkaline side of the pH also. Both the metals ions show a negligible tendency to combine with the functional groups of the protein containing donor nitrogen atom.

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C_H_A_P_T_E_R II.

Equilibrium dialysis studies on the interaction of magnesium, nickel and cobalt with transfusion gelatin.

I_N_T_R_O_D_U_C_T_I_O_N

Many experimental techniques have been employed for the study of metal-protein complexes. The choice of the technique is governed by the availability of the equipment as well as by the nature of the problem. Out of the different techniques the equilibrium dialysis technique is extensively employed because it is found to be generally applicable to the quantitative study of metal-protein complexes. As any other method it gives the greater part of the most accurate data on the binding of metal ions with proteins. The other advantages are that the method gives amount of binding directly so that the properties indirectly related to the extent of complex formation do not interfere and also the less elaborate equipment is required.

Obsrne(249) was the first who pointed out the great utility of this technique about a half century ago. Its wide potentialities were emphasised especially by Northrop and Kunitz(61) in the study of metal-protein complexes,in the year 1924-25. This method was put to more productive use by Klotz and Curme(66). With the development of the theory of multiple equilibria as applicable to protein interaction,the dialysis technique has now been applied in the studies of a number of systems, and quite useful informations(250-252) have been obtained regarding the binding data and relevant intrinsic

association constants of the metal-protein interactions as well as the number of reactive sites available with a protein for binding. Copper, zinc and cadmium (63-69, 81, 86, 95, 253) have been extensively studied by several workers, as the metal ions bound, by this technique; the proteins mostly employed for such studies are the globular proteins because of the availability of large fractions of highly purified plasma proteins. It would be a real contribution to the knowledge of metal ion binding to proteins if ~~a~~ productive techniques like equilibrium dialysis be applied for the study of the fibrilla-protein complexes of metal ions of physiological interest other than copper, zinc and cadmium.

The present chapter deals with the equilibrium dialysis studies on the interaction of magnesium, nickel and cobalt with transfusion gelatin (bone gelatin, used as plasma expender). Transfusion gelatin has been selected because gelatin preparations of high purity are now available as are reliable data on amino acid analysis (186).

E_X_P_E_R_I_M_E_N_T_A_L

In the equilibrium dialysis method the two solutions, the protein and the metal salt solution are separated by means of a semipermeable membrane. This membrane permits only the water and the metal ions to

pass through while the colloidal particles do not. The two solutions are shaked by means of an electric driven stirrer to reach the equilibrium,and at equilibrium the activity of the free metal ions is the same on both sides of the membrane. Therefore,if the total amount of the metal ions in the system is know and the concentration of the free metal ions outside the protein solution is measured, the amount of metal bound can be computed. Actually if the protein binds metal ions,then at equilibrium the total metal concentration in the protein compartment is greater than that in the outside one (protein free). The difference in the two concentrations is a measure of the metal ion bound to the protein. The concentration of the metal ion in the outer compartment is analysed by the standard analytical methods.

In practice there are some possible sources of error in this technique viz.,(i) some assymetry in the distribution of metal ions may exist because of the Donnan effect,and so the allowance should be made for this in the calculation,or an inert electrolyte may be added to eliminate it(ii) some adsorption of metal ions may occur on the membrane itself or they bound themselves with the membrane as well as by the protein. In this case suitable corrections can be made by setting up controlled dialysis,in which the protein is absent and then measuring the depletion of the metal ions from the solution by

usual methods.

Apparatus.

It consists of an electric driven stirrer, carrying a bridge suspended in a thermostat. The bridge is provided with holes to carry the dialysing tubes and the temperature of the thermostat was adjusted to 30 ± 0.1°C. The speed of the stirrer was controlled by putting a resistance variant in the circuit and the same speed was adjusted in all the experiments. The pyrex tubes were closed by means of rubber stoppers (the rubber stoppers are covered with thin sheets of polyethylene so that the rubber may not come in contact of the solutions) and these were used as the dialysing vessels.

pH-measurements were carried out by means of a Cambridge Bench pH-meter,with the glass electrode, reading upto 0.02 pH unit.

Preparation of the dialysis bags.

The cellulose sausage casing(23/32 inch in diameter) was obtained from the Visking Co.,Chicago, Illinois* under the trade name ,Nojax, and they were used as the dialysis bags. 15 to 16 cms.length of these Casings were cut out from main strip and put into beaker of distilled water and heated on a steam bath

* obtained by the courtsey of Mr.K.P.Singh(Research Scholar,Boston University,Boston).

for about an hour. Heating was repeatedly done with distilled water. The casings were next soaked in distilled water at room temperature for about six hours. This procedure was repeated several times until the final washings were free from sulphur. The casings were again soacked for about 6 hours in a solution of supporting electrolyte to be used in the binding measurements.

Filling of the dialysis bag:

The dialysis casing was taken out of the electrolyte solution in which it was soaked, and the excess of liquid was removed by stripping out between the first two fingers. Then a square knot was tied at one end of the casing and 5.0 mls. of the protein solution was pipetted in. Most of the air was forced out between the fingres, and the tube was closed with two overhead knots, one above the other and the tube was suspended in protein free solution.

Solutions and reagents.

Transfusion gelatin(6.0% concentration, molecular weight 75,000) was obtained from the Director,N.C.L., Poona, was used throughout these investigations. A stock solution of the protein in the supporing electrolyte (0.15 M potassium chloride) was prepared and the pH was adjusted to the desired value.

The metal salt solutions were prepared by dissolving A.R.samples of magnesium chloride,nickel

chloride and cobalt chloride in double distilled water. Their metal content was estimated volumetrically using EDTA titrations. M/50 solutions of these metal salts were then prepared by their subsequent dilution. 1.M potassium chloride solution was used as the supporting electrolyte. Dilute solution of potassium hydroxide was prepared by dissolving A.R.samples in double distilled water. This was used to maintain the pH of the protein solution. Solutions of EDTA (B.D.H.) ,E.Black T and murexide were prepared as recommended by Schwarzenbach(254).

Procedure.

Varying volumes of magnesium chloride(M/50) viz., 1.0,2.0,3.0,4.0,5.0,6.0,7.0,8.0 were taken in different pyrex glass tubes. The total volume was made upto 20 mls. first by adding potassium chloride, to maintain the ionic strength 0.15 and then by adding requisite amount of water. 5.0 mls.of each of these solutions were taken out in separate pyrex glass tubes(25 x 200 mm.),designated as dialysing tubes. Now the dialysis bag containing the protein solution(of a definite pH and ionic strength) was suspended by means of a thread(in absence of fibre glass) in each tube carefully so that the thread did not come in contact with the metal solution. The other 5.0 mls.of the metal salt solutions were used for blank experiments under strictly identical set of conditions,

(the dialysis bag contained 5.0 mls.protein free supporting electrolyte solution of the same pH).

A similar set was also prepared containing the same metal concentration with the only difference that the pH of the protein solution was kept at 7.5 in lieu of pH 5.5,as in the previous case.

Varying volumes of nickel chloride or cobalt chloride(M/50) viz.,1.0,2.0,3.0,4.0,5.0,6.0,7.0 and 8.0 mls. were taken in different pyrex tubes. The total volume was made upto 20 mls.by adding potassium chloride and the water as described above in the case of magnesium chloride.

The dialysis tubes were placed on the shaker, which was driven electrically,for the required period. The time to attain equilibrium was found to be 44 hours for all the three,magnesium,nickel and cobalt metal ions. After the required period the dialysis bags were withdrawn from the tubes and the strength of the protein free solution was determined by titration with EDTA. For this an accurately known volume(2.0 mls.) was withdrawn from the solutions and was titrated as recommended for the particular metal ions. The blank tubes were employed to determine the amount of metal ions bound to the material of dialysis bag. The amounts for magnesium,nickel and cobalt were found to be negligibly small.

T_A_B_L_E 1

Concentration of transfusion gelatin =2.4 %.

Total volume inside the bag =5.0 mls.

Total volume outside the bag =5.0 mls.

Ionic strength inside and outside the bag. =0.15

Temperature $30 \pm 0.1^{o}C$

Time of equilibration = 40 hrs.

pH of the protein solution= 5.5

Initial conc. of metal ions x 10^{-3}M($MgCl_2$)	Conc.of metal ions after equilibration x 10^{-3}M	pH of the protein solution.
0.5	0.18	5.49
1.0	0.375	5.48
1.5	0.51	5.46
2.0	0.69	5.45
2.5	0.85	5.43
3.0	1.05	5.42
3.5	1.345	5.40
4.0	1.70	5.40

T_A_B_L_E 2

Concentration of transfusion gelatin =2.4 %

Total volume inside the bag =5.0 mls.

Total volume outside the bag =5.0 mls.

Ionic strength inside and outside the bag. =0.15

Temperature 30°C

Time of equilibration = 40 hrs.

pH of the protein solution= 7.5

Initial conc.of metal ions x 10^{-3}M($MgCl_2$)	Conc.of metal ions after equilibration x 10^{-3}M	pH of the protein solution.
0.50	0.165	7.48
1.0	0.305	7.46
1.5	0.45	7.45
2.0	0.51	7.43
2.5	0.575	7.41
3.0	0.590	7.39
3.5	0.850	7.37
4.0	1.20	7.30

T_A_B_L_E 3

Concentration of transfusion gelatin = 2.4%

Total volume inside the bag =5.0 mls.

Total volume outside the bag =5.0 mls.

Ionic strength inside and outside the bag. =0.15

Temperature $30^{o}C$

Time of equilibration = 44 hrs.

pH of the protein solution= 5.5

Initial conc.of metal ions x 10^{-3}M($NiCl_2$)	Conc.of metal ions after equilibration x 10^{-3}M	pH of the protein solution.
0.50	0.255	5.48
1.00	0.560	5.48
1.50	0.830	5.46
2.00	1.205	5.45
2.50	1.475	5.43
3.00	2.700	5.41
3.50	2.025	5.40
4.00	2.40	5.40

T_A_B_L_E 4.

Concentration of transfusion gelatin = 2.4 %

Total volume inside the bag = 5.0 mls.

Total volume outside the bag = 5.0 mls.

Ionic strength inside and outside the bag. = 0.15

Temperature $30^{o}C$

Time of equilibration = 44 hrs.

pH of the protein solution = 7.5

Initial conc.of metal ions. x 10^{-3}M($NiCl_2$)	Conc.of metal ions after equilibration x 10^{-3}M	pH of the protein solution.
0.50	0.245	7.49
1.00	0.445	7.47
1.50	0.635	7.45
2.00	0.715	7.41
2.50	1.00	7.38
3.00	1.33	7.35

T_A_B_L_E 5

Concentration of transfusion gelatin =2.4%

Total volume inside the bag. =5.0 mls.

Total volume outside the bag =5.0 mls.

Ionic strength inside and outside the bag. =0.15

Temperature $30^{\circ}C$.

Time of equilibration = 44 hrs.

pH of the protein solution = 5.5

Initial conc.of metal ions x $10^{-3}M(CoCl_2)$	Conc.of metal ions after equilibration x $10^{-3}M$	pH of the protein solution.
0.5	0.280	5.48
1.0	0.420	5.47
1.5	0.605	5.45
2.0	0.70	5.44
2.5	0.915	5.42
3.0	1.305	5.40
3.5	1.770	5.37
4.0	2.235	5.35

T_A_B_L_E 6

Concentration of transfusion gelatin = 2.4 %

Total volume inside the bag = 5.0 mls

Total volume outside the bag = 5.0 mls.

Ionic strength inside and outside the bag. = 0.15

Temperature $30^{o}C$

Time of equilibration = 44 hrs.

pH the protein solution = 7.5

Initial conc.of metal ions x $10^{-3}M(CoCl_2)$	Conc.of metal ions after equilibration x $10^{-3}M$	pH of the protein solution.
0.5	0.240	7.48
1.0	0.360	7.45
1.5	0.550	7.42
2.0	0.650	7.41
2.5	0.895	7.40
3.0	1.270	7.40
3.5	1.645	7.36
4.0	2.020	7.30

D_I_S_C_U_S_S_I_O_N

From the data given in tables 7 to 12, the number of moles of metal bound per mole of protein is evaluated by the help of the relation $V_M = \frac{Mp}{P_T}$ where Mp is the number of moles of protein bound metal in a dialysis tube and P_T ,is the total number of moles of protein per tube. If the dialysing membrane does not bind any of the ligand (metal or protein),and if the Donnan effect is negligible, the calculation of V_M would be extremely simple. Mp being calculated by the relationship.

$$Mp = MT - VT\ (M)$$

where MT is the total amount of metal ions in the tube, VT is the total volume of the solution and (M) is the concentration of metal ions in the protein free external solution. Actually most ligand are bound by the dialysing bags,so ~~the~~ the above equation is modified in the form,

$$Mp = MT - VT\ (M) - MB$$

where MB is the number of moles of metal bound to the bag. Three methods have been used for the determination of MB. The simplest being the comparision method(255).

Direct comparision method.

A blank tube containing the same amount of each non-protein component is equilibrated, and from the knowledge of difference in concentration of metal inside and outside the bag,the amount of total metal bound to the bag(MB) is calculated as,

$$MB = M_T - VT (M)^-$$

where $(M)^-$ is the concentration of unbound metal in the blank tube. Since the binding is sifficiently small (in the range 0.5×10^{-5} to 1×10^{-6} M in the present case) direct comparision method has been utilised.

The binding data have been represented graphically for the metal-protein complex formation, as a function of free metal concentration on a semi-logarithm graph(extent of binding/is plotted against the logarithm of the concentration of unbound metal ions).

T_A_B_L_E 7

The equilibrium between magnesium and transfusion gelatin at pH 5.5

Concentration of transfusion gelatin $= 3.2 \times 10^{-4}$ M

Ionic strength = 0.15 Temperature 30°C

pH	Bound metal ions $\times 10^{-3}$ M	Free metal ions $\times 10^{-3}$ M	V_M	log K
5.49	0.320	0.18	1	1.836
5.48	0.625	0.375	1.9	1.802
5.46	0.990	0.51	3.1	1.886
5.45	1.310	0.69	4.1	1.882
5.43	1.650	0.85	5.2	1.900
5.42	1.950	1.05	6.1	1.883
5.40	2.155	1.345	6.8	1.829
5.40	2.300	1.70	7.2	1.752

Mean log K = 1.846

T_A_B_L_E_S 8

The equilibrium between magnesium and transfusion gelatin at pH 6.5.

Concentration of transfusion gelatin $=3.2 \times 10^{-4}$ M

Ionic strength = 0.15 Temperature 30°C

pH	Bound metal ions $\times 10^{-3}$ M	Free metal ions $\times 10^{-3}$ M	V_M	log K
7.48	0.335	0.165	1.04	-
7.46	0.695	0.305	2.2	-
7.45	1.05	0.45	3.3	3.289
7.43	1.49	0.51	4.7	3.35
7.41	1.925	0.575	6.0	3.419
7.39	2.41	0.59	7.2	3.372
7.37	2.650	0.85	8.2	3.530
7.30	2.80	1.20	8.8	3.222

Mean log K = 3.363

T_A_B_L_E 9

The equilibrium between nickel and transfusion gelatin at pH 5.5

Concentration of transfusion gelatin = 3.2×10^{-4} M

Ionic strength = 0.15 Temperature 30°C

pH	Bound metal ions $\times 10^{-3}$ M	Free metal ions $\times 10^{-3}$ M	V_M	log K
5.48	0.245	0.255	0.8	1.586
5.48	0.440	0.560	1.4	1.492
5.46	0.670	0.830	2.1	1.500
5.45	0.895	1.105	2.8	1.506
5.43	1.025	1.475	3.2	1.440
5.41	1.30	1.700	4.1	1.490
5.40	1.475	2.025	4.6	1.470
5.40	1.60	2.40	5.0	1.460

Mean log K = 1.493

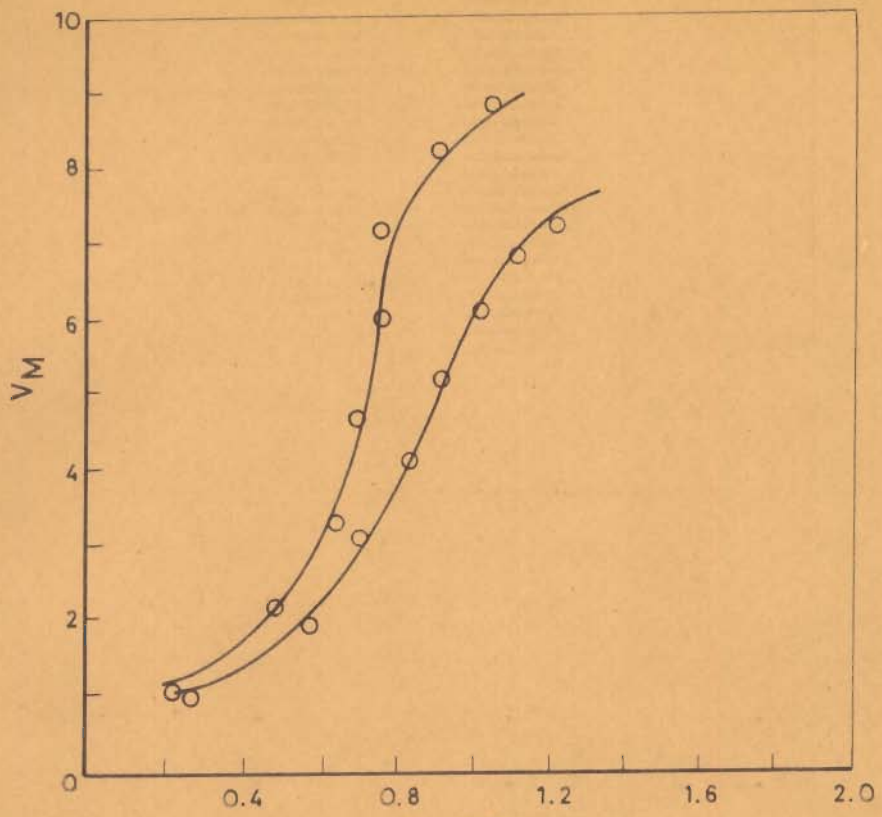

FIGURE 1

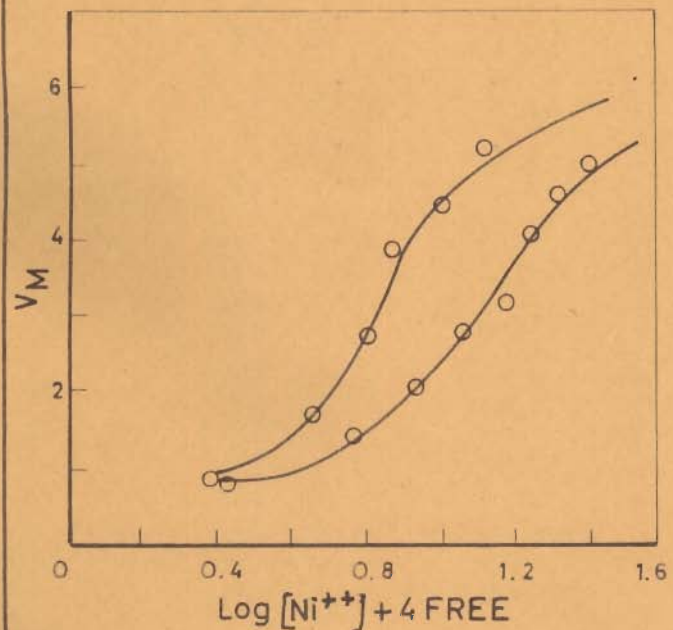

FIGURE 2

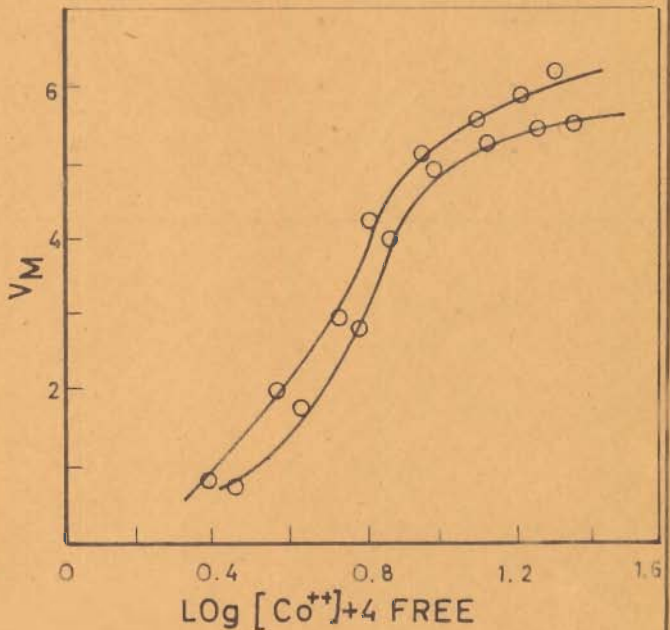

FIGURE 3

T_A_B_L_E 10

The equilibrium between nickel and transfusion gelatin at pH 6.5.

Concentration of transfusion gelatin = 3.2×10^{-4}M

Ionic strength = 0.15 Temperature 30°C

pH	Bond metal ions $\times 10^{-3}$M	Free metal ions $\times 10^{-3}$M	V_M	log K
7.49	0.255	0.245	0.5	-
7.47	0.555	0.445	1.7	3.306
7.45	0.865	0.635	2.7	3.100
7.41	1.285	0.715	4.0	3.383
7.38	1.50	1.00	4.4	3.204
7.35	1.670	1.33	5.2	3.177

Mean log K = 3.234

T_A_B_L_E 11

The equilibrium between cobalt and transfusion gelatin at pH 5.5.

Concentration of transfusion gelatin = 3.2×10^{-4}M

Ionic strength = 0.15 Temperature 30°C

pH	Bound metal ions $\times 10^{-3}$M	Free metal ions $\times 10^{-3}$M	V_M	log K
5.48	0.220	0.280	0.7	-
5.47	0.580	0.420	1.8	1.729
5.45	0.895	0.605	2.8	1.768
5.44	1.30	0.70	4.1	1.876
5.42	1.585	0.915	4.9	1.842
5.40	1.695	1.305	5.3	1.722
5.37	1.730	1.77	5.4	1.598
5.35	1.765	2.235	5.5	-

Mean log K = 1.756

T_A_B_L_E 12

The equilibrium between cobalt and transfusion gelatin at pH 6.5

Concentration of transfusion gelatin = 3.2×10^{-4} M

Ionic strength =0.15 Temperature 30°C

pH	Bound metal ions $\times 10^{-3}$ M	Free metal ions $\times 10^{-3}$ M	V_M	log K
7.48	0.260	0.240	0.8	-
7.45	0.640	0.360	2.0	-
7.42	0.950	0.550	2.9	3.485
7.41	1.350	0.650	4.2	3.333
7.40	1.605	0.895	5.0	3.701
7.40	1.730	1.270	5.4	3.595
7.36	1.855	1.645	5.8	3.495
7.30	1.980	2.020	6.2	3.544

Mean log K 3.526

The interpretation of the experimental results on the studies of the metal-protein system,are fraught with difficulties due to complex physico-chemical nature of the protein molecule(141). Several general properties of the protein must be taken into account, before any attempt is made to arrive at a definite conclusion. First of all the length of the peptide chain is much greater than in most compounds (amino-acids and peptides etc.) which are employed as a model for the comparision and interpretation of the data on the metal-protein interaction(e.g. the affinity of a particular metal ion for acetate,imidazole and ammonia has been frequently compared to the carboxyl, imidazole and amino groups of the protein). Due to this long peptide chain,the side chain groups have got much more importance in binding metal ions than the terminal amino or carboxyl groups. Secondly, there are usually several number of a given class of groups in a protein molecule,with the result,that the binding of metal ions is dependent both on the intrinsic affinity and with number of groups available for interaction. Thirdly,the ligand groups in a protein molecule are not free to move,therefore do not behave as a simple classical ligand. Finally the protein molecule normally bears a considerable number of positive and negative charges,whose net potential

field may ~~be~~ favour or hinder the approach of the metal ion. This means that the apparent affinity constant changes as more and more ions are taken-up or given off by the protein molecule.

Apart from this, one has to consider the factors associated with the chemistry of simple metal complexes, e.g., coordination number, hydration as well as the hydrolysis of metal ions, competition with hydrogen ions and chelate formation. Such data are summarised in the following table(25).

T_A_B_L_E 13

System	Coordination number.	log K	App. pH, onset of pption.
Mg-imidazole	6	small	10
Mg-acetate	-	1.05[246]	-
Ni-imidazole	6	3.27[256]	8
Ni-acetate	-	-	-
Co-imidazole	6	7.52[257]	8
Co-acetate	-	-	-

Magnesium-transfusion gelatin system.

In the preceeding chapter on the basis of the pH-metric studies on the interaction of magnesium with transfusion gelatin we concluded that magnesium has got considerable affinit towards the carboxylate side chain of transfusion gelatin. This fact is further confirmed by equilibrium dialysis studies.

Two different pH levels were selected for equilibrium studies; one at which the carboxyl groups are expected to be fully deprotonated and the other where the imidazole groups lose their positive charge. A series of observations were made at pH 5.5 and 6.5 over a wide range of metal concentration. A plot (Fig.1) between log C (free metal ion) and V_M (the extent of binding) reveals that when nearly 7 magnesium ions are bound to the protein,the extent of binding becomes independent of the metal concentration. This shows that at this limiting concentration the saturation limit is being attained,i.e.,by increasing the free metal concentration no more metal ions seem to be bound to the protein. Similar studies at pH 7.3 gives a maximum binding limit of 8.8. If a comparision be made at corresponding free metal concentration at both pH levels, the values are V_M = 6.8 at 1.34 x 10^{-4}M Mg^{++}; at pH 5.4 and V_M = 8.8 at 1.20 x 10^{-4}M Mg^{++} at pH 7.3. It is

clear that by changing pH from 5.3 to 7.3 the maximum binding limit is shifted from 6.8 to 8.6. It may, therefore, be concluded that two additional magnesium ions are bound to the imidazole sites. These data are employed for the calculation of intrinsic association constant applying Scatchard's equation(258),

$$K = \frac{V_M}{(n - V_H - V_M)\, C_F}$$

where V_M and V_H are the number of active sites covered by the metal and hydrogen ions respectively, n is the free metal concentration. V_H and n are taken from literature(185). The log K values for the magnesium-carboxyl and magnesium-imidazole interaction come out to be (1.846 $\pm$ 0.06) and (3.363 $\pm$ 0.14) respectively. The corresponding value of log K for magnesium-carboxyl group interaction as calculated from pH measurements is 1.812(preceeding chapter).

The log K values for magnesium-carboxyl group interaction as calculated from pH metric and equilibrium dialysis studies, closely resemble with the logarithm of the first association constant of magnesium and acetate nucleus. This close agreement shows that the stoichometry of the reaction is nearly 1:1.

Cobalt and nickel-transfusion gelatin systems.

Similar studies have been carried out on the

interaction of nickel and cobalt with transfusion gelatin, selecting two different pH levels. The binding data obtained for these two systems(tables 9,10) are utilized to calculate the intrinsic association constants. Both the metal ions show a close similarity in the mode of binding with transfusion gelatin. At pH 5.5 cobalt and nickel combine with the carboxyl groups. The saturation limit is being attained at metal concentration equal to 2.00×10^{-3}M for cobalt and 2.399×10^{-3}M for nickel when 5.4 and 4.8 metal ions are bound to the protein. This limit is shifted to greater value of V_M i.e.,6.2 and 5.8 when the pH is changed from 5.5 to 7.3 for the above concentrations of cobalt and nickel respectively(metal concentration at V_M corresponding to pH 5.5 and 7.3 are taken from Fig.2,3). This indicate that approximately one ion of each metal gets bound to the imidazole groups of the histidine residue,whereas 5 ions of each metal combine with carboxylate side chains at given metal concentration(as indicated in tables 9 to 12). The intrinsic association constants of both the system for carboxyl as well as imidazole groups interaction thus calculated are shown in the following table alongwith the previous value and log K values of some model compounds for comparision.

T_A_B_L_E 14

Ligand	Method	log K(carboxyl)		(log K(imidazole)	
		Co^{2+}	Ni^{2+}	Co^{2+}	Ni^{2+}
T.gelatin	Equilibrium dialysis[a]	1.756	1.493	3.52	3.23
do	pH metric[b]	2.02	1.860	-	-
Serum albumin	--	2.30	-	-	-
Acetate		2.4[260]	2.1[261]	-	-
Imidazole		-	-	7.52[257]	3.27[256]

a. Present studies.

b. Reference No.259.

The log K values for cobalt and nickel-carboxyl group interaction thus obtained and those of computed earlier from pH-metric results compare fairly well with those of the logrithms of the first association constant of the cobalt and nickel and acetate nuceus. The close agreement between these values constitutes a strong evidence for a 1:1 stoichiometry of the reaction. The same analogy could be applied to the cobalt-and nickel-imidazole groups interaction.

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C_H_A_P_T_E_R III.

pH-metric studies on the interaction of copper, zinc, magnesium, manganese and strontium with α_s-casein and casein.

I_N_T_R_O_D_U_C_T_I_O_N

pH-metric titration method has been employed for the study of hydrogen ion equilibria and also for the study of metal-protein complexes. There arise many complexities in the study of these metal-protein complexes which are further multiplied in the case of caseins, as they contain amount of phosphoric acid in addition to other amino acid residues. The uncertainty about the molecular weight of these proteins discourages to approach any systematic quantitative study.

Since Mallenders(256) demonstration that casein is a complex mixture containing atleast three components the separation of these components and their characterisation offered one of the most intresting and useful fields of study in casein chemistry. Following Mallender, Hipp et al (214) separated these components into α -, β -, and γ - casein and made an attempt to corelate the properties of these components with that of casein. Recently Wake and Baldwin(262) have shown that casein is a mixture of atleast 20 components. D.F. Waugh(263, 264) achieved great success in separating the pure $\alpha_{s1,2}$ caseins and their molecular weight have been determined with certainty employing light scattering method. A A review of the present literature has revealed that though much work has been done on casein much more remains to be done, particularly because the fractions

and better characterisation of casein components will help in understanding the complex chemical nature of casein as well as the interaction of these different components with each other.

The present chapter deals with both the qualitative and quantitative studies performed using casein and α_s-casein. In qualitative studies the interaction of magnesium, strontium and beryllium is discussed while the quantitative part deals with the interaction of physiologically important metal ions like copper, zinc, magnesium, manganese and strontium with casein and α_s-casein, employing pH-metric method. In the case of whole casein the binding has been calculated per 10^5 gms of casein while in the case of α_s-casein the values are per mole of the protein, taking the molecular weight of α_s-casein equal to 27,000 as found out by D.F.Waugh(263).

E_X_P_E_R_I_M_E_N_T_A_L

Protein solutions.

α_s-casein used was obtained by using its differential solubility in 50% aqueous alcohol(265) with changes in pH and temperature. The procedure was as follows.

The pure casein(E.Merk) was dissolved in dilute alkali and made to contain 50% alcolhol and 0.2M ammonium acetate. The pH of this solution was maintained at 6.5 by the addition of 1.0 M acetic acid in 50% alcohol. At this stage about one third of the total casein is precipitated. The pH of the filtrate was adjusted to 5.7 by adding 50%

alcoholic acetic acid. The precipitate obtained at this stage corresponds to two fifths of the total casein.

The α-casein was prepared from first fraction. The precipitate obtained was dissolved in dilute alkali and was reprecipitated at pH 7.2 in the presence of ammonium acetate in 50% alcohol. This process was repeated again and the final product was washed with water, till it was free from ions and finally with alcohol. Finally it was dried at low temperature and used for the studies.

The whole casein used was an E.Merk product. Solutions of this protein were prepared by soaking dry protein in water for several hours and then the mixture was stirred mechanically by the addition of known amount of 0.1 M potassium hydroxide solution and ths stirring was continued till the solution is obtained. Its pH was adjusted to 12.00.

The concentration of the protein solution was further checked up by drying an aliquot to constant weight at 105°C and then correcting for potassium. This method gave the same value for protein concentration as was calculated on the basis of anhydrous weight of the protein used.

Metal salts, viz., magnesium chloride and strontium nitrate were A.R. products, while the berylium nitrate was an E.Merk product. These were dissolved in doubly disstilled water and their strength was determined by usual

methods. M/50 solutions were then prepared by subsequent dilutions of their stock solutions and the pH maintained at 2.00 by the addition of requisite amount of 0.1 M hydrochloric acid solution.

Hydrochloric acid and potassium hydroxide solutions were prepared by their subsequent dilution and their pH adjusted to 2.00 and 12.00 respectively.

Procedure:

Varying volumes viz., 1.0,2.0,4.0,6.0,8.0,10.0,12.0, 14.0,16.0 mls. of the metal salt solutions or hydrochloric acid were taken in different pyrex tubes. Two such sets were arranged. In one set constant volume (4.0 mls.) of the protein solution was added while in the other set 4.0 mls of potassium hydroxide solution of the same pH as that of the protein (12.00) was added and the total volume was then made upto 20.0 mls.by adding the requisite amount of water.

pH-measurements were made with the Cambridge Bench pH meter with the glass electrodes at 30°C . The special blue glass electrode 'Alki' was used for more alkaline solutions(pH above 9.00). The buffer solutions used for the staindardisation of the pH-meter were 0.05M potassium acid phthalate and 0.05M borax solution for the two acid and basic ranges of pH.

The pH of these solutions was measured immediately and after 24 hours. The pH remained the same in both the

cases.

RESULTS AND DISCUSSIONS

The interaction of metal ions can take place either by competing with hydrogen ions or the hydroxyl ions, depending on the pH of the metal-protein system. If (H_1) and (H_2) represent the concentration of free hydrogen ions in the potassium hydroxide and anionic protein interaction with metal ions then $\triangle$pH (the difference between the two) indicates the extent of binding, the greater the $\triangle$pH the greater is the binding of the metal with the protein.

The results are summarised in the following tables.

T_A_B_L_E 1

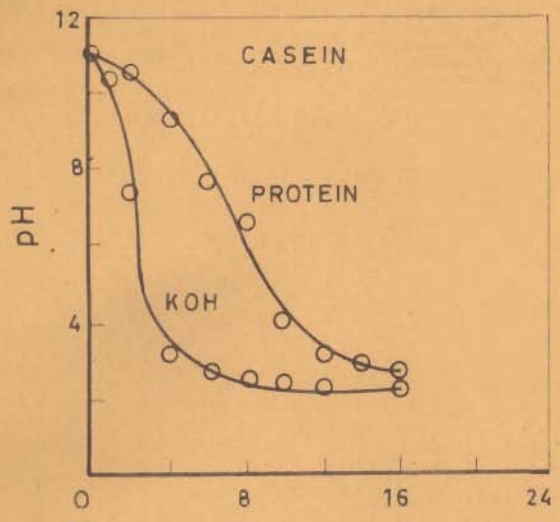
Concentration of casein solution = 2.0%, pH 12.00

Hydrochloric acid solution pH 2.00

Potassium hydroxide solution pH =12.00

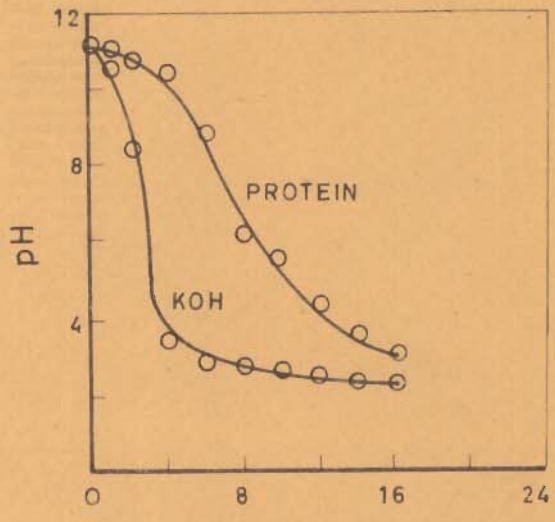
Total volume = 20 mls. Temperature 30°C

Volume of HCl added	pH_1 with KOH	pH_2 with protein	$\triangle$pH
0.0	11.00	11.00	-
1.0	10.44	10.70	0.26
2.0	7.34	10.56	3.22
4.0	3.08	9.34	6.26
6.0	2.66	7.74	5.08
8.0	2.46	6.55	4.09
10.0	2.36	4.00	1.64
12.0	2.24	3.10	0.85
14.0	2.16	2.80	0.64
16.0	2.08	2.56	0.48



VOL. OF ACID ADDED.

FIGURE 1



VOL. OF ELECTROLYTE ADDED.

