MICROPOTENTIOMETRIC STUDY OF CHLORIDE BINDING TO MYOGLOBIN

BY

HASSAN A. TAYIM

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A C K N O W L E D G M E N T

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A B S T R A C T

The present work deals with the detection and evaluation of the binding of chloride ions to sperm whale ferrimyoglobin in aqueous solution.

A micropotentiometric technique was developed which uses a highly sensitive micropotentiometer in conjunction with a locally constructed null detector sensitive to D.C. signals of few microvolts. The method involves measurement of E.M.F. attained by silver/silver chloride electrodes placed in a concentration cell comprising two chloride solutions of the same concentration, with myoglobin in one solution but not the other. A modified design of platinum electrodes has been used and the optimum conditions for silver and silver chloride deposition have been determined. A new and more efficient method for cleaning the electrodes has been developed. Different cell types were tried leading to a new design of a semi-micro cell with capillary liquid junction, which is simple to handle and thermostat.

Experiments were carried out under varying conditions of salt and protein concentrations, pH, ionic strength and temperature.

The results indicate that myoglobin not only binds chloride ions but also produces a large negative liquid junction potential in the cell. The data obtained could best be fitted by a model with three classes of chloride binding sites: one site with very strong, six sites with weak, and thirteen sites with very weak affinity for chloride ions. The binding (association) constants have been computed, and the results correlated with the known chemical structure of the protein.

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I N T R O D U C T I O N

The subject of protein interactions covers a wide range of phenomena which are of fundamental chemical and biological significance. Interactions vary from highly specific ones, as between an antigen and its antibody or an enzyme and its substrate, to the general nonspecific binding of small molecules and ions, and from the exceptionally strong binding, as between metal and protein in hemoproteins, to the very weak binding of certain ions and molecules such as chloride and iodide to albumin. It is in this latter type of relatively weak reversible interaction that the anion binding to protein is normally regarded.

The binding of inorganic ions to proteins was once thought to be non-existent or negligible and hence unimportant. However, recent work has shown that this is not the case and there has been a growing need for precise determinations of the number of ions bound, their free energies of binding, the sites of interaction on the protein surface as well as the nature of the forces involved and their dependence on the medium and other factors. Scatchard[1] first drew attention to this need in an early review of the subject in which he stated: "We want to know of each molecule or ion which can combine with a protein molecule, How many? How tightly? Where? Why?"

The reasons for this growing interest in the subject are not far to seek. From the physico-chemical point of view, a protein molecule in solution is a highly complex but ordered structure, with

various hydrophilic and mostly charged side groups protruding into the medium, equilibrated with hydrogen ions, hydroxyl ions and water molecules as well as other ions in solution, and attracting electrostatically a sheath of counter-ions which forms an electrical double layer around the protein. Solutions of neutral salts are needed to preserve the protein molecule in its native state. Any interaction between protein and ions which exceeds the simple electrostatic balance at the double layer will result in binding through ion-pair formation or some other mechanism. This interaction will alter the ionic strength of the medium as computed on the basis of the total molar concentration of the neutral salt, and will also alter the nature and the extent of charge distribution on the protein surface, thereby affecting the activity coefficient of ionic species involved, as well as their electrochemical properties.

Such effects of binding have indeed been encountered in practice. They were manifested as anomalous results in the physicochemical studies of proteins in salt solutions. In order to preserve the protein molecule in its native state during investigation, it has been common practice to carry out investigations on protein solutions in the presence of varying salt concentrations, most commonly potassium and sodium chlorides. Anomalous observations obtained under these conditions could only be interpreted by assuming that salt ions are bound by the protein molecules. For instance, Scatchard's first experience with combination of protein with molecules other than hydrogen or hydroxyl ions came with measurements of the osmotic pressure of serum albumin in solutions of sodium chloride.[2,1] Edmundson

and Hirs[3] had to assume the binding of anions by myoglobin in order to explain the low weight recovery of the protein from chromatographic columns. These and similar observations[4] made it essential to understand the nature of small ion interactions with proteins.

The problem is further complicated by the fact that, from the very nature of the equilibria, the proportion of ions bound is greater the smaller the concentration of the ions. Hence, for accurate measurements the technique used should be of extremely high precision, and this is by no means simple.

A variety of methods have been applied to the study of protein interactions. These have been conveniently reviewed by Klotz[5] who classified them into two main categories: Those dependent on changes in the behaviour of the protein, and those dependent on changes in the properties of the interacting molecules (small ions).

Under the first category he includes methods of investigation of changes in pH, optical properties (spectrophotometry, refractometry, light scattering and optical rotation), osmotic pressure, sedimentation, electrophoresis, precipitation, viscosity, surface tension, magnetic properties and biological activity.

Under the second category are included methods utilizing investigation of changes in solubility, migration in electric field, polarographic reduction, diffusion, changes in spectra, biological activity and reduction in the thermodynamic activity of the combining small molecules or ions, as determined by measurement of free ion concentration or activity by equilibrium dialysis, ultrafiltration, ultracentrifugation, distribution between phases or electromotive force

methods.

Among these methods only few have been applied to the study of small ion binding to proteins. The investigation of changes in pH has been applied most extensively to the study of acid-base properties of proteins, i.e. to the study of hydrogen ion and hydroxyl ion binding. However, its application as a tool for the investigation of the binding of other ions was only a byproduct of acid-base investigations. In fact, ion binding (other than hydrogen and hydroxyl ions) had to be assumed in order to account for some anomalous results observed in protein titration curves. Among classical contributions in this respect one may mention the work of Steinhardt[6] on wool, where the author interpreted his data on the basis that anions as well as hydrogen ions are bound by the proteins in the acid portion of the titration curve; the investigations of Cannan, Kibrick and Palmer[7] on egg albumin and on β-lactoglobulin in the presence of potassium chloride, the extensive studies of Tanford[8], and the recent review of Scatchard, Hughes, Gurd and Wilcox.[9]

The method of equilibrium dialysis is by far the most commonly applied method for the investigation of the binding of ions, other than hydrogen and hydroxyl ions, to proteins. It has been extensively used by Klotz, Walker and Pivan,[10] and Scatchard and coworkers.[11,12] A disadvantage of this method is that the measured binding represents total cation and anion binding. Furthermore Donnan equilibria complications limit the validity of the application of this method under certain conditions.

The method of osmotic equilibria is one that has been applied

almost exclusively by Scatchard and co-workers[13,14] to the study of small ion binding to proteins.

Electrophoretic investigations as applied to binding measurements have appeared in a few recent papers, for instance the work of Luck,[15] who also used ultrafiltration. His studies, however, were only qualitative. Velick[16] applied electrophoresis and obtained results in agreement with those obtained by the method of equilibrium dialysis in his work on the enzyme aldase in phosphate and acetate buffers.

Spectrophotometric methods have been applied by Klotz[17] as well as by Klotz, Triwush and Walker.[18]

Of all the methods, the electromotive force method is the most accurate and gives direct measurement of the binding of a particular ionic species, namely the one to which the electrode used is reversible. Nevertheless, it should be noted that it cannot be applied reliably at very low electrolyte concentration, or at high electrolyte concentration where E.M.F. changes are very small and inaccurate.

This method has been used most widely in quantitative studies of acid-base properties of proteins. Bugarszky and Liebermann (1898) were the first investigators to demonstrate by E.M.F. studies that proteins combine with acids and bases.[19] They measured the E.M.F. between two hydrogen electrodes, one immersed in a cell containing 0.05N hydrochloric acid, the other in a cell containing the same concentration of the acid and varying amounts of egg albumin. From the initial concentration of the acid and the E.M.F. measurements

they could calculate the concentration of free acid, and hence the bound acid. Their values are only slightly different from those obtained later by the most careful investigators.[20] The same method was adopted by Cohn and Berggren in 1924[21] in their studies on casein in alkaline solutions, and later by Hitchcock (1932-1933),[22] who used silver/silver chloride reference electrodes and came to the conclusion that chloride ions are bound to casein, edestin, and gelatin molecules.

However, the first quantitative study of anion (other than hydroxyl ions) binding to proteins by direct E.M.F. measurements, is that of Scatchard (1950).[11] He used two silver/silver chloride electrodes immersed in sodium chloride solutions of equal concentrations but one containing, in addition, human serum albumin solution. He calculated the extent of binding of chloride and thiocyanate ions to albumin and by comparing results obtained by E.M.F. measurements to those obtained on the same solutions by equilibrium dialysis method, he came to the conclusion that albumin does not bind sodium cations.

Scatchard has recently[23,24] developed E.M.F. cells for the investigation of binding, which utilize anion exchangers as electrodes. It is claimed that these electrodes give more reliable results than conventional electrodes. However, conventional electrodes still have the advantage of being reversible only to one ionic species, while anion exchangers have not yet been made extremely selective. Results of investigations of anion binding to albumin, as reported by various investigators, are controversial. Even reports

of the same investigator regarding the same problem are controversial.[11,23] This calls for both refinement of the experimental technique and more care in experimental procedure; two factors which have been kept in mind in carrying out the present work.