FIGURE 2

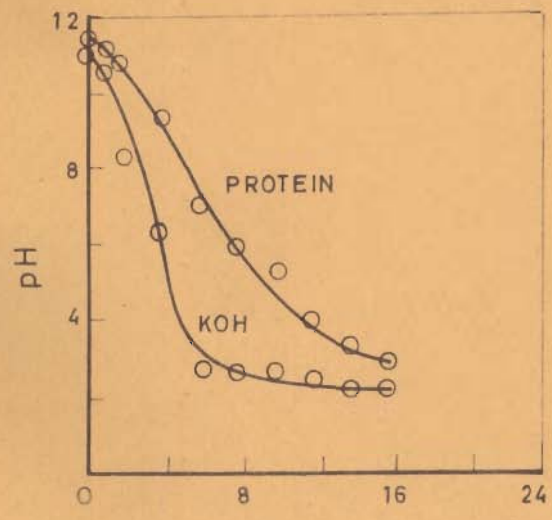
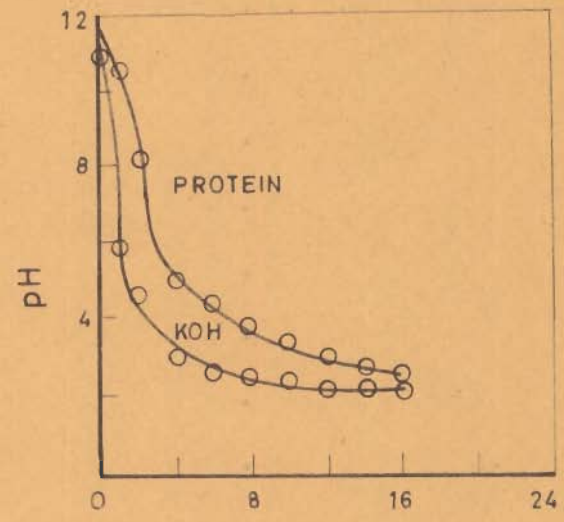


FIGURE 3



VOL. OF ELECTROLYTE ADDED.

FIGURE 4

T_A_B_L_E 2

Concentration of casein solution = 2.0%, pH 12.00

Concentration of magnesium chloride solution = M/50, pH 2.00

Potassium hydroxide solution pH 12.00

Total volue 20 mls. Temperature 30°C

Electrolyte added in mls.	pH_1 with KOH	pH_2 with protein	pH
0.0	11.00	11.00	-
1.0	10.58	11.00	0.42
2.0	8.26	10.62	2.36
4.0	3.32	10.28	6.96
6.0	2.84	8.80	5.96
8.0	2.62	6.10	3.48
10.0	2.50	5.46	2.96
12.0	2.40	4.34	1.94
14.0	2.32	3.56	1.24
16.0	2.24	3.00	0.76

Curve No.2

T_A_B_L_E 3

Concentration of casein solution = 2.00 %, pH 12.00

Concentration of strontium nitrate solution = M/50, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20 mls. Temperature 30°C

Volume of electrolyte added.	pH1 with KOH	pH_2 with protein	△ pH
0.0	11.0 0	11.20	0.20
1.0	10.52	10.94	0.42
2.0	8.20	10.72	2.52
4.0	6.26	9.30	6.04
6.0	2.74	7.02	4.28
8.0	2.56	5.86	3.30
10.0	2.46	5.16	2.70
12.0	2.36	4.00	1.64
14.0	2.24	3.24	1.00
16.0	2.20	2.84	0.64

Fig.No.3

T_A_B_L_E 4

Concentration of casein solution = 2.0%, pH 12.00

Concentration of beryllium nitrate solution = M/50, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20 mls. Temperature 30°C

Electrolyte added in mls.	pH_1 with KOH	pH_2 with protein	$\triangle$pH
0.0	11.00	11.20	0.20
1.0	5.82	10.60	4.78
2.0	4.60	8.20	3.60
4.0	3.00	5.02	2.02
6.0	2.68	4.40	1.72
8.0	2.54	3.90	1.36
10.0	2.36	3.54	1.18
12.0	2.24	3.00	0.76
14.0	2.20	2.74	0.54
16.0	2.12	2.58	0.46

Fig. 4

T_A_B_L_E 5

Concentration of α-casein = 2.0%, pH 12-00

Hydrochloric acid solution pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20 mls. Temperature 30°C

HCl.added in mls.	pH_1 with KOH	pH_2 with protein	$\triangle$pH
0.0	11.10	11.30	0.20
1.0	10.60	11.10	0.50
2.0	8.64	11.08	2.44
4.0	3.24	10.50	7.26
6.0	2.80	9.72	6.92
8.0	2.60	8.04	5.44
10.0	2.50	5.80	3.30
12.0	2.48	4.20	1.72
14.0	2.38	3.40	1.02
16.0	2.24	3.00	0.76

Fig. 5.

T_A_B_L_E 6

Concentration of α-casein solution = 2.00%, pH 12.00

Concentration of magnesium chloride = M/50, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20 mls. Temperature 30°C

Electrolyte added in mls.	pH_1 with KOH	pH_2 with protein	ΔpH
0.0	11.30	11.30	-
1.0	10.36	10.76	0.40
2.0	8.20	10.44	2.24
4.0	3.10	8.90	5.80
6.0	2.70	6.44	3.74
8.0	2.58	5.58	3.00
10.0	2.38	4.58	2.20
12.0	2.36	3.70	1.34
14.0	2.22	3.06	0.84
16.0	2.18	2.70	0.52

Fig. 6.

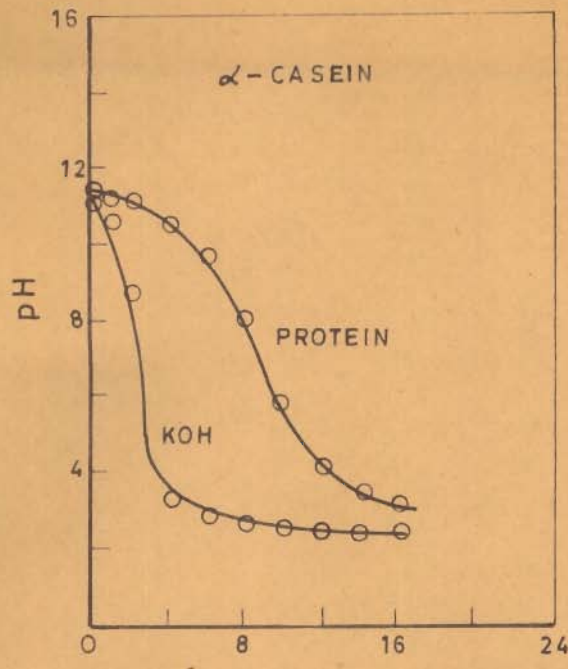


FIGURE 5

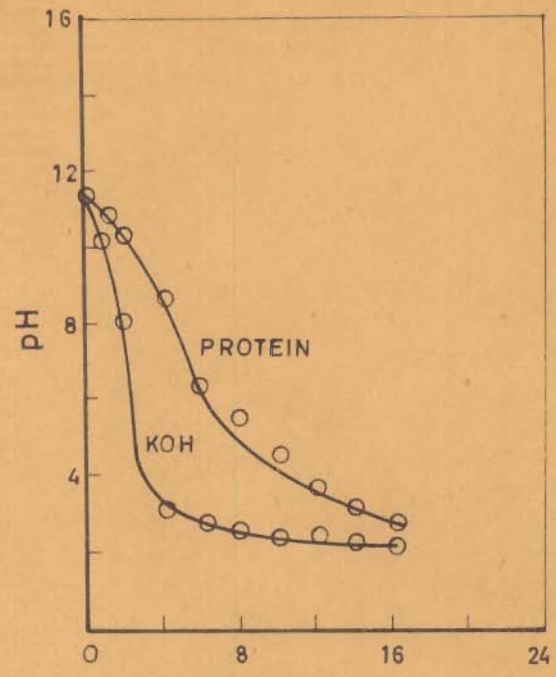


FIGURE 6

VOL. OF ELECTROLYTE ADDED.

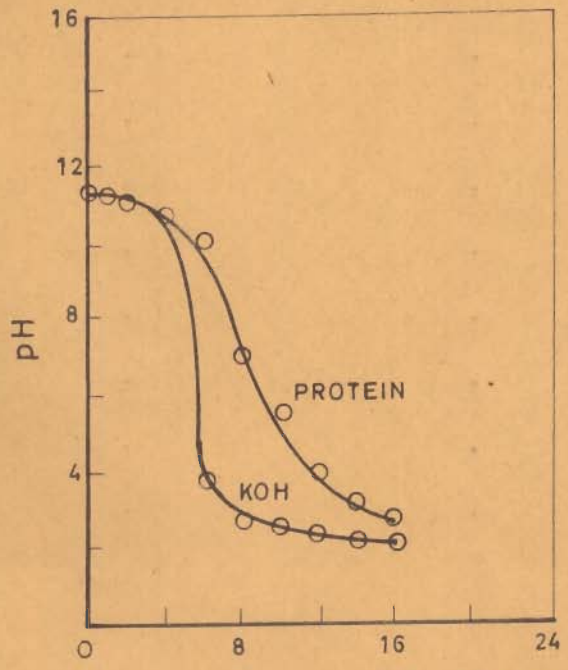


FIGURE 7

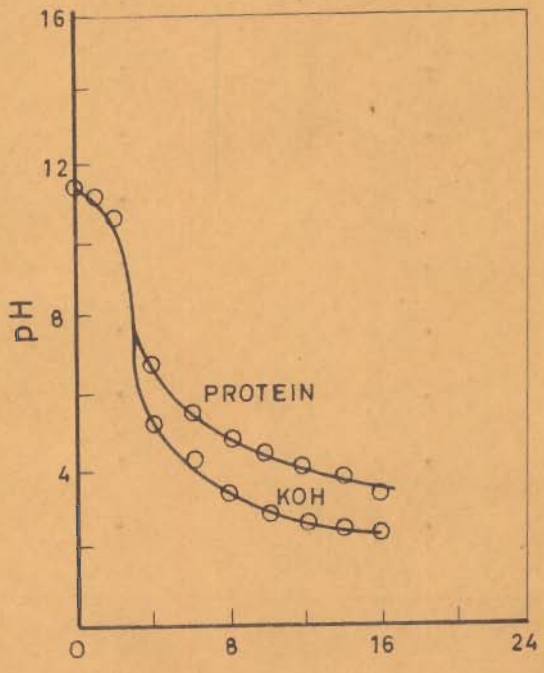


FIGURE 8

VOL. OF ELECTROLYTE ADDED.

T_A_B_L_E 7

Concentration of α-casein solution = 2.0 %, pH 12.00

Concentration strontium nitrate = M/50, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume=20 mls. Temperature 30°C

Electrolyte added in mls.	pH_1 with KOH	pH_2 with protein	ΔpH
0.0	11.30	11.30	-
1.0	11.30	11.30	-
2.0	11.20	11.20	-
4.0	10.80	10.80	-
6.0	3.80	10.14	6.34
8.0	2.82	7.10	4.28
10.0	2.62	5.60	2.98
12.0	2.42	4.00	1.58
14.0	2.28	3.20	0.92
16.0	2.20	2.80	0.60

Fig. 7

T_A_B_L_E 8

Concentration of α-casein solution = 2.0%, pH 12.00

Concentration of beryllium nitrate = M/50, pH 2.00

Potassium hydroxide solution = pH 12.00

Total volume = 20 mls. Temperature 30°C

Electrolyte added in mls.	pH_1 with KOH	pH_2 with protein	ΔpH
0.0	11.30	11.30	-
1.0	11.20	11.20	-
2.0	10.70	10.80	0.10
4.0	5.08	6.80	1.72
6.0	4.34	5.48	1.06
8.0	3.40	4.74	1.34
10.0	2.90	4.40	1.50
12.0	2.64	4.06	1.42
14.0	2.50	3.80	1.30
16.0	2.44	3.30	0.86

Fig. 8.

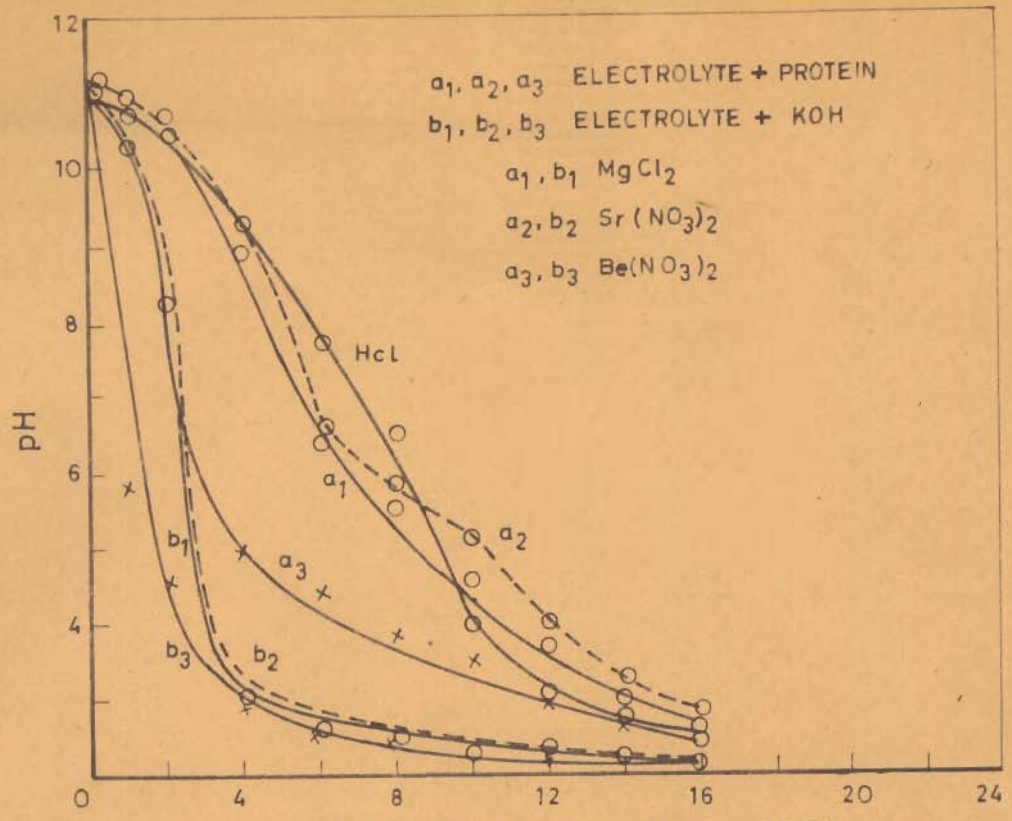


FIGURE 9

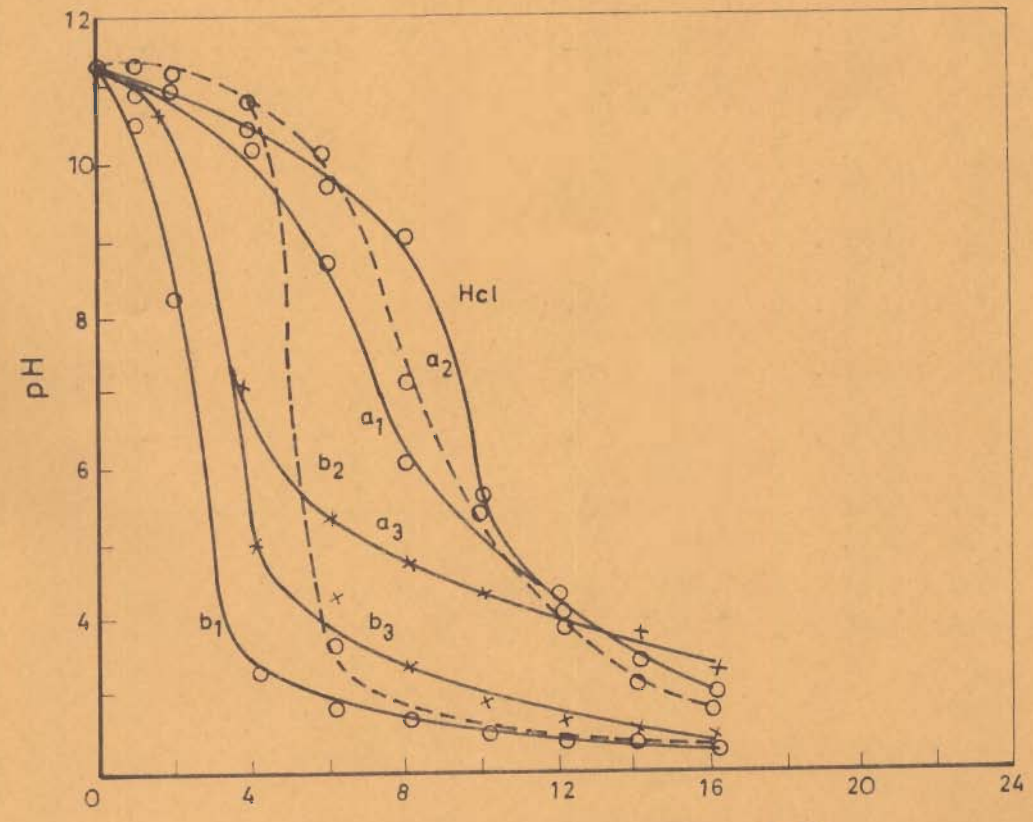


FIGURE 10

D_I_S_C_U_S_S_I_O_N

The pH-metric titrations carried out in the presence of magnesium,strontium and berylium ions with casein and α-casein give some conclusive evidence of the binding of these ions with the available sites of the protein. Since casein does not show any detectable chloride ion binding tendency,the only explaination which may be offered for the variation in pH is the possible chemical combination of metal ions with the protein.

It is evident from the Fig.9 and 10 that the titration curves of magnesium,strontium and beryllium for casein and α-casein respectively,lie above the corresponding curves for the metal ions and alkali. Between pH 3.0-5.5 the ionised carboxyl groups of casein play significant role in the fixation of magnesium,beryllium and strontium. The extent to which the electrolyte-alkali curves are displaced by the addition of protein may be taken as a quantitative measure of the extent of metal protein combination.

From Fig.9 the flat portion of the curve lie in the pH range 2.5 to 5.0 indicating thereby that the combination is mostly with the carboxyl groups of the protein. In this case strontium shows greater binding than magnesium to the carboxyl groups of the casein.

At higher pH values, it is evident from the curves, that beryllium goes in combination only with casein;and in the case of α-casein no appreciable combination of beryllium is visible above pH 8.0. So in case of α-casein

beryllium combines mostly with the carboxyl groups.

Magnesium and strontium show the tendency to combine with proteins even at higher pH values. Probably they enter in combination either with imidazole groups of histidine residue or amino groups of lysyl residue.

A different behaviour is seen in the case of α-casein(Fig.10) from that of casein. Here magnesium shows greater binding tendency over the whole pH range as compared to strontium. This information is contradictory to the results of casein where strontium shows greater binding tendency to the protein molecule.

According to Hipp,Groves and McMeekin(214) at pH 9.3 the titration curve for α-casein gives the number of carboxyl group + 2 equivalents of phosphorous + imidazole groups. At this pH magnesium shows greater binding than strontium,in the case of α-casein,while in the case of casein the order is just reversed.

We conclude the above results by the remark that the binding of beryllium takes place through the carboxyl groups of α-casein only while with casein groups other than carboxyl are also involved. Magnesium and strontium combine through the imidazole and amino groups beside the carboxyl groups of both α -casein and casein. The order of binding for casein and α-casein are: strontium $>$ magnesium $>$ beryllium and magnesium $>$ strontium $>$ beryllium respectively.

E_X_P_E_R_I_M_E_N_T_A_L

α_s-casein was prepared by the method of Waugh(263). Whole casein(E.Merk) was soaked for several hours in water and then dissolved in dilute sodium hydroxide solution and pH was maintained at 7.5. This was precipitated by the addition of 1 M HCl at pH 4.6. After filtration the product was washed with distilled water and finally suspended in sufficient water. The supernatant water was removed by decantation and the whole process was repeated four times. Then the washed casein was suspended in water and the pH was adjusted to 4.0 with 1.0M acetic acid and was stirred in a stirrer for several hours. Then the casein was filtered. In this way phosphatase, proteolytic enzyme, red protein and another casein components are removed.

Then the casein was dissolved in dilute sodium hydroxide and the pH was not allowed to proceed 7.0. Then the container was cooled in the refrigerator and diluted with cold water. The casein was again precipitated by adding 0.1M HCl until a pH 4.3-4.4 was reached. It was again kept over night in the refrigerator at 4°C. The precipitate was removed by filtration and it was again dissolved in dilute sodium hydroxide as before. This process was repeated about five times to remove β-casein completely. In this last precipitation the α_s-casein was precipitated with hydrochloric acid at pH 4.70 and washed free of chloride ions by decantation and centrifugation.

Casein (soluble at pH 6.8) for the quantitative studies was prepared in the following manner.

The pure E.Merk product was soaked for several hours in distilled water and then was dissolved in dilute ammonia solution(266,267); adding ammonia slowly during mechanical agitation so as to have no pronounced alkalinity at any time in any part of the solution(the pH of the solution was kept below 7.0). After dilution with water the casein was precipitated by the calculated amount of dilute acetic acid. The precipitate was washed with water. The casein was beaten up with water,first to a thin paste by means of an electrically driven stirrer and then more water was added and the stirring continued until the casein was well distributed. It was then centrifused from the wash water and this method of washing was repeated for about 5 to 6 times. The washed casein,after centrifugation finally being stored under water with toluene at $5^{o}C$.

The casein so obtained was dissolved in minimum amount of 0.1M KOH so that the pH remained between 6.5 to 7.0, and the whole mass was stirred. Finally the solution was centrifused and the product obtained was stored in a stoppered pyrex flask which was kept in a refrigerator. The solution after centrifugation was used for the quantitative studies.

The concentration of the protein solutions was checked up by taking an aliquot (10.0 mls.) in a weighed

crucible and drying it to a constant weight at 105°C . The correct concentration was calculated by applying correction for potassium(added in the form of potassium hydroxide).

The metal salts viz.,cupric chloride, zinc chloride, magnesium chloride,manganese chloride and strontium nitrate were all A.R.products. They were dissolved in doubly distilled water and their strength was determined gravimetrically. The solutions of requisite strength (M/50) of various metal salts were made by the subsequent dilution of the stock solution.

Hydrochloric acid and potassium hydroxide solutions were prepared by the dilution of their stock solutions, which were standardised by usual methods.

Procedure. Varying volumes viz., 2.0,1.6,1.0,0.8,0.7,0.5, 0.3,0.1 and 0.02 mls. of hydrochloric acid (0.0861 M) and 0.2,0.3,0.4,0.6,1.0,1.1 and 1.5 mls of potassium hydroxide (0.0605 M) were taken in different pyrex tubes. Two such sets were prepared. Equal amount (1.0 ml.) of the metal salt was added in each set. Now in one set the α_s-casein (2.0 mls. of 4.2%) solution was added while the total volume was made upto 10.0 mls.,first by adding potassium chloride,to maintain the ionic strength 0.15,and then by adding requisite amount of water.

The following sets were prepared, for the study of the binding of different metal ions with α_s-casein.

1. Acid or base alone.
2. Acid or base + α_s-casein.
3. Acid or base + cupric chloride.
4. Acid or base + cupric chloride + α_s-casein.
5. Acid or base + zinc chloride.
6. Acid or base + zinc chloride + α_s-casein.
7. Acid or base + magnesium chloride.
8. Acid or base + magnesium chloride + α_s-casein.
9. Acid or base + manganese chloride.
10. Acid or base + manganese chloride + α_s-casein.
11. Acid or base + strontium nitrate.
12. Acid or base + strontium nitrate + α_s-casein.

Exactly similar sets were prepared with casein also with the only difference that the α_s-casein was replaced by casein.

The pH-measurements were made by the help of a Cambridge Bench Type pH meter with the glass electrode, reading upto 0.02 pH unit. Special blue 'Alki' glass electrode was used for the more alkaline solutions (above pH 9.00). The buffers used for the standardisation were 0.05 M potassium acid phthalate and 0.05 M borax solutions for the two acid and basic ranges of pH.

The calculation were made by the equations discussed in chapter I of this thesis.

The results are summarised in the following tables.

T_A_B_L_E 9

Concentration of α_s-casein solution = 0.84%
Total volume = 10 mls. Ionic strength =0.15
Temperature 30°C

HCl added in mls.	pH values			
	With acid or alkali	With protein.	With M/500 Cu^{++}	With M/500 Cu^{++} +protein.
2.0	1.90	2.30	1.92	2.31
1.6	2.00	2.50	2.00	2.44
1.0	2.18	3.10	2.16	3.20
0.8	2.29	3.85	2.28	3.58
0.7	2.34	4.13	2.34	3.76
0.5	2.50	4.79	2.48	4.08
0.3	2.71	5.22	2.70	4.45
0.1	3.24	5.66	3.20	4.82
0.02	4.00	5.90	3.82	5.02
0.0	4.60	6.00	5.38	5.10
KOH added in mls.				
0.2	10.43	6.43	5.14	5.50
0.3	10.74	6.67	5.35	5.67
0.4	10.84	6.90	5.50	5.88
0.6	11.07	7.56	8.00	6.35
1.0	11.34	9.57	10.66	7.20
1.1	11.40	9.68	10.91	7.51
1.5	11.48	10.18	11.24	8.22

Fig. 11

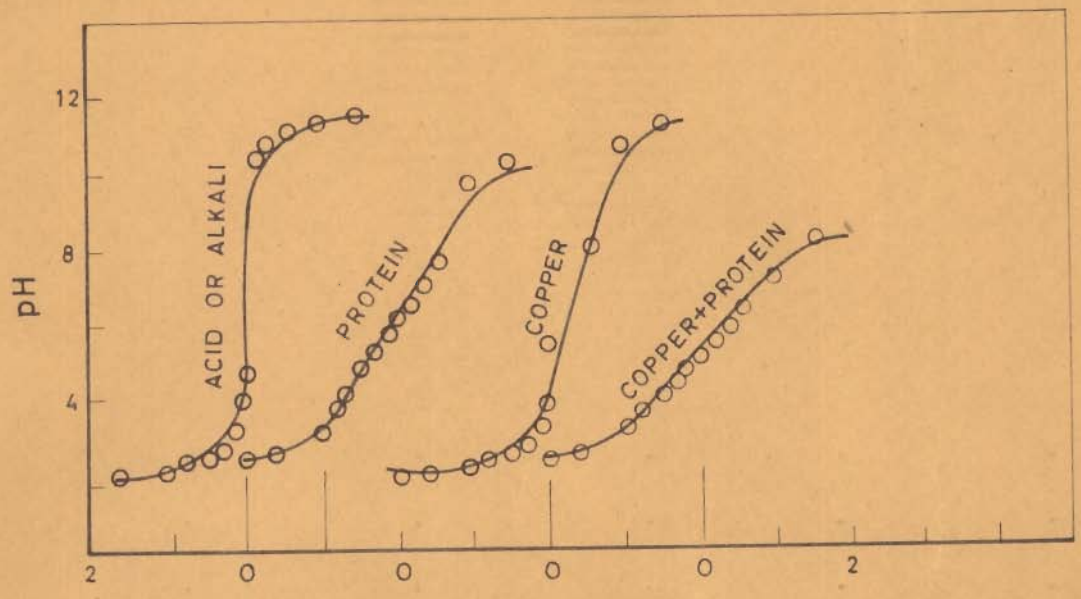


FIGURE 11

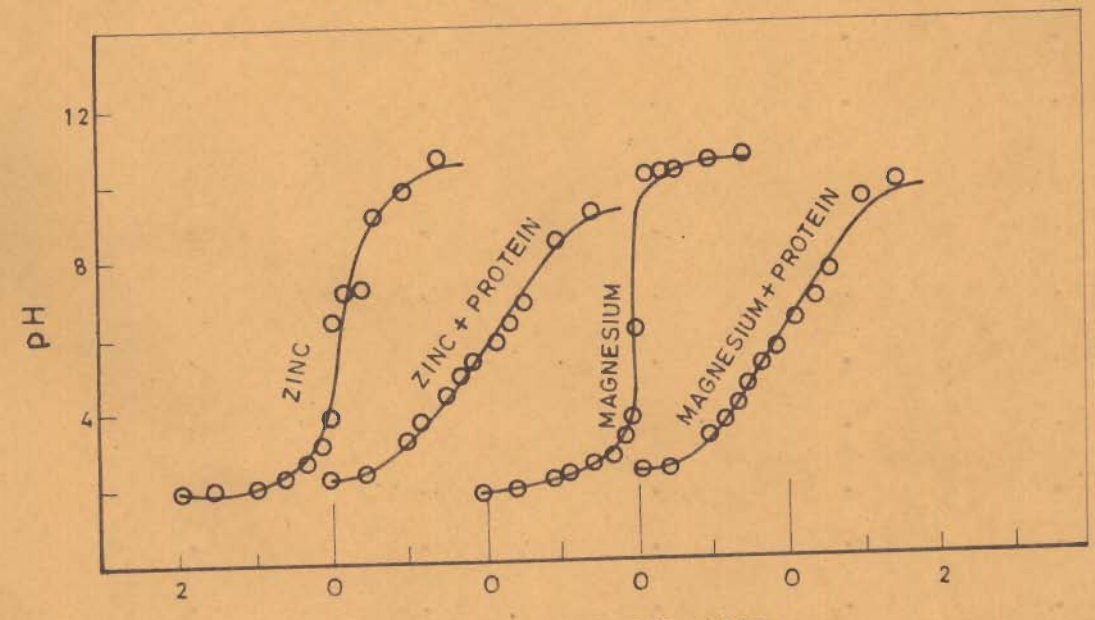


FIGURE 12

T_A_B_L_E 10

Concentration of α_s-casein solution =0.84%

Total volume =10 mls. Ionic strength =0.15

Temperature = 30°C

HCl added in mls.	pH values			
	With M/500 Zn^{++}	With M/500 Zn^{++} +protein	With M/500 Mg^{++}	With M/500 Mg^{++} +protein.
2.0	1.94	2.30	1.90	2.29
1.6	1.98	2.44	1.98	2.39
1.0	2.12	3.26	2.15	3.29
0.8	2.26	3.76	2.26	3.72
0.7	2.31	4.02	2.32	4.10
0.5	2.44	4.44	2.56	4.62
0.3	2.72	4.90	2.67	5.17
0.1	3.20	5.26	3.18	5.57
0.02	3.85	5.50	3.72	5.75
0.0	6.40	5.56	6.14	5.84
KOH added in mls.				
0.2	7.18	5.94	10.28	6.38
0.3	7.25	6.10	10.28	6.48
0.4	7.32	6.30	10.26	6.88
0.6	9.20	6.80	10.28	7.56
1.0	9.84	8.48	10.46	9.49.
1.1	-	8.53	10.46	9.58
1.5	10.72	9.34	10.72	9.90

Fig. 12

T_A_B_L_E 11

Concentration of α_s-casein solution =0.84%

Total volume = 10 mls. Ionic strength = 0.15

Temperature =30°C

HCl added in mls.	pH values			
	With M/500 Mn^{++}	With M/500 Mn^{++} +protein	With M/500 Sr^{++}	With M/500 Sr^{++} +protein
2.0	1.92	2.32	1.88	2.33
1.6	2.00	2.48	1.98	2.47
1.0	2.16	3.25	2.14	3.24
0.8	2.26	3.70	2.24	3.80
0.7	2.30	4.06	2.28	3.98
0.5	2.47	4.78	2.44	4.80
0.3	2.70	5.12	2.69	5.19
0.1	3.18	5.54	3.12	5.56
0.02	4.22	5.72	3.82	5.84
0.0	6.84	5.76	5.98	5.84
KOH added in mls.				
0.2	7.54	6.19	10.41	6.37
0.3	7.52	6.35	10.65	6.57
0.4	7.72	6.45	10.80	6.78
0.6	8.23	6.83	11.00	7.22
1.0	9.36	7.66	11.23	9.37
1.1	10.24	7.90	11.30	9.60
1.5	10.70	8.94	11.43	10.00

Fig. 13

These pH values are used to calculate the number of proton liberated. Actually the number of hydrogen ions bound per protein molecule is obtained by the difference of hydrogen ions added and the hydrogen ions liberated after mixing the metal ions and the α_s-casein. Titration curves were obtained by plotting ' r ' (the number of hydrogen ions dissociated per mole of α_s-casein) against pH.

Due to the uncertainties about the molecular weight of the casein the binding was calculated per 10^5 gm.of protein,instead of per mole of protein.