The present work deals with the binding of chloride ions to ferrimyoglobin, a system not hitherto examined by any of the methods described above. Apart from its general interest, there are three reasons for the choice of this system:

(1) The detailed structure of crystalline myoglobin including its amino acid sequence has been made available by X-ray analysis at about 2Å resolution.[25] It remains to find out how its structure in solution is related to its structure in the crystalline solid state.

(2) Potassium and sodium chlorides are the salts usually present in protein solutions, under physiological conditions as well as in experimental work. Hence the need for precise data of the extent of binding of the ions of these salts to protein. The study of chloride binding may also throw light on the question of charge distribution on the surface of the protein.

(3) Fortunately, silver/silver chloride electrodes are reasonably stable and free from trouble, hence accurate E.M.F. data can be obtained.

If this technique succeeds, and reliable concordant data are

obtained on myoglobin, then, similar experiments carried out on hemoglobin, globin, as well as on their modified proteins should be of help in determining differences in charge distribution on protein surfaces. The extent to which this is possible can be deduced from the results obtained in the following chapters.

THEORETICAL

In the potentiometric method of measuring chloride binding to proteins, the object is to determine the concentration of free (unbound) chloride ions in a solution of a chloride salt (potassium chloride) which also contains a known concentration of the protein. For this purpose, a concentration cell is set up comprising two silver/silver chloride electrodes which, being of the second order, are reversible only to chloride ions. With equal potassium chloride concentrations (activities) in the two half-cells the electrodes should develop identical potentials. However, if the protein (which is in the solution of one of the two half-cells) binds chloride at some or all of its free cationic side groups, the concentration of chloride in that solution inevitably decreases, and as a consequence a potential difference between the two electrodes develops. This cell E.M.F. gives a direct and sensitive measure of the free energy of the binding process if it is assumed that the protein produces no other effect on the electrode and junction potentials in the cell.

E.M.F. of Concentration Cell

The Nernst equation for the reversible potential of a metal-metal ion electrode is:

$$E_{M,M^{n+}} = E^{\circ}_{M,M^{n+}} + \frac{RT}{nF} \ln a_{M^{n+}} \qquad (1)$$

where $a_{M^{n+}}$ is the activity of the metal ion and $E^{\circ}_{M^{n+}}$ is the standard electrode potential i.e. at standard state of unit metal ion activity.

The potential of silver/silver chloride electrode, reversible to chloride ions, may be derived by considering first that the electrode is reversible to silver ions. Thus,

$$E_{Ag,Ag^+} = E^{\circ}_{Ag,Ag^+} + (RT/F)\ln a_{Ag^+} \qquad (2)$$

But, the activity of silver ion is controlled by the solubility equilibrium of solid silver chloride. Thus,

$$a_{Ag^+} = K_s/a_{Cl^-}$$

where K_s is the activity solubility product of silver chloride. Substituting above, we may write the potential of silver/silver chloride electrode as,

$$E_{Ag,AgCl} = E^{\circ}_{Ag,Ag^+} + (RT/F)\ln K_s - (RT/F)\ln a_{Cl^-}$$
$$= E^{\circ}_{Ag,AgCl,Cl^-} - (RT/F)\ln a_{Cl^-} \qquad (3)$$

The standard potential of silver/silver chloride electrode, namely, $E^{\circ}_{Ag,AgCl,Cl^-}$ in equation (3), defined following the Stockholm Convention, is the E.M.F. of the hypothetical cell

$$H_2(\text{ideal, 1atm}),Pt^-/HCl(\text{aq.ideal, m=1})/AgCl/Ag^+$$

in which all the reactants are in their standard states. Bates & Bower[26] have determined the E.M.F. of this cell for the temperature range of 0°-$95^{\circ}C$

$$E^{\circ} = 0.23659 - 4.856\times10^{-4}t - 3.4205\times10^{-6}t^2 - 5.869\times10^{-9}t^3 \qquad (4)$$

If two identical silver/silver chloride electrodes are placed in two chloride solutions of different activities, a_1 and a_2 say, then electrode potentials attained in solution (1) and solution (2) are:

$$E_1 = E^{\circ} - (RT/F)\ln a_1$$

$$E_2 = E^{\circ} - (RT/F)\ln a_2$$

respectively. For a hypothetical cell of the form

$$Ag/AgCl/KCl_{aq}\,a_1 \;\vdots\; KCl_{aq}\,a_2/AgCl/Ag$$

a_2 greater than a_1

(the dotted vertical line indicating the liquid-liquid junction), the E.M.F. is given by:

$$E_1-E_2 = E^o-(RT/F)\ln a_1 - E^o+(RT/F)\ln a_2 + E_j$$

$$= (RT/F)\ln (a_2/a_1) + E_j \qquad (5)$$

where E_j is the liquid-liquid junction potential. It is clear that the sign of the E.M.F. depends on that of E_j, but if the latter is assumed to be zero, then E_1-E_2 will be positive.

For a concentration cell of this type, there is no complete theoretical treatment of the effect of the protein on the activity coefficients of the small ions. However, for dilute solutions of the protein the usual practice has been to assume that the protein has very little or no effect on the activity coefficient, f, of chloride,[11] so that, on this basis

$$f_1 = f_2 \qquad (6)$$

since

$$a = cf \qquad (7)$$

where c is concentration in moles/litre, then equation (5) may be written as:

$$E = (RT/F)\ln(c_2/c_1) + E_j \qquad (8)$$

The potential of the electrode in the more dilute solution being positive with respect to the other electrode, if $E_j = 0$.

If E_j is not zero, which is certainly the case in practice, it should be estimated by controlling experimental conditions. It is not possible to calculate it theoretically, however, with such an unknown ionic and molecular atmosphere as chloride/protein systems.

Theory of Anion Binding to Proteins

It is assumed[27] that when macromolecules combine reversibly with smaller molecules or ions the usual laws of chemical equilibria govern the reaction. The combination of a protein P with an anion A may thus be described by n association constants:

$$K_1=(PA)/(P)c, \quad K_2=(PA_2)/(PA)c, \quad K_n = (PA_n)/(PA_n -1)c \qquad (9)$$

where brackets represent concentrations, c being the concentration of free (unbound) anion A, and n the total number of combining sites on the protein.

The equilibrium constants K_1, K_2, K_n are not true constants but depend on the solvent, salt concentration, temperature, etc., as do all experimentally determined equilibrium constants. True thermodynamic constants, expressed in terms of the activities of the combining species, may be obtained from these constants in the usual way, for instance by extrapolation to infinite dilution of all solutes. The usual practice, however, is not to determine the true thermodynamic constants, because the accuracy of experimental data rarely justifies this refinement[27].

Normally, the concentrations of individual species (P), (PA), (PA_n) are not experimentally determinable. Instead, it is usually sufficient to determine only the average number of ions of A associated with each protein molecule. This number, $\bar{v}$, is defined as the ratio of the number of moles of A bound, to the total number of moles of protein P, i.e.

$$\bar{v} = ((A)_{total} - c) / (P)_{total} \qquad (10)$$

In order to relate $\bar{v}$ to the equilibrium constants, we note that:

$$(P)_{total} = (P) + (PA) + \dots + (PA_n) \quad (11)$$

$$(A)_{total} = c+(PA) + 2(PA_2) + \dots + n(PA_n) \quad (12)$$

and that

$$(PA) = K_1c(P)$$

$$(PA_2) = K_1K_2c^2(P)$$

$$(PA_n) = K_1K_2 \dots K_nc^n(P) \quad (13)$$

Hence, the average binding is given by:

$$\bar{v} = (K_1c+2K_1K_2c^2 +\dots+nK_1K_2\dots K_nc^n)/(1+K_1c+K_1K_2c^2+\dots+K_1K_2\dots K_nc^n) \quad (14)$$

In practice, three general cases are encountered in the treatment of the theory of binding (see for instance Tanford[27]). In the simplest case, all binding sites on the protein molecule are identical and independent; in the second case, the binding sites are identical but interacting; the third, which is the most common, involves different classes of sites as well as interaction between sites.

For the simplest case, there are identical independent sites with n identical equilibrium constants. They may be related to an overall association constant by the equation:

$$\bar{v}/(n-\bar{v}) = kc \quad (15)$$

For the second case, there are n identical sites but binding at any one site affects the binding affinity at other sites. Such an interaction has to be taken into consideration in deriving a theory for binding. This has been done by Tanford[27] in the following manner: First define an intrinsic association constant, K_{int}, which will be the limiting value of k when $\bar{v} = 0$, i.e. before any interaction due to binding has occured. Then define an arbitrary function, $Q(\bar{v})$, such that for $\bar{v}$ more than o,

$$k = k_{int} \exp(-Q(\bar{v})) \tag{16}$$

If $Q(\bar{v})$ is an increasing function of $\bar{v}$, occupation of some sites makes binding at other sites more difficult; if it is a decreasing function of $\bar{v}$, occupation of some sites facilitates binding at other sites.