T_A_B_L_E 12

Concentration of α_s-casein solution = 0.84%
Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/Mole of protein	Moles of H^+ dissociated per mole of protein$.
17.22	2.30	39	9
13.776	2.50	30	18
8.61	3.10	24	24
6.888	3.85	20	28
6.027	4.13	19	29
4.305	4.79	14	34
2.583	5.22	8	40
0.861	5.66	3	45
0.172	5.90	1	47
-	6.00	0	48
Base (OH^-) added Moles/L x 10^{-3}	pH	Bound (OH^-) Moles/Mole of protein	Moles of H^+ dissociated per mole of protein.
1.210	6.43	4	52
1.815	6.67	6	54
2.420	6.90	8	56
3.360	7.56	12	60
6.05	9.57	19	67
6.655	9.68	21	69
9.075	10.18	28	76

Fig. 14

T_A_B_L_E 13

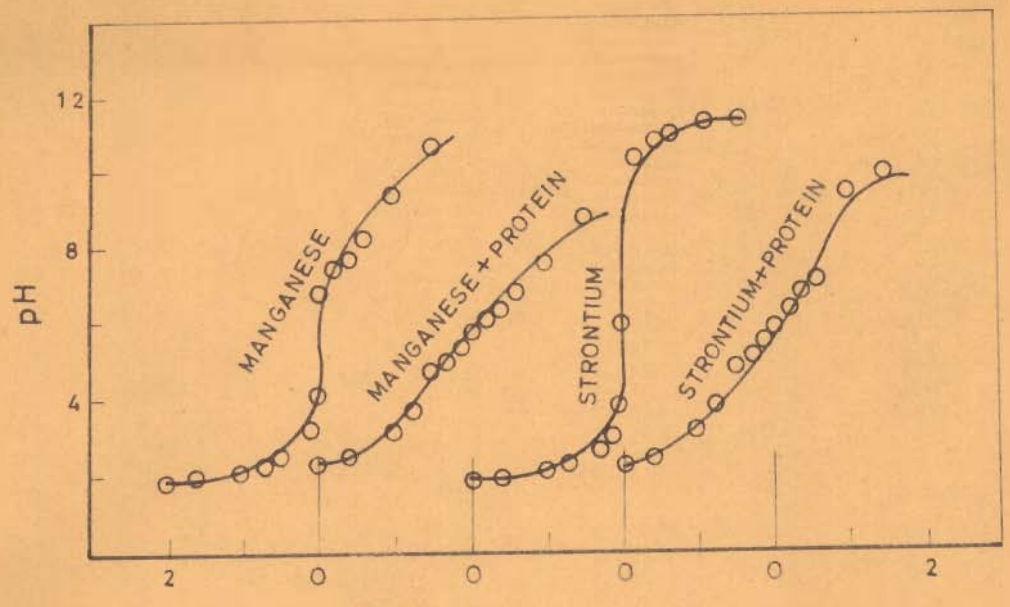
Concentration of α_s-casein = 0.84 %

Concentration of cupric chloride = M/500

Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L x 10^{-3}	pH	Bound H^+ Moles/Mole of protein	Moles of H^+ dissociated per mole of protein.
17.22	2.31	39	9
13.776	2.44	28	20
8.61	3.20	25	23
6.888	3.58	21	27
6.027	3.76	20	28
4.305	4.08	13	35
2.583	4.45	8	40
0.681	4.82	2	46
0.172	5.02	-	48
-	5.10	-	48
$(OH)^-$ added Moles/L x 10^{-3}	pH	Bound $(OH)^-$ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
1.210	5.50	2	50
1.815	5.67	3	51
2.420	5.88	5	53
3.630	6.35	11	59
6.05	7.20	19	67
6.655	7.51	21	69
9.075	8.22	29	77

Fig. 14



VOL. OF Hcl OR KOH

FIGURE 13

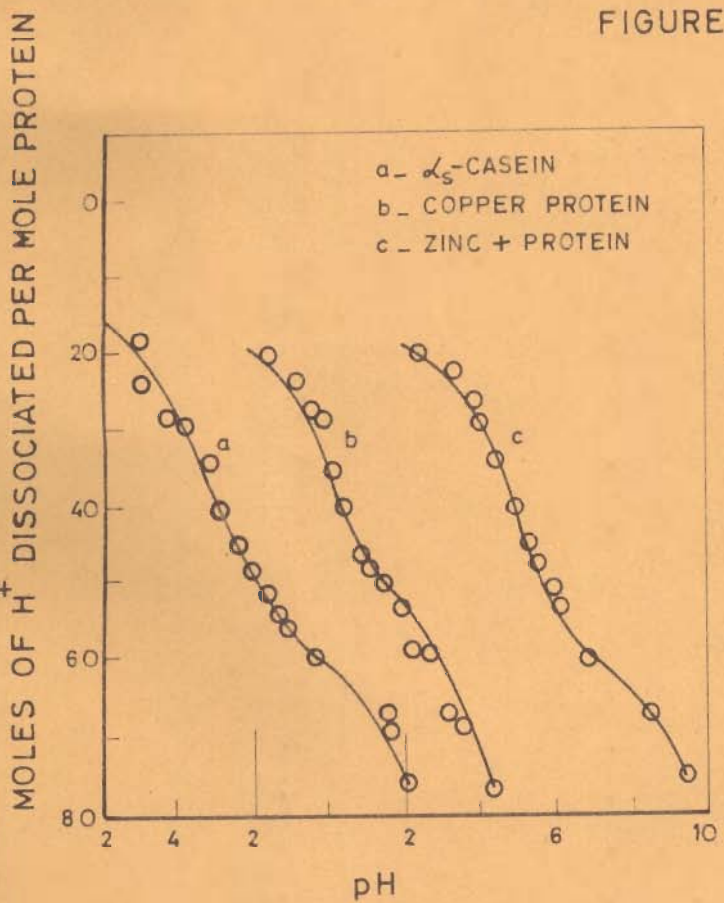


FIGURE 14

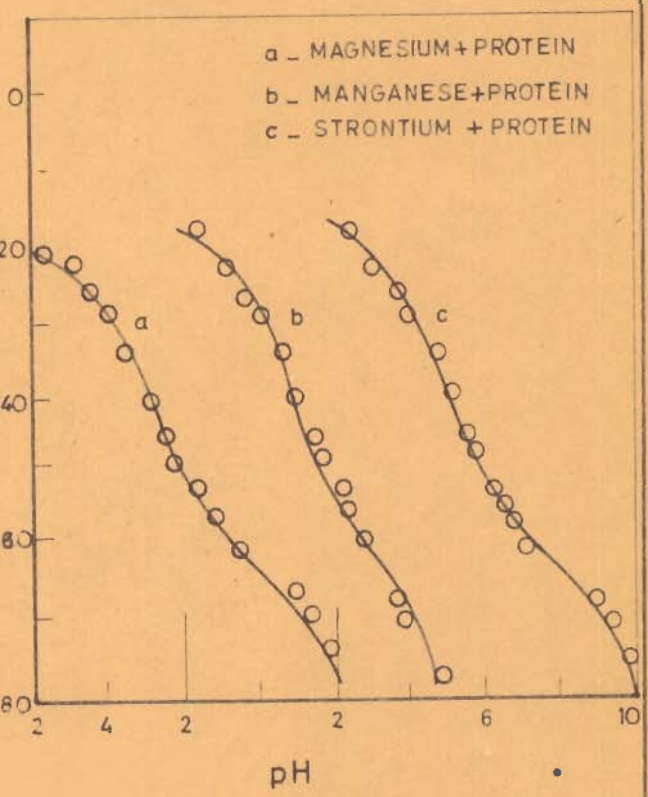


FIGURE 15

T_A_B_L_E 14

Concentration of α_s-casein solution = 0.84%

Concentration of zinc chloride solution = M/500

Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L x 10^{-3}	pH	Bound H^+ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
17.22	2.30	39	9
13.776	2.44	28	20
8.61	3.26	26	22
6.888	3.76	22	26
6.027	4.02	19	29
4.305	4.44	14	34
2.583	4.90	8	40
0.861	5.26	3	45
0.172	5.50	1	47
-	5.56	-	48
$(OH)^-$ added Moles/L x 10^{-3}	pH	Bound $(OH)^-$ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
1.210	5.94	3	51
1.815	6.10	5	53
2.420	6.30	7	55
3.630	6.80	12	60
6.05	8.48	19	67
6.655	8.53	-	-
9.075	9.54	27	75

Fig. 14

T_A_B_L_E 15

Concentration of α_s-casein solution = 0.84%
Concentration of magnesium chloride = M/500
Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
17.22	2.29	38	10
13.776	2.39	27	21
8.61	3.29	26	22
6.888	3.72	22	26
6.027	4.10	19	29
4.305	4.62	14	34
2.583	5.17	8	40
0.861	5.57	3	45
0.172	5.75	1	47
-	5.84	-	48
$(OH)^-$ added Moles/L x 10^{-3}	pH	Bound $(OH)^-$ Moles/Mole protein	Moles of H^+ dissociated per mole of protein
1.210	6.38	4	52
1.815	6.48	6	54
2.420	6.88	8	56
3.630	7.56	12	60
6.05	9.49	17	65
6.655	9.58	19	67
9.075	9.90	25	73

Fig. 15

T_A_B_L_E 16

Concentration of α_s-casein solution = 0.84%
Concentration of manganese solution = M/500
Ionic strength = 0.15 Temperature 30°C

H^+added Moles/L.x10^{-3}	pH	Bound H^+ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
17.22	2.32	39	9
13.776	2.48	30	18
8.61	3.25	25	23
6.888	3.70	21	27
6.027	4.06	19	29
4.305	4.78	14	34
2.583	5.12	8	40
0.861	5.54	3	45
0.172	5.72	1	47
-	5.76	-	48
$(OH)^-$added Moles/L.x10^{-3}	pH	Bound$(OH)^-$ Moles/Mole protein	Moles of H^+ dissociated per mole of protein.
1.210	6.19	4	52
1.815	6.35	5	53
2.420	6.45	7	55
3.630	6.83	11	59
6.05	7.66	19	67
6.655	7.90	21	69
9.075	8.94	29	77

Fig. 15

T_A_B_L_E 17

Concentration of α_s-casein solution = 0.84%
Concentration of strontium nitrate = M/500
Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/Mole protein	Moles H^+ dissociated per mole of protein.
17.22	2.33	39	9
13.776	2.47	30	18
8.61	3.24	25	23
6.888	3.80	22	26
6.027	3.98	19	29
4.305	4.80	14	34
2.583	5.19	8	40
0.861	5.56	3	45
0.172	5.84	1	47
-	5.84	-	48
$(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/Mole protein	Moles H^+ dissoci- per mole of protein
1.210	6.37	4	52
1.815	6.57	6	54
2.420	6.78	8	56
3.630	7.22	12	60
6.05	9.37	19	67
6.655	9.60	21	69
9.075	10.00	28	76

Fig. 15

T_A_B_L_E 18

Copper α_s-casein system.

Concentration cupric chloride = 2.0×10^{-3}M

Concentration of α_s-casein =(0.311×10^{-3}M)

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	Free metal ions $\times 10^{-3}$M	V_M	log K
3.0	22	20	-	2	-
4.0	33	28	-	5	-
5.0	45	40	-	5	-
5.50	48	43	0.445	5	2.272
6.35	56	51	-	5	-
7.00	62	56	-	6	-
8.00	67	61	0.134	6	3.572
9.30	-	68	-	-	-

T_A_B_L_E 19

Zinc α_s-casein system

Concentration of zinc chloride = 2.0×10^{-3}M

Concentration of α_s-casein = 0.84%(0.311×10^{-3}M)

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	Free metal ions $\times 10^{-3}$M	V_M	log K
3.0	22	20	-	2	-
4.0	30	28	-	2	-
5.0	44	40	-	4	-
5.50	47	43	0.756	4	1.936
6.35	56	51	-	5	-
7.0	61	56	-	5	-
7.5	64	59	0.445	5	3.051
8.0	66	61	0.445	5	-
9.30	74	68	-	6	-

T_A_B_L_E 20

Magnesium α_s-casein system

Concentration of magnesium chloride = 2.0×10^{-3} M

Concentration α_s-casein = (0.311×10^{-3} M)

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	Free metal ions $\times 10^{-3}$ M	V_M	log K
3.0	21	20	-	1	-
4.0	29	28	-	1	-
5.0	41	40	-	1	-
5.75	45	43	1.378	2	1.362
6.35	53	51	-	2	-
7.0	58	56	-	2	-
7.5	61	59	1.378	2	-
8.0	63	61	-	2	-
9.30	71	68	-	3	-

T_A_B_L_E 21

Magnesium α_s-casein system.

Concentration of manganese chloride = 2.0×10^{-3}M

Concentration of α_s-casein = (0.311×10^{-3}M)

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	Free metal ions $\times 10^{-3}$M	V_M	log K
3.0	21	20	-	1	-
4.0	29	28	-	1	-
5.0	42	40	-	2	-
5.50	46	43	1.067	3	1.655
6.35	54	51	-	3	-
7.0	59	56	-	3	-
7.5	63	59	0.756	4	2.820
8.0	65	61	0.756	4	2.820
9.0	-	68	-	-	-

T_A_B_L_E 22

Strontium α_s-casein system

Concentration of strontium nitrate = 2.0×10^{-3} M

Concentration of α_s-casein = (0.311×10^{-3} M)

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	Free metal ions $\times 10^{-3}$ M	V_M	log K
3.0	20	20	-	-	-
4.0	29	28	-	1	-
5.0	41	40	-	1	-
5.50	45	43	1.378	2	1.328
6.35	53	51	-	2	-
7.0	58	56	-	2	-
7.5	62	59	1.067	3	2.670
8.0	64	61	1.067	3	2.670
9.0	72	68	-	4	-

T_A_B_L_E 23

Concentration of casein solution = 0.78%

Total volume = 10 mls. Ionic strength =0.15
Temperature 30°C

HCl added in mls.	pH values			
	With acid or alkali	With protein.	With M/500 Cu^{2+}	With M/500 Cu^{2+} +protein.
2.0	1.86	2.26	1.92	2.65
1.6	1.96	2.46	2.00	3.16
1.0	2.15	3.38	2.16	3.86
0.8	2.26	3.96	2.28	4.15
0.7	2.28	4.20	2.34	4.21
0.5	2.44	4.82	2.48	4.55
0.3	2.64	5.26	2.70	4.88
0.1	3.24	5.83	3.20	5.18
0.02	3.70	6.03	3.82	5.36
0.0	-	6.16	5.40	5.34
KOH added in mls.				
0.2	10.54	6.73	5.05	5.60
0.3	10.92	7.05	5.21	5.72
0.4	11.10	7.30	-	-
0.6	11.24	8.48	8.40	6.09
1.0	11.56	10.06	10.68	7.00
1.0	11.62	10.30	10.90	7.27
1.5	11.76	10.76	11.24	7.96

Fig. 16

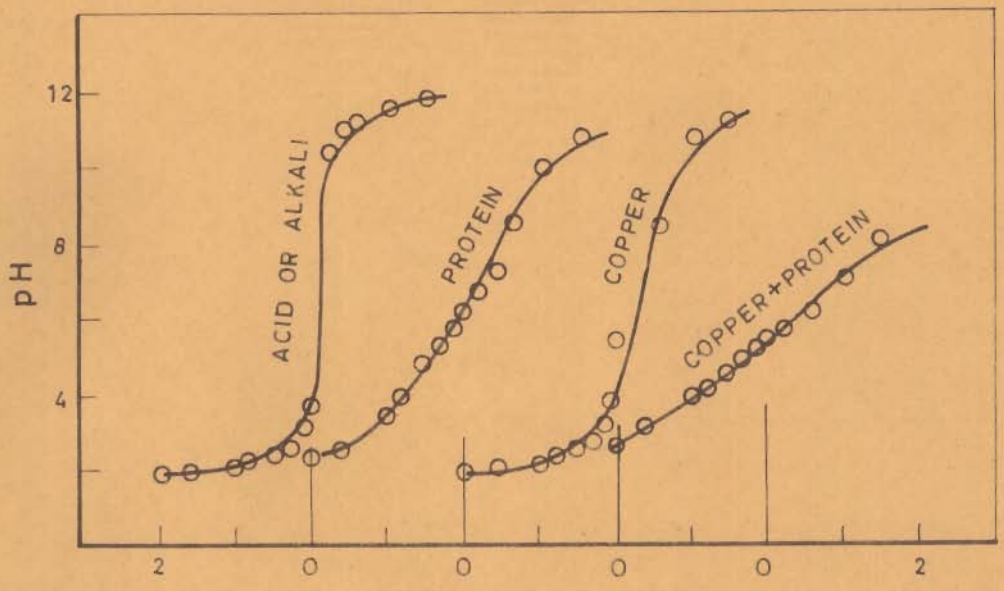


FIGURE 16

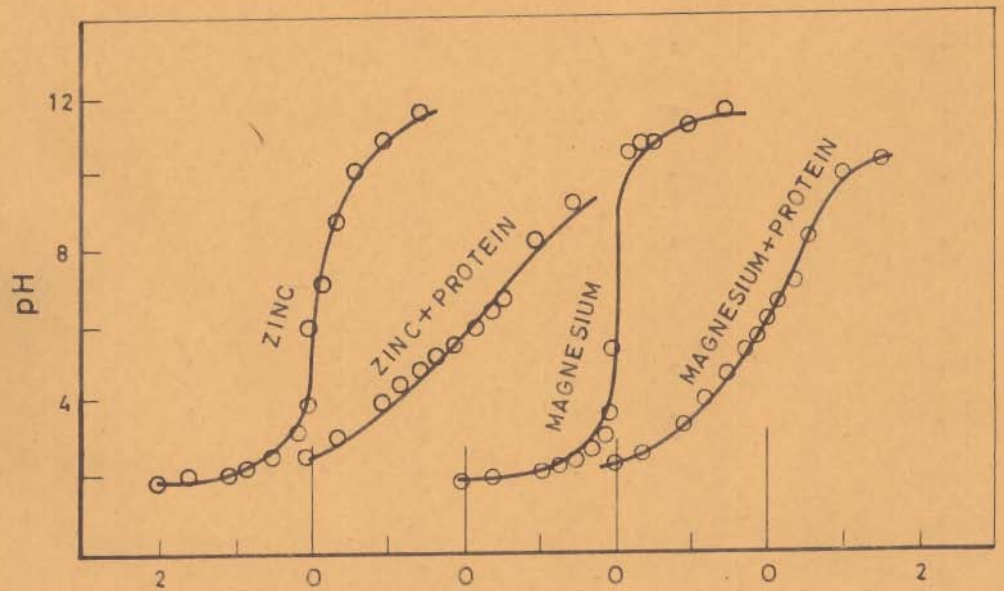


FIGURE 17

T_A_B_L_E 24

Concentration of casein solution = 1.194%

Total volume = 10 mls. Ionic strength = 0.15

Temperature = 30°C

HCl added in mls.	pH values			
	With M/500 Zn^{2+}	With M/500 Zn^{2+} +protein	With M/500 Mg^{++}	With M/500 Mg^{2+} +protein
2.0	1.90	2.70	1.88	2.28
1.6	1.98	3.12	1.96	2.46
1.0	2.16	4.06	2.12	3.43
0.8	2.26	4.47	2.22	3.98
0.7	2.33	4.60	2.26	4.12
0.5	2.49	4.90	2.42	4.82
0.3	2.70	5.28	2.66	5.26
0.1	3.24	5.52	3.10	5.74
0.02	3.96	5.64	3.66	6.03
0.0	6.00	5.64	5.44	6.08
KOH added in mls.				
0.2	7.08	5.98	10.64	6.62
0.3	7.40	6.14	10.44	6.90
0.4	8.84	6.40	10.76	7.24
0.6	10.17	6.76	10.80	8.40
1.0	10.88	8.27	11.24	10.00
1.1	10.96	8.57	11.36	10.10
1.5	11.72	9.27	11.64	10.28

Fig. 17

T_A_B_L_E 25

Concentration of casein solution = 0.78%

Total voume = 10 mls Ionic strength = 0.15

Temperature 30°C

HCl added in mls.	pH values			
	With M/500 Mn^{2+}	With M/500 Mn^{2+} +protein	With M/500 Sr^{2+}	With M/500 Sr^{2+} +protein
2.0	1.84	2.24	1.86	2.26
1.6	1.96	2.46	1.96	2.50
1.0	2.16	3.36	2.10	3.62
0.8	2.24	3.96	2.26	3.86
0.7	2.30	3.96	2.28	4.04
0.5	2.43	4.72	2.46	4.82
0.3	2.68	5.12	2.68	5.20
0.1	3.13	5.50	3.18	5.62
0.02	3.77	5.70	3.94	5.90
0.0	5.88	5.80	5.80	5.96
KOH added in mls.				
0.2	6.60	6.25	10.80	6.50
0.3	6.84	6.50	11.02	6.72
0.4	7.20	6.80	11.20	6.94
0.6	7.60	7.60	11.37	7.50
1.0	10.56	9.00	11.62	9.80
1.1	10.88	9.22	11.62	10.10
1.5	11.40	9.70	11.76	10.60

Fig. 18

T_A_B_L_E 26

Concentration of casein solution = 0.78%

Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/10^5 gms.protein	Moles of H^+ dissociated per 10^5 gms protein.
17.22	2.26	129	28
13.776	2.46	122	35
8.61	3.38	104	53
6.888	3.96	86	71
6.027	4.20	76	81
4.305	4.82	55	102
2.583	5.26	33	124
0.861	5.83	11	146
0.172	6.03	2	155
-	6.16	-	157
Base $(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5 gms. protein	Moles of H^+ dissociated per 10^5 gm.protein.
1.210	6.73	15	172
1.815	7.05	23	180
2.420	7.30	29	186
3.630	8.48	46	203
6.05	10.06	75	232
6.655	10.30	81	238
9.075	10.76	104	261

Fig. 19

T_A_B_L_E 27

Concentration of casein solution = 1.194%
Concent. of cupric chloride solution = M/500
Ionic strength = 0.15 Temperature 30°C

H^+ added Moles/L.x 10^{-3}	pH	Bound H^+ Moles/10^5gm protein.	Moles of H^+ dissociated per 10^5 gm. protein.
17.22	2.65	120	37
13.776	3.16	115	42
8.61	3.86	71	86
6.888	4.15	58	99
6.027	4.21	50	107
4.305	4.55	36	121
2.583	4.88	21	136
0.861	5.18	7	150
0.172	5.36	2	155
-	5.34	-	157
$(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5gm proteins	Moles of H^+ dissociated per 10^5gm protein
1.210	5.60	7	164
1.815	5.72	8	165
2.420	-	-	-
3.630	6.09	31	188
6.05	7.00	51	208
6.655	7.27	56	213
9.075	7.96	76	233

Fig. 19

T_A_B_L_E 28

Concentration of casein solution = 1.194%
Concentration of zinc chloride solution = M/500
Ionic strength =0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/10^5 gm. protein	Moles of H^+ dissociated per 10^5 gm. protein.
17.22	2.70	123	34
13.776	3.12	109	48
8.61	4.06	72	85
6.888	4.47	58	99
6.027	4.60	51	106
4.305	4.90	36	121
2.583	5.28	22	135
0.861	5.52	7	150
0.172	5.64	2	155
-	5.64	-	157
$(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5gm protein.	Moles of H^+ dissociated per 10^5 gm. protein.
1.210	5.98	9	166
1.815	6.14	15	172
2.420	6.40	20	177
3.630	6.76	31	188
6.05	8.27	51	208
6.655	8.57	55	212
9.075	9.27	75	232

Fig. 19

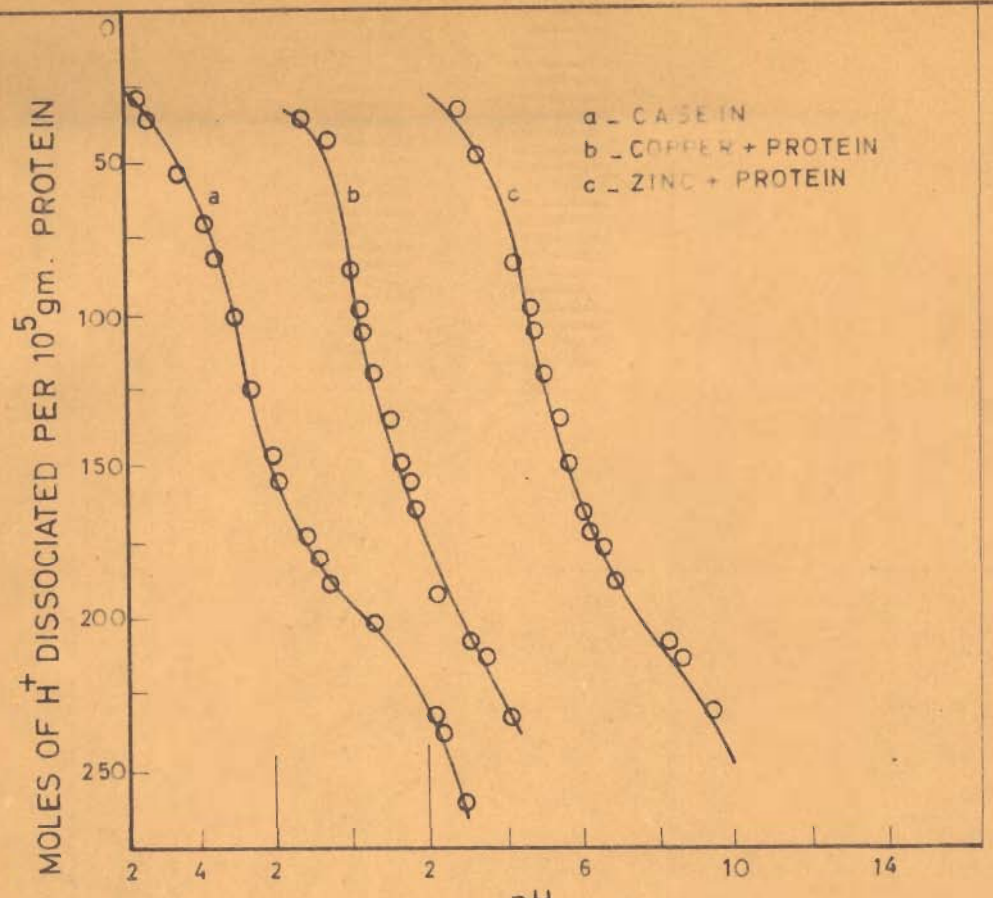


FIGURE 19

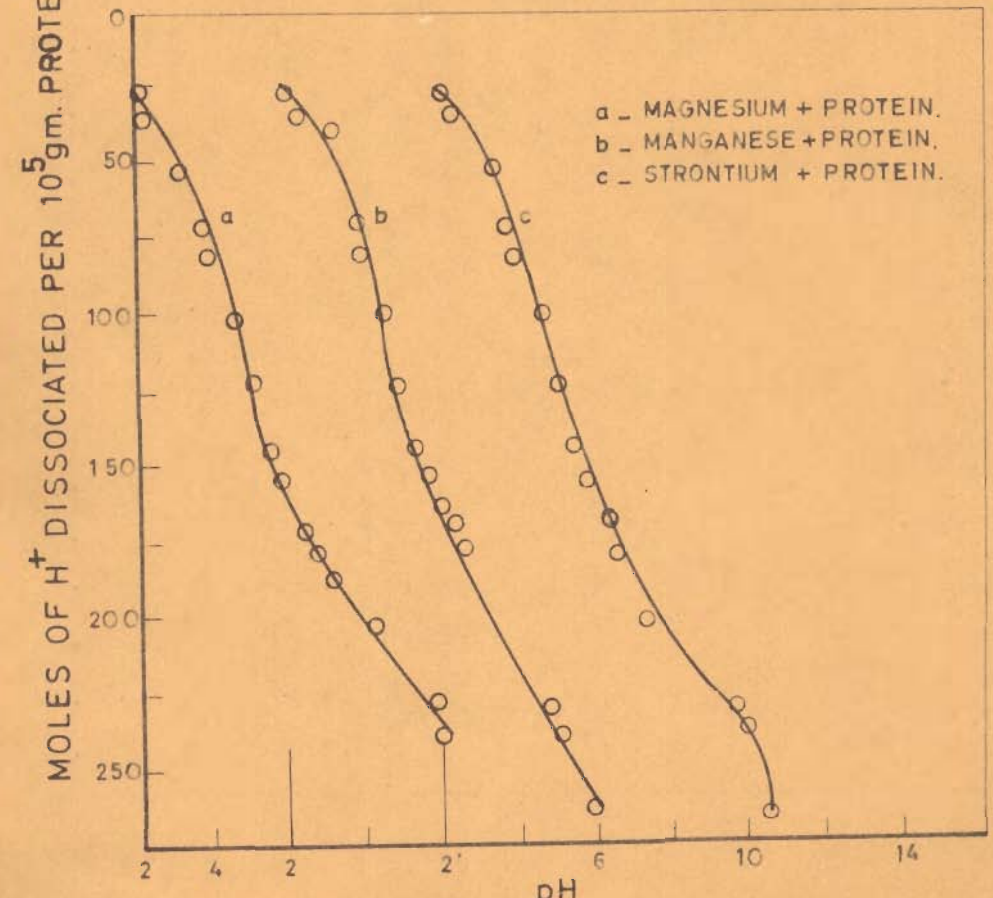


FIGURE 20

T_A_B_L_E 29

Concentration of casein solution = 0.78%

Concentration of magnesium chloride solution. = M/500

Ionic strength = 0.15 Temperature 30°C

H^+added Moles/L.x10^{-3}	pH	Bound H^+ Moles/10^5 gm. protein.	Moles of H^+ dissociated per 10^5 gms. protein.
17.22	2.28	129	28
13.776	2.46	122	35
8.61	3.43	104	53
6.888	3.98	86	71
6.027	4.12	76	81
4.305	4.82	55	102
2.583	5.26	33	124
0.861	5.74	11	146
0.172	6.03	2	155
-	6.08	-	157
$(OH)^-$added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5gm. protein.	Moles of H^+ dissociated per 10^5 gms. protein.
1.210	6.62	15	172
1.815	6.90	23	180
2.420	7.24	31	188
3.630	8.40	46	203
6.05	10.00	73	230
6.655	10.10	81	238
9.075	10.68	104	261

Fig. 20

T_A_B_L_E 30

Concentration of casein solution = 0.78%

Concentration of manganese chloride solution. = M/500

Ionic strength =0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/10^5 gm. protein.	Moles of H^+ dissociated per 10^5 gm. protein.
17.22	2.24	129	28
13.776	2.46	122	35
8.61	3.36	104	53
6.888	3.96	86	71
6.027	3.96	76	81
4.305	4.72	56	101
2.583	5.12	33	124
2.861	5.50	11	146
0.172	5.70	2	155
-	5.80	-	157
$(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5 gm protein	Moles of H^+ dissociated per 10^5 gm. protein.
1.210	6.25	8	165
1.815	6.50	12	169
2.420	6.80	19	176
3.630	7.60	-	-
6.05	9.00	75	232
6.655	9.22	84	241
9.075	10.02	107	264

Fig. 20

T_A_B_L_E 31

Concentration of casein solution = 0.78%

Concentration of strontium nitrate = M/500

Ionic strength =0.15 Temperature 30°C

H^+ added Moles/L.x10^{-3}	pH	Bound H^+ Moles/10^5 gm protein.	Moles of H^+ dissociated/10^5 gm protein.
17.22	2.26	129	28
13.776	2.50	122	35
8.61	3.62	104	53
6.888	3.86	84	73
6.027	4.04	74	83
4.305	4.82	55	102
2.583	5.20	32	125
0.861	5.62	11	146
0.172	5.90	2	155
-	5.96	-	157
$(OH)^-$ added Moles/L.x10^{-3}	pH	Bound $(OH)^-$ Moles/10^5 gm protein	Moles of H^+ dissociated per 10^5gm protein.
1.210	6.50	15	172
1.815	6.72	23	180
2.420	6.94	30	187
3.630	7.50	45	202
6.05	9.80	75	232
6.655	10.10	81	238
9.075	10.60	109	266

Fig. 20

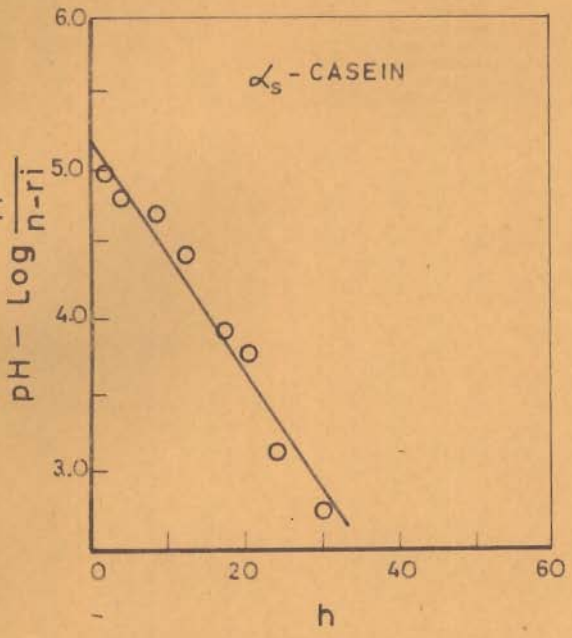


FIGURE 21

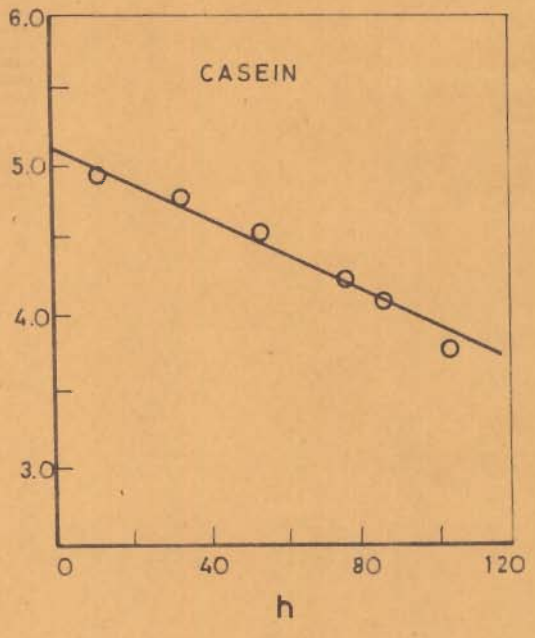


FIGURE 22

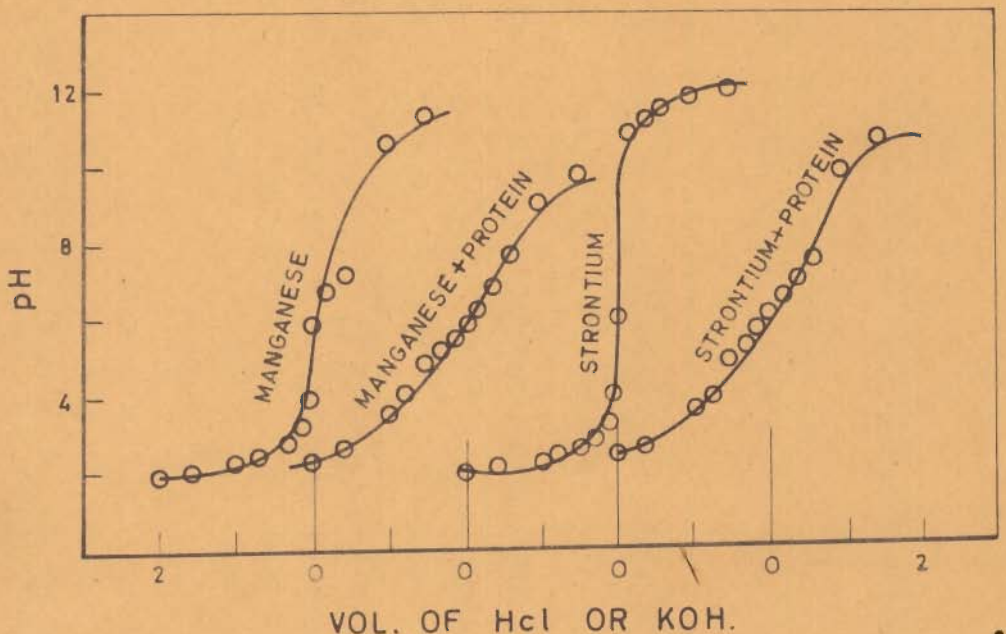


FIGURE 18

T_A_B_L_E 32

Copper- casein system.

Concentration of casein = 1.194 %

Concentration of cupric chloride = 2.0×10^{-3} M

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M
3.0	43	40	3
4.0	90	68	22
5.0	144	120	24
5.50	163	138	25
6.35	190	165	25
7.0	208	180	28
8.0	226	196	30

T_A_B_L_E 33

Zinc-casein system.

Concentration of casein = 1.194%

Concentration of zinc chloride = 2.0×10^{-3} M

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M
3.0	43	40	3
4.0	73	68	5
5.0	130	120	10
5.75	150	138	12
6.35	178	165	13
7.0	194	180	14
8.0	212	196	16
9.30	232	215	17

T_A_B_L_E 34

Magnesium -casein system.

Concentration of casein = 0.78%

Concentration of magnesium chloride = M/500

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M
3.0	42	40	2
4.0	73	68	5
5.0	125	120	5
5.50	145	138	7
6.35	171	165	6
7.0	186	180	6
8.0	203	196	7
9.30	223	215	8

T_A_B_L_E_35

Manganese - casein system

Concentration of casein = 0.78%

Concentration of manganese chloride= 2.0×10^{-3} M

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M
3.0	42	40	2
4.0	71	68	3
5.0	125	120	5
5.50	145	138	7
6.35	172	165	7
7.0	188	180	8
8.0	204	196	8
9.30	-	215	-

T_A_B_L_E 36

Strontium -casein system.

Concentration of casein = 0.78%

Concentration of strontium nitrate = 2.0×10^{-3} M

pH	H^+ dissociated in presence of metal ions.	H^+ dissociated in absence of metal ions.	V_M
3.0	42	40	2
4.0	70	68	2
5.0	123	120	3
5.50	142	138	4
6.35	170	165	5
7.0	187	180	7
8.0	203	196	7
9.00	224	215	9

T_A_B_L_E 37

Binding data calculated from titration curves for α_s-casein.

pH	H^+ dissociated in presence of metal ion.	H^+ dissociated in absence of metal ion.	V_M	Free metal at equilibrium x 10^{-3}M	log K
		Cu^{2+}- α_s-casein system			
5.50	48	43	5	0.445	2.272
7.0	62	56	6	0.134	3.572
7.5	65	59	6	0.134	3.572
8.0	67	61	6	0.134	-
		Zn^{2+}-$\alpha_s$casein system			
5.50	47	43	4	0.756	1.930
7.0	61	56	5	0.445	3.056
7.5	64	59	5	0.445	3.056
8.0	66	61	5	0.445	-
		Mg^{2+}- α_s- casein system			
5.50	46	43	3	1.067	1.655
7.0	58	56	2	1.378	-
7.5	61	59	2	1.378	-
8.0	64	61	3	1.067	-
		Mn^{2+}- α_s-casein system			
5.50	46	43	3	1.067	1.655
7.0	59	56	3	1.067	-
7.5	63	59	4	0.756	2.820
8.0	65	61	4	0.756	-
		Sr^{2+}- α_s-casein system			
5.50	45	43	2	1.378	1.328
7.0	58	56	2	1.378	-
7.5	62	59	3	1.067	2.670
8.0	64	61	3	1.067	-

T_A_B_L_E 38.

Binding data calculated for casein on the basis of 10^5 gm. of protein.

pH	H^+ dissociated in presence of metal ions.	H^+ dissociation in absence of metal ions.	V_M
		Cu^{2+} casein system	
5.50	163	138	25
6.35	190	165	25
7.5	218	190	28
8.0	226	196	30
		Mg^{2+} casein system	
5.50	145	138	7
6.35	171	165	6
7.50	196	190	6
8.0	203	196	7
		Zn^{2+} casein system	
5.50	150	138	12
6.35	178	165	13
7.50	205	190	15
8.0	212	196	16
		Mn^{2+} casein system	
5.50	145	138	7
6.35	172	165	7
7.50	198	190	8
8.0	204	196	8
		Sr^{2+} casein system	
5.50	142	138	4
6.35	170	165	5
7.50	196	190	6
8.0	203	196	7

T_A_B_L_E 39

Intrinsic association constants of combination.

Ligand	Method	log K(carboxyl groups)					log K(imidazole groups)				
		Cu^{2+}	Zn^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}	Cu^{2+}	Zn^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}
T.Gelatin	pH metric	2.10[a]	1.87[a]	1.812[b]	1.485[b]	1.334[b]	3.28[a]	2.74[a]	3.194[b]	3.119[b]	2.77[b]
T.Gelatin	Equilibrium dialysis.	2.18	1.87	1.846	-	-	3.40	2.91	3.363	-	-
α_s-Casein	pH-metric	2.272	1.936	1.655	1.655	1.328	3.57	3.056	-	2.82	2.67
α_s-Casein	Polarography[g]	2.23	-	-	-	-	3.25	-	-	-	-
Acetate		2.16[f]	1.03[f]	1.05[d]	-	0.97[c]	-	-	-	-	-
Imidazole		-	-	-	-	-	4.36[f]	2.58[f]	-	4.23[e]	-

a. Reference No. 268
b. Reference to chapter 1.
c. Reference No. 247.
d. Reference No. 246
e. Reference No. 248
f. Reference No. 141
g. Reference to chapter 4.

D_I_S_C_U_S_S_I_O_N

The general principles on which the interpretation of experimental data obtained by pH measurements of metal-protein mixtures is based, have been fully discussed in Chapter 1. These may be summarised as:

1. The hydrogen ion equilibria of metal protein system is bound to shift towards the basic side of the functional groups (in comparision with the hydrogen ion equilibria of protein alone) if a reversible chemical binding occurs between the metal ions and the active sites of protein. This shift indicates a competitive phenomena in the interaction process and also gives a qualitative test of the metal ion binding to the protein.

2. If a η assumption be made as suggested by Gurd and Murray(64) and Malik and Co-workers(75,83,92,93,259,268-272) of one-to-one binding in most of the cases,then the number of protons displaced during the interaction process gives directly the number of active sites covered by the metal ions.

3. Considering the electro-chemical nature of protein and the intrinsic pK-values of proton binding,studies under a specific pH range providenecessary information regarding the nature of the active sites involved in the interaction process.

The successful application of these assumptions to the transfusion gelatin-metal systems have given invaluable informations,and helped considerably in the calculation of

It is evident that the potentiometric titration method yeilds data of interest in case of metal complexes of α_s-casein also. The results have been summarised in tables (9-22).

The titration curves of copper,zinc,megnesium, mangnese and strontium-α_s-casein systems differ from that of α_s-casein alone. In the vicinity of pH 5.5 the titration curves of these systems are generally shifted towards the basic side of the functional groups and a limited number of protons (i.e.,5,4,3,3 and 2 for copper,zinc,magnesium, mangnese and strontium respectively) are displaced. These values are equal to V_M in each case and intrinsic association constants have been calculated applying Scatchard's equation(258),

$$K = \frac{V_M}{(n - V_M - V_H)C_F}$$

where V_M and V_H are the active sites covered by the metal and hydrogen ions respectively,n is the total number of such sites and C_F is the metal concentration at equilibrium. Since accurate values of V_H are not available in the literature,separate experiments were performed to evaluate it. The α_s-casein fraction was isolated following the method of D.F.Waugh and the molecular weight is taken to be 27,300. The hydrogen ion titration curve of α_s-casein closely resemble the hydrogen ion titration curve of α_s-casein of Waugh(Fig.14). From this curve the intrinsic pK value of carboxyl as

well as imidazole groups were calculated as 5.1 and 7.0 respectively(Fig.21,22). These values closely resemble with the pK values calculated for these groups by Waugh. The number of each type of active sites carboxyl,imidazole, amino etc. as calculated by Waugh are as follows:

Charged groups in α_s-casein

Groups	Waugh et al		Hipp et al amino acid analysis.
	Titration	Amino acid analysis.	
α-Carboxyl		3	
Side chain carboxyl	40 ± 3	46 ± 3	33
Phosphate		20	18
Imidazole	5	6	5
α-NH_2(ϵ-amino)		1	
Phenolic	28 ± 2	12	11
Side chain NH_2		18	16
Guanidyl	10 ± 2	9	6
Total anionic	63 ± 3	69	
Total cationic	34 ± 2	34	

The values of V_H at a desirable pH are taken from the hydrogen ion titration curve(Fig.14). These values and those of V_M for each metal ion have been inserted in Scatchard's equation and log K value calculated are given

in table 37.

Since the protons are released in the vicinity of pH 5.0 by the addition of metal ions to the titration mixture it is concluded that all the metal under investigation combine with the carboxyl groups.

The interesting feature of the present studies is that these intrinsic association constant are nearly equal to the logarithm of the first association constant of each metal and acetate nucleus and these values also closely resemble with the log K values obtained by other workers in case of transfusion gelatin by different methods (table 39). It is,therefore, quite evident that the stoichiometry of the reaction is 1:1 in all the cases. The order of binding capacity as indicated by the log K values is as follows:

$$Cu^{2+} > Zn^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+}$$

In the neutral pH region except magnesium all the metals show combination with the imidazole groups replacing hydrogen ions. The metals copper,zinc,manganese and strontium show a decreasing tendency to bind themselves with the imidazole groups. The relevant intrinsic association constants are given in table 37. A comparision of these data show that manganese yeilds very low log K values for imidazole groups interaction,in comparision with the log K values of manganese and free imidazole nucleus. The other metal ions give values which compare favourably well

with log K values of corresponding metal-free imidazole interaction. It is,therefore,concluded that probably the affinity of manganese towards imidazole groups has been reduced considerably due to the competition of carboxyl and imidazole groups for manganese. Since log K values have been calculated by means of Scatchard's equation,and this equation does not account for such competitive effects. Therefore application of Scatchard's equation yeilds log K values which are not comparable with the log K values of free imidazole groups interaction in most of the cases and specially in case of manganese.

Similar studies are undertaken with whole casein also. Here the binding data obtained by pH measurements (Table 38) are not of much quantitative value but they still provide some qualitative informations which strongly support the assumption derived on the basis of quantitative studies on metal - α_s -casein systems. These studies remain of qualitative nature since the molecular weight of whole casein is not known with certainty and also the mode of combination of different fractions of casein with each other is not fully known. Keeping in view these limitations the binding data has been calculated per 10^5 gms.of casein. But as far as qualitative informations are concerned, these studies fully support the conclusions derived on the binding of these metals with α_s - casein.

From the data summarised in table 38 it is evident

that copper has got the maximum binding with the carboxyl groups of casein followed by zinc,magnesium,manganese and strontium. The affinity of magnesium and manganese seems to be equal,as they both liberate equal number of protons. This fact is in complete agreement with results derived on the binding of these metal ions with α_s-casein. The order of their binding tendency with carboxyl groups is copper > zinc > magnesium > manganese > strontium which is exactly similar to the order obtained with α_s -casein.

In the neutral pH range, pH 7.50,the imidazole groups are available for the binding. The maximum number of groups available for binding is 20. At this pH all the metals liberate limited number of protons,showing their binding with the imidazole groups except magnesium. The behaviour of the magnesium with casein is similar as obtained in the case of α_s-casein. The number of metal ions bound to the imidazole groups are 3,3,1 and 2 in case of copper,zinc,manganese and strontium respectively. These data also support the fact that copper and zinc have strong affinity for the binding with the imidazole groups.

It is further interesting that these results are in accordance with the results of α_s-casein. Magnesium shows no binding with the imidazole groups of both casein and α_s-casein. However, these data are different from the results obtained on the binding of magnesium with

transfusion gelatin studied by the pH-metric method (chapter 1) and equilibrium dialysis technique (chapter II) . In case of transfusion gelatin,magnesium shows binding with the imidazole groups.

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C_H_A_P_T_E_R IV

Polarographic studies on the interaction of copper with α_s-casein.

I_N_T_R_O_D_U_C_T_I_O_N

Since its invention in 1922 the polarography(269) has found varied applications to almost all branches of chemistry including biology and medicine. This technique has scintillated as an invaluable tool for research and industry. In addition to the inorganic reactions,it has been successfully used in reaction involving organic compounds,and products of biological importance. A review of the present literature on amino acids, polypeptides, native and denatured proteins stands testimony to the accuracy of the polarographic estimations in various fields. The method even claims its utility in the diagonisis of diseases,especially in cancer(270).

Besides the analytical importance of polarography, the technique has been recognised for its application to abstract problems of inorganic and electro-chemistry such as to workout the structural problems,especially in the chemistry of coordination compounds and also chemical kinetics. The technique has been successfully employed for the determination of stability as well as the free energy of such compounds. Recent work(271-274) on the metal complexes of simple organic substances,amino acids and peptides has established the superiority of the method over other prevalent physico-chemical methods.

The proteins exert a two-fold influence on the reduction waves of metal ions,namely the suppression of

the polarographic maxima and marked reduction in the diffusion current. The latter effect has been ascribed to a number of factors,viz., adsorption of proteins on mercury drops (76,275,276), increase in viscosity of the medium(277) and complex formation between metal and protein. According to Zuman (275) and also Tanford (76), the decrease in diffusion current is primarily due to complex formation,provided the investigations are carried out in the higher pH range where the possibility of adsorption of the negatively charged protein molecule on mercury drop is very remote. Scatchard (258) gave the following relationship for metal-protein interaction:

$$Ke^{-2ZM^{Z}P} \quad W = \frac{V_M}{(n - V_M - V_H)\, C_F}$$

where V_M and V_H are the number of metal and hydrogen ions bound per protein molecule, n is the total number of reactive sites, C_F is the free metal concentration and K is the intrinzic association constant. If the viscosity factor is not involved ,V_M when computed polarographically (considering the depression of diffusion current) would be independent of protein concentration; a test which has proved to be valid in a number of cases.

On the basis of the above reasoning Tanford(76-79), for the first time, successfully applied the polarographic

method to investigate the nature of the metal-protein interaction. The salient feature of these investigations are: (i) for a given metal and protein concentration, the ratio $(id)/(id)_0$, where (id) and $(id)_0$ are the diffusion current of metal ion in presence and absence of protein, should decrease with increase in pH and attain a limiting value;(ii) for a given metal concentration a ratio $(id)/(id)_0$ should decrease with increasing amount of protein and attain a limiting value not zero (277) and (iii) the ratio should decrease with increasing metal ion. The validity of Tanford's method(which mainly deals with the interaction of heavy metal ions with serum albumin and insulin) is found in the work of Rao and Lal (80) (zinc and cadmium complexes of bovine serum albumin in buffer media) and Saroff and Mark(81) (zinc and mercury-serum albumin complexes). The complex formation was accompanied by a detectable shift in half wave potential and the binding data calculated from diffusion current measurements were agreed well with equilibrium dialysis studies.

Recently Malik and Co-workers (82-84) have made some advances in this field of study,using a collagen type of protein like transfusion gelatin. They obtained some very interesting results and were able to show the binding of zinc to amino groups of transfusion gelatin

employing polarographic technique. The study of lead indicate that the lead ions are bound only with the carboxyl groups of transfusion gelatin.

The polarographic investigations carried out by Malik and Co-workers have been extended to the phosphoproteins. The binding of the metals with protein as yet could not be studied quantitatively due to the uncertainty about its composition. However, recent work of Waugh on its fractionation and molecular weight determination has provided some useful data which can be applied for the study of the reaction. In this chapter the results of the binding of cupric chloride α_s-casein by polarographic technique are described.

E_X_P_E_R_I_M_E_N_T_A_L

α_s-casein used during these investigations was prepared by the method described in chapter III. Its concentration was found to be 6.5%.

Cupric chloride (A.R.) was dissolved in doubly distilled water and its strength was checked gravimetrically.

Walpole acetate and ammonia chloride-ammonia buffers were prepared by mixing sodium acetate and acetic acid (each 0.2M) and ammonia chloride and ammonia (each 1.0 M) respectively.

A.R.sample of potassium chloride was employed to prepare 1.0 M solution. This solution was used to maintain constant ionic strength (0.15).

Apparatus. The polarograph used was Toshniwal(India) make in conjunction with a Pye Galvanometer in the external circuit. An H-shaped polarographic cell was designed as recommended by Tanford to hold 2 to 3 mls. of solution. It was found to be suitable for deareation of protein solution without denaturation and subsequent measurement at dropping mercury electrode. An inert atmosphere was ensured by passing hydrogen (obtained after bubbling through alkaline pyrogallol,chromous chloride,dilute sulphuric acid and water) for about 15 to 20 minutes for each run.Triply distilled mercury was used for the dropping electrode. The diffusion current was measured for each

increament of potential keeping the cell in a constant temperature bath, $30 \pm 0.1^{\circ}C$. The capillary used had a flow rate of m = 5.54 mgm/sec. as determined by Lingane method with a drop time of 3.5 seconds and $m^{2/3} t^{1/6} = 3.88$. Since the capillary characteristics (m , t) exert a pronounced effect on diffusion current according to Ilkovic equation, these factors were, therefore, controlled carefully throughout these investigations.

The pH of the buffers and mixtures of metal and protein solutions were measured on a Backman Model H pH-meter using a general perpose glass electrode.

The results are summarised in the following tables.

T_A_B_L_E 1

Concentration of cupric chloride = 1.0×10^{-3} M

Concentration of α_s-casein = 0.12×10^{-3} M

Total volume = 10 mls. Total ionic strength = 0.15

Acetate buffer Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential	pH 5.57		pH 7.00	
	$(id)_0$	$(id)_0$	$(id)_0$	$(id)_0$
0.0	3.0	5.0	5.0	4.5
0.02	3.5	5.5	6.0	5.0
0.05	5.0	6.0	8.0	5.5
0.08	18.0	7.5	11.5	7.0
0.10	21.5	8.5	15.0	7.5
0.12	23.0	10.0	19.0	8.0
0.15	25.5	12.0	22.5	9.5
0.18	27.5	13.0	25.5	11.5
0.20	28.5	14.0	27.5	12.5
0.25	28.5	16.0	29.5	15.0
0.30	28.5	17.0	29.5	15.0
0.35	28.5	17.5	30.0	16.0
0.40	28.5	17.5	30.0	16.0
0.45	28.0	17.5	30.0	16.0
0.50	28.0	17.5	30.0	16.5
0.60	28.0	17.5	30.2	16.5

T_A_B_L_E 2

Concentration of cupric chloride $= 1.0 \times 10^{-3}$ M

Concentration of α_s-casein $= 0.12 \times 10^{-3}$ M

Total volume = 10 mls. Ionic strength = 0.15

Acetate buffer Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (volts).	pH 7.90 (id)	pH 8.50 (id)
0.0	2.50	7.00
0.02	5.0	8.5
0.05	9.0	9.5
0.08	11.0	10.5
0.10	12.0	10.5
0.12	12.5	10.5
0.15	13.0	11.5
0.18	13.0	12.0
0.20	13.0	13.0
0.25	15.0	17.0
0.30	18.5	18.5
0.35	21.5	19.5
0.40	22.5	20.0
0.45	23.5	20.5
0.50	23.5	20.5
0.60	23.5	20.5

Fig. 1 and 2

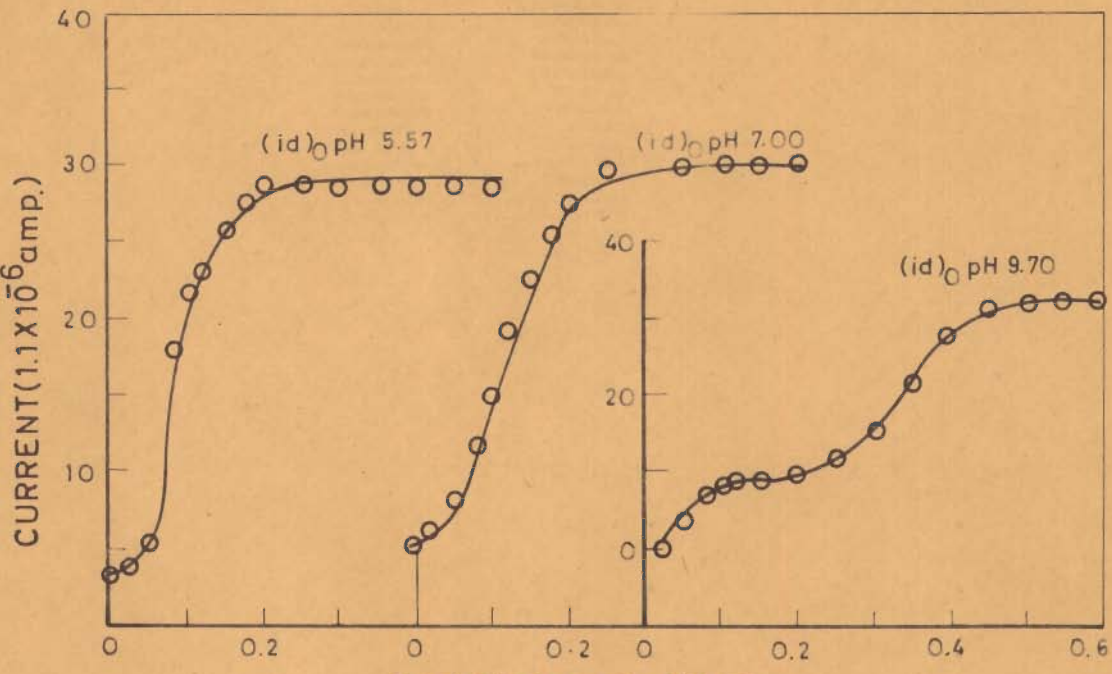

FIGURE 1

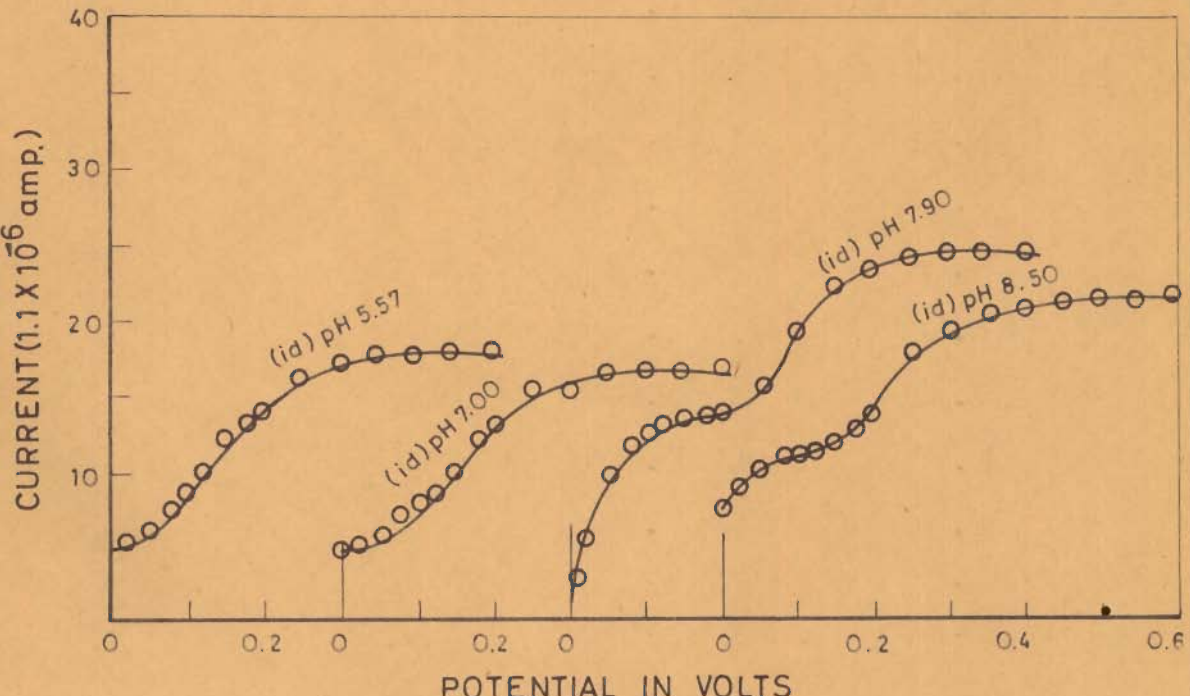

FIGURE 2

T_A_B_L_E_ 3

Concentration of cupric chloride = 1.0×10^{-3} M

Concentration of α_s-casein = 0.12×10^{-3} M

Total volume = 10 mls. Ionic strength = 0.15

Ammonium chloride -ammonia buffer. Temperature = 30^{o} C

Current 1.1×10^{-6} Amp.

Applied potential (volts)	pH 9.20		pH 9.70	pH 10.20
	$(id)_o$	(id)	(id)	(id)
0.0	-10.0	-11.0	-27.0	-30.0
0.02	-1.0	- 2.0	- 9.5	-14.0
0.05	+3.0	+ 1.5	- 1.0	- 7.5
0.08	7.5	5.0	+ 2.0	- 3.5
0.10	8.0	7.0	3.5	- 1.5
0.12	8.5	8.5	5.5	+ 1.0
0.15	8.5	10.5	8.0	3.0
0.18	8.5	11.5	9.5	4.5
0.20	9.0	11.5	10.5	5.5
0.25	11.0	11.5	11.0	5.5
0.30	15.0	12.5	11.5	6.5
0.35	21.5	15.0	13.5	9.5
0.40	27.5	19.0	16.0	10.0
0.45	31.0	19.5	16.5	10.5
0.50	32.0	19.5	16.5	10.5
0.60	32.0	19.5	16.5	10.5

Fig. 3

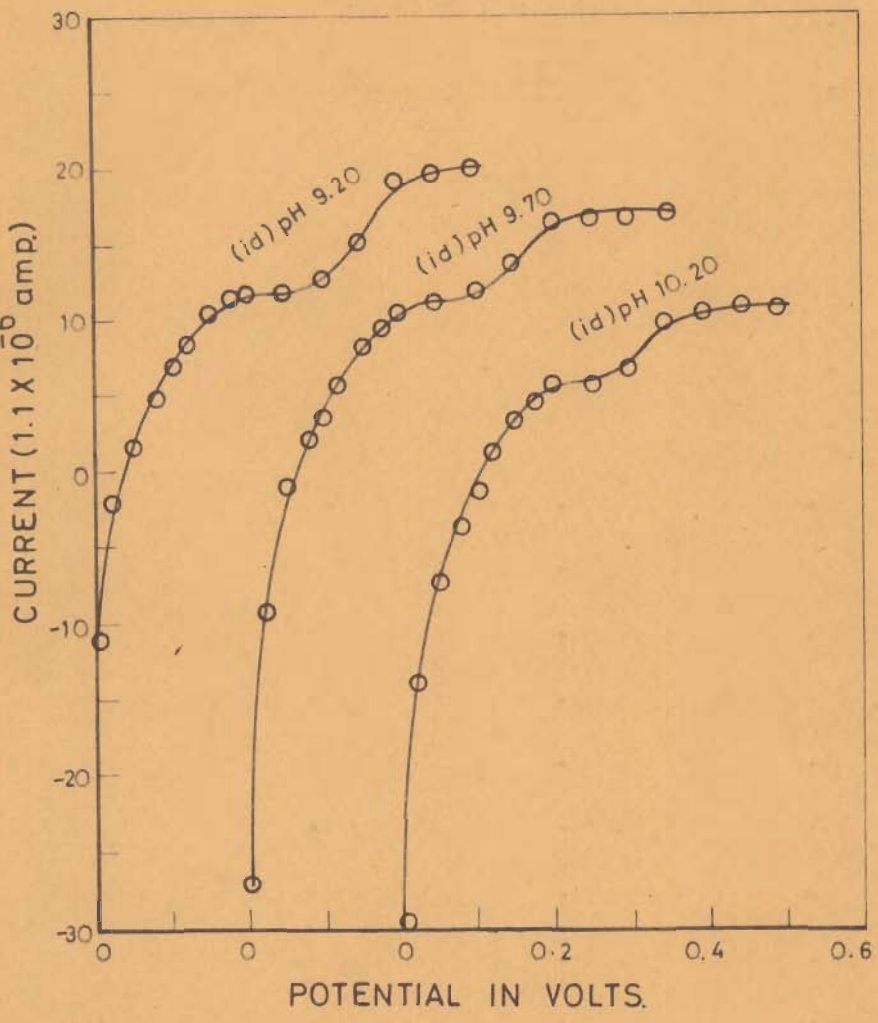
30
20
10
0
-10
-20
-30
CURRENT (1.1 X 10⁻⁶ amp.)
(id) pH 9.20
(id) pH 9.70
(id) pH 10.20
0
0
0
0.2
0.4
0.6
POTENTIAL IN VOLTS.

FIGURE 3

T_A_B_L_E 4

Concentration of protein varied.

Concentration of cupric chloride = 1.0×10^{-3} M

pH of acetate buffer = 5.57

Total volume = 10 mls. Ionic strength = 0.15

Temperature $30^{\circ}C$

Current 1.1×10^{-6} Amp.

Applied potential volts.	(id) 0.0%	(id) 0.65%
0.0	12.0	5.5
0.02	12.5	6.0
0.05	14.0	10.0
0.08	18.0	17.0
0.10	21.5	18.5
0.12	23.0	22.0
0.15	25.5	25.0
0.18	27.5	26.5
0.20	28.5	27.0
0.25	28.5	27.5
0.30	28.5	28.0
0.35	28.5	29.0
0.40	28.5	29.0
0.45	28.0	29.0
0.50	28.0	29.0
0.60	28.0	29.0

Fig. 4.

T_A_B_L_E_ 5

Concentration cupric chloride = 1.0×10^{-3} M

pH of acetate buffer = 5.57

Total volume = 10 mls. Ionic strength = 0.15

Temperature $30^{\circ}C$ Current 1.1×10^{-6} Amp.

Applied potential (volts).	0.0975%	0.1625%	0.325%
0.0	6.0	3.5	5.0
0.02	6.5	4.0	15.5
0.05	9.5	5.0	6.0
0.08	15.0	10.5	7.5
0.10	17.0	12.5	8.5
0.12	19.0	14.0	10.0
0.15	21.5	16.0	12.0
0.18	23.5	17.0	13.0
0.20	24.0	18.0	14.0
0.25	25.0	19.5	17.0
0.30	25.5	19.5	17.5
0.35	26.5	20.5	17.5
0.40	26.5	20.5	18.5
0.45	26.5	20.5	18.5
0.50	26.5	20.5	18.5

Fig. 4

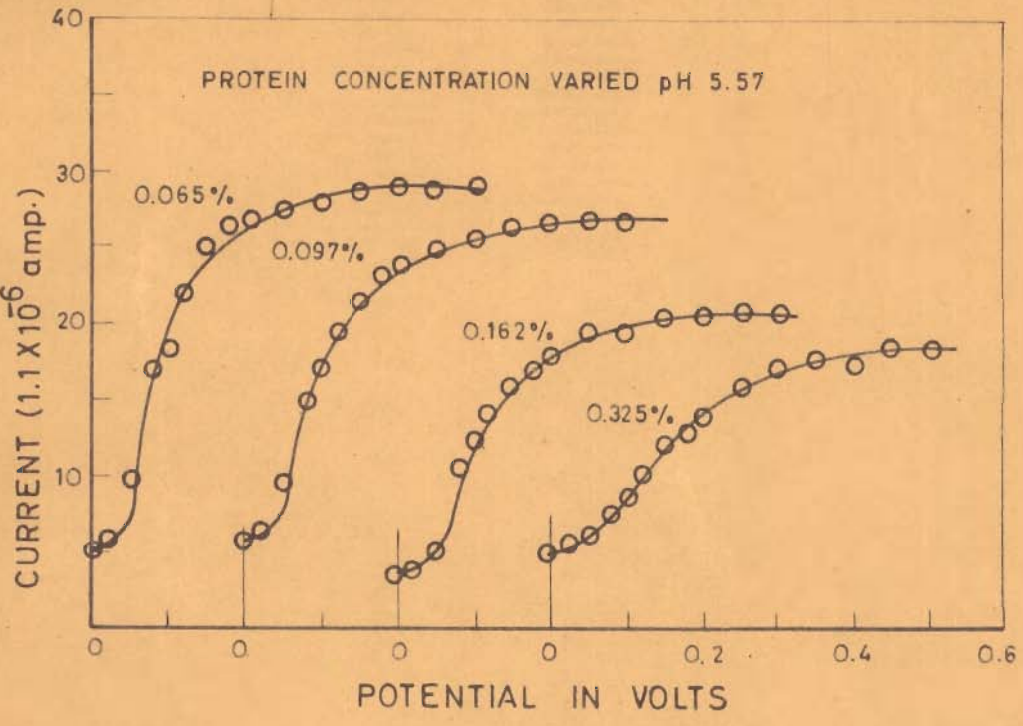

FIGURE 4

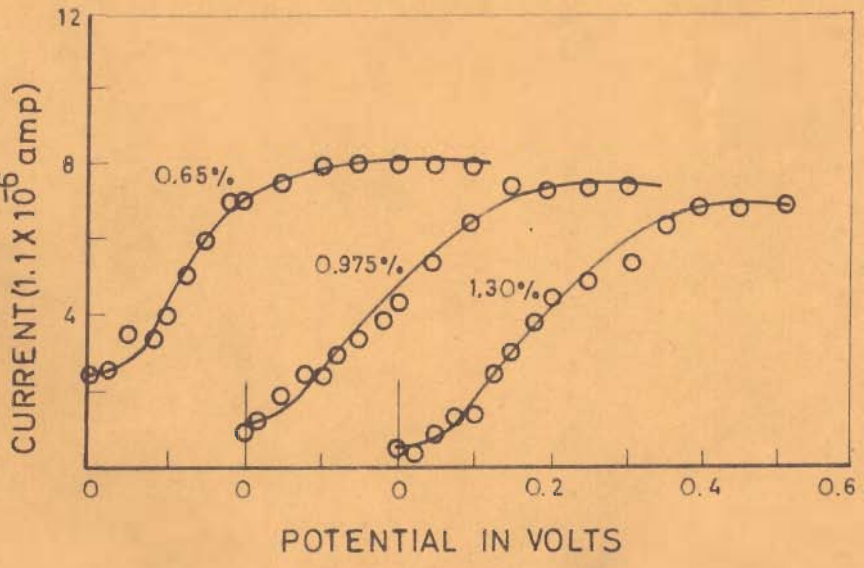

FIGURE 5

T_A_B_L_E 6

Concentration of cupric chloride = 1.0×10^{-3}M

pH of acetate buffer = 5.57

Total volume = 10 mls. Ionic strength =0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (volts)	0.65%	0.976%	1.30%
0.0	2.5	1.0	0.5
0.02	2.5	1.2	0.5
0.05	3.5	2.0	1.0
0.08	3.5	2.5	1.5
0.10	4.0	2.5	1.5
0.12	5.0	3.0	2.5
0.15	6.0	3.5	3.0
0.18	7.0	4.0	4.0
0.20	7.0	4.5	4.5
0.25	7.5	5.5	5.0
0.30	8.0	6.5	5.5
0.35	8.0	7.5	6.5
0.40	8.0	7.5	7.0
0.45	8.0	7.5	7.0
0.50	8.0	7.5	7.0

Fig. 5

T_A_B_L_E 7

Effect of Metal Concentration.

Concentration of α_s-casein $= 0.12 \times 10^{-3}$ M

pH of acetate buffer = 5.57

Total volume 10 mls. Ionic strength = 0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Potential applied (volts).	0.4×10^{-3} M Cu^{2+}		0.6×10^{-3} M Cu^{2+}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	6.0	1.5	8.5	2.5
0.02	7.0	1.5	9.0	2.5
0.05	8.5	1.5	12.0	2.5
0.08	11.0	2.5	15.0	3.5
0.10	13.5	2.5	18.0	3.5
0.12	15.0	3.0	20.5	4.0
0.15	17.0	3.5	22.5	5.0
0.18	17.5	4.5	23.0	5.5
0.20	17.5	5.0	23.0	6.0
0.25	17.5	5.5	23.0	6.5
0.30	17.5	6.0	23.0	7.0
0.35	18.0	6.0	23.0	7.5
0.40	18.0	6.0	23.0	7.5
0.45	18.0	6.0	23.0	7.5
0.50	18.0	6.5	23.0	7.5
0.60	18.0	6.5	23.0	7.5

Fig. 6

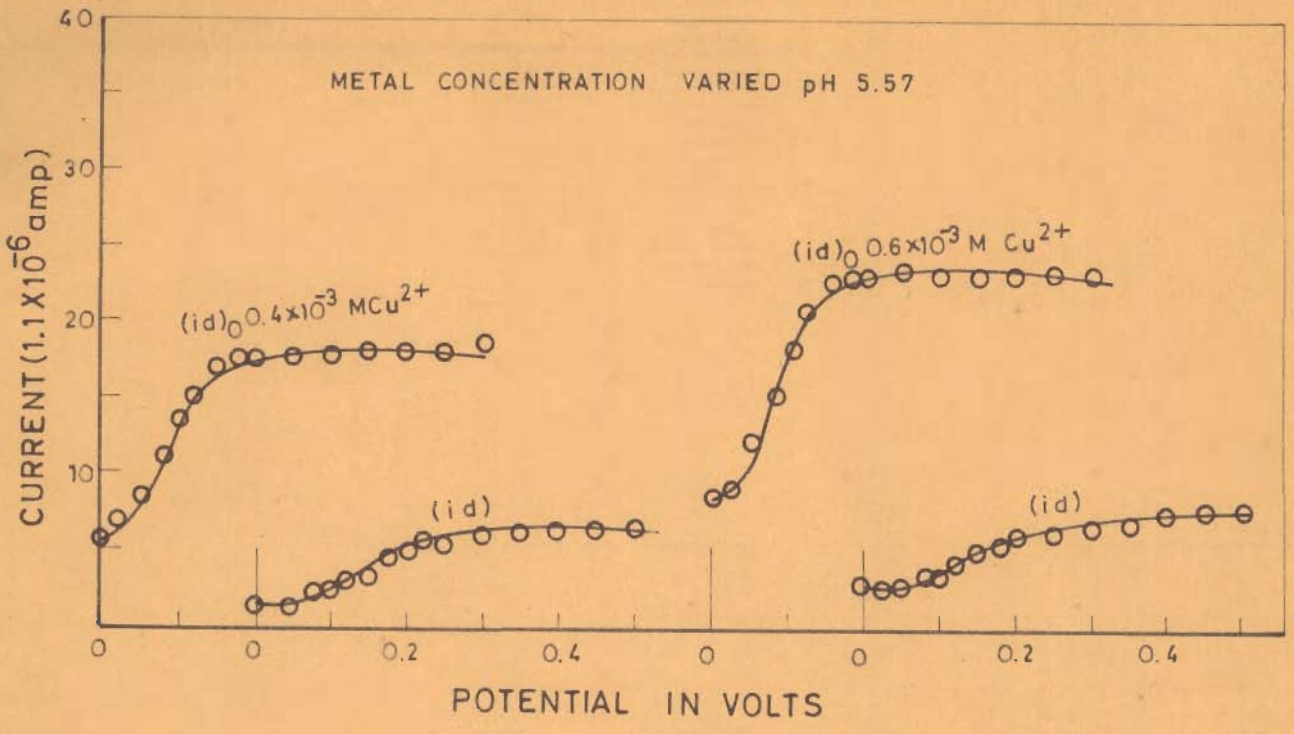

FIGURE 6

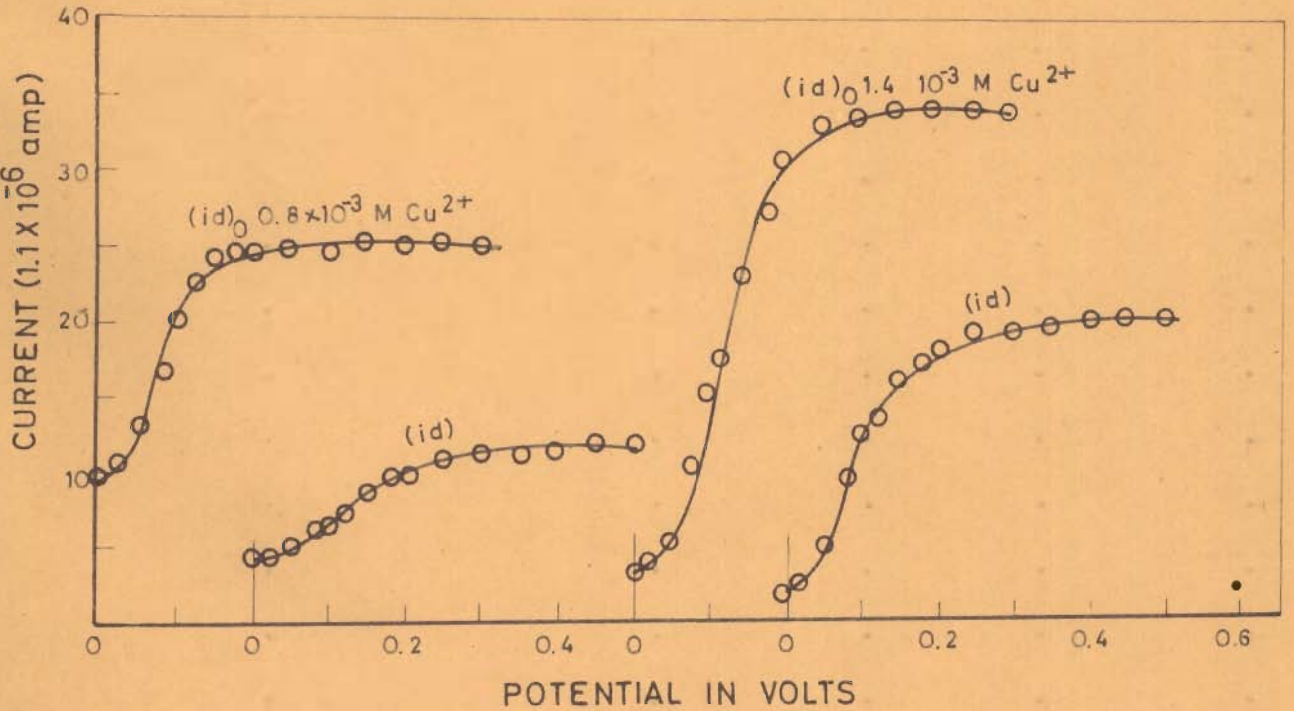

FIGURE 7

T_A_B_L_E 8

Concentration of α_s-casein = 0.12×10^{-3} M

pH of acetate buffer. = 5.57

Temperature 30°C Ionic strength =0.15

Current 1.1×10^{-6} Amp.