Substituting the value of k from equation (16) in equation (15), we get:

$$\bar{v}/(n-\bar{v}) = k_{int} \exp(-Q(\bar{v}))\, c \tag{17}$$

It remains to determine the nature of the function $Q(\bar{v})$. In the case of combination of simple ions (e.g. chloride ions) with large protein molecules, the predominent form of interaction between sites is likely to be electrostatic in nature; i.e. $Q(\bar{v})$ is directly related to the effect of charged sites on the chemical potential of the reacting species. This means that $Q(\bar{v})$ can be replaced by $Q(\bar{Z})$ where $\bar{Z}$ is the average net charge per molecule, and the intrinsic properties of a site are redefined as to represent those which it would have in the absence of any charges rather than when $\bar{v} = 0$. $Q(\bar{Z})$ has been calculated[28] from free energy considerations and shown to be:

$$Q(\bar{Z}) = 2ANz_i\bar{Z}/RT \tag{18}$$

where

$$A = (e^2/2DR)\,(1-rR/(1+ra)) \tag{19}$$

and N represents Avogadro's number, R and T having their usual significance, e is the protonic charge, z_i being the charge of the small ion, D the dielectric constant of the solvent and r Debye-Hückel's parameter given by:

$$r = (4\pi e^2/DkT)^{\frac{1}{2}}\, N^{\frac{1}{2}} I^{\frac{1}{2}} \tag{20}$$

where k is Boltzmann's constant, and I is ionic strength.

Putting $w = AN/RT$ we get:

$$w = (e^2/2DkTR)\ (1 - rR/(1 + ra)) \qquad (21)$$

Utilizing the fact that $z_i = -1$ for monovalent anions, and combining equations (17), (18) and (21) one gets:

$$(\bar{v}/c)\ \exp\ (2w\bar{v}) = k_{int}\ (n-\bar{v}) \qquad (22)$$

Scatchard[4,11], however, uses a more accurate expression for w, which is,

$$w = \frac{e^2}{2DkT}\ (\frac{1}{b} - \frac{r}{1 + ra}) \qquad (23)$$

where a represents the distance of closest approach, and is always larger than b, the radius of the hypothetical sphere representing the protein molecule.

Introducing the values of $a = 20\text{Å}$, $b = 18\text{Å}$, which are the accepted values for myoglobin in chloride solutions, and of other constants occuring in equation (23) one gets,

$$w = 0.1981 - 1.174\ I^{\frac{1}{2}}/(1+6.586\ I^{\frac{1}{2}}) \qquad (24)$$

for $25^{\circ}C$.

Since RTlnf is, by definition, the difference between the electrostatic contribution to the chemical potential at any ionic strength and the same contribution calculated at infinite dilution of all ions (I = r = o) i.e.

$$RT\ln f = -Z^2e^2r/2D\ (1+ra) \qquad (25)$$

then the activity coefficient of chloride ions may be calculated from the following equation obtained by substitution in equation (25) of the values of constants, taking $a = 6.2\text{Å}$ for chloride ions[11].

$$-\log f = 0.5I^{\frac{1}{2}}/(1 + 2I^{\frac{1}{2}}) \qquad (26)$$

It is now possible to apply the above results to the more

complicated and general case of a protein having different classes of sites. It can be shown that for n_1 sites with intrinsic association constant $k^{(1)}_{int}$, n_2 sites with intrinsic constant $k^{(2)}_{int}$, etc., in the absence of interaction between sites, equation (15) gives:

$$\bar{v} = \frac{n_1 k^{(1)}_{int} c}{1+k^{(1)}_{int} c} + \frac{n_2 k^{(2)}_{int} c}{1+k^{(2)}_{int} c} + \cdots\cdots \qquad (27)$$

and that if there is interaction between sites, equation (22) gives,

$$\bar{v} = \frac{n_1 k^{(1)}_{int} c \exp(2w\bar{v})}{1+k^{(1)}_{int} c \exp(2w\bar{v})} + \frac{n_2 k^{(2)}_{int} c \exp(2w\bar{v})}{1+k^{(2)}_{int} c \exp(2w\bar{v})} + \cdots\cdots \qquad (28)$$

Representation of Experimental Results

The first and second cases of the above treatment of binding theory are rarely met in work with proteins. The most probable situation is that a number of classes of sites is present, each class having a different intrinsic association constant, and that electrostatic interaction does take place between sites.

Equation (15), representing the simple law of mass action, can be inverted and solved for $\bar{v}$, following some investigators[5], to give:

$$\frac{1}{\bar{v}} = \frac{1+kc}{knc} = \frac{1}{n} + \frac{1}{knc} \qquad (29)$$

then $1/\bar{v}$ is plotted against $1/c$ and the best straight line is drawn; its intercept is $1/n$ and its slope $1/kn$.

According to Scatchard[1], this method has the disadvantages of concealing deviations from the ideal laws, and of tempting one

to plot straight lines where there should be curvature. He prefers solving equation (15) for c to get:

$$\frac{\bar{v}}{c} = k\,(n-\bar{v}) \qquad (30)$$

and to plot $\bar{v}/c$ against $\bar{v}$ to get a straight line if k is constant. The intercept on the $\bar{v}/c$ axis is kn, giving the classical first association constant; and the intercept on the $\bar{v}$ axis is n. Curvature would indicate different intrinsic constants or deviations from independent probabilities. In this case equation (22) is utilized and $\frac{\bar{v}\,\exp(2w\bar{v})}{cf}$ is plotted against $\bar{v}$. If one class of sites is present, then a straight line is obtained with a slope =k and intercept = kn. Curvature, again, indicates the presence of more than one class of sites. Nevertheless, the two intercepts still give the classical first association constant and the total number of sites. However, any curve resulting from this plot has to be represented by equation (27) or (28). Utilizing the known total number of sites and first association constant from the intercepts of the curve, and any information available regarding the structure of the protein, theoretical curves are plotted representing equation (27) or (28) for different assumed values of k and n; the one which fits the experimental curve most closely, most likely has the answer to the questions concerning the number of sites, classes and their association constants.

Effect of Salts on the pH of Protein Solution

The effect of salts on the pH of protein solutions has been investigated by Scatchard and co-workers[4, 23]. If $(pH)_o$ is the isoionic pH of the protein, $\bar{v}_i$ is the number of salt ions of charge

z_i bound to each protein molecule at a given salt concentration, then

$$pH = (pH)_o - 0.868w\sum_i \bar{v}_i z_i \qquad (31)$$

It can be noticed that pH rises when anions are bound and falls when cations are bound. A plot of the measured pH versus $0.868w\bar{v}$ would give a straight line according to equation (31), with a unit slope, the intercept being the isoionic pH.

EXPERIMENTAL

1. Chemicals

Water: Deionized redistilled water was used in making all solutions and in washing the electrodes and apparatus. For this purpose, tap water was passed through a two-bed ion-exchanger (Deminerolit Mark 4E, United Water Softeners Ltd., London), and was then redistilled from a commercial glass still (Laughborough Glass Co., England). It was stored in polyethylene containers. The conductance was frequently checked and found to be less than 2×10^{-6} ohm^{-1} cm^{-1}.

Hydrochloric Acid: A stock solution was made of constant boiling hydrochloric acid (density 1.096 gm/ml). This was prepared from micro-analytical grade hydrochloric acid (British Drug Houses, density 1.18 gm/ml, free chlorine content 0.0001% maximum). The B.D.H. acid was diluted with an equal volume of water and then glass distilled; the middle 50% of the fraction boiling at 108 - 109°C at atmospheric pressure was collected in the original bottle supplied by the manufacturer. The procedure of distillation was again repeated twice, in every case only the middle portion of the distillate being collected.

0.1N hydrochloric acid was used in the chloridization of electrodes. This was prepared from constant boiling hydrochloric acid by diluting 4.15 ml of the latter with water to a volume of 250 ml.

Nitric Acid: Concentrated nitric acid was used for washing and

cleaning the electrodes. The chemical was Merck's pro-analysi grade, minimum guaranteed reagent 65%, density 1.40 gm/ml, maximum chloride content 0.0001%. Purity of the acid was considered satisfactory for washing and anodization purposes.

Ammonia: Concentrated ammonia solution was used in the washing process. It was purchased from Hopkin and Williams (London), specific gravity 0.92, ammonia content 22% w/w.

Potassium Chloride: The crystalline solid was used for the preparation of potassium silver cyanide and for the preparation of all chloride solutions. It was Merck's pro-analysi guaranteed reagent labelled potassium chloride M. Wt. 74.56 (bromide and iodide contents not reported). No further purification of this reagent was attempted since such purification, unless extremely carefully done, might lead to further contamination of the reagent.