Applied potential (volts)	0.8×10^{-3} M Cu^{2+}		1.0×10^{-3} M Cu^{2+}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	9.5	4.0	3.0	5.0
0.02	10.5	4.0	3.5	5.5
0.05	13.0	5.0	5.0	6.0
0.08	16.5	6.0	18.0	7.5
0.10	20.0	6.0	21.5	8.5
0.12	22.5	7.0	23.0	10.0
0.15	24.0	8.5	25.5	12.0
0.18	24.5	9.5	27.5	13.0
0.20	24.5	9.5	28.5	14.0
0.25	24.5	10.5	28.5	16.0
0.30	24.5	11.0	28.5	17.0
0.35	25.0	11.0	28.5	17.5
0.40	25.0	11.5	28.5	17.5
0.45	25.0	11.5	28.5	17.5
0.50	25.0	11.5	28.0	17.5

Fig. 7.

T_A_B_L_E 9

Concentration of α_s-casein = 0.12×10^{-3}M

pH of acetate buffer = 5.57

Total volume 10 mls. Ionic strength =0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (volts)	1.4×10^{-3} M Cu^{2+}	
	$(id)_o$	(id)
0.0	3.5	2.0
0.02	4.0	2.5
0.05	5.0	5.0
0.08	10.5	9.5
0.10	15.10	12.5
0.12	17.5	13.5
0.15	23.0	16.0
0.18	27.0	17.0
0.20	30.5	18.0
0.25	32.5	19.0
0.30	33.0	19.0
0.35	33.5	19.5
0.40	33.5	20.0
0.45	33.5	20.0
0.50	33.5	20.0

Fig. 7

T_A_B_L_E 10

Effect of protein concentration of pH 7.00

Concentration of cupric chloride = 1.0×10^{-3} M

pH of ammonia buffer = 7.00

Total volume = 10 mls. Ionic strength =0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	Protein concentration		
	0.0 %	0.13 %	0.195 %
0.0	5.0	8.0	7.0
0.02	6.0	8.5	7.5
0.05	8.0	9.0	8.0
0.08	11.5	10.0	9.0
0.10	15.0	10.5	9.5
0.12	19.0	11.0	10.0
0.15	22.5	14.5	11.5
0.18	25.5	17.0	13.5
0.20	27.5	19.5	15.5
0.25	29.5	20.5	18.0
0.30	29.5	20.5	19.0
0.35	30.0	20.5	19.0
0.40	30.0	20.5	20.0
0.45	30.0	21.0	20.0
0.50	30.0	21.0	20.0

Fig. 8

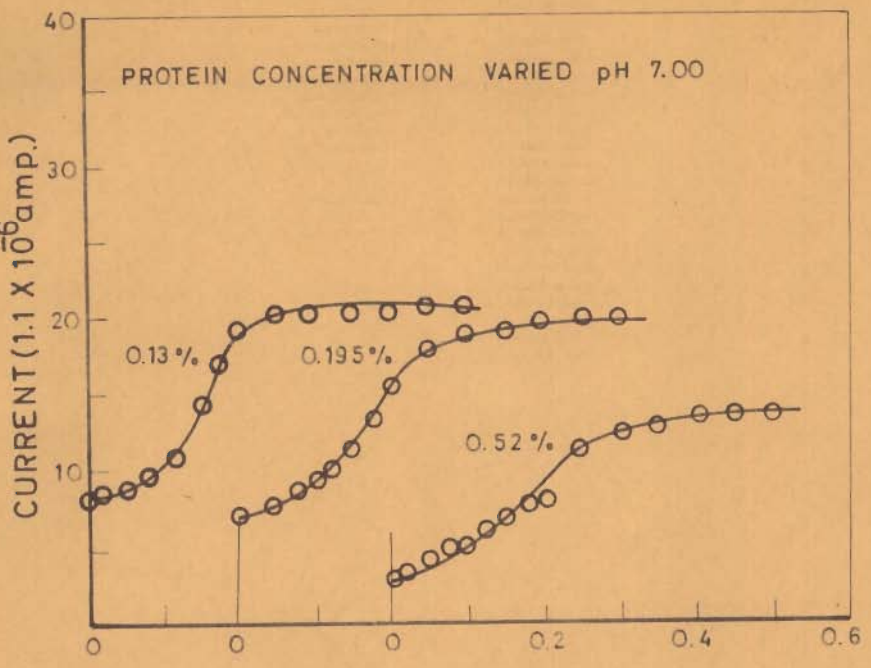

FIGURE 8

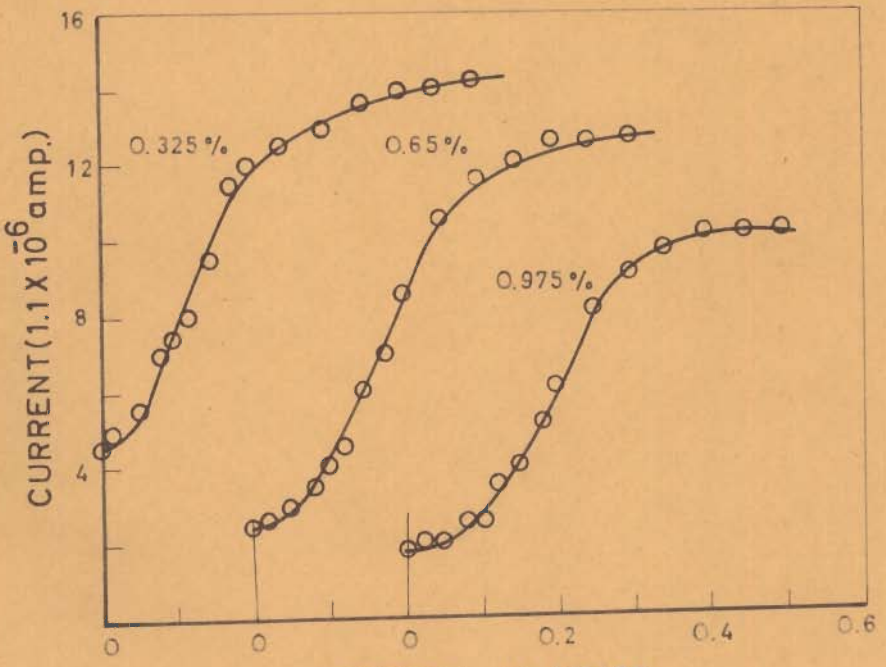

FIGURE 9

T_A_B_L_E 11

Concentration of cupric chloride = 1.0×10^{-3} M

pH of ammonia buffer = 7.00

Total volume = 10 mls. Ionic strength=0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	Protein concentration	
	0.325 %	0.520 %
0.0	4.5	3.0
0.02	5.0	3.5
0.05	5.5	4.0
0.08	7.0	4.5
0.10	7.5	5.0
0.12	8.0	6.0
0.15	9.5	7.0
0.18	11.5	8.5
0.20	12.0	8.0
0.25	12.5	11.5
0.30	13.0	12.5
0.35	13.5	13.0
0.40	14.0	13.5
0.45	14.0	13.5
0.50	14.5	13.5

Fig. 9

T_A_B_L_E 12

Concentration of cupric chloride = 1.0×10^{-3} M

pH of ammonia buffer = 7.00

Total volume = 10 mls Ionic strength = 0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	Protein concentration	
	0.65 %	0.975 %
0.0	2.5	2.0
0.02	2.5	2.0
0.05	3.0	2.0
0.08	3.5	2.5
0.10	4.0	2.5
0.12	4.5	3.5
0.15	6.0	4.0
0.18	7.0	5.0
0.20	8.5	6.0
0.25	10.6	8.0
0.30	11.5	9.0
0.35	12.0	9.5
0.40	12.5	10.5
0.45	12.5	10.0
0.50	12.5	10.0

Fig. 9

T_A_B_L_E 13

Effect of metal concentration at pH 7.00

Concentration of α_s-casein = 0.12×10^{-3} M

pH of ammonia buffer = 7.0

Total volume = 10 mls. Ionic strength = 0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	0.4×10^{-3} M Cu^{2+}		0.6×10^{-3} M Cu^{2+}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	6.0	0.0	8.5	2.0
0.02	7.0	0.5	9.0	2.0
0.05	8.5	0.5	12.0	2.5
0.08	11.0	1.0	15.0	3.0
0.10	13.5	1.5	18.0	3.5
0.12	15.0	2.5	20.5	4.0
0.15	17.0	3.0	22.5	5.0
0.18	17.5	3.5	23.0	6.0
0.20	17.5	4.0	23.0	7.0
0.25	17.5	5.0	23.0	8.5
0.30	17.5	6.5	23.0	9.5
0.35	17.5	6.5	23.0	9.5
0.40	18.0	6.5	23.50	9.5
0.45	18.0	6.5	23.0	9.5
0.50	18.0	6.5	23.5	9.5

Fig. 10

T_A_B_L_E 14

Concentration of α_s-casein = 0.12×10^{-3} M

pH of ammonia buffer = 7.00

Total volume = 10 ml. Ionic strength = 0.15

Temperature 30°C

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	0.8×10^{-3} M Cu^{2+}		1.0×10^{-3} M Cu^{2+}	
	$(id)_0$	(id)	$(id)_0$	(id)
0.0	9.5	3.5	5.0	4.5
0.02	10.5	4.0	6.0	5.0
0.05	13.0	4.5	8.0	5.5
0.08	16.5	4.5	11.5	7.0
0.18	20.0	5.0	15.0	7.5
0.12	22.5	5.5	19.0	8.0
0.15	24.0	6.0	22.5	9.5
0.18	24.5	7.5	25.5	11.5
0.20	24.5	9.0	27.5	12.5
0.25	24.5	10.5	29.5	15.0
0.30	24.5	11.5	29.5	15.5
0.35	25.0	11.5	30.0	16.0
0.40	25.0	11.5	30.0	16.0
0.45	25.0	11.5	30.0	16.5
0.50	25.0	12.0	30.0	16.5

Fig. 10 and 11

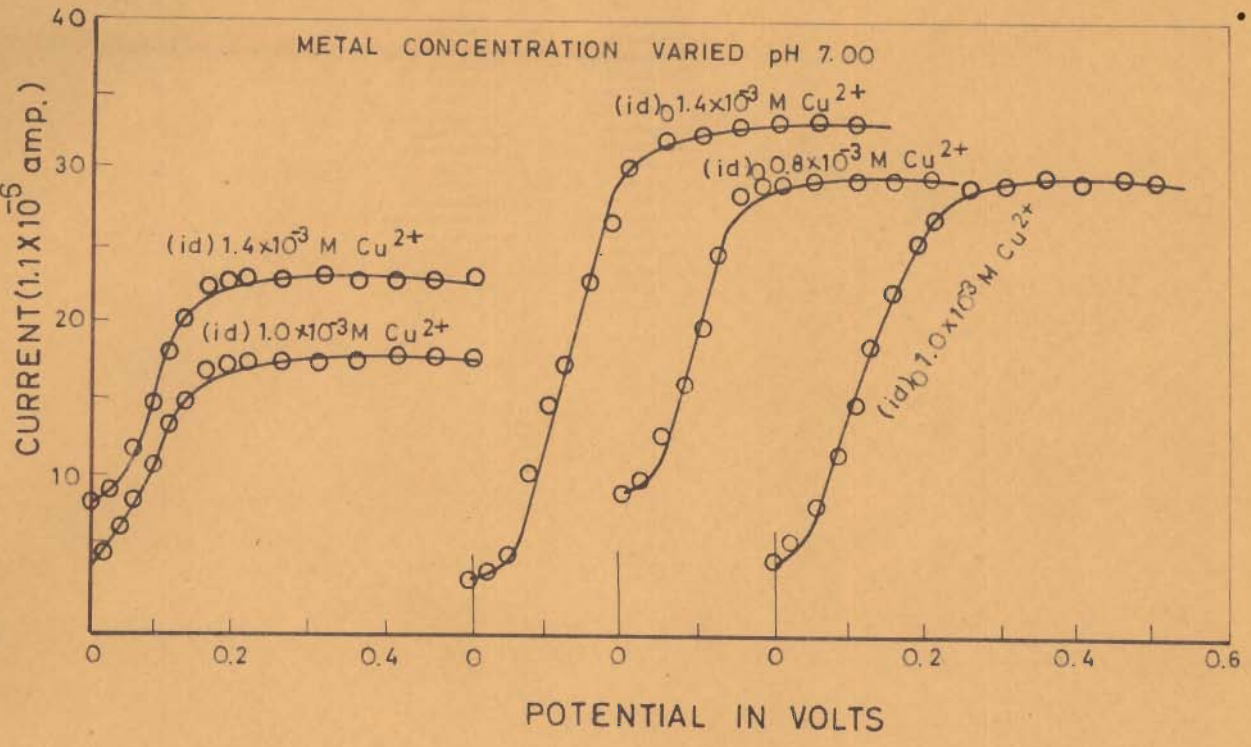

FIGURE 10

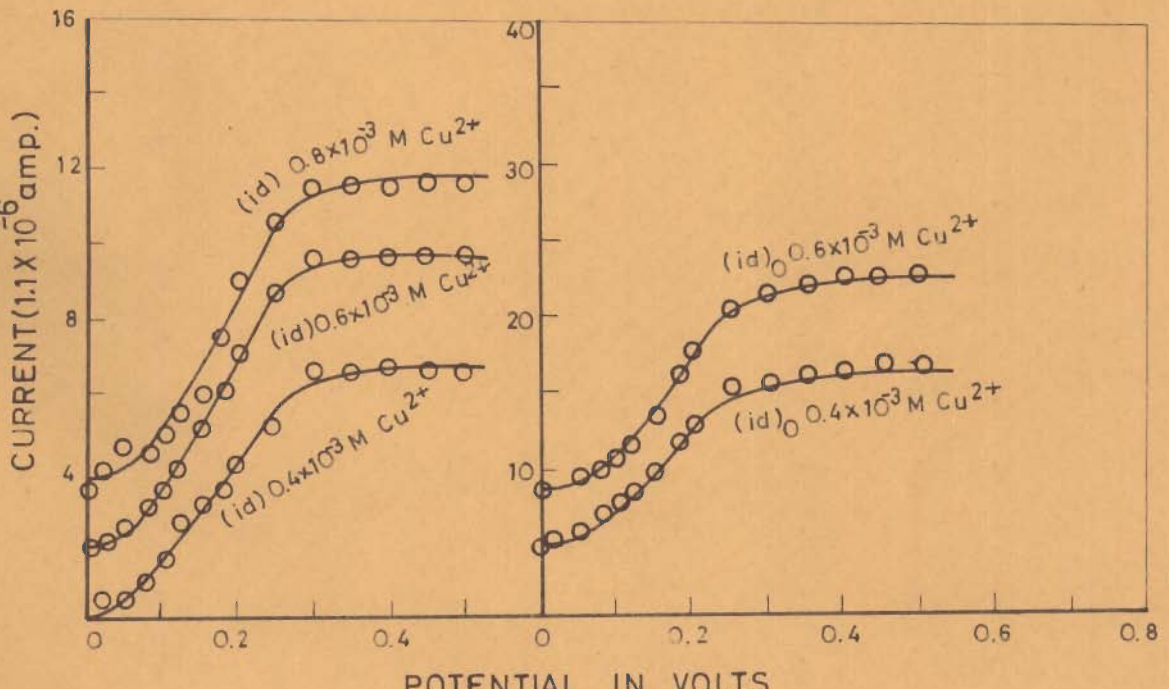

FIGURE 11

T_A_B_L_E 15

Concentration α_s-casein = 0.12×10^{-3} M

pH of ammonia buffer = 7.00

Total volume = 10 mls. Ionic strength=0.15

Temperature $30^{\circ}C$

Current 1.1×10^{-6} Amp.

Applied potential (Volts)	1.4×10^{-3} M Cu^{2+}	
	$(id)_o$	(id)
0.0	3.5	8.5
0.02	4.0	8.5
0.05	5.0	9.0
0.08	10.5	10.0
0.10	15.0	10.5
0.12	17.5	11.5
0.15	23.0	13.5
0.18	27.0	16.0
0.20	30.5	17.5
0.25	32.5	20.5
0.30	33.0	21.5
0.35	33.5	22.0
0.40	33.5	22.5
0.45	33.5	22.5
0.50	33.5	22.5

Fig. 11

T_A_B_L_E 16

Effect of pH

Concentration of cupric chloride = 1.0×10^{-3} M

Concentration of α_s-casein = 0.119×10^{-3} M

Total volume = 10 mls. Ionic strength = 0.15

Temperature 30°C

pH	$(id)_0$	(id)	$(id)/(id)_0$	Cb $\times 10^{-3}$ M	V_M
5.57	25.0	13.0	0.52	0.631	5.3
7.00	24.5	11.0	0.45	0.723	6.1
7.90	24.5	10.5	0.43	0.750	6.3
8.50	23.0	9.0	0.39	0.802	6.8
9.20	23.0	7.5	0.33	0.881	7.4
9.70	23.0	6.0	0.26	0.973	8.2
10.20	23.0	5.5	0.24	0.993	8.4

Fig.12

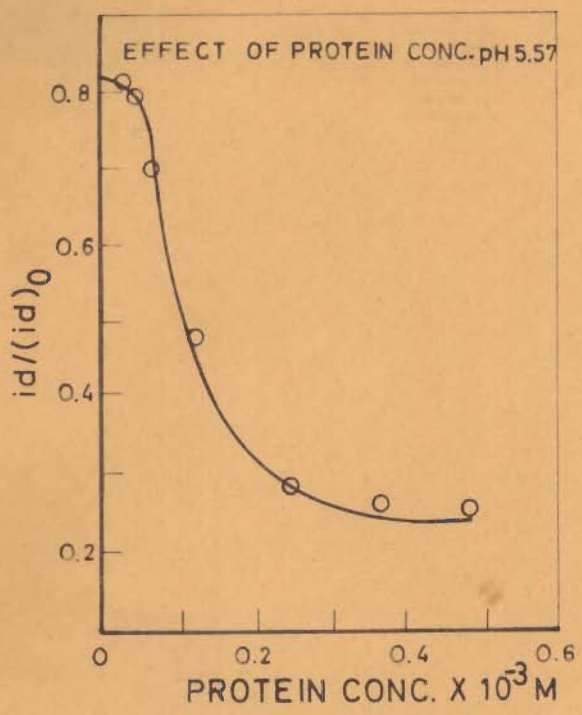

FIGURE 13

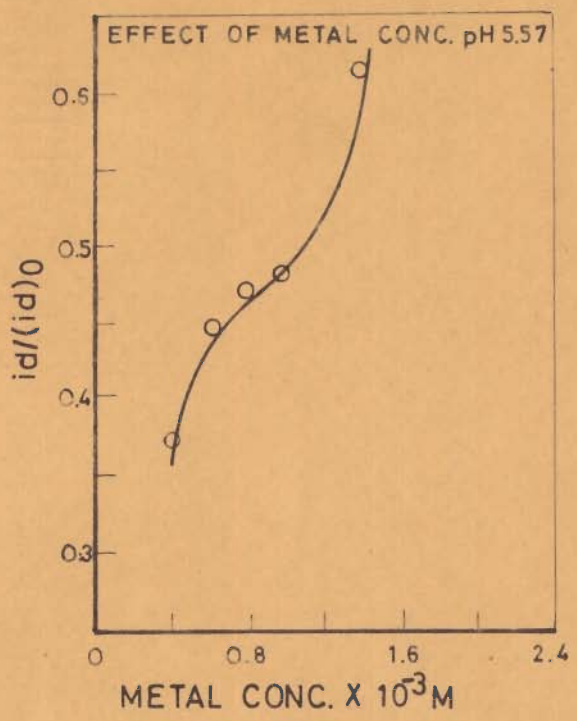

FIGURE 14

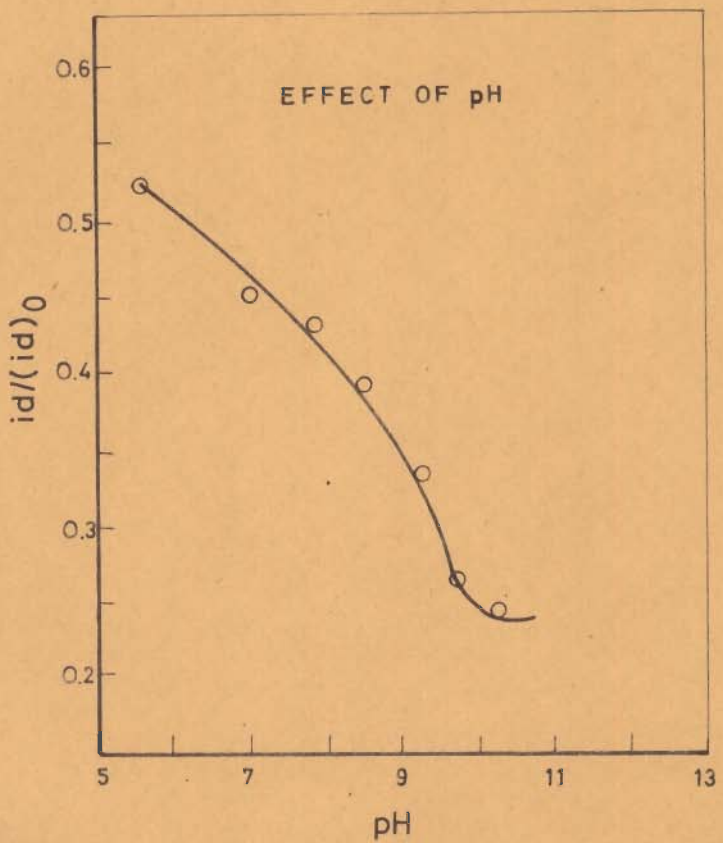

FIGURE 12

T_A_B_L_E 17

Effect of protein concentration.at pH 5.57.

Conc.of protein x 10^{-3}M	$(id)_o$ $1.1x10^{-6}$ Amp.	(id) $1.1x10^{-6}$ Amp.	$(id)/(id)_o$	Cb. $x10^{-3}$M	V_M
0.0	25.0	-	-	-	-
0.024	25.0	23.0	0.812	0.247	10.0
0.036	25.0	20.0	0.80	0.263	7.3
0.06	25.0	17.5	0.70	0.40	6.7
0.12	25.0	12.0	0.48	0.684	5.7
0.24	25.0	7.0	0.28	0.947	4.0
0.36	25.0	6.5	0.26	0.974	2.7
0.48	25.0	6.5	0.26	0.974	2.7

Fig. 13

T_A_B_L_E 18

Effect of metal concentration at pH 5.57.

Conc.of metal ions x 10^{-3}M	$(id)_o$ $1.1x10^{-6}$ Amp.	(id) $1.1x10^{-6}$ Amp.	$(id)/(id)_o$	Cb $x10^{-3}$M	V_M
0.4	12.0	4.5	0.375	0.329	2.7
0.6	14.5	6.5	0.448	0.436	3.6
0.8	17.0	8.0	0.470	0.558	4.7
1.0	25.0	12.0	0.480	0.684	5.7
1.4	30.0	18.5	0.616	0.707	5.9

Fig. 14

T_A_B_L_E 19

Effect of protein concentration at pH 7.00.

Protein conc. x 10^{-3}M	$(id)_o$ $1.1x10^{-6}$ Amp.	(id) $1.1x10^{-6}$ Amp.	$(id)/(id)_o$	Cb $x10^{-3}$M	V_M
0.0	25.0	-	-	-	-
0.048	25.0	15.0	0.60	0.526	10.9
0.072	25.0	12.0	0.48	0.684	9.5
0.12	25.0	11.0	0.44	0.737	6.1
0.192	25.0	10.0	0.40	0.789	4.1
0.240	25.0	9.5	0.38	0.816	3.5
0.36	25.0	9.0	0.36	0.842	2.5

Fig. 15

T_A_B_L_E 20

Effect of metal concentration at pH 7.00.

Metal x 10^{-3}M	$(id)_o$ $1.1x10^{-6}$ Amp.	(id) $1.1x10^{-6}$ Amp.	$(id)/(id)_o$	Cb $x10^{-3}$M	V_M
0.4	12.0	6.5	0.542	0.241	2.0
0.6	14.0	7.5	0.536	0.366	3.0
0.8	17.0	9.0	0.530	0.494	4.1
1.0	75.0	11.0	0.440	0.737	6.1
1.4	30.0	12.5	0.416	1.076	9.0

Fig. 16

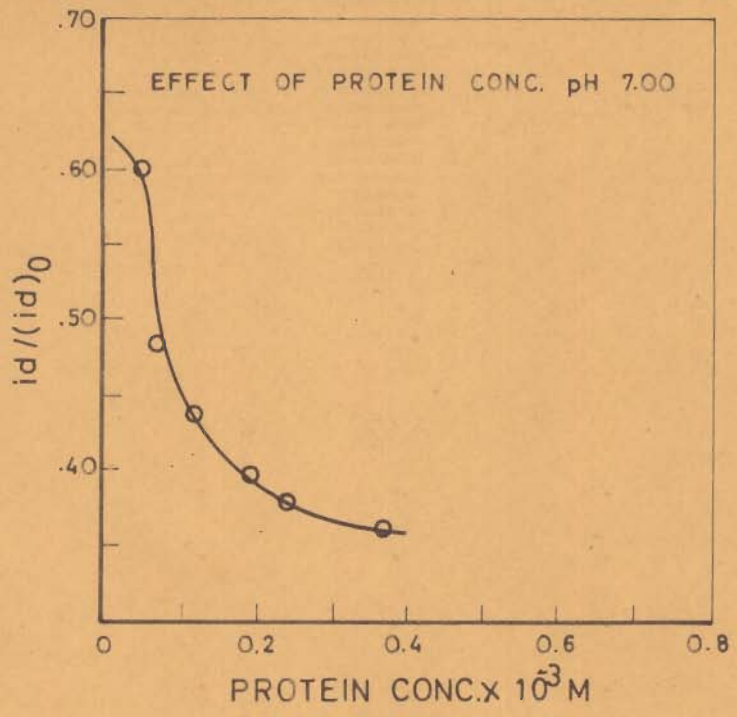

FIGURE 15

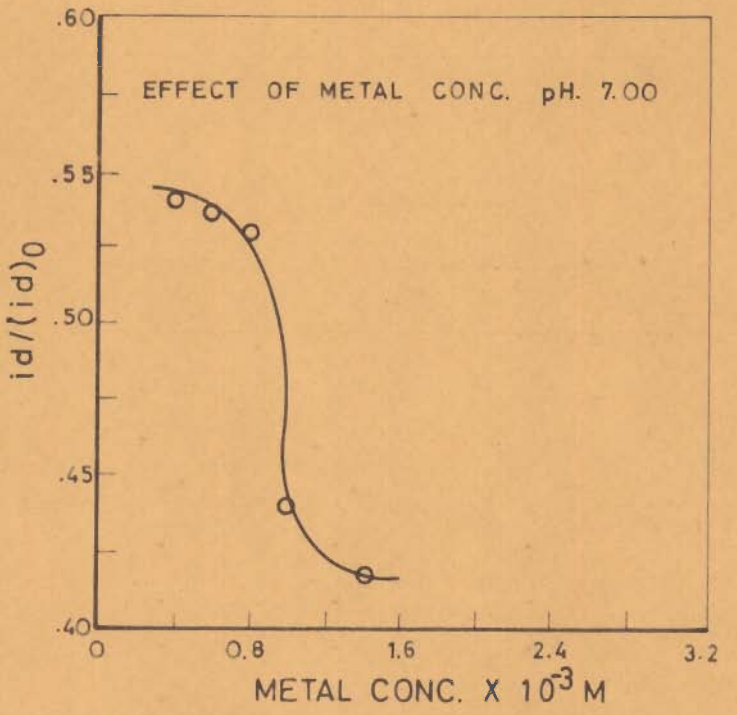

FIGURE 16

D_I_S_C_U_S_S_I_O_N

If the protein concentration is quite high(higher than required for the suppression of the polarographic maxima) then the change in the diffusion current of the metal ions in presence of protein(id values) has been ascribed due to; (i) the probable complex formation between metal and protein;(ii) adsorption and(iii) Viscosity effects. Since the present investigations were carried out at pH 5.57 and above and every care was taken to keep the protein in its native state,the factors like adsorption and viscosity can very well be ruled out and the relative dippression in diffusion current can, therefore,be taken as a measure of metal-protein combination.

The interaction of copper with α_s-casein could not be studied at a pH lower than 5.57,since the protein got precipitated below this pH. As described in the preceeding chapter the pK value for the dissociation of the carboxyl groups of α_s-casein comes out to be 5.1. The pH range for the ionisation of the carboxyl groups will be in the visinity of 5.5. So the pH 5.57 can be taken as the pH at which all the carboxyl groups of α_s-casein are available for binding. Since at this pH a large decrease in the diffusion current is observed,therefore the combination of metal ions with the carboxyl groups is

indicated. These results also provide evidence of the fact that the carboxyl groups offer principal site for the binding of copper ions. The intrinsic association constant, calculated by applying Scatchard's equation(258) comes out to be 2.47.

One of the interesting question on the combination of metals with proteins is : can the affinity be explained on the basis of combining power of amino acid residues? If the reactivity of carboxylate ion in α_s-casein is taken to be roughly equivalent to that of acetate ion,(log K for copper acetate 2.47) it becomes clear that the copper ions have lesser affinity for carboxylate ions in α_s-casein. Such a behaviour is not unlikely in view of the competition which exists between the carboxylate ions of the protein, and the anions (chloride ions) of the supporting electrolyte. The effective concentration of carboxylate ions at pH 5.5 is 0.0062M(taking the molecular weight of α_s-casein 27,300 and 52 carboxyl groups are completely dissociated) in 0.119 x 10^{-3}M. protein,where as the effective concentration of supporting electrolyte is 0.15M. Hence it may be assumed that such a competition exists. Further-more electrostatic effects may be operative for second oncoming copper ion when an adjacent carboxyl group has already been occupied by a metal ion.

At higher pH values(above pH 5.5), imidazole groups from histidine residue lose their proton and are thus

available for the binding of metal ions and a further drop in diffusion current would be expected with the increase in pH. This fact is confirmed by the studies performed at higher pH values and a clear indication of the binding of copper ions with the imidazole groups of α_s-casein is obtained. In all α_s-casein contains 6 imidazole groups. The observations made at pH 7.00 and 7.5 give conclusive evidence that at least one imidazole group is bound by the copper ions. The effect of protein and metal concentration on the binding at pH 5.57 and pH 7.00 are summarised in tables 16 - 20. These results are in complete agreement with those of the pH metric studies on copper - α_s -casein system. The log K value comes out to be 3.20(for copper - imidazole binding) . This value is also in agreement with the value(3.50) obtained by the pH-metric studies (chapterIII).

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C_H_A_P_T_E_R V

pH-metric studies on the mutual interaction of silicic acid sol and tabashir sol with transfusion gelatin.

I_N_T_R_O_D_U_C_T_I_O_N

A large amount of work has been done on the mutual interaction of hydrophobic sols. Amongst those who have substantially contributed to the problem the names worth mentioning are those of Billitzar(278),Lottermoser(279), Blitz(280),Thomos(281),Nathanson(282),Thomos and Jhonson (283),Weiser and Chapman(284) and Hazel and McQueen(285). Weiser and Chapman(286) have suggested that the following factors influence the mutual coagulation:(i) adsorption of colloidal particles,(ii) the presence of precipitating ions as impurities in the sol and (iii)interaction between the stabilising ions. Hazel and McQueen (285) pointed out that the mutual adsorption of the oppositely charged sol would result in the unequal distribution of charges around the particles,with a consequent coagulation of the two sols. Weiser and Milligan(287) also have shown that mutual coagulation was accompanied by the lowering of zeta potential of the two sols and the displacement of their counter ions.

Another equally important aspect of the problem,viz., the study of the colloidal conditions existing in the mixtures of hydrophobic and hydrophilic sols has not yet been critically examined. Until recently this problem has been examined purely from the physical stand point and little emphasis has been laid on the role of chemical forces in such interactions. Freendlich(288,289),Pauli(290,291)

and Andrieve(292) observed that the unstable hydrophobic sols were stabilised in excess of protein where as small amounts of protein sensitise or even coagulate the sol. The sensitisation of the gold sol(negatively charged) by acidified gelatin may be explained interms of flocculation whereas the sensitisation of the same sol above pH 7.0 may be due to the formation of agglomerates of gold sol. The agglomerates are formed due to the partial covering of the hydrophobic colloid by gelatin,while protection is affected by the complete covering. This idea,however, was rejected by William and Chang. Jirgensons(293),and Poeter and Matalon(294) demonstrated that the protective power of the hydrophilic substances was somewhat dependent upon its structure. He observed that the protective action was due to the adsorption of gelatin on the surface of the sol particles through the undissociated carboxyl groups. Any-how the principle underlying protection has been utilised in preparing hydrophobic sols of high degree of dispersion.

Recently Bull and Co-workers(297-299) have investigated the problem of the interaction of cationic proteins with negatively charged glass particles.According to them mutual interaction was affected through the partial neutralisation of the negative charge on the glass particle by means of the positive protein molecule.

However,they did not take into account the role of possible ionising groups of the protein.

Investigations in this field became all the more fascinating when the hydrophilic colloid is a protein with its large number of reactive groups available at various pH values. Pauli(300) for the first time,drew attention to the chemical aspect of the problem during the course of his studies on the mutual interaction of albumin with congo blue. He observed that the equal concentrations of the sols resulted in their mutual coagulation whereas the greater concentration of protein caused a change in colour from blue to red. The possible explaination offered by Pauli was that complex formation takes place between the nitrogen atom (NH_3^-) of the dye and the carboxyl groups of the protein. Another work worth mentioning in this connection is that of Kvyat(301),who carried out extensive studies on the effect of a number of organic substances including amino acids and proteins on the formation of hydrous oxide sols of iron and aluminium.

Apart from the studies which can be carried out between hydrophilic and hydrophobic colloids of the hydrous oxide or sulphide types,the other systems worth considering are those involving interaction between the silicic acid and hydrophilic sols,particularly the proteins. Such a system was first considered by Graham(302) who

observed that gelatin combined with silicic acid to give an insoluble precipitate,Mylius and Groschuff(303) observed that silicic acid of very low molecular weight did not precipitate egg albumin,but as soon as the silica polymrised to a small degree it coagulated the protein.

Lesley(304) found that gelatin and albumin were precipitated by silicic acid only below pH 6.0. According to him below this pH both the sols are negatively charged so it was not a case of coagulation by neutralisation. This was also confirmed by the fact that there was no change in pH when the precipitate was formed.

Recently systematic and more comprehensive studies on the problem of mutual interaction of hydrous oxide sols with proteins have been taken by Malik and Co-workers(176-179). Considering the protein as a multivalent electrolyte and the availability of the various reactive groups at different pH values for interaction,these authors treated the problem in the light of possible combination of the protein with the metal ions from the inner part of the electrical double layer of the hydrous oxide sols. These studies have now been extended to protein-silicic acid sol mixtures. The present chapter deals with the results on the interaction of silica and tabashir sol (of great medicinal importance in indigenious system of medicine) with transfusion gelatin.

Preparation of silicic acid sol(305).

The silicic acid sol was prepared by mixing slowly a clear solution of 30.0% sodium silicate to 100 mls. of doubly diluted concentrated hydrochloric acid. The resulting mixture was put to dialysis through parchment paper till the desired pH(between 2.0 -3.0) value was obtained. The concentration of silica in the sol was determined by taking a 10 mls. aliquot of the sol and drying it in an electric oven to dryness at 110°C for 5-6 hours. After cooling the crucible,the precipitate was washed (to remove electrolyte etc.) in a Weighed sintered glass crucible. The amount of pure silica was determined after drying the crucible in the oven at 105-110°C. This gave the concentration of silica present in the sol in gms./litre.

Preparation of tabashir (vanshlochan) sol.

The pure and fresh tabashir for this purpose was obtained from the Gurukul Kangri Pharmacy,Haridwar. Its sol was prepared by boiling the 25 gms tabashir with concentrated alkali. The silica content(the chief constituent of tabashir) goes in the solution. This solution was centrifuged and the clear solution was collected. 100 mls of the solution was mixed with 50 cc. of dilute hydrochloric acid and then dialysed as before. The product thus obtained was designated as 'tabashir sol'. Its silica content was determined as described previously in the case of silicic acid sol. The sol was diluted by

adding water to obtain the different concentrationsof the sol.

A 3.0% solution of transfusion gelatin was prepared by diluting the stock solution(6.0% conc.). The pH of the solution was adjusted to 12.00 by the addition of dilute potassium hydroxide solution.

Solution of potassium hydroxide of pH 12.00 was prepared by adding dilute potassium hydroxide solution drop by drop into double distilled water. The pH of the water being continuously checked with the pH meter till it reached the desired value.

The pH meter used was a Beckman H-2 model,with glass electrodes. The potassium acid phthalate and Borax buffers were used for the standardisation of the pH-meter for the two acid and basic ranges of pH.

Procedure.

Varying volumes viz.,0.0,1.0,2.0,3.0,4.0,6.0, 8.0,10.0,12.0,14.0 and 16.0 mls.of the silicic acid sol and the tabashir sol(of 2.0 and 2.5 pH) were taken in different pyrex tubes. Two identical sets were prepared each containing the same amounts of silicic acid or tabashir sol, but one having 4.0 mls.of transfusion gelatin solution (pH 12.00) in it while the other containing 4.0 mls.of potassium hydroxide solution of the same pH as that of protein solution. The total volume was made upto 20.0 mls. in each case by adding

the requisite amount of water.

Viscometric methods, for the study of the interaction of these sols with transfusion gelatin, could not be applied because at concentration above 0.3% of the silicic acid coagulation set in and hence the viscometric studies could not be made. When silicic acid or tabashir sol of lesser concentration were employed no appreciable change in viscosity was observed.

The results have been summarised in the following tables.

T_A_B_L_E 1

Concentration of transfusion gelatin = 3.0%, pH 12.00

Concentration of silicic acid sol = 2.0%, pH 2.00

Potassium hydroxide solution pH = 12.00

Total volume = 20 mls. Temperature 30°C

Vol. of sol added. in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.10	11.00	0.10
1.0	9.70	9.10	0.60
2.0	9.30	7.95	1.35
3.0	9.00	3.75	5.25
4.0	7.80	3.30	4.50
6.0	6.70	2.80	3.90
8.0	5.10	2.60	2.50
10.0	4.20	2.50	1.70
12.0	3.75	2.35	1.40
14.0	3.40	2.25	1.15
16.0	2.95	2.15	0.80

Fig. 1

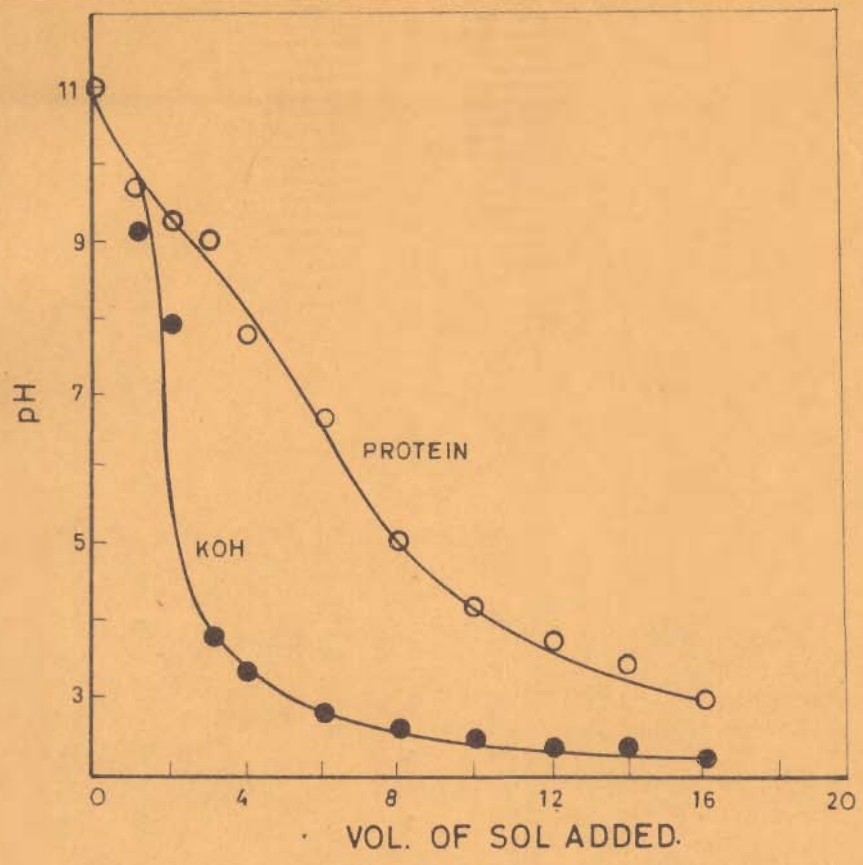

FIGURE 1

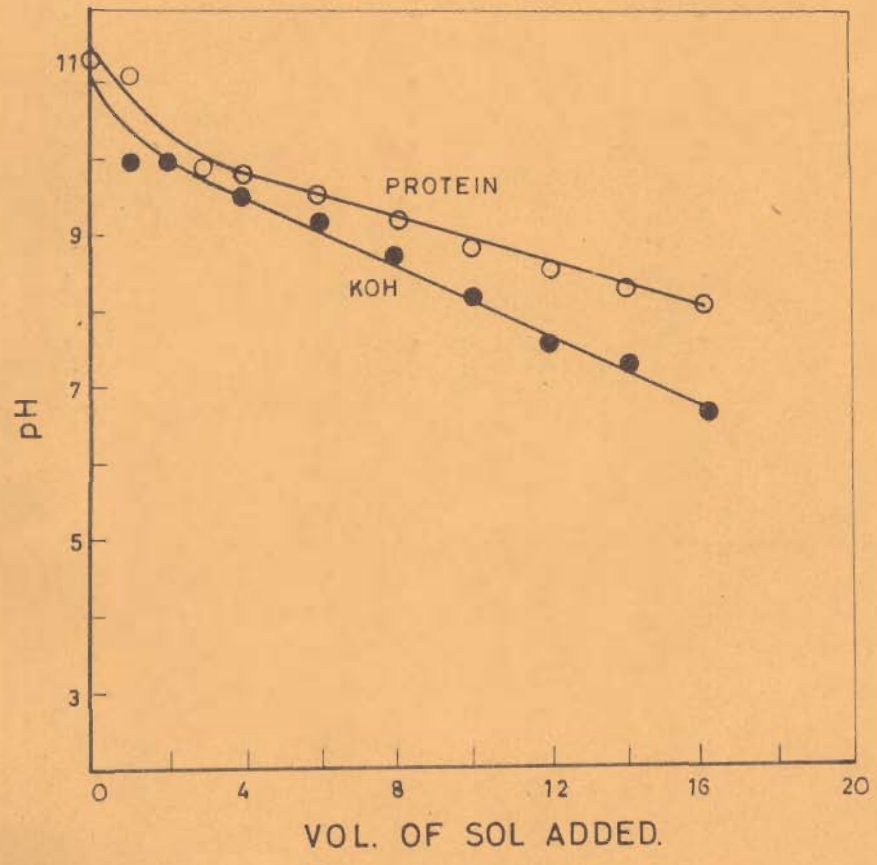

FIGURE 2

T_A_B_L_E 2

Concentration of transfusion gelatin =3.0%, pH=12.00

Concentration of silicic acid sol =2.0%, pH= 2.5

Potassium hydroxide solution = pH 12.00

Total volume = 20.0 mls. Temperature 30°C

Vol.of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.30	11.30	-
1.0	11.10	9.90	1.20
2.0	10.00	9.90	0.10
3.0	9.90	9.90	-
4.0	9.75	9.55	0.20
6.0	9.50	9.20	0.30
8.0	9.15	8.70	0.45
10.0	8.80	8.10	0.70
12.0	8.50	7.50	1.00
14.0	8.30	7.20	1.10
16.0	8.00	6.60	1.40

Fig. 2

T_A_B_L_E 3

Concentration of transfusion gelatin = 3.0%, pH 12.00

Concentration of silicic acid sol = 1.0%, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20 mls. Temperature 30°C

Vol. of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	10.80	10.80	-
1.0	9.45	9.20	0.25
2.0	9.20	8.75	0.45
3.0	9.10	7.90	1.20
4.0	8.55	4.30	4.25
6.0	7.70	2.90	4.80
8.0	6.10	2.70	3.40
10.0	5.00	2.55	2.45
12.0	4.25	2.40	1.85
14.0	3.80	2.35	1.45
16.0	3.50	2.25	1.25

Fig. 3.

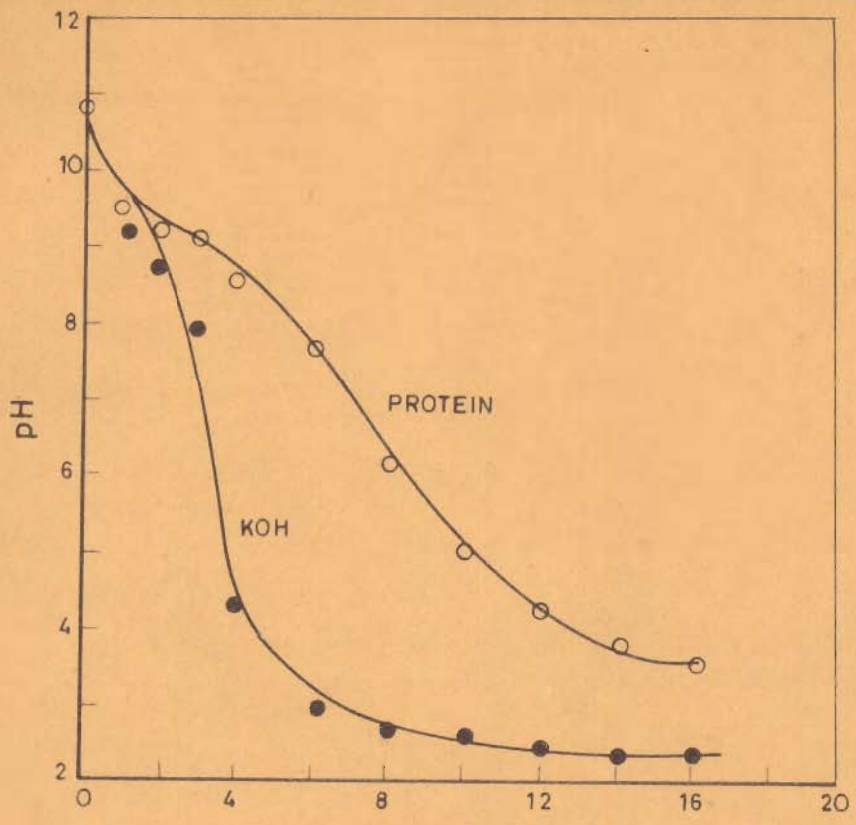

FIGURE 3

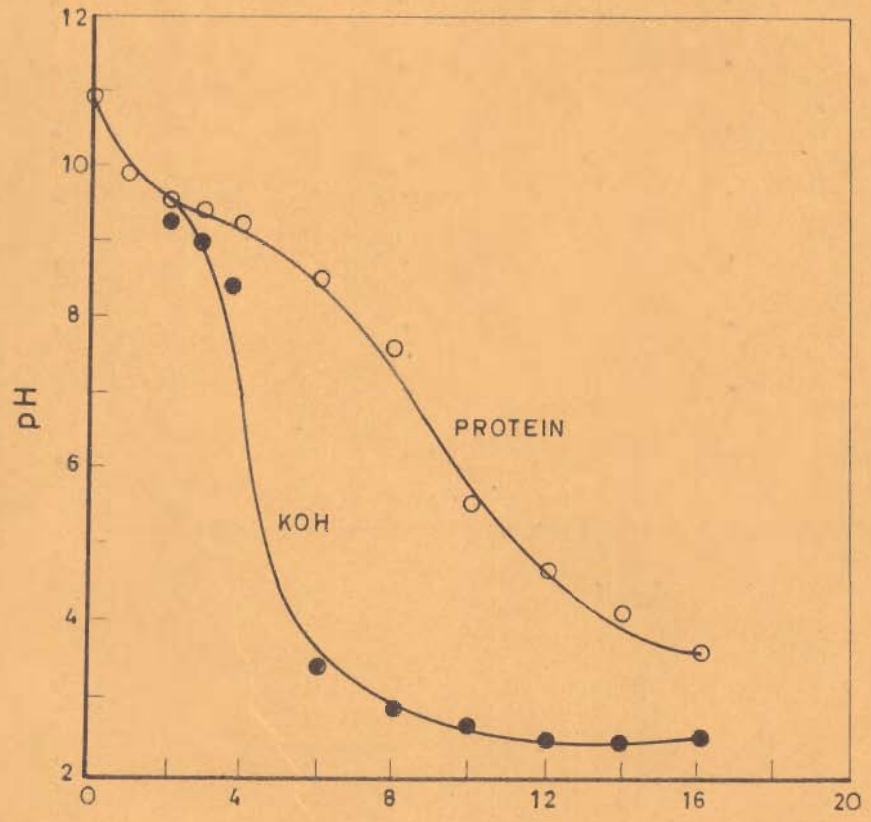

FIGURE 4

T_A_B_L_E 4

Concentration of transfusion gelatin = 3.0%, pH 12.00

Concentration of silicic acid sol = 0.5%, pH 2.00

Potassium hydroxide solution pH 12.00

Total volume = 20.0 mls. Temperature 30°C

Vol. of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.00	11.00	-
1.0	9.90	9.80	0.10
2.0	9.50	9.30	0.20
3.0	9.45	9.00	0.45
4.0	9.25	8.50	0.75
6.0	8.55	3.30	5.25
8.0	7.60	2.80	4.80
10.0	5.55	2.60	2.95
12.0	4.65	2.45	2.20
14.0	4.10	2.40	1.70
16.0	3.55	2.40	1.15

Fig. 4.

T_A_B_L_E 5

Concentration of transfusion gelatin = 3.0%, pH 12.00

Concentration of silicic acid sol = 0.5%, pH 2.50

Potassium hydroxide solution pH 12.00

Total volume = 20.0 mls. Temperature 30°C

Vol. of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.10	10.90	0.20
1.0	10.60	10.30	0.30
2.0	10.35	9.60	0.75
3.0	9.55	9.50	0.05
4.0	9.85	9.20	0.65
6.0	9.25	-	-
8.0	9.30	9.10	0.20
10.0	9.40	8.20	1.20
12.0	9.30	7.55	1.75
14.0	9.20	4.00	5.20
16.0	8.80	3.20	5.60

Fig. 5.

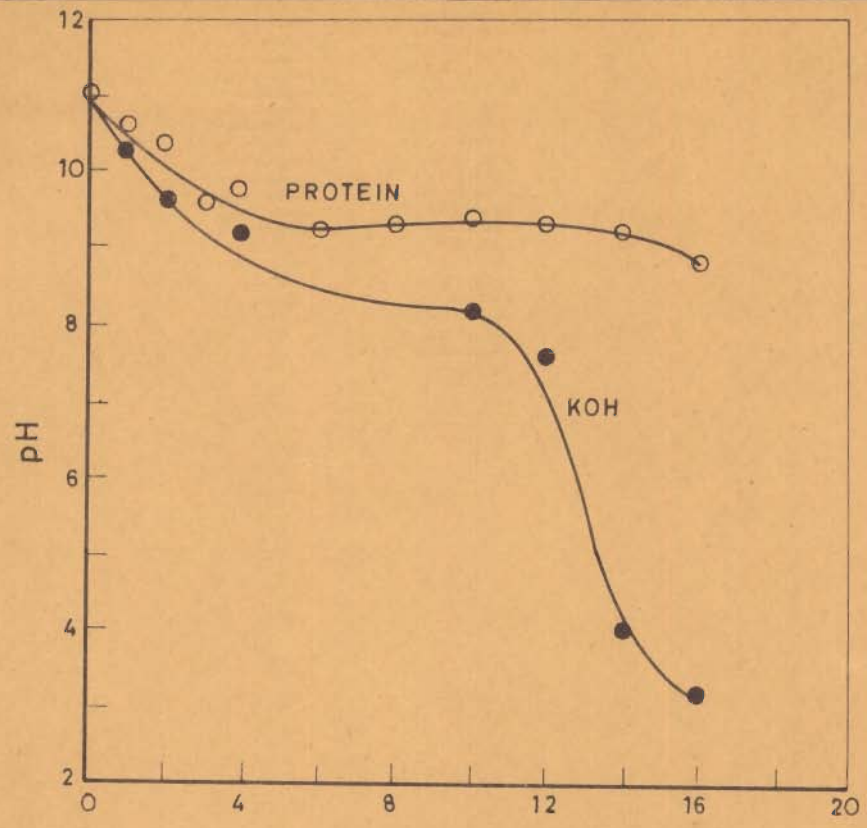

FIGURE 5

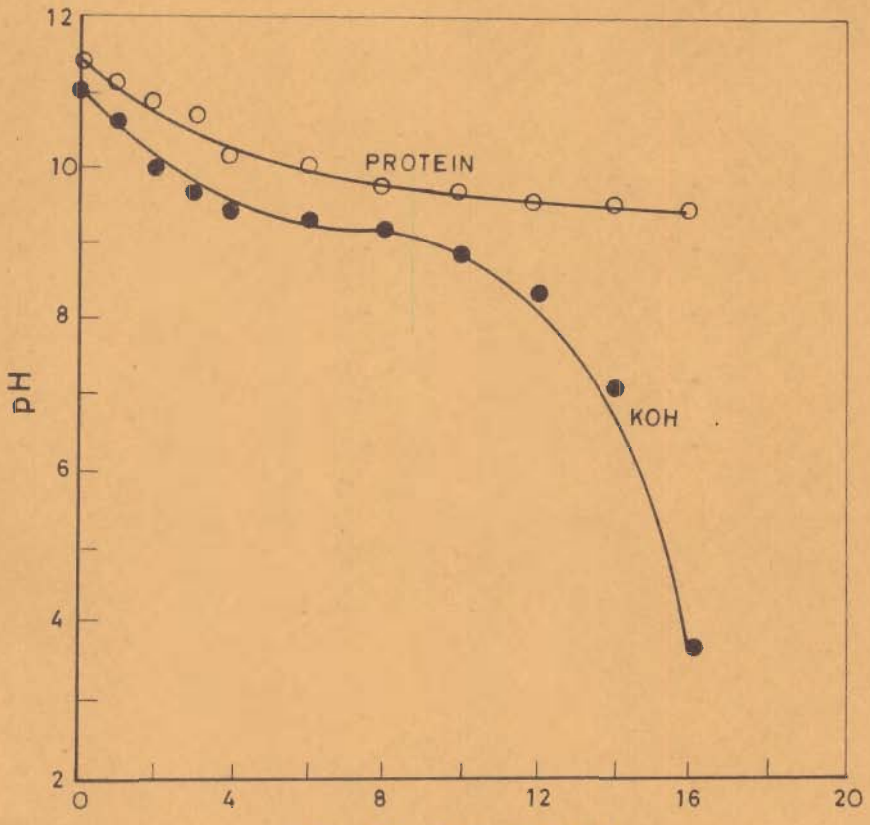

FIGURE 6

T_A_B_L_E 6

Concentration of transfusion gelatin =3.0%, pH 12.00

Concentration of tabashir sol =0.5%, pH 2.50

Potassium hydroxide solution pH 12.00

Total volume 20.0 mls. Temperature 30°C

Vol.of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.30	11.00	0.20
1.0	11.10	10.60	0.50
2.0	10.85	9.95	0.70
3.0	10.65	9.70	0.95
4.0	10.10	9.40	0.70
6.0	10.00	9.30	0.70
8.0	9.75	9.15	0.60
10.0	9.65	8.80	0.85
12.0	9.50	8.30	1.20
14.0	9.50	7.00	2.50
16.0	9.40	3.60	5.80

Fig. 6.

T_A_B_L_E 7

Concentration of transfusion gelatin = 3.0%, pH 12.00

Concentration of tabashir sol = 1.0%, pH 3.00

Potassium hydroxide solution pH 12.00

Total volume = 20.0 mls. Temperature 30°C

Vol. of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.60	11.50	0.10
1.0	11.30	11.20	0.10
2.0	10.55	10.35	0.20
3.0	10.55	10.10	0.45
4.0	10.50	10.00	0.50
6.0	10.45	9.90	0.55
8.0	10.00	9.75	0.25
10.0	9.60	9.50	0.10
12.0	9.30	9.25	0.05
14.0	9.15	9.15	-
16.0	9.00	8.90	0.10

Fig. 7.

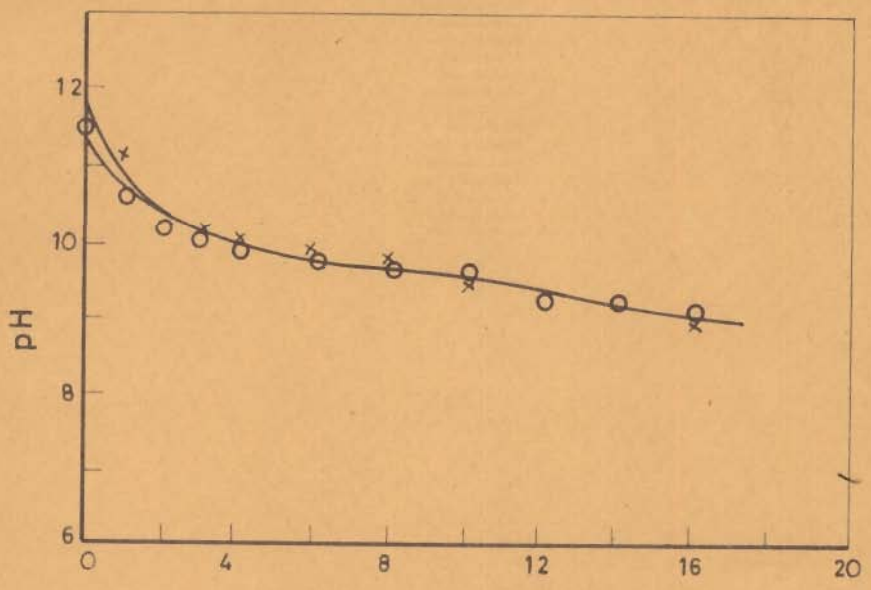

FIGURE 7

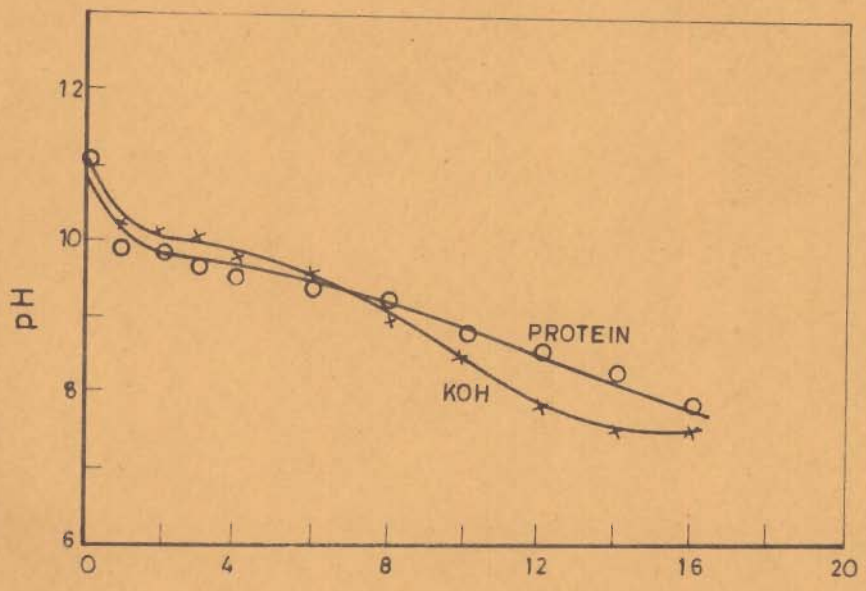

FIGURE 8

T_A_B_L_E 8

Concentration of transfusion gelatin =3.0%, pH 12.00

Concentration of tabashir sol =1.5%, pH 2.50

Potassium hydroxide solution pH 12.00

Total volume = 20.0 mls. Temperature 30°C

Vol.of sol added in mls.	pH_1 with protein	pH_2 with KOH	pH
0.0	11.60	11.60	-
1.0	10.40	10.20	0.20
2.0	10.25	10.05	0.20
3.0	10.10	9.75	0.35
4.0	9.80	9.65	0.15
6.0	9.65	9.50	0.15
8.0	9.10	9.05	0.05
10.0	8.75	8.50	0.25
12.0	8.50	7.80	0.70
14.0	8.25	7.50	0.75
16.0	7.80	7.50	0.30

Fig. 8.

D_I_S_C_U_S_S_I_O_N

Unlike metal hydroxide-protein interaction, the corresponding reaction involving silicic acid sol is of a fairly complex nature. The system changes from a hydrophobic-hydrophilic one to that made up of almost hydrophilic constituents. Further intricacies set in due to the complex nature of the particles of silicic acid sol.

According Carman(306) SiO_2 in contact with water tries to complete its unsatisfied surface charges through the hydrogen and the hydroxyl ions resulting in the formation of strong Si-OH bonds. This state of affairs in the neutral medium is disturbed on the addition of alkali due to the replacement of H^+ ions by Na^+ ions and since the latter cannot enter into the electronic structure of the $\bar{O}$ ions, the resulting colloidal solution becomes negatively charged.

Controversy also exists as to the pH at which silica particles would be negatively charged, neutral or positively charged. Losenbench(307) considers the iso-electric point of silica sol to lie between pH 2 and 3 while Lesley(308) believes that silica is negatively charged even down to pH 2.0. Gordon(309) has reported that the silica gel membrane is negative at pH 3.17 and is positive at pH 1.22. The true picture, regarding the nature of silica sol, however, appears to be as follows:

In the highly acidic range the silica particles are positively charged due to the adsorption of H^+ ions with the probable formation of SiH groups on the surface particle. A decrease in acidity,say by the addition of the alkali would result in the ionisation of the SiOH group due to the replacement of the protons by the sodium ions. A gradual transition from the positive to the negative charge thus takes place by the increase in the pH. At no stage,however,the existence of a completely uncharged silicic acid sol can be visualised although zwitter ions may exist at a certain pH.

The results on the interaction of transfusion gelatin with silicic acid and tabashir sols between pH 2 and 3, support the above viewpoint.

On the addition of the increasing amountsof silicic acid sol of pH 2.0 to a fixed amount of transfusion gelatin of pH 12.0,a resulting pH of 2.95 is reached. A similar change is observed on the addition of silicic acid sol to caustic potash of the same pH as the protein (Table 1,3,4). The shapes of the two curves are,however, different(Fig.1,3,4) and from the shift in the flat portion of the curve evidence for the interaction of silicic acid with the carboxyl groups of the protein is obtained. The mechanism involved a competition between the hydrogen of the SiH groups on the silicic acid sol and the carboxyl groups of the protein(deprotonation takes place in the pH range where the shift has been observed) resulting in

the binding of the silica to the protein. This mechanism finds support from the modern view which envisages the combination by the formation of a multiplisity of hydrogen bonds between the relatively large molecules of protein and silicic acid so that a mixed net work of the molecules is formed. The concentration of the silicic acid sol do not effect the binding as can be seen from the comparision of the data in Tables 1,3,4(Fig.1,3,4).

The behaviour of the silica sol of pH 2.5 is entirely different from that of pH 2.0. There the resulting pH comes out to be 8.0 and 8.8 on the addition of sols of concentration 2.0% and 0.5% respectively(Table 2 and 5) as against 2.95 and 3.55 for the sol of pH 2.0. The variations in pH for corresponding mixtures of silicic acid and caustic potash are from pH 12.0 to 6.6 and 3.2 respectively(Table 2 and 3). The shapes of the curves with and without transfusion gelatin are different and exhibit a shift in the pH range 8 to 10 (Fig.2 and 3). Since in this pH range the nitrogen containing groups,viz.,the amino,imidazole and guanidino groups of the protein offer sites for combination. As for the mechanism of interaction it appears that the combination would involve the formation of hydrogen bonds between the nitrogen of the transfusion gelatin and the hydrogen of the silanol group of the silicic acid.

Another interesting fact emerges out from these

studies. It is seen that the pH variations with and without the protein on the addition of concentrated silicic acid sol(2.0 %) lie within almost the same pH range. The caustic potash curve is not like an acid-base titration curve as obtained with dilute sols. This difference in behaviour is due to the polymerisation of silica when concentrated solutions are employed. However, this tendency, i.e.,polymerisation of the silica(and subsequent formation of an insoluble net work) takes place even at low concentrations of silicic acid if a little protein is present.

Tabashir sol.

The medicinal importance of tabashir may be ascribed to its combination with nitrogen of the proteins.Since combination with the amino,imidazole and other nitrogen containing groups takes place with silicic acid sol near-about of pH 2.5 and above, transfusion gelatin-tabashir sol interaction was studied at pHs 2.5 and 3.0. The variation in pH and the shifts in the curves are the same as with silicic acid of pH 2.5 (Table 6,8;Fig.6,8). It means here too,the mechanism of combination is the same, involving the formation of hydrogen bonds between the nitrogen of the transfusion gelatin and the hydrogen of the silanol groups of the silicic acid.

There is a significant difference between the behaviour of silicic acid and tabashir sols as far as their concentrations are concerned . Concentrated sols of

tabashir undergo gelation readily and the interaction with transfusion gelatin is not so effective with a concentrated sol(1.5 %) than with the dilute sol (0.5 %).From the curves (Fig.6 to 8) it may be seen that typical curves pointing towards definite combination in the pH range 8 to 10(where imidazole and other nitrogen containing groups offer sites for combination) are realised. This anamoly may be due to the difference in the properties of commercial silica and the naturally occuring one(Tabashir).

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C_H_A_P_T_E_R VI.

Viscosity variations during sol-gel transformation of casein-formaldehyde mixtures, in presence of sodium meta silicate or sodium hexyl sulphate.

I_N_T_R_O_D_U_C_T_I_O_N

The problem of the interaction of proteins with polymeric substances and surface active compounds present many interesting aspects for fundamental studies. The reactionsworth considering are those between the proteins and silicic acid or detergents and organic compounds. The reaction between silicic acid and gelatin was first observed by Graham(303). Lesley(304) worked on the precipitation of gelatin and albumin by silicic acid while Merill and Spencer(310) studied the sorption of sodium silicate and silicic sol on the cellulose fibres. Reactions involving polysilic acids and proteins have also been used for the analysis of silica and in the tanning industry(311).

The reactions involving proteins and surface active agents have been considered in view of their importance in industry and technology. With globular proteins reactions with detergents (soap solutions) or organic solvents have proved to be of immense interest to the technologist. The presence of detergents unfolds the native structure of the protein resulting in the formation of elastic and highly double refracting fibres(312). This was achieved with egg albumin,wheat glutamin,casein, zein and blood albumin. Many laboratories have used highly concentrated organic solvents in place of detergents (313). Synthetic detergents,especially the anionic ones, are found to influence proteins in a number of ways,

especially,in bringing about their denaturation(314, 315),precipitation and solubilisation(316,317). In many cases these effects have been interpreted in terms of protein detergent complex (318,319) and form the basis of many interesting physico-chemical studies.

The interaction of aldehydes (320) and particularly formaldehyde with proteins is of particular interest. These compounds not only get bound with the $-NH_2$,NH and SH groups of the protein but also exert their reducing property. In aqueous solutions,more alkaline than pH 6.0 the aldehydes generally react with the amino groups of the protein by the formation of ($- N = CH_2$) linkage.

The conversion of casein into artificial horn or fibres also fall under the class of the complicated reactions mentioned above. Many patents (321-326) employing the alkaline solutions of casein and formaldehyde appear in the literature but very little is known about the actual mechanism leading to the formation of these products. Even the researches of Ferreti(327) on the spinning of casein fibre, or those of Braida(328) on their tensile strength,hardly provide the required informations. The aim of the present studies is to get some idea about the nature of the reactions by considering the variations in viscosity during the gelation of casein-formaldehyde mixtures mixed in presence of big molecules like sodium meta-silicate and sodium hexyl sulphate.

E_X_P_E_R_I_M_E_N_T_A_L

Casein solutions.

The casein used in the experiments was a pure E.Merk product. The required amount of the protein was soaked in about half the amount of water and the swollen mass,after 5-6 hours was dissolved in the appropriate amount of sodium meta silicate,dissolved in the other half amount of water, until clear solutions were obtained.

The α-casein and β-casein used were obtained from the whole casein,by using their differential solubilities in 50 % alcohol with variation in pH and temperature. The method of separation is described in details in chapter III,dealing with the studies of casein andα-casein with metal ions.

Sodium meta silicate was an A.R.,B.D.H. product. The formal-dehyde used was also a B.D.H.product containing 37-41 % solution of formaldehyde in water.

Sodium hexyl sulphate (soap) was prepared by refluxing 100 cc.of n - hexyl alcohol(B.D.H.) with 200 cc.of concentrated sulphuric acid(C.P., Sp.gr. 1.8) for about 8 hours. The cooled mass was poured in about 200 cc.of water. The excess of sulphuric acid was neutralised by adding just sufficient amount of calcium carbonate(B.D.H.) till effervascence stopped. The filtrate consisting of the calcium soap was treated with small amounts of saturated solution of sodium carbonate. The slightly alkaline solution so obtained was evaporated on a water bath until a drop

withdrawn on glass rod crystalises on cooling. It was then allowed to crystalise and the dark brown crystals were filtered at the pump,drained and dried over anhydrous calcium chloride in a desiccator. Solutions of requisite strength were prepared in double distilled water.

Viscosity measurements.

Scarpa method(329) as modified by farrow,Mehta, Prasad and Desai(334) was employed for measuring the viscosity. The constant,K, for the viscometers was determined at a particular pressure(25.5 cms.of water) and temperature(30 $\pm$ 0.1^{o}C) from the relationship,

$$\eta = K \frac{t_1 t_2}{t_1 + t_2}$$

where t_1 and t_2 are the time of fall and rise of the liquid of known viscosity,through the capillary of an Ostwald viscometer. Half a dozen viscometers of different capillary size were employed depending upon the mixture to be investigated.

The following sets of mixtures were used for determining the variations in viscosity with time.

Set I: 6.0 % casein + 0.9 % concentration of sodium meta silicate + varying concentrations of formaldehyde(2.0, 3.0,4.0 and 5.0 %).

Set II: 6.0 % casein + 1.0 % sodium meta silicate + varying concentrations of formaldehyde (2.0,3.0,4.0 and 5.0 %).

Set III: Varying concentrations of casein(5.0,5.5,5.7, 5.8,5.9,6.0 %) + 1.0 % sodium meta silicate + 2.0 %

formaldehyde.

Set IV : 6.0 % casein + varying concentration of sodium meta silicate(0.9,1.0,1.1 and 1.2 %) + 2.0 % formaldehyde.

Set V: 6.0 % casein + varying concentration of sodium hexyl sulphate(1.0,1.1,1.2 and 1.3 %) + 2.0 % formaldehyde.

Set VI: 6.0 % casein + 1.0 % sodium meta silicate + varying concentration of sodium hexyl sulphate(0.1,0.2, 0.25 and 0.3 %) + 2.0 % formaldehyde.

Set VII: 6.0 % casein + 1.0 % sodium hexyl sulphate + varying concentrations of sodium meta silicate(0.1,0.2, 0.25 and 0.3 %) + 2.0 % formaldehyde.

The above sets were repeated with α-casein also with the only difference that 5.0 % α-casein was employed as compared to the 6.0 % concentration of the casein. The concentrations of the protein,varied according to set III,were, 4.7, 4.8, 4.9 and 5.0 %.

The point at which abrupt change in viscosity was observed in the viscosity- time curve was taken as the time of gelation.

Experiments were also performed with β-casein. The variation in viscosity for time were quite small.

The results are summarised in the following tables.

T_A_B_L_E 1

Concentration of casein solution = 6.0 %

Concentration of sodium meta silicate = 0.9 %

Concentration of formaldehyde = 2.0 %

Formaldehyde 2.0%		Formaldehyde 3.0%	
Time in mts.	η	Time in mts.	η
18.0	20.545	22.0	20.174
56.0	21.954	50.0	20.526
95.0	24.694	100.0	27.115
150.0	40.309	145.0	47.750
Time of gelatinn=108 mts.		Time of gelation=122 mts.	

T_A_B_L_E 2

Concentration of casein =6.0 %

Concentration of sodium meta silicate=0.9 %

Formaldehyde 4.0 %		Formaldehyde 5.0 %	
Time in mts.	η	Time in mts.	η
20.0	36.0	18.0	10.666
50.0	41.9	52.0	12.431
95.0	55.5	96.0	15.649
135.0	76.2	140.0	21.627
190.0	-	235.0	53.018
Time of gelation=140 mts.		Time of gelation=200 mts.	