All weighings of potassium chloride were made on a single pan Mettler B5 balance.

Potassium Silver Cyanide $KAg(CN)_2$: Two samples of this salt were used; one which was already available and another which was prepared in the laboratory. The first sample was tried in the early stages of the work. However, its color was not homogeneous and it was much less soluble in water than is reported in the literature(*). A fresh batch of potassium silver cyanide was therefore prepared

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(*) 10 gm of the available sample could hardly be dissolved in one liter of water, while solubility in cold water as reported in "Handbook of Chemistry and Physics" 42nd edition, 1961, pp. 628-629 is 25 gm/100 ml.

and purified according to the following procedure, originally suggested by Fray and Porter[29] and later modified by Bassett and Corbett[30]:-

> Dissolve 20 gm of potassium cyanide in 100 ml of water, boil, add 41 gm of silver cyanide, boil, filter, cool. Add one third volume of ethyl alcohol and cool to crystallize the product. Filter, wash with ice-cold water, air-dry. Evaporate the filtrate to incipient crystal formation, filter, begin to cool, add one third volume of ethyl alcohol, cool and collect the crystals. The process of evaporation through filtration of crystals may be repeated to get a third crop. The whole product collected is doubly crystallized from boiling water. The crystals are air-dried at a temperature lower than 55°C. The yield is about 50%.

The yield, after the final crystallization was about 60%, which is better than the above yield. The product was white and readily soluble in water. It was kept in a brown glass bottle. No change in color or solubility of the salt was observed over a period of about eight months.

Proteins:

(1) Albumin: a sample of human serum albumin was kindly provided by Dr. Fouad Farah, American University of Beirut. It had been purchased from Nutritional Biochemicals Corporation, California, U.S.A., as the

fraction V powder preparation, and it was used without further treatment except for exhaustive dialysis of its aqueous solution against water just before use.

(2) Myoglobin: a sample of sperm whale ferrimyoglobin was purchased from Seravac Laboratories, Colnbrook, England, as the batch 2 preparation from skeletal muscle, lyophilized and salt free. It was used in three forms: untreated, dialyzed, or passed through a Sephadex G-25 column.

2. Apparatus

The apparatus used in the present work consisted of three main parts; the electrodes, the cell, and the potentiometer-electrometer set-up.

A. Silver/Silver Chloride Electrodes:

Silver/Silver halide electrodes in general have the advantages, as secondary electrodes, of being relatively easily prepared, compact, reproducible, and directly used in aqueous solutions of halide ions, thereby avoiding the uncertainties of junction potentials. However, liquid junction potentials still pose a problem with silver/silver halide electrodes when many ionic species are involved as will be seen later.

The most suitable form of silver/silver chloride electrodes is the one which consists of a platinum wire on which silver is deposited and then partly converted into silver chloride.

The design of silver/silver chloride electrodes for the present work is a modification of one described by Brown and MacInnes[31]. A

platinum wire, 0.6 mm in diameter, is flattened close to one end and sealed into a glass tube of 3 mm internal diameter. This end is connected with a nickel-plated copper wire through a Wood's metal junction. The other end of the platinum wire extends beyond the sealed end of the tube to a length of 1 cm within a glass sleeve (tube) at the top of which are two holes. A diagram of the platinum electrode is shown in Figure 1.

This arrangement was found most satisfactory in protecting the electrode surface from mechanical disturbance, allowing immediate contact with the electrolyte, and preventing formation of air bubbles at the electrode surface.

Deposition of Silver and Silver Chloride: Silver/silver chloride electrodes are classified[32] according to the methods of silver and silver chloride deposition on the platinum wire into:

(i) Electrolytic type, in which silver is deposited and chloridized electrolytically.

(ii) Thermal, where silver/silver chloride mixture is formed thermally on a platinum base in one operation, for instance by heating an aqueous paste of 7 parts silver oxide and one part silver perchlorate in an electric furnace, to decomposition. This type has the disadvantage of being massive, spongy and requiring longer period of time to equilibrate with solution.

(iii) Thermal-Electrolytic type, where silver chloride is formed electrolytically on a thermally reduced silver oxide paste.

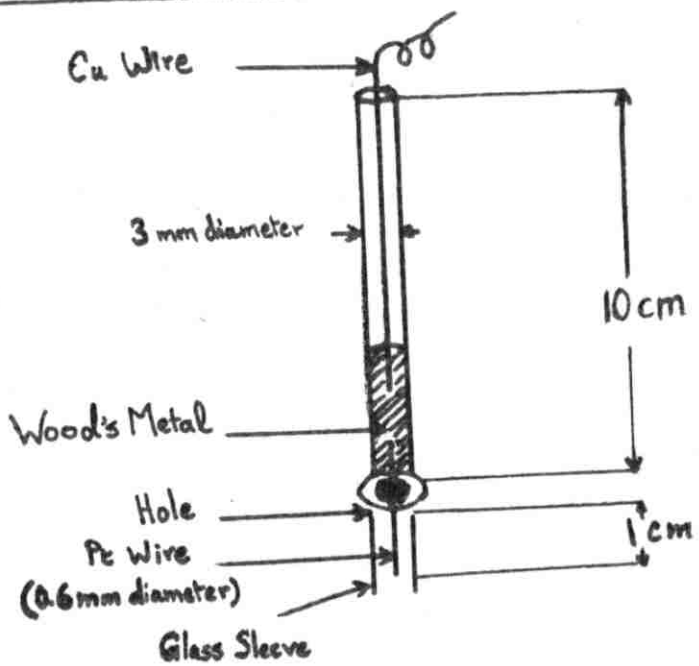

Figure 1. Diagram of Platinum Electrode.

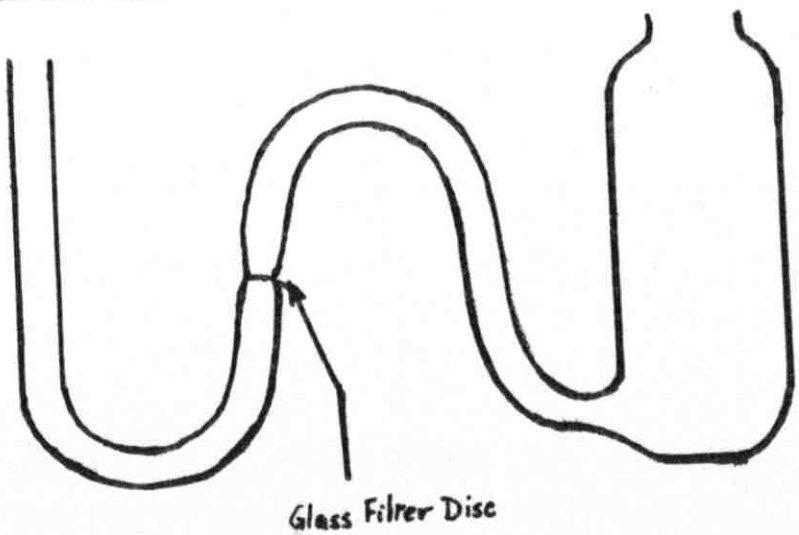

Figure 2. Diagram of Electrolysis Cell.

The first type, namely, the completely electrolytic electrode was found to be the most convenient for our purposes, combining ease of preparation and good reproducibility. It has been used here throughout.

The preparation of electrodes involves five stages: cleaning, silver-plating, washing, chloridization, and final washing. Some technical aspects of every stage are described below.

(a) Cleaning: The classical method of cleaning platinum electrodes involves boiling in concentrated nitric acid for 10 minutes followed by anodization in concentrated nitric acid for 30 minutes at a current density of 0.5 milliamperes per electrode (Ives and Janz)[33]. When this method was applied to silver/silver chloride electrodes which had already been used, it was found that at least 30 minutes of boiling and some two hours of anodization were required to remove completely the solid layer of silver and silver chloride. The time could be reduced, if one scratched the surface of the electrodes to remove the solid layer loosened by previous boiling in concentrated nitric acid. However, this is certainly not a good idea in practice, because of its effect on the surface of the platinum wire.
A much more convenient method of cleaning was developed in the course of our work. This method consisted of placing silver/silver chloride electrodes in concentrated ammonia solution for 10 minutes to dissolve the silver chloride, and then in concentrated nitric acid for 15 minutes to dissolve the silver layer. Following this the

electrodes were placed in boiling concentrated nitric acid for 10 minutes. Finally anodization for 30 minutes at 0.5 ma/electrode was easy to perform and gave very clean electrodes.

The current was supplied by a Heathkit D.C. power supply (The Heath Co., Michigan, U.S.A.). Anodization circuit was a simple one comprising the "electrodes" as anode, a simple platinum wire as cathode, concentrated nitric acid as electrolyte, a milliammeter, potential divider and the power supply.

The electrodes were washed thoroughly with water and were then ready for silver-plating.

(b) Silver-Plating: The cell which was used in electroplating, and actually in all steps of electrode preparation, is shown in Figure 2.

The long path between the two compartments and the presence of the glass filter disc assure good separation between anode and cathode and, hence, avoidance of contamination of the deposit at one electrode with byproducts formed at the other.

Silver was electrolytically deposited on the platinum cathode (usually a group of 4 electrodes) from an aqueous solution of potassium silver cyanide (10 gm/liter), to which a few drops of dilute silver nitrate solution were added just to effect slight turbidity of the solution indicating the removal of any free cyanide which would otherwise be included

in the deposited silver. The most convenient current density was found to be 2.5 ma/cm^2 or 0.5 ma/electrode, electrolysis proceeding for four hours. This resulted in a smooth coherent layer of silver. The theoretical quantity of silver deposited, assuming 100% current efficiency, is 42.05 mg/cm^2. Taking into account the fact that the exposed electrode is approximately a solid cylinder 10 mm long and 0.6 mm in diameter, this corresponds to a layer of silver 0.04 mm thick.

Electrodes were plated in sets of four at a time, the same solution of potassium silver cyanide being used for two successive electrolytic processes only.

This silver plating process was carried out in darkness inside a wooden box designed for the purpose.

The platinum wire used as anode developed a white deposit of silver cyanide, which could be easily washed away by dissolving it in potassium cyanide solution.

(c) Washing: After silver-plating, the electrodes were rinsed with water, then washed in concentrated ammonia solution and kept in it for eight hours. The ammonia solution was changed twice at about equal intervals. The electrodes were then washed with water and kept in water for 24 hours. Water was changed twice at about equal intervals.

(d) Chloridization: The electrodes were next chloridized by being made anode in the electrolytic cell described above, with 0.1N hydrochloric acid as electrolyte. This process

converts part of the silver on the platinum wire into silver chloride, thereby forming a solid mixture of silver and silver chloride which can equilibrate with chloride ions in solution. The most suitable current density was found to be 0.375 ma/electrode, for 60 minutes. This, assuming 100% current efficiency, would be enough to change 18.8% of the deposited silver into silver chloride; in practice, 10 to 25% conversion is usually regarded as sufficient[32].

Washing and chloridization processes were carried out in darkness. The resulting electrodes were light pink in color; when the color was not so, the electrodes were rejected, and the process of cleaning etc. started again.

(e) Final Washing and Storage: The electrodes were finally washed in water, and kept in water for 24 hours. They were then stored in potassium chloride solution of a concentration equal to that in which the electrodes were to be used. They were then considered ready for use. The need for storing electrodes before use is considered below under the section "Effect of Time".

The final washing and the storing were carried out in darkness. The electrodes were never kept for more than one or two weeks. After that period, regardless of whether they were used or not, they were cleaned and fresh silver/silver chloride layer was electrolytically deposited as described above.

B. Concentration Cell:

The main requirements of the cell used in this work were:

feasibility of construction; ease of handling and thermostating; provision of reproducible liquid junction; and economy in the use of solutions. The extent to which one of these requirements could be fulfilled was found to increase only at the expense of some other requirement. Consequently, in weighing the merits of each of the cells tried in the work, due consideration was given to all these factors. Altogether, four cells of different design were constructed and used.

First Cell: The glass cell which was tried in the early stages of the work is shown schematically in Figure 3. It was designed in 1963 by Dr. Cameron, Department of Chemistry, American University of Beirut. The cell consisted of five parts: two U-tubes containing the electrode compartments, each connected to the middle U-tube salt-bridge through a 3-way tap as shown in the figure. All connections were of ground glass. The middle U-tube and the two inverted U-tubes containing taps were filled with saturated potassium chloride solution, each separately, and then connected while full. The other two U-tubes were filled each with the solution for which the E.M.F. was to be measured. They were then connected to the free ends of the inverted U-tubes, and the junction formed by use of the taps.

The main merit of this design was thought to be the possibility of producing a good cylindrical liquid junction surface, and the fact that with tap controls at the tope, easy manual adjustments could be carried out while the cell partly dips in a thermostated bath. The cell was used extensively in the early stages of

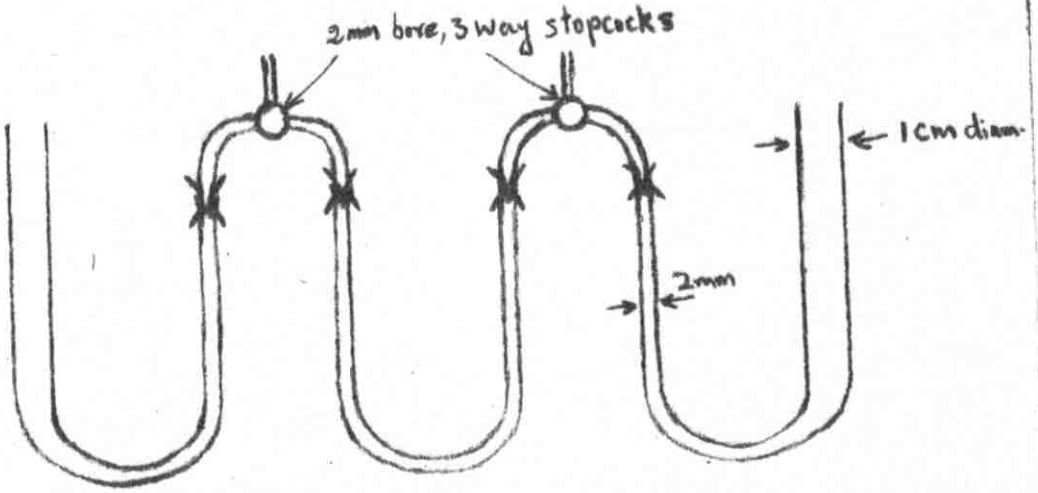

Figure 3. First Concentration Cell.

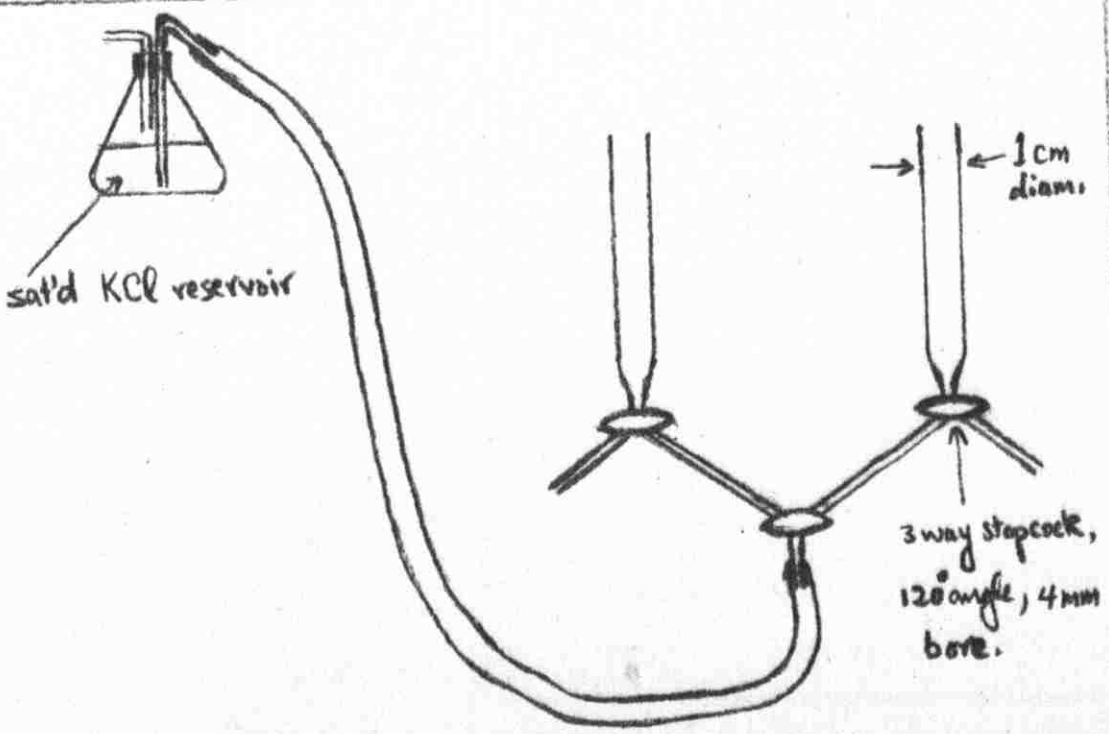

Figure 4. Second Type Concentration Cell (with Salt Bridge).

the work and was very useful as a guide to the technical difficulties which one encounters in this sort of work. Actually, the cell was by no means easy to set up, and the levelling of the liquids posed another problem because the cell consisted of so many parts. As a result, the liquid junctions formed between saturated potassium chloride solution in the middle compartment and very dilute potassium chloride solutions in each of the electrode compartments were very difficult to establish and preserve.

Second Cell (with Salt Bridge): A cell designed after Scatchard[11,12] was next tried. This is schematically shown in Figure 4. It consists of two glass tubes 1 cm in diameter held vertically and connected symmetrically by three 3-way glass stopcocks, 0.4 cm inner diameter, making 120° angle. The middle tap is connected to a saturated potassium chloride solution reservoir. After rinsing the whole cell, the two oblique arms were flushed with saturated potassium chloride solution from the reservoir. A few drops of this solution were allowed to flow out to the waste through the side arms to get rid of any trapped air bubbles. The vertical arms were then filled with the electrode solutions and the electrodes were placed in them through air-tight rubber stoppers. Junction was then made by the taps.

Although reproducible results were obtained using this cell, no liquid junction of cylindrical symmetry could be guaranteed. Furthermore, trapped air bubbles in the taps frequently resulted in a loss of a good part of the solution in the process of eliminating air bubbles. Also hazards of contaminating very dilute potassium

chloride solutions with saturated potassium chloride solution presented a drawback of this cell. Finally the cell was not convenient for use in a thermostat for obvious reasons.

Third (Capillary Junction) Cell: A modified design of an entirely different cell was next tried. The cell was originally described by Smith and Speakman[34], then modified by Dunsmore and Speakman[35]. The design used in this work is shown in Figure 5. No saturated potassium chloride bridge was used with this cell which permitted the formation of a liquid junction at the end of a short capillary tube.

The cell was used as follows: Dilute potassium chloride solution was introduced into reservoir A with tap D closed. Arm B was filled with the same solution and closed by an air-tight rubber stopper through which a silver/silver chloride electrode passed. Tube H was filled with the same solution, which was pulled down through the capillary C by applying suction at the opening of reservoir A. When tube H was emptied and the level of the solution was well below C, chloride solution of the same concentration but containing the protein under investigation was placed in tube H, in which a silver/silver chloride electrode was inserted through a rubber stopper. Tap D was then used to control the level of the solution below C. When that level was just below the end of capillary C, the capillary tap F was opened to allow the solution in H to pass through capillary C in such a way as to form contact between the two solutions through a drop.

It was found, however, that the drop forming the contact

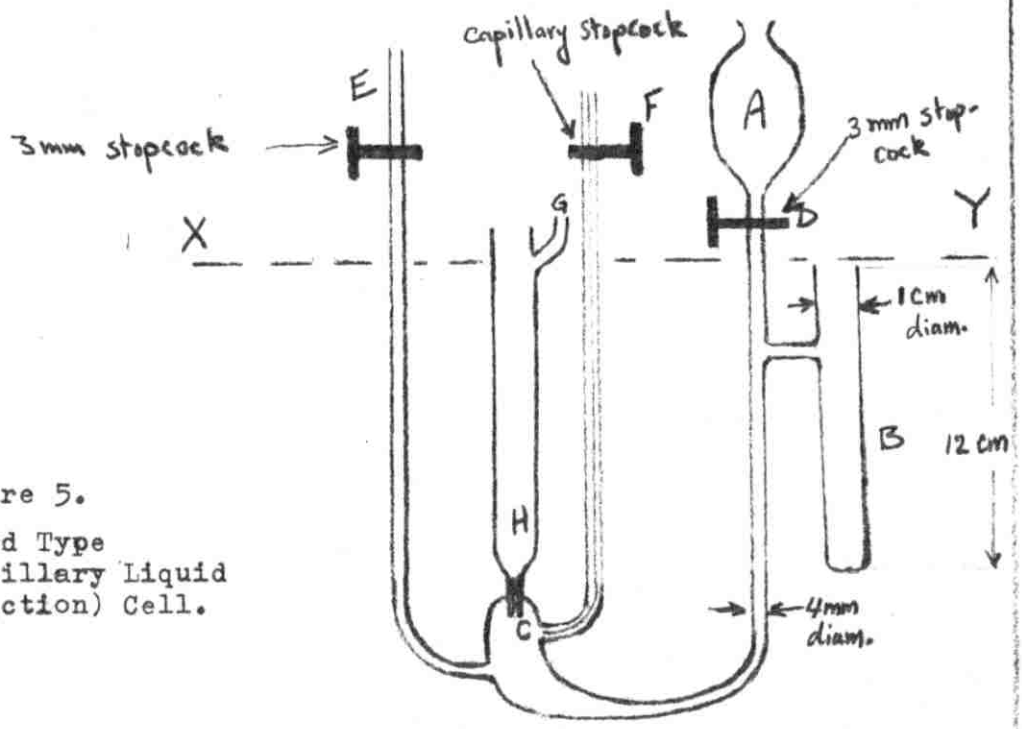

Figure 5.

Third Type (Capillary Liquid Junction) Cell.

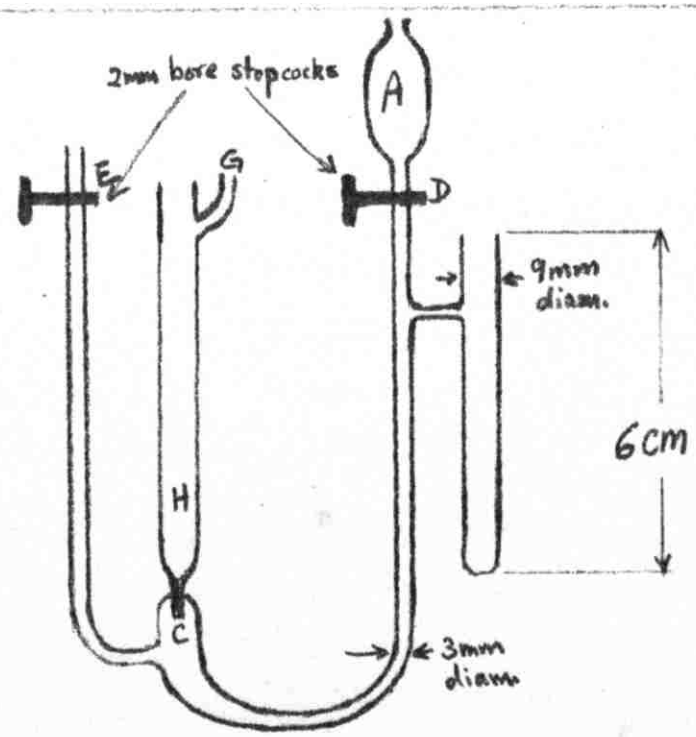

Figure 6. Fourth Type (Junior Capillary Liquid Junction) Cell.

provided a relatively large surface area across which diffusion took place. This was easily checked when the colored myoglobin was placed in compartment H. Junction was therefore formed at the upper end of capillary C by using only tap D as controller. This resulted in less diffusion and better reproducibility. Tap E was used for cleaning by suction.

The advantages of this cell over the previous one include its ease of use, reduced chance of contamination, and the fact that liquid junction is formed under conditions closer to cylindrical symmetry than in previous designs. The whole cell below line XY could be thermostated by immersing it in a liquid bath, since all controlling taps are above this line.

Fourth (Junior Capillary Junction) Cell: A further simplification was introduced into the design of the third cell in the course of this work. The arm ending with tap F was eliminated, and the one ending with tap E was retained (see Figure 5). In addition, the general dimensions of the cell were reduced so as to make it possible to make an E.M.F. measurement with only 10 ml of protein solution, the protein being placed in either one or both electrode compartments. The cell is shown in Figure 6.

The cell was used in the following manner: Tube B was filled with potassium chloride/protein solution while tap D was closed. Silver/silver chloride electrode was inserted into tube B through an air-tight rubber stopper. Reservoir A was partially filled with potassium chloride/protein solution, then taps D and E were opened

while tube H and its side arm were air-tightly closed. Solution was allowed to fall from reservoir A through tap D in the tube ending with tap E, but was not allowed to rise beyond tap E. By suction through A and E any air trapped in the path DE could be got rid of, and, likewise, the tube ending with tap E could be emptied of any solution without any waste. Solution was then forming a continuous column extending from tap D or somewhere above the junction to tube C, to a level well below capillary C. Taps D and E were then closed and tube H opened and filled with potassium chloride solution. A silver/silver chloride electrode was placed in it through a rubber stopper. While side arm of tube H was open, tap D was used to control the surface of solution below capillary C, and to form a contact between the two solutions above and below capillary C as in the previous cell. The whole cell was then placed in a thermostated bath to a level below XY. It was then ready for taking measurements.

C. Potentiometer - Electrometer Set-up:

The main requirement of the potentiometer that was needed for this work was that it should be sensitive enough to read E.M.F.'s with a precision of 10 microvolts (± 0.01 mv) or better. The electrometer (galvanometer) had to be sufficiently sensitive to respond to such low E.M.F.'s, and to give reproducible readings not drifting appreciably with time.

For initial purposes it was considered good enough to use a pH meter (Model PHM4, Radio Co., Copenhagen) comprising both potentiometer and galvanometer circuits, and with a precision of 0.5 mv.

However, the problem of electrical pick up posed a serious limitation to the use of this instrument. This could be solved partially by shielding the cell, by placing it in a Faraday cage which was connected to the pH-meter ground contact. But, the difficulty persisted especially at higher internal resistance of the cell i.e. at higher dilution of the electrolyte.

Although more sensitive commercial electrometers are available, it was decided to construct locally an instrument that fulfils the required limits of sensitivity. This was achieved by the kind co-operation of Dr. Douglas and Dr. Wilcox of the Department of Physics, American University of Beirut, and their co-workers. They designed and constructed a vibrating reed electrometer, to be used as a null-indicator sensitive to small E.M.F.'s (about 5 μv D.C. when input is sufficiently noise free) from high impedence source; in conjunction with an available highly sensitive K3 micropotentiometer (Leeds & Northorp) that had a precision of 0.5 μv to 50 μv corresponding to full scale settings of 16 mv to 1.600 volts respectively.

The vibrating reed electrometer comprises the following units:

(i) Vibrating Capacitor and Preamplifier:

A D.C. voltage difference (the E.M.F. of the cell under consideration) is applied between a and b (see Figure 7), across the vibrating capacitor C_v (about 10 μμf). A 500 cps voltage proportional to V_{ab} appears at c and is amplified by a factor of a few hundred before appearing at the output. The output signal consists of noise generated in the pre-amplifier plus the 500 cps signal.

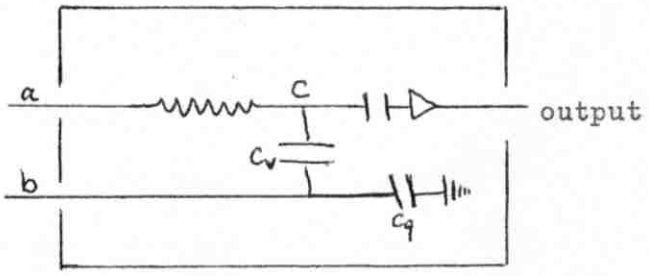

Figure (7) Vibrating Capacitor
and Preamplifier Connection

(ii) Narrow Band Amplifier:

This amplifier is tuned to amplify only frequencies near 500 cps, thus enhancing the desired signal relative to the background noise by a factor of about 100.

(iii) Lock-in Detector:

The 500 cps signal from the narrow band amplifier is demodulated (converted to a D.C. voltage) and appears across a meter on the front panel. The lock-in is sensitive to only a narrow band of frequencies around the frequency at the vibrating capacitor (provided the input signal is not large enough to override the detector). The band of frequencies can be made as narrow as desired, with the consequent increase in rejection of noise signals, but only at the expense of an increased response time. The response time was adjusted to about 4 seconds, giving a band width of $\frac{1}{4}$ cps.

(iv) Power Supply Unit:

This provides regulated 300 volt D.C. plate supply and 6.3V A.C. for the filaments of the lock-in and narrow

band amplifier valves, and filtered 100 V and 5.8V D.C. for preamplifier. This power supply unit is similar to the one used in the electrolysis circuit. Due to contact potentials and inherent 500 cps pick-up in the vibrating capacitor, a 500 cps signal is generated if $V_{ab}=0$. To obtain null output a voltage V_{ab} of several millivolts must be applied. This is easily done by connecting a potentiometer between ground and input b, shorting input a to ground (with the shorting cap) and adjusting for null indication. The potentiometer reading for this setting is called ground level E.M.F. If an E.M.F. is now connected to input a (from ground) the potentiometer must be re-adjusted to give null indication. The E.M.F. is the difference between potentiometer settings.

The ground level E.M.F. was usually 11 to 12 mv. It was found more correct to take the potentiometer reading with the cell connected and call this V_1, reverse the connections of the electrodes and take the new potentiometer reading V_2. The E.M.F. of the cell was then $(|V_1 - V_2|)/2$. The sign was assigned in such a way as to indicate that the positive electrode was the one which was grounded when the potentiometer gave the higher reading. When the E.M.F. of the cell was higher than 12 mv i.e. higher than the ground level E.M.F., only one potentiometer reading, namely the higher one, could be obtained

and the E.M.F. of the cell in this case was simply the difference between the potentiometer reading and the ground level E.M.F., the grounded electrode being the negative one.

3. Investigation of Experimental Conditions

In electrochemistry, E.M.F. measurements are notoriously susceptible to change under the influence of experimental conditions. Hence, before the present experimental set-up could be used to obtain the required data, it was considered necessary to investigate the various experimental factors which might alter the measured E.M.F.'s, and thus to establish definite experimental conditions. In this section are discussed the preliminary effects of light, shielding, polarity, time, temperature, pH and chloride traces in the protein.

Effect of Light: Several investigators[36,37,38] in the field of electrochemistry have reported that light has no effect on the potential of silver/silver chloride electrodes. The color of the electrodes, however, is definitely affected by exposure to light. In this work, the color of freshly prepared electrodes was light pink and turned brown within a week or two even under conditions of minimal exposure to light. Although no effect of occasional exposure of electrodes to subdued day light on the potential of these electrodes, beyond the experimental error, could be detected in the course of this work, all processes involving preparation of the electrodes and their use were carried out in darkness or

allowing of subdued light to a minimal extent. The electrolysis set-up was placed in a light-tight wooden box, the concentration cell was placed in another light-tight metal box which also served the purpose of a Faraday cage. Electrodes were always stored in darkness.

Effect of Shielding: The problem of electrostatic pick-up formed a major difficulty in the early stages of this work, when pH meter was used as a potentiometer. The problem was partially solved by shielding the concentration cell, by placing it in a Faraday cage (metal box) which was grounded in common with the pH meter ground contact. However, at chloride concentrations less than 0.001M, the pick-up problem persisted; with the pH meter plugged to the mains (which served only to feed a filament heater for reducing humidity) a discrepancy of several millivolts in the reading of the meter was noted.

It was then essential to have an instrument insensitive to pick-up frequencies. This requirement was taken care of by the vibrating reed electrometer that was designed and constructed to match the micropotentiometer available at the laboratory, in its sensitivity. The potentiometer reads E.M.F.'s with a precision of 0.1 uv, but the electrometer had a precision of 10 uv (0.01 mv) which was the practical limit of sensitivity of the null detector. No pick-up problems were encountered with this instrument. The cell used in conjunction with it was placed in a Faraday cage, especially designed and constructed, to provide enough room for

refilling and washing inside the box which was painted black from inside and was light-tight so as to eliminate the effect of light (if any) on the electrode potential.

Polarity: Since measurements of the order of a few millivolts were being made, with no prior knowledge of the relative potentials of the two electrodes comprising the cell, it was necessary to determine unequivocally the sign of the measured E.M.F. The polarity of the cell was checked in a number of independent ways. For instance, when the potentiometer was being calibrated against a Weston cadmium standard cell (E.M.F. = 1.0186 volt at 20°C), the polarity of the experimental cell could be compared with that of the standard cell. Furthermore, the polarity could also be checked by measuring the E.M.F. of the cell on the pH meter which has the polarity of its input terminals and potentiometer readings clearly set.

However, as a further check, a cell was constructed comprising two silver/silver chloride electrodes dipping in 0.01M and 0.001M potassium chloride solutions respectively. The theoretical potential of the electrode with the more dilute chloride solution should be 59 mv (at 25°) positive with respect to the other electrode. This was found to be the case, and subsequently all E.M.F. measurements were made on the basis of the checked polarity of the potentiometer circuit.

With the micropotentiometer, the electrode which was connected to the ground of the instrument when a reading higher than the

ground level potential(*) was obtained had a negative potential with respect to the other electrode and vice versa.

Effect of Time: It is known that freshly prepared electrodes show an aging effect i.e. the electrode potential changes with time. Smith and Taylor[39] have pointed out that the aging period of freshly prepared electrodes may vary from a few minutes to 1 to 20 days, while Hornibrook, Janz, and Gordon[36] found no indication of aging after periods of 24 to 40 minutes. Experience with the present work has shown that the electrodes usually take about one hour to attain a constant potential difference. The technique followed, however, was to keep the electrodes, short-circuited, for an overnight in a chloride solution in which they were to be used the following day. They were found to give a constant zero potential difference in less than 10 minutes.

When these electrodes were used in chloride solutions containing protein, the drift of E.M.F. with time was higher and persisted for a longer time. It was observed that the drift was less for higher chloride concentrations at constant protein concentration. The E.M.F. for chloride solutions of concentration higher them 0.001M took about 30 minutes to come to a fairly constant value. For more dilute chloride solutions, however, they took about one hour. The measured E.M.F. was considered to be the last reading before the E.M.F. came to a fairly constant value, i.e. drifting at a rate of less than 0.01 mv/minute.

(*) The ground level potential is the reading of the potentiometer when both terminals are grounded.

One factor which may account for drift of E.M.F. with time is the diffusion of ions and molecules across the liquid junction. It has already been mentioned (section on Concentration Cells) that diffusion of colored myoglobin could be observed at the liquid drop junction in the capillary junction cell, and that to reduce this effect, liquid junction was formed at the top rather than the bottom end of the capillary (Figures 5 and 6). As a further check on this problem, experiments were performed in which protein/chloride solution was placed below the capillary and junction formed as usual at the top of the capillary column. Two observations were noticed in these experiments:

(1) The equilibrium E.M.F. was equal, within experimental error, to the E.M.F. obtained when protein/chloride solution was placed above capillary.

(2) The drift of E.M.F. with time was about equal in both cases, but the equilibrium point was approached from opposite directions.

Effect of Temperature: Measurements of E.M.F. were made in an air-conditioned laboratory (temperature 25 $\pm$ 1°C). In order to improve junction conditions and reduce the effects due to heat of solution or dilution at the liquid junction, a thermostat was designed and constructed to allow a water stream of constant temperature to flow in a glass jacket around a vessel containing the cell in water bath. Measurements could thus be taken over a range of temperature of 5°C to 25°C, the minimum being limited by the freezing of water and the higher limit fixed by the danger

of denaturation of the protein.

Effect of pH: Most of the E.M.F. measurements were made on solution of potassium chloride versus protein/potassium chloride, within the pH range of 6 to 7. Owing to a buffering effect of the protein the two electrode solutions necessarily had different pH values, and the difference was greater the higher the protein concentration. However, since hydrogen and hydroxyl ion concentrations are extremely small in this pH range, the effect was considered negligible. Nevertheless, an attempt was made to control pH by the addition of a suitable counter buffer; but experimental results indicated that the buffer introduced greater effects than could be accounted for in terms of pH changes. This suggests that buffer anions compete with chloride ions for binding to protein, and also raises the question of ionic atmosphere in solutions. The observation is in qualitative agreement with the findings of Klotz and Urquhart[40], who found that anions were bound by bovine serum albumin in the increasing order: acetate, chloride, nitrate. They also found that at pH less than 9, the degree of binding was a function of the nature of the buffer.

The effect of varying pH over a wide range on the measured E.M.F. was investigated in this work. It is to be expected that at sufficiently high pH, the binding of chloride to protein is reduced to a minimum. Consequently, E.M.F.'s measured at high pH values should be either zero, or have some value attributable to an effect of the protein on the E.M.F. other than its being a

source of binding. The pH of the protein/chloride system was varied between pH6 and 11 by adding sodium hydroxide solution to effect the required pH change and in quantities so small as not to introduce appreciable dilution effects. The pH of the chloride solution forming the other electrolyte was controlled in a similar manner. The procedure adopted was to measure the pH of every solution for which an E.M.F. is measured, then to add sodium hydroxide to make the pH about 11 and measure the corresponding E.M.F. This pH is the limit of the pH which the protein/chloride solution could be made to attain, because of the danger of denaturation at high pH values. Discussion of the variation of E.M.F. with pH will be dealt with in a later section.

Salt Content of Proteins: Although the myoglobin used was reported to be salt free, the possiblity of the presence of any chloride that may affect the significance of E.M.F. measurements was investigated. This was done by running a set of measurements under similar conditions: first on a solution prepared simply by dissolving myoglobin in chloride solution of the required concentration, and filtering the solution; secondly on aqueous solution of myoglobin that had been dialyzed against water for 24 hours, then added to chloride solution to make up a solution of myoglobin in chloride of the required concentration; and thirdly on an aqueous solution of myoglobin that had been passed through a chromatographic column packed with Sephadex G-25 molecular sieve resin, then added to a chloride solution to make up a solution of myoglobin in chloride of the

required concentration. It was found that the resulting E.M.F.'s did not differ in the three cases from one another by more than ± 0.2 mv (ca. 4%). This was taken as a proof that, at least for the purposes of this work, the myoglobin available was chloride free, and, hence, all myoglobin solutions were made directly from crystalline myoglobin without further treatment other than filtration.

Since only a sample of crystalline human serum albumin sufficient for one E.M.F. measurement was available, no investigation of its possible salt content was tried. It was dissolved in water and dialyzed against water for three days, then added to chloride solution of the proper concentration.

4. Procedure

On the basis of the above preliminary investigation, the following procedure for the preparation of electrodes and for measuring E.M.F.'s was developed.

Preparation of Electrodes:

(1) Place electrodes in concentrated ammonia solution for 10 minutes, rinse with water, then place them in concentrated nitric acid for 15 minutes.

(2) Transfer electrodes to boiling concentrated nitric acid for 10 minutes, wash with water and air-dry.

(3) Anodize electrodes in groups of four in concentrated nitric acid for 30 minutes at 0.5 ma/electrode, wash with water.

(4) Electroplate electrodes, in groups of four, with silver, by making them cathode in an electrolytic cell with aqueous solution of potassium silver cyanide (10 gm/l) as electrolyte at 0.5 ma/electrode for four hours. Wash platinum anode with potassium cyanide solution.

(5) Wash electrodes in concentrated ammonia solution for eight hours changing solution twice, then in water for 24 hours, changing water twice.

(6) Chloridize electrodes by making them anode in electrolytic cell with 0.1N hydrochloric acid as electrolyte at a current density of 0.375 ma/electrode for sixty minutes.

(7) Wash with water for 24 hours.

(8) Store in chloride solution of concentration equal to that in which they are to be used.

(9) The above procedure is followed for used silver/silver chloride electrodes. For platinum electrodes being plated for the first time, step (1) is omitted.

Zero Potential: Before using electrodes, they should be placed in chloride solution of the concentration in which they will be used. However, electrodes that have been so treated for at least one hour, may be tried, and thereafter used if they show no significant drift.

Before every single E.M.F. measurement, and after every set of measurements, zero potential of the particular pair of electrodes

should be determined according to the following procedure:

(1) Fill both arms of the cell with potassium chloride of the same concentration, place electrodes in the arms of the cell.

(2) Connect electrodes to the terminals of vibrating capacitor, adjust micropotentiometer to bring electrometer needle to zero and take micropotentiometer reading.

(3) Interchange electrodes-to-vibrating capacitor connections and similarly take another reading.

(4) Half the difference between reading (2) and reading (3) gives the potential difference between electrodes.

(5) Empty the cell and refill with the same chloride concentration and repeat steps (2) through (4).

(6) The value of E.M.F. as measured in (4) above and confirmed in (5) is the zero potential for that particular pair of electrodes and is applied as a zero correction for any E.M.F. measured with them.

(7) A pair of electrodes should be rejected, and hence, cleaned and replated, if its zero potential is more than $\pm$ 0.5 mv, or if no two values of its zero potential as measured in (4) and (5) above agreed within $\pm$ 0.02 mv, or if the electrodes do not come to equilibrium in less than 15 minutes.

(8) Zero potentials measured between experiments, however,

are allowed to exceed ± 0.5 mv provided that such a value is reproduced by successive refillings and measurements.

E.M.F. Measurement: The procedure of measuring E.M.F. has been developed in the course of this work into the following routine. It should be noted that every single value of E.M.F. reported in a table or in the context or included in a graph, was determined by going through all the steps of this procedure.

(1) Determine zero potential for a pair of electrodes in the chloride solution under consideration. Repeat determination, if necessary, for other pairs until a pair is found which has acceptable zero potential.

(2) Empty the cell and refill one compartment with chloride solution and the other with chloride/protein solution, place electrodes in the cell and make contact between solutions (i.e. form the liquid junction) as previously described.

(3) Connect electrodes to terminals of vibrating capacitor, bring electrometer pointer to null and take micropotentiometer reading.

(4) Interchange connections in (3), nullify galvanometer and take micropotentiometer reading.

(5) If in connection (3) or (4), the galvanometer could not be nullified, short-circuit the vibrating capacitor terminals, nullify galvanometer and take micropotentiometer reading.

(6) The E.M.F. measured is either half the difference between readings (3) and (4), or as the case may be, the difference between either (3) or (4) and (5).

(7) Repeat steps (3) through (6) every 5 to 10 minutes, until a fairly constant E.M.F. as determined in (6) above is obtained. This usually takes 30 to 70 minutes.

(8) Disconnect electrodes, remove the chloride/protein solution with a pipette, wash the cell and rinse with the chloride solution under consideration.

(9) Refill as in step (2) above and repeat steps (2) through (7) until two values of E.M.F. are obtained that agree with each other within 0.1 mv.

(10) Apply zero potential correction to the accepted value of E.M.F. in (9) to get the net required E.M.F. with the proper sign (see section on Potentiometer-Electrometer Set-up).

(11) Place the chloride/protein solution for which E.M.F. was measured into a pH