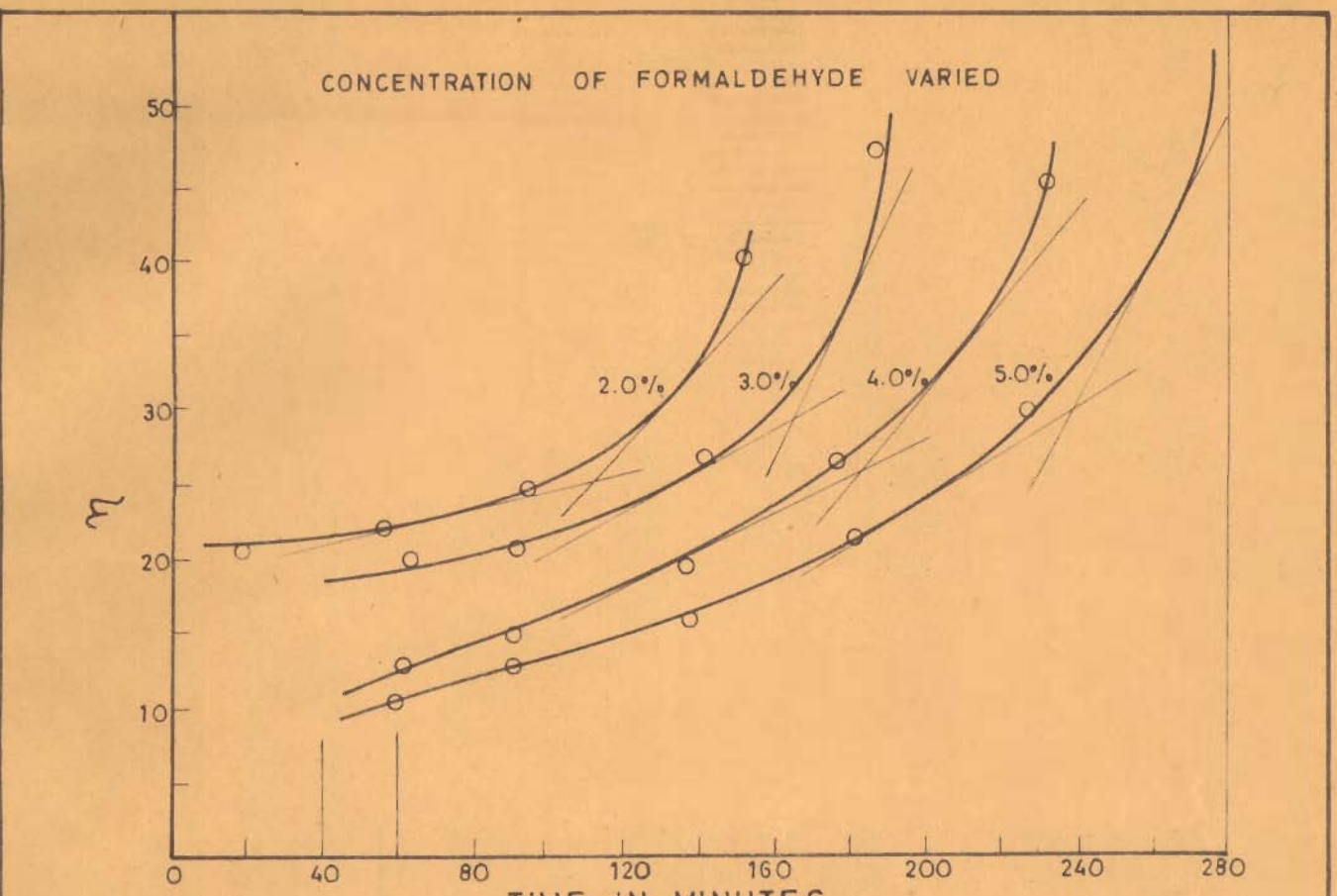


FIGURE 1

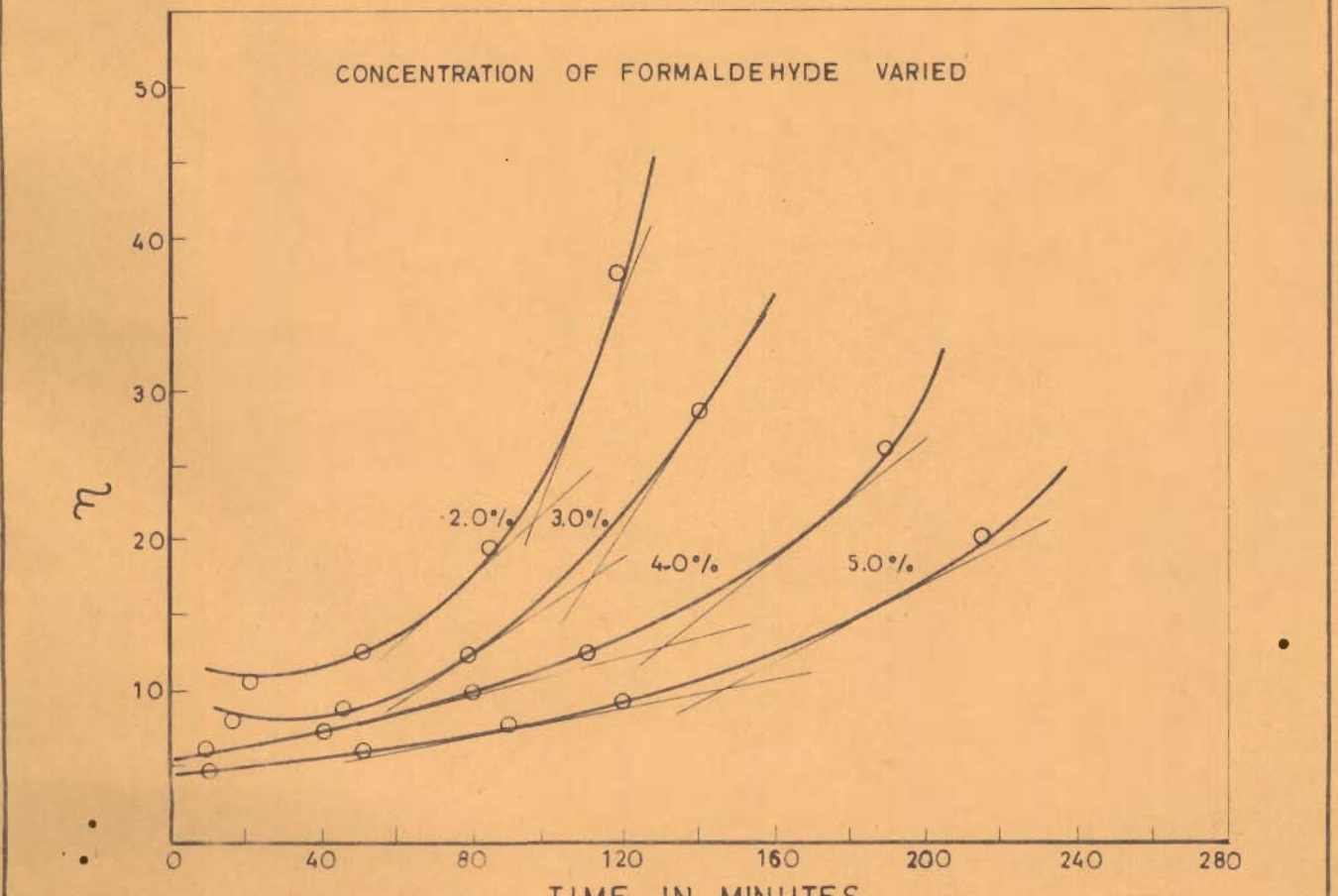


FIGURE 2

T_A_B_L_E 3

Concentration of casein solution = 6.0 %

Concentration of sodium meta silicate. = 1.0 %

Formaldehyde 2.0 %		Formaldehyde 3.0 %	
Time in mts.	η	Time in mts.	η
20.0	10.522	16.0	7.772
50.0	12.218	45.0	8.855
85.0	19.543	80.0	12.274
120.0	38.074	140.0	28.596
Time of gelation=96 mts.		Time of gelation =112 mts.	

T_A_B_L_E 4

Concentration of casein solution = 6.0 %

Concentration of sodium meta silicate. = 1.0 %

Formaldehyde = 4.0 %		Formaldehyde = 5.0 %	
Time in mts.	η	Time in mts.	η
10.0	6.008	10.0	4.806
40.0	7.171	50.0	5.809
80.0	9.473	90.0	7.642
110.0	12.204	120.0	9.275
190.0	26.236	216.0	20.179
Time of gelation= 132 mts.		Time of gelation=142 mts.	

Fig. 2.

T_A_B_L_E_ 5

Concentration of sodium silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

Concentration of casein=5.0%		Concentration of casein=5.5%	
Time in mts.	η	Time in mts.	η
36.0	5.142	10.0	8.892
50.0	5.818	36.0	9.936
94.0	6.330	78.0	11.995
114.0	6.530	110.0	14.534
155.0	7.238	150.0	20.027
174.0	7.602	194.0	27.606
200.0	8.078	224.0	46.946
No gelation		Time of gelation=180 mts.	

T_A_B_L_E 6

Concentration of sodium meta silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

Conc.of casein =5.7 %		Conc.of casein =5.8 %	
Time in mts.	η	Time in mts.	η
20.0	10.321	22.0	12.534
44.0	11.574	46.0	14.201
82.0	14.706	84.0	18.866
105.0	17.482	110.0	21.568
152.0	27.791	148.0	33.389
172.0	37.059	166.0	48.051
Time of gelation =144 mts.		Time of gelation =130 mts.	

T_A_B_L_E 7

Concentration of sodium meta silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

Conc.of casein =5.9 %		Conc. of casein =6.0 %	
Time in mts.	η	Time in mts.	η
36.0	13.868	20.0	10.522
48.0	16.293	50.0	12.218
90.0	23.313	85.0	19.543
108.0	28,249	108.0	30.100
120.0	36.212	120.0	38.074
Time of gelation=112mts.		Time of gelation =96 mts.	

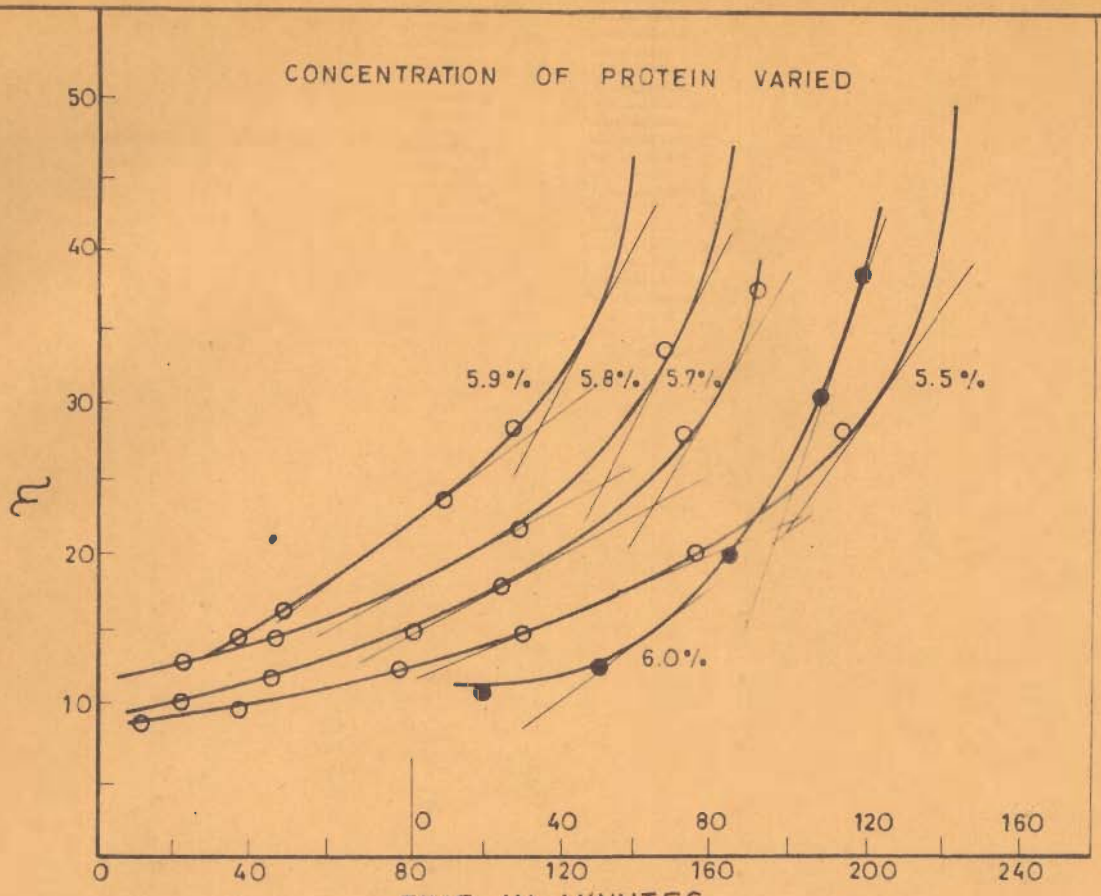


FIGURE 3

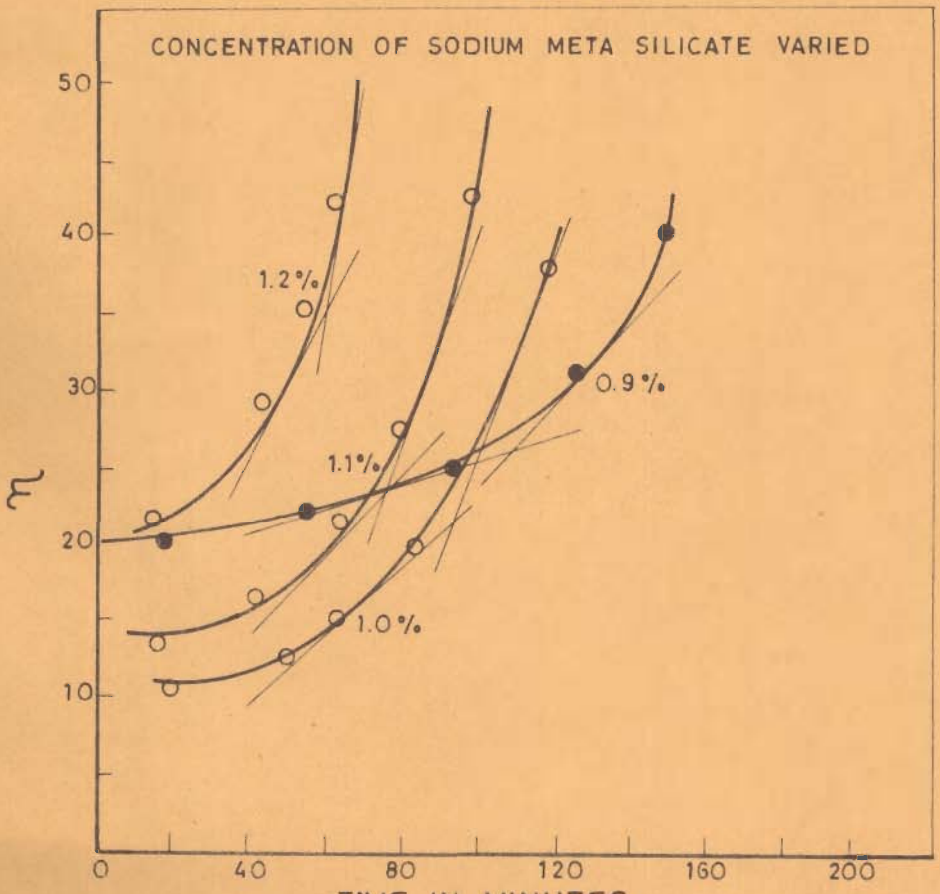


FIGURE 4

T_A_B_L_E 8

Concentration of casein solution =6.0 %

Concentration of formaldehyde =2.0 %

Sodium meta silicate =0.9 % Sodium meta silicate=1.0 %

Time in mts.	η	Time in mts.	η
18.0	20.545	20.0	10.522
56.0	21.954	50.0	12.218
95.0	24.694	65.0	15.312
128.0	31.230	85.0	19.543
150.0	40.309	120.0	38.074
185.0	-		
Time of gelation =108 mts.		Time of gelation =96 mts.	

T_A_B_L_E 9

Concentration of casein solution = 6.0 %

Concentration of formaldehyde = 2.0 %

Conc. of sodium meta silicate=1.1% Sodium meta silicate=1.2%

Time in mts.	η	Time in mts.	η
16.0	13.431	16.0	21.717
42.0	16.323	44.0	29.080
66.0	21.330	56.0	35.645
80.0	27.180	63.0	38.516
100.0	42.576	70.0	42.539
Time of gelation =80 mts.		Time of gelation =62 mts.	

Fig. 4.

T_A_B_L_E 10

Concentration of casein =6.0 %

Concentration of formaldehyde =2.0 %

Sodium hexyl sulphate=1.0%		Sodium hexyl sulphate=1.1 %	
Time in mts.	η	Time in mts.	η
24.0	7.586	10.0	7.939
50.0	7.901	45.0	8.050
75.0	8.235	65.0	8.559
90.0	8.551	90.0	8.790
115.0	8.896	120.0	9.125
150.0	9.344	160.0	10.046
190.0	9.889	180.0	12.857
250.0	10.568	250.0	25.778
No gelation		Gelation after 4 hrs.	

T_A_B_L_E 11

Concentration of casein = 6.0 %

Concentration of formaldehyde = 2.0 %

Sodium hexyl sulphate =1.2 %		Sodium hexyl sulphate=1.3 %	
Time in mts.	η	Time in mts.	η
15.0	8.584	5.0	22.941
45.0	9.734	28.0	23.174
75.0	11.738	50.0	32.449
120.0	15.603	60.0	-
150.0	20.004		
180.0	30.542		
Time of gelation=160 mts.		Time of gelation=55 mts.	

Fig. 5.

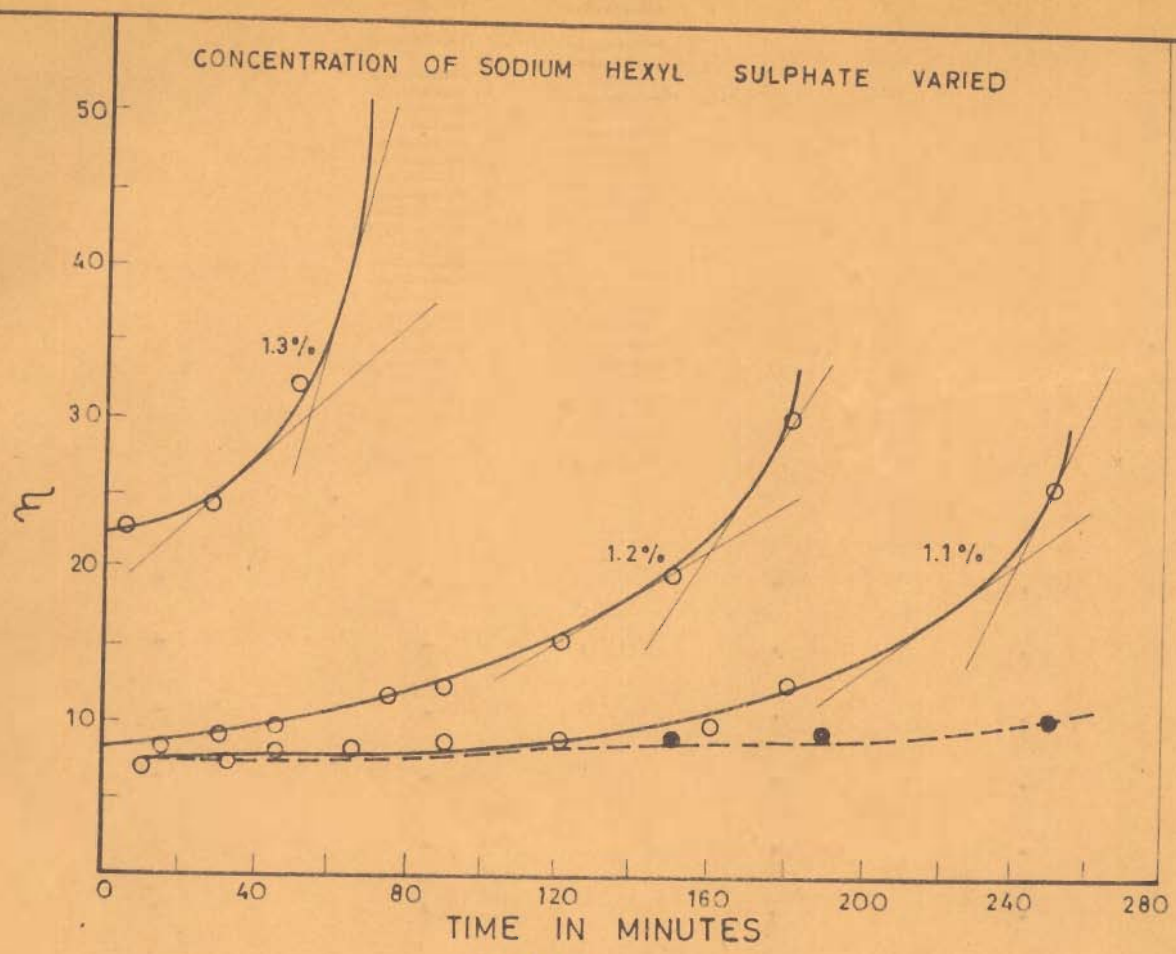


FIGURE 5

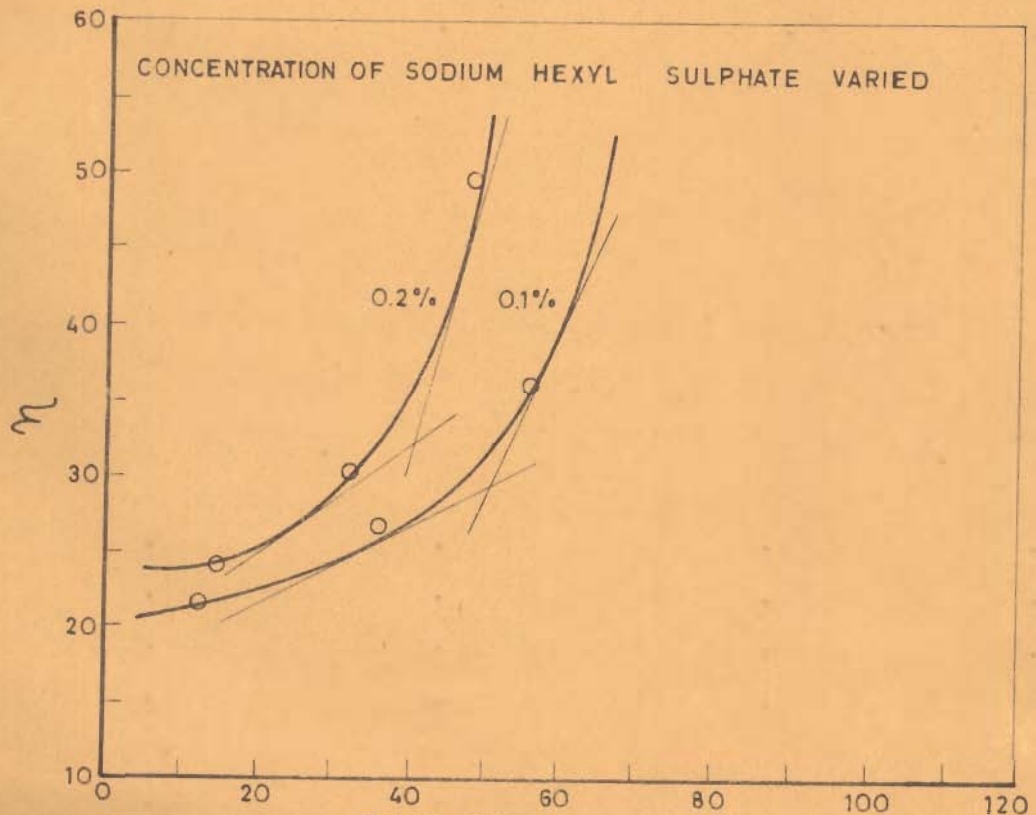


FIGURE 6

T_A_B_L_E 12

Concentration of casein = 6.0 %

Concentration of sodium meta silicate. = 1.0 %

Concentration of formaldehyde = 2.0 %

Sodium hexyl sulphate =0.1%		Sodium hexyl sulphate=0.2%	
Time in mts.	η	Time in mts.	η
12.0	21.757	15.0	24.272
36.0	26.996	32.0	30.444
55.0	36.509	48.0	50.298
70.0	-		
Time of gelation=50 mts.		Time of gelation 40 mts.	

Fig. 6.

On adding higher concentrations of sodium hexyl sulphate (0.25 % and 0.3 %) the whole mass coverns into a gel within 30.0 and 5.0 minutes respectively. Hence the measurements on the variation in viscosity could not be made.

T_A_B_L_E 13

Concentration of casein = 6.0 %

Concentration of sodium hexyl sulphate = 1.0 %

Concentration of formaldehyde =2.0%

Sodium metal silicate = 0.1% Sodium meta silicate =0.2 %

Time in mts.	η	Time in mts.	η
10.0	22.439	8.0	15.798
20.0	18.467	18.0	15.889
35.0	20.296	38.0	18.880
45.0	22.418	50.0	24.905
60.0	28.900	70.0	33.189
70.0	38.205		
Time of gelation = 65 mts.		Time of gelation =60 mts.	

T_A_B_L_E 14

Concentration of casein = 6.0 %

Concentration of sodium hexyl sulphate = 1.0 %

Concentration of formaldehyde = 2.0 %

Sodium meta silicate =0.25 % Sodium meta silicate =0.3%

Time in mts.	η	Time in mts.	η
6.0	18.768	15.0	24.260
24.0	21.395	30.0	29.900
38.0	24.711	39.0	30.231
55.0	30.160	47.0	50.321
70.0	45.231		
Time of gelation =50 mts.		Time of gelation =40 mts.	

Fig.7.

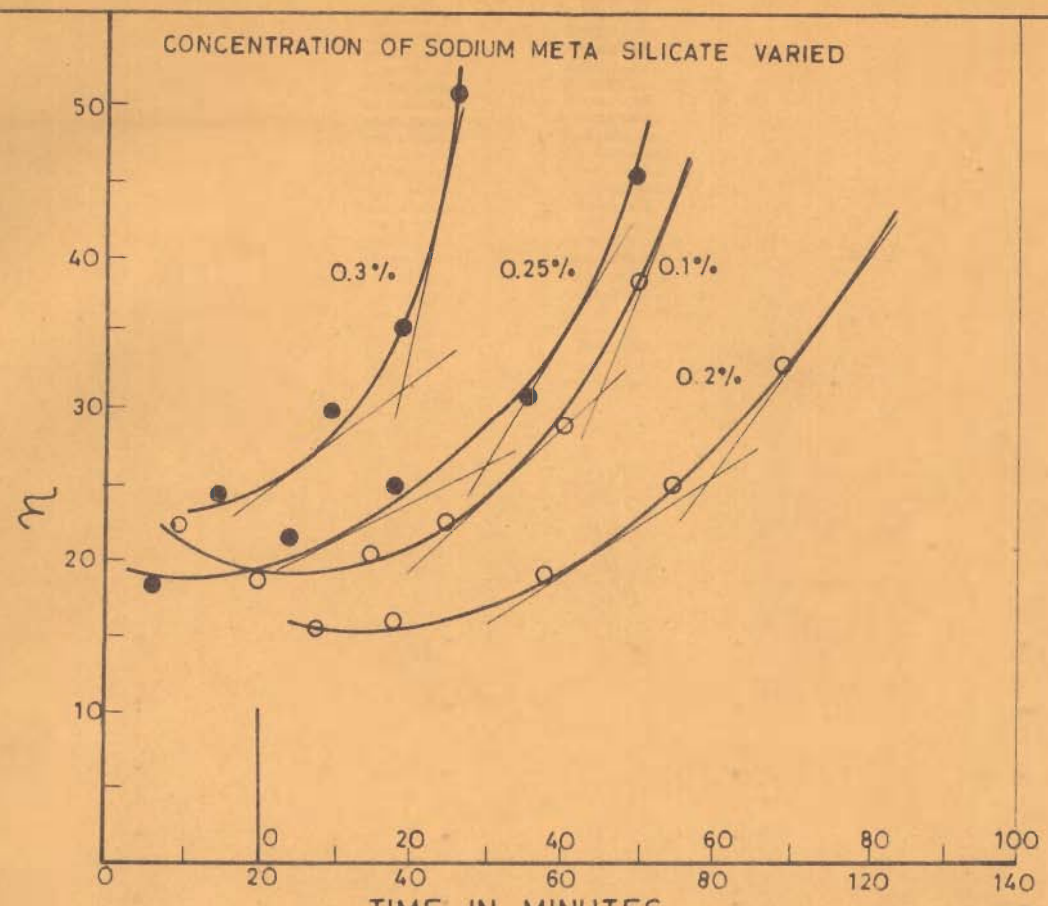


FIGURE 7

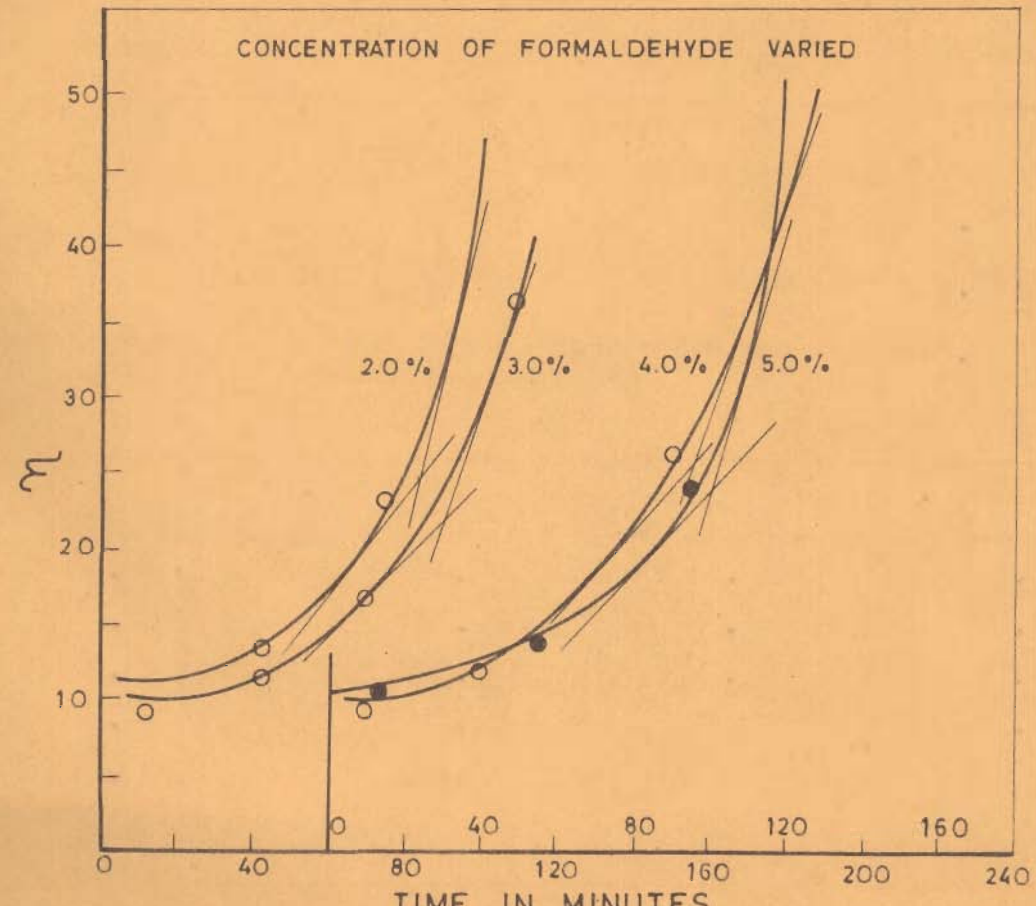


FIGURE 8

T_A_B_L_E 15

Concentration of α-casein =5.0 %

Concentration of sodium meta silicate =1.0 %

Formaldehyde =2.0 %		Formaldehyde =3.0%	
Time in mts.	η	Time in mts.	η
8.0	10.620	12.0	9.40
44.0	13.010	42.0	11.82
75.0	23.404	70.0	16.50
100.0	-	110.00	36.43
Time of gelation=86 mts.		Time of gelation = 92 mts.	

T_A_B_L_E 16

Concentration of α-casein = 5. 0 %

Concentration of sodium meta silicate = 1.0 %

Formaldehyde =4.0 %		Formaldehyde 5.0 %	
Time in mts.	η	Time in mts.	η
14.0	10.31	10.0	9.486
54.0	13.75	40.0	11.928
95.0	24.125	90.0	26.461
120.0	-	130.0	-
Time of gelation =96 mts.		Time of gelation= 100 mts.	

Fig. 8.

T_A_B_L_E 17

Concentration of sodium meta silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

α-casein =4.7 %		α-casein =4.8 %	
Time in mts.	η	Time in mts.	η
20.0	12.034	35.0	13.763
45.0	14.200	50.0	16.463
80.0	18.531	90.0	23.310
110.0	21.460	105.0	28.012
148.0	33.560	140.0	53.260
160.0	47.868		
Time of gelation = 128 mts.		Time of gelation=112 mts.	

T_A_B_L_E 18

Concentration of sodium meta silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

α-casein =4.9 %		α - casein = 5.0 %	
Time in mts.	η	Time in mts.	η
15.0	12.480	8.0	10.621
40.0	13.532	44.0	13.010
65.0	16.012	75.0	23.404
100.0	21.421	100.0	-
140.0	42.190		
Time of gelation = 98 mts.		Time of gelation = 86 mts.	

Fig.9.

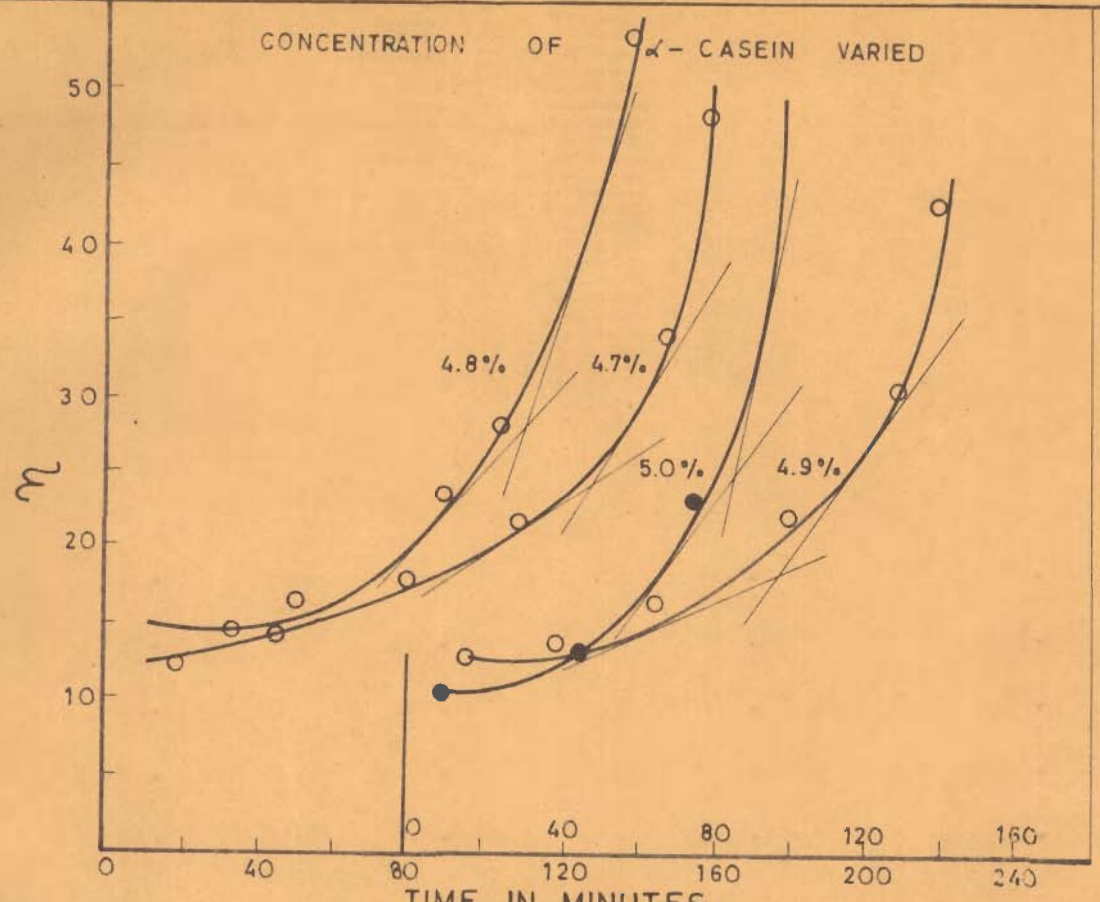


FIGURE 9

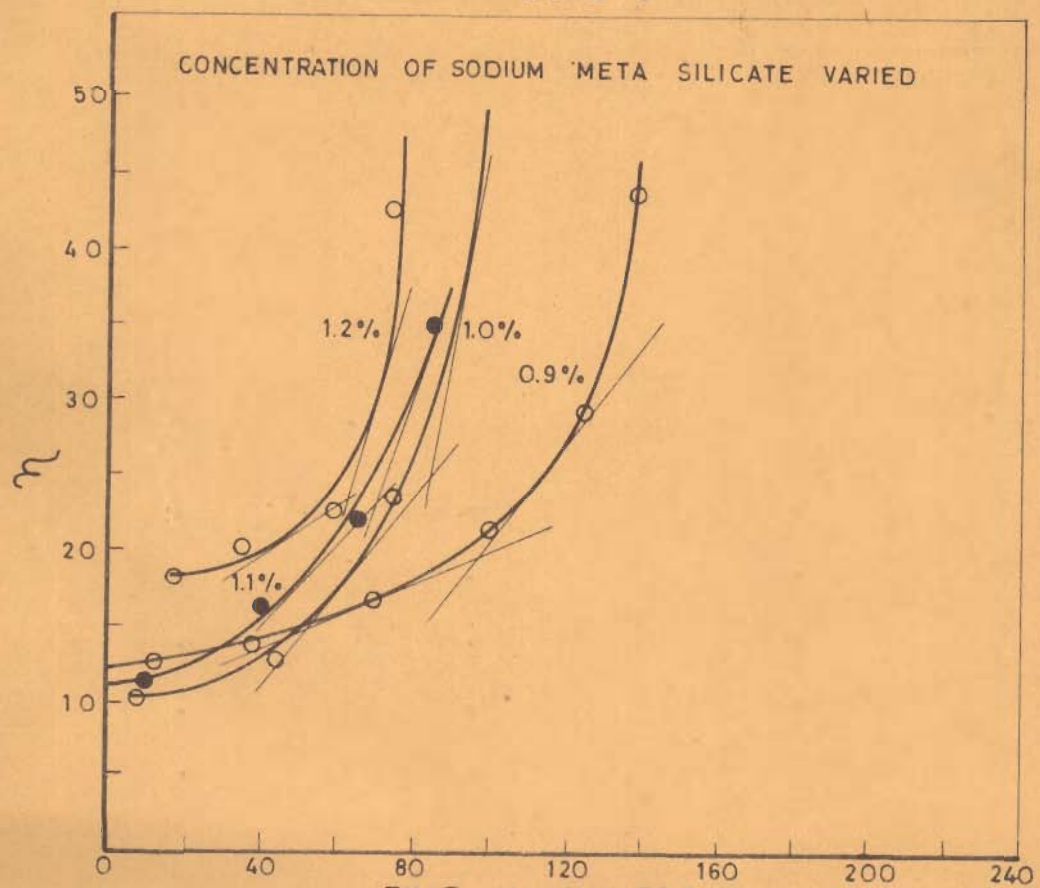


FIGURE 10

T_A_B_L_E 19

Concentration of α- casein = 5.0 %

Concentration of formaldehyde = 2.0 %

Sodium meta silicate =0.9 %		Sodium meta silicate=1.0%	
Time in mts.	η	Time in mts.	η
12.0	12.520	8.0	10.621
38.0	13.735	44.0	13.010
70.0	16.521	75.0	23.404
100.0	21.467	100.0	-
125.0	29.633		
140.0	43.401		
Time of gelation = 96 mts.		Time of gelation = 86 mts.	

T_A_B_L_E 20

Concentration of α-casein = 5.0 %

Concentration of formaldehyde = 2.0 %

Sodium meta silicate =1.1 %		Sodium meta silicate 1.2 %	
Time in mts.	η	Time in mts.	η
10.0	11.656	18.0	18.330
40.0	15.921	36.0	20.321
65.0	22.235	60.0	21.965
85.0	34.951	75.0	42.421
Time of gelation = 72 mts.		Time of gelation=64 mts.	

Fig. 10.

T_A_B_L_E 21

Concentration of α-casein =5.0 %

Concentration of formaldehyde =2.0 %

Sodium hexyl sulphate=1.0% Sodium hexyl sulphate=1.1 %

Time in mts.	η	Time in mts.	η
10.0	5.396	10.0	5.639
40.0	5.981	40.0	6.831
90.0	6.552	100.0	7.895
150.0	6.986	160.0	9.935
200.0	7.321	240.0	12.528
280.0	7.326	280.0	16.250
360.0	8.537	314.0	25.105
No gelation		Gelation after 5 hrs.	

T_A_B_L_E 22

Concentration of α-casein =5.0 %

Concentration of formaldehyde =2.0 %

Sodium hexyl sulphate = 1.2 % Sodium hexyl sulphate =1.3%

Time in mts.	η	Time in mts.	η
10.0	8.800	15.0	13.420
35.0	9.931	40.0	16.323
80.0	12.010	65.0	21.321
120.0	14.872	86.0	30.036
150.0	20.327	105.0	55.561
205.0	27.961		
230.0	47.261		
Time of gelation =180 mts.		Time of gelation =80 mts.	

Fig. 11.

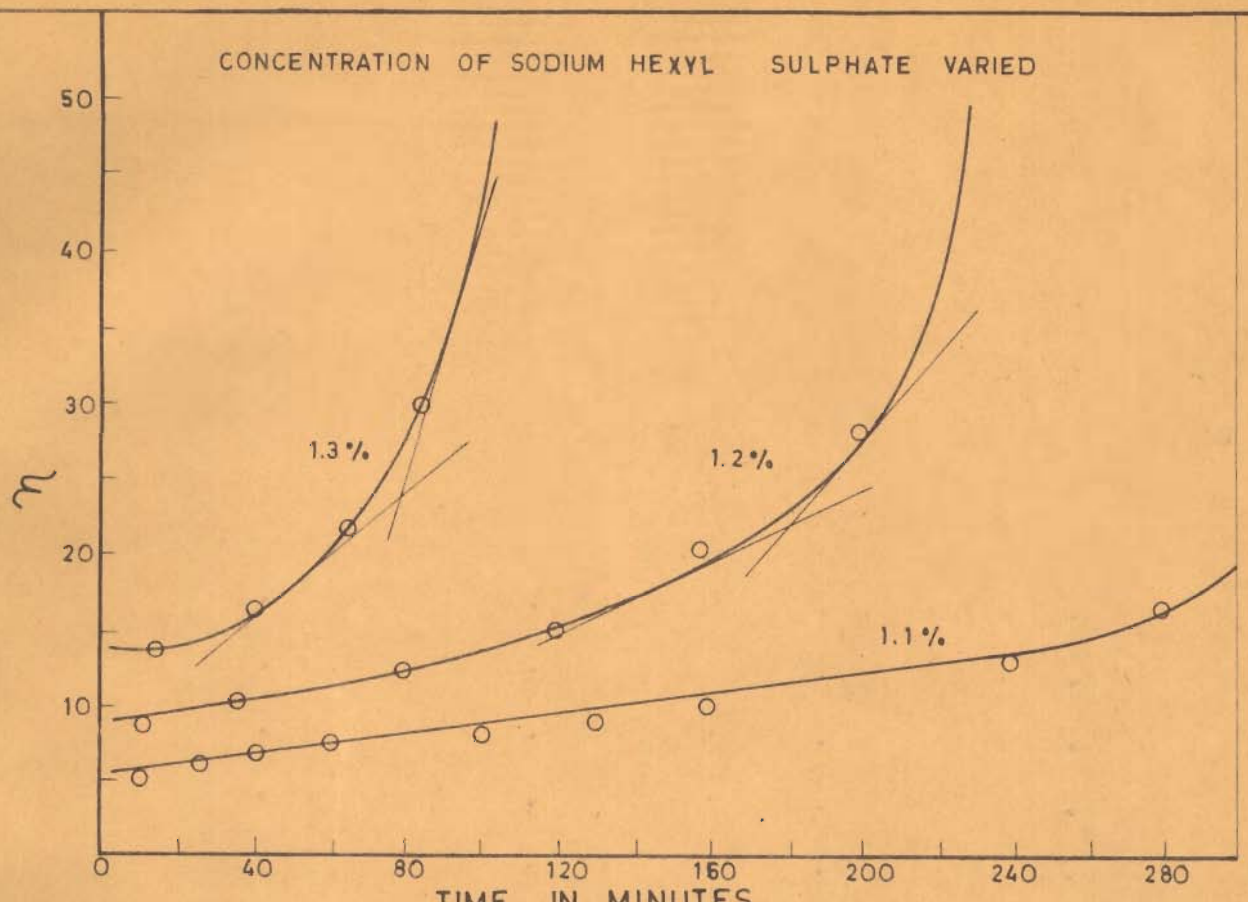


FIGURE 11

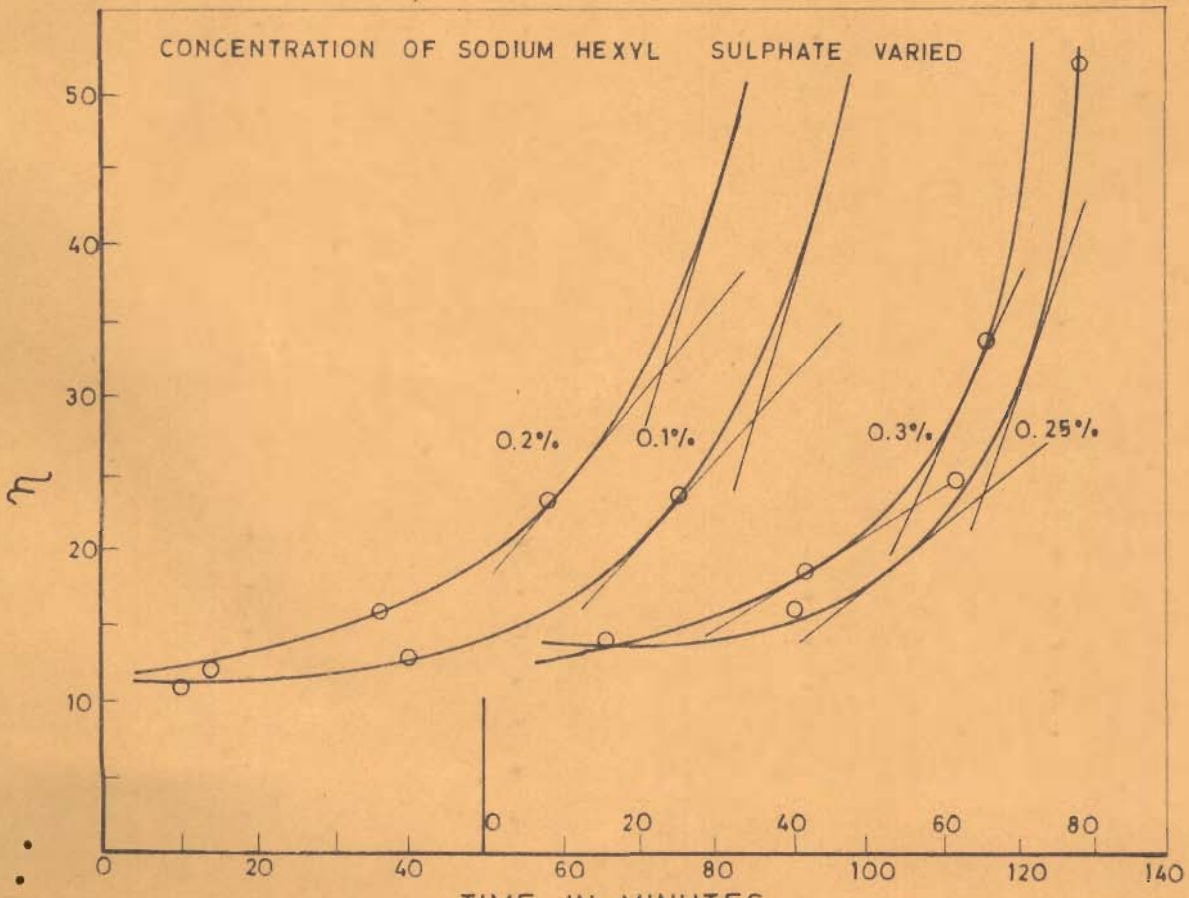


FIGURE 12

T_A_B_L_E 23

Concentration of α-casein = 5.0 %

Concentration of sodium meta silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

Sodium hexyl sulphate = 0.1 % Sodium hexyl sulphate=0.2%

Time in mts.	η	Time in mts.	η
10.0	10.746	14.0	11.808
40.0	12.890	36.0	15.672
75.0	23.389	58.0	22.856
98.0	-	84.0	-
Time of gelation = 86 mts.		Time of gelation =74 mts.	

T_A_B_L_E 24

Concentration of α-casein =5.0 %

Concentration of sodium meta silicate =1.0 %

Concentration of formaldehyde =2.0 %

Sodium hexyl sulphate=0.25 % Sodium hexyl sulphate=0.3 %

Time in mts.	η	Time in mts.	η
16.0	13.655	17.0	13.222
40.0	19.838	42.0	18.134
62.0	24.706	66.0	33.551
78.0	-	72.0	-
Time of gelation = 66 mts.		Time of gelation = 56 mts.	

Fig. 12.

T_A_B_L_E 25

Concentration of α-casein =5.0 %

Concentration of sodium hexyl sulphate =1.0 %

Concentration of formaldehyde =2.0 %

Sodium meta silicate=0.1 % Sodium meta silicate=0.2%

Time in mts.	η	Time in mts.	η
15.0	16.786	15.0	20.861
30.0	18.311	31.0	25.320
60.0	26.521	45.0	35.731
80.0	40.301	70.0	44.028
Time of gelation = 62 mts.		Time of gelation = 50 mts.	

T_A_B_L_E 26

Concentration of α-casein = 5.0 %

Concentration of sodium hexyl sulphate = 1.0 %

Concentration of formaldehyde = 2.0 %

Sodium metal silicate = 0.25 % Sodium meta silicate =0.3%

Time in mts.	η	Time in mts.	η
8.0	15.798	8.0	16.358
20.0	15.992	20.0	17.363
40.0	19.014	38.0	21.832
70.0	35.022	65.0	-
Time of gelation = 44 mts.		Time of gelation =34 mts.	

Fig. 13.

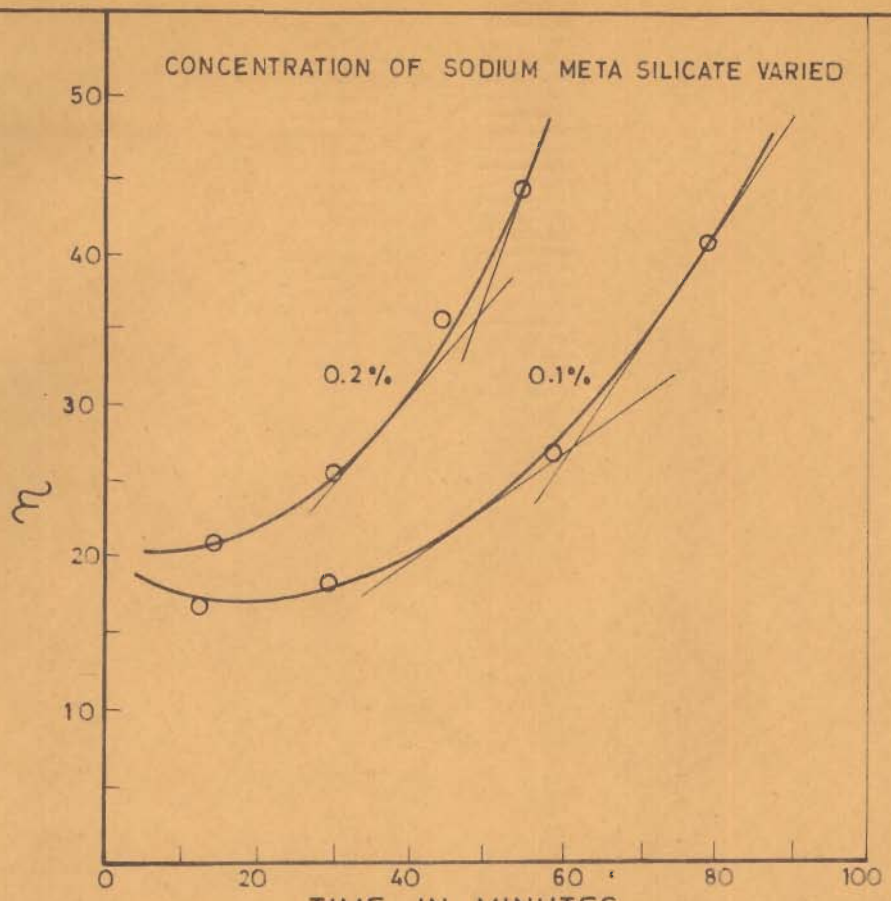


FIGURE 13

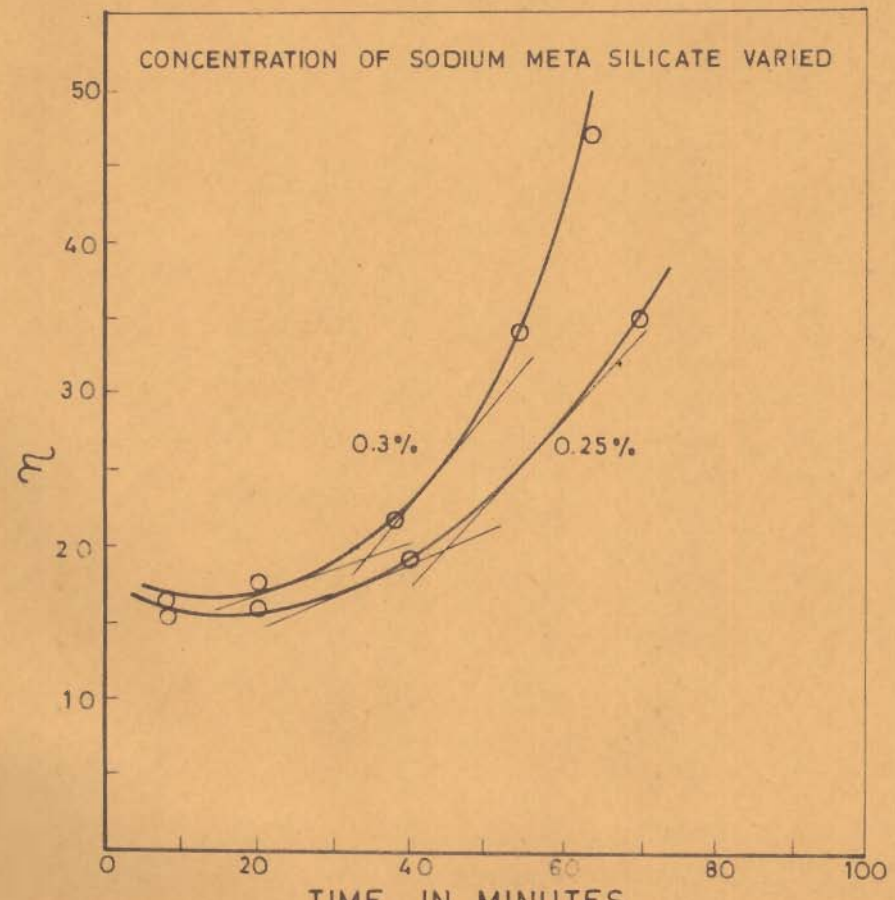


FIGURE 14

T_A_B_L_E 27

Concentration of β-casein = 5.0 %

Concentration of sodium metal silicate = 1.0 %

Concentration of formaldehyde = 2.0 %

Time in mts.	
22.0	3.319
44.0	3.678
68.0	3.963
90.0	4.050
125.0	4.255
161.0	4.732

No gelation

T_A_B_L_E 28

Concentration of formaldehyde = 2.0 %

Casein = 5.4 % Casein = 5.3 %

Sod.meta-silicate	Time of gelation	Sod.meta-silicate	Time of gelation
1.0 %	Abount 10 hrs.	1.0 %	About 16 hrs.
1.5 %	About 8 hrs.	1.5 %	About 14 hrs.
2.0 %	No gelation	2.0 %	No gelation
3.0 %	"	3.0 %	"
4.0 %	"	4.0 %	"
5.0 %	"	5.0 %	"

Below 5.3 % casein no gelation takes place

T_A_B_L_E 29

Concentration of formaldehyde = 2.0 %

Casein =5.4 %		Casein = 5.3 %	
Soap	Time of gelation	Soap	Time of gelation
1.0 %	13 hrs.	1.0 %	20 hrs.
2.0 %	8 hrs.	2.0 %	15 hrs.
3.0 %	7 hrs.	3.0 %	14 hrs.
4.0 %	6 hrs.	4.0 %	10 hrs.
5.0 %	5 hrs.	5.0 %	9 hrs.

At 5.2 % of casein and soap concentrations of 4.0 to 5.0 % gelation takes place in about 24 hours.

T_A_B_L_E 30

Set II % of formaldehyde	Time of gelation in mts.	Set III % of casein	Time of gelation in mts.	Set IV % of sod. meta silicate	Time of gelation in mts.	Set V % of soap	Time of gelation in mts.	Set VI % of soap	Time of gelation in mts.	Set VII % of sod. meta silicate	Time of gelation in mts.
2.0	96	5.0	9 No	0.9	108	1.0	No	0.1	50	0.1	65
3.0	112	5.5	180	1.0	96	1.1	4 hrs.	0.2	40	0.2	60
4.0	132	5.7	144	1.1	80	1.2	160	0.25	30	0.25	50
5.0	142	5.8	120	1.2	62	1.3	55	0.3	5	0.3	40
		5.9	112								
		6.0	96								
					α - casein						
2.0	86	4.7	128	0.9	96	1.0	No	0.1	86	0.1	62
3.0	92	4.8	112	1.0	86	1.1	5 hrs.	0.2	74	0.2	50
4.0	96	4.9	98	1.1	72	1.2	180	0.25	66	0.25	44
5.0	100	5.0	86	1.2	64	1.3	80	0.3	56	0.3	34

D_I_S_C_U_S_S_I_O_N

The results on the viscosity of the mixtures of protein(casein or α-casein) with formaldehyde,sodium meta silicate or sodium hexyl sulphate go to show that the time of gelation is highly dependent on the concentration of the constituents of the mixture.

For example, the addition of gradually increasing amount of formaldehyde to a mixture of protein and sodium meta silicate or sodium hexyl sulphate increases the time of gelation(table 30,set II). On the otherhand the effect of the increasing concentration of the other constituents (including casein) in mixtures containing fixed amount of formaldehyde is to decrease the time of gelation. In the latter case,that is for mixtures containing fixed concentration of formaldehyde,the optimum condition for gelation are: 5.5 % casein + 1.0 % sodium meta silicate + 2.0 % formaldehyde;and 6.0 % casein + 1.1 % sodium hexyl sulphate + 2.0 % formaldehyde. It is further interesting to note that the gelation does not take place at a lower protein concentration(5.5 %) in the above two sets but if the concentration of the sodium meta silicate or sodium hexyl sulphate is increased then gelation is achieved with mixtures containing casein upto 5.3 %(table 28 and 29). Below this concentration gelation is not possible on increasing the concentration of sodium meta silicate. On the other hand addition of more than 1.5 % of the reagent

brings about the peptisation of the mixture.

The behaviour of sodium hexyl sulphate is,however, altogether different. Here higher concentration of the soap helps in gelation(table 29). This effect is so marked that mixture containing 5.2 % casein can be brought into a semi-gel state on prolonged keeping(24 hrs) by the addition of 4 to 5 % of the soap.

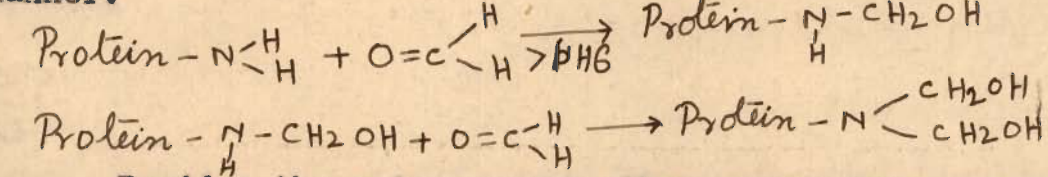
From the above results it is evident that sodium meta silicate as well as sodium hexyl sulphate are responsible for bringing about the gelation of casein which had been modified by the addition of formaldehyde. To check up whether the high alkalinity of the mixture due to the presence of these additives effects gelation,experiments under similar conditions were performed by adding caustic soda solution of about the same pH as sodium meta silicate and sodium hexyl sulphate. Under this condition no gelation was observed.

Far more interesting results are obtained when a a mixture of sodium hexyl sulphate and sodium meta silicate is added to casein formaldehyde mixture instead of using these reagents alone. From the results summarised in table 30 it may be seen that presence of extremely small amount of one reagent in the other,decreases the time of gelation considerably. For example,the addition of 0.1% soap to a mixture of 6.0 % casein + 1.0 % sodium meta silicate + 2.0 % formaldehyde reduces the time of gelation from 96 mts.

to 50 mts. The addition of larger amount of soap solution further decreases the time of gelation until immediate gelation is realised at 0.3 % concentration of soap solution.

Similar effects are observed when small amounts of sodium meta silicate are mixed with sodium hexyl sulphate(table 30,Set VII). Thus it is observed that the mixture containing 6.0 % casein,1.0 % sodium hexyl sulphate and 2.0 % formaldehyde does not undergo gelation. But the mere addition of 0.1 % sodium silicate changes it into a gel in about 65 minutes. On increasing the concentration of sodium meta silicate the time of gelation decreases, the values being 60,50 and 40 minutes in presence of 0.2%, 0.25% and 0.3% of the reagent.

The role of formaldehyde is well understood. The formation of modified protein is based on the formation of methylene linkages with the amino groups in the following manner:



Besides the amino groups other groups have also been reported to combine e.g., -SH of the denatured egg albumin and the imidazole group of the protein but this is not well established. Nevertheless the hardening of the casein fibre with formaldehyde depends on the formation of cross linkages as a result of the coupling of the amino

groups by the methylene bridges.

While considering the transformation of casein into some other material to be work out as substitute for fibre, the following factors should be taken into consideration: (i) complete denaturation of the protein; (ii) its proper dispersion so that smooth transformation from the sol to the gel state may takes place;(iii) formation of a three dimensional structure importing sufficient tensile strength to the fibre.

Sodium meta silicate particle due to the negative charge on their surface would be favourable to the maximum dispersion of negatively charged casein particles. However, when added in large amount interaction with protein involving the formation of hydrogen bonds between the nitrogen of the protein and the hydrogen of the silanol group would take place(329). This would be followed by the polymerisation of silica forming an in soluble net work throughout the silica-protein complex.

The role of sulphonated detergents is more or less the same as that of sodium meta silicate. These soaps besides denaturating the protein are known to form viscous solutions in which the protein molecules are present as extended chains.

The need of having sodium meta silicate or sodium hexyl sulphate in casein-formaldehyde mixture to make it suitable for gelation and the decrease in the time of

gelation with the increase in their concentration as observed in these investigations find support in the above mechanism.

It appears that the soap micelles rather than the soap anions are responsible for the gelation of casein formaldehyde mixture. This viewpoint finds support from the fact that the critical concentration at which soap micelles begin to form is lower in the presence of the silicate (330). It is for this reason that the presence of extremely small amount of sodium silicate in sodium hexyl sulphate brings about the gelation with smaller conentrations of the soap than when used as such(table 30,Set VII).

Attention has already been drawn to the fact that after the interaction of the silicic acid with the protein the next important step is the polymerisation of the silica-protein complex.The presence of soap would help in the formation of this net work. As to why the presence of a little soap in sodium meta silicate enhances the process of gelation can be explained on the basis.

To sum up the role of the different constituents in a casein fibre bath is as follows: The formaldehyde modifies the protein with the formation of cross-linkages by the coupling of the amino groups by the methylene bridges. Sodium meta silicate is useful both for dispersing the casein and its subsequent gelation to give an in soluble silica-protein net work. The part played by sodium hexyl

sulphate is more or less the same. Best results can be achieved when mixtures of both the additives are employed since the presence of each of them in small quantity remarkably influences the property(critical micelle concentration or degree of polymerisation) of the other.

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R_E_S_U_M_E

The advancements made in the field of metal-protein interactions are of relatively recent origion. Many well known workers,viz.,Linderstrom-Lang,Cannan, Scatchard,Tanford etc.(1922,1945,1949,1952,1954-58) have contributed to the theoretical as well as applied aspects of the problem,and in many cases results of far reaching importance have been achieved. The results obtained with simple glabular proteins,based on the use of different physico-chemical techniques,may be divided under three sub-heads:(i) the investigation of Klotz (1942-48) and co-workers on the binding of cupric ions to bovine serum albumin;(ii) Tanford's work (1952-54) on the polarography of Copper,Zinc,Cadmium,Lead and Thallium complexes with bovine serum albumin,(iii) the investigation of Gurd and coworkers(1948-52) on metal-protein interaction using the technique of equilibrium dialysis.

Until recently very little work was done to study the binding of metal ions with fibrous or collagen type of proteins. Malik and Co-workers(1960-65) made some contribution in this direction using transfusion gelatin(obtained from bone gelatin and used as a plasma expender). With this well characterised fibrous protein they had successfully studied the binding of a large number of heavy metal ions employing the polarographic,pH metric,equilibrium dialysis and spectrophotometric methods. These authors have

investigated another aspect of the problem of metal-protein binding, viz., the availability of metal ions from hydrous oxide sols for interaction and which according to them might provide the basis for the use of metal oxides used (Bhasmas) by the Indians for the treatment of many ailments, especially pertaining to the nervous system.

The work described in the thesis deals mainly with the binding of metal ions of physiological importance with transfusion gelatin (extension of the earlier work) and α_s-casein and whole casein (studies taken up for the first time). Another portion of the thesis is devoted to the studies on the interaction of silicic acid sol and Tabashir (naturally occuring form of pure silica used as a medicine in the country) with transfusion gelatin while the remaining part deals with the studies on the gelation of casein formaldehyde mixtures in presence of sodium meta silicate and sodium hexyl sulphate.

Part A.

Transfusion gelatin:- The interactions of magnesium, manganese and strontium by pH metric method and magnesium, nickel and cobalt by equilibrium dialysis studies have been studied. The hydrogen ion titration curves of transfusion gelatin in presence and absence of metal ions are quite different from each other indicating thereby the binding of these metal ions with the protein. The following conclusions have been arrived at (i) the amount of hydrogen

ions given out by the protein are greater in presence of metal ions than with the protein alone. This would be the case if replacement of hydrogen ions by metals is visualised from the site under consideration;(ii)the extent which a particular metal ion displaces the hydrogen ion equilibria of transfusion gelatin towards the basic side of the functional groups,may be taken as a measure of metal-protein binding. Assuming that one-to-one binding is favoured(according to Gurd and Murray) in preference to intermolecular cross-linking than the number of hydrogen ions displaced per protein molecule,directly yields the binding data V_M ,(iii) the results reveal that these metals combine mainly with the carboxyl groups of transfusion gelatin. The log K values between these metal and carboxyl groups comes out to be nearly equal to the first association constant between the acetate nucleus and the corresponding metal ions. This similarity indicates a 1:1 stoichiometry of these interactions.

Magnesium,nickel and strontium show binding with the imidazole groups of transfusion gelatin while manganese and cobalt seem to have little affinity as their log K values for metal-imidazole groups are quite low as compared to the log K of the interaction of these metals with free imidazole nucleus.

α_s-casein and casein:- The interaction of copper,zinc, magnesium,manganese and strontium with both casein and α_s-casein has been studied employing the pH-metric method.

These studies reveal that all these metals combine with the carboxyl groups of the α_s-casein, just as in the case of transfusion gelatin, their log K values compare favourably with the corresponding log K values for the combination of these metal with free acetate nucleus and also the log K values obtained in case of transfusion gelatin with these metal ions. The close similarity regarding the binding of the metals with the carboxylic groups in the two cases (α_s-casein and transfusion gelatin) is significant. With α_s-casein the log K value obtained in the case of manganese - imidazole binding is quite low as compared to the value of the first association constant between manganese and free imidazole nucleus. This shows that in the interaction of proteins, manganese shows little tendency to bind with the imidazole groups-a behavour different from that of transfusion gelatin. This dis-similarity in behaviour is much more marked when considering the interaction of magnesium with α_s-casein in the higher pH range. In α_s-casein it does not show any binding to the imidazole groups, while in the transfusion gelatin a definite binding (confirmed by pH-metric and equilibrium dialysis methods) is exhibited.

The polarographic studies were applied to the study of the interaction between α_s-casein and the copper ions. Tanford's method was found to be suitable and yielded useful results. For calculating binding data Tanford's equation was employed,

$$(id)/(id)_0 = \frac{CF+K\ Cb}{Co}$$

where (id) and $(id)_0$ are the diffusion currents in presence

and absence of metal ions, C_O, C_F and Cb are the total, free and bound metal ions. The intrinsic association constant were calculated by applying Scatchard's equation,

$$K = \frac{V_M}{(n - V_H - V_M)C_F}$$

where V_M and V_H are the sites covered by metal and hydrogen ions respectively, n is the number of such sites, and C_F is the concentration of free metal ions at equilibrium.

The log K values drived for various metal ions by different methods are summarised below:

The log K values for metal-carboxyl groups

Method	Protein	Cu^{2+}	Zn^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}	Ni^{2+}	Co^{2+}
pH-metric	T.gelatin	2.10	1.87	1.81	1.48	1.33	1.86	2.02
Equi-dialysis	do	2.18	1.87	1.85	-	-	1.49	1.75
pH-metric	α_s-casein	2.27	1.94	1.65	1.65	1.33	-	-
Polaro-graphy	do	2.23	-	-	-	-	-	-

log K for metal-imidazole group binding

pH-metric	T.gelatin	3.28	2.74	3.19	3.12	2.77	-	-
Equi-dialysis	do	3.40	2.91	3.36	-	-	3.23	3.52
pH-metric	α_s-casein	3.57	3.06	-	2.82	2.67	-	-
Polaro-graphy	do	3.25	-	-	-	-	-	-

The binding in case of whole casein was calculated per 10^5 gms of protein due to the uncertainty about its molecular weights. Though these results are not of much quantitative value,but even then they gave clear indication of the binding of these metal ions with the carboxyl groups and the imidazole groups of casein,except the case of magnesium.In which the magnesium-imidazole binding is not favoured(table 38,chapter 3).

Part B.

In this part the studies on the interaction of silicic acid and tabashir sols with transfusion gelatin carried out pH metrically has been described. The most interesting aspect of these studies is that the reaction undergoes a radical change within a small pH range.It is thus observed that silicic acid sol of pH 2.0 combines with the carboxyl group of the protein and that of pH 2.5 shows binding with the imidazole,amino and guanidinium groups of the protein. This difference in behaviour has been attributed to the nature of the charged particles in the two cases. At pH 2.00 or below,the H^+ ions are adsorbed on the silicic acid sol with possible SiH groups on its surface. The hydrogen ions on the sol particles compete with the hydrogen ions of the carboxyl groups of the protein,thereby making themselves available for binding (page 176,chapter 5)..

At higher pH values,2.5 and above,the silanol groups(SiOH) on the surface of the silica particles would combine with the protein. Since the shift in the curves

is realised in the pH range 8 to 10, strong evidence of the binding of silicic acid with the nitrogen of the protein is obtained. This combination involves hydrogen bonding between the hydrogen of the silanol group and the nitrogen from the amino, imidazole or other nitrogen containing groups of transfusion gelatin.

The results with tabashir sols of pH 2.5 and 3.0 are just the same as for the silicic acid sol of pH 2.5. There is, however, one point of some significance which differentiates the behaviour of the two. In the case of silicic acid better binding is realised only at higher concentrations whereas in the case of tabashir it is possible even at lower concentrations (table 1 to 8 chapter v) . This has been attributed to the greater purity of tabashir sol as compared to the silicic acid sol obtained from the commercial meterial.

The second chapter of Part B deals with the viscometric studies on the sol-gel transformation of casein-formaldehyde mixtures in the presence of sodium meta silicate or sodium hexyl sulphate or in the mixture of the two. These studies were undertaken to setforth the role of various constituents of the casein mixtures used for the manufacture of casein fibre. The possibility of the use of sodium silicate in place of sodium aluminate in obtaining the viscose solution of casein has been indicated. Both sodium meta silicate and sodium hexyl

sulphate can be employed for obtaining the viscous material: the composition of the mixtures giving optimum results are as follows:

6.0 % casein + 2.0 % formaldehyde and 1.0 % sodium meta silicate.

6.0 % casein + 2.0 % formaldehyde and 1.1 % sodium hexyl sulphate.

The role of sodium meta silicate or sodium hexyl sulphate appears to be twofold, viz., proper dispersion of the protein modified by formaldehyde and its denaturation. The process of denaturation involves interaction of the nitrogen of the casein with these additives. This is followed by the formation of an insoluble net work structure(page 204, chapter VI).

The other interesting aspect of these studies is that the presence of small quantities of either of the two additives helps in the gelation of the casein mixtures (table 30, chapter VI). This may be due to the decrease in the c.m.c. of the soap in presence of sodium meta silicate, when the soap is the main additive and an increase in the degree of polymerisation of silicic acid in presence of small amounts of soap when the former is used to bring about gel formation.

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LIST OF PAPERS PUBLISHED

1. pH-metric studies on the interaction of magnesium, strontium and beryllium with casein and α-casein. J.I.C.S. 42, No.10, 1965, p. 681.

2. pH-metric studies on the interaction of magnesium, manganese and strontium with transfusion gelatin. (In press).

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2. Studies on the interaction of magnesium, nickel and cobalt with transfusion gelatin.

3. Interaction of metal ions with α_s-casein.

4. Viscosity changes during sol-gel transformation of casein or α-casein and formaldehyde mixtures.

5. Interaction between silicic acid or tabashir sols with transfusion gelatin.

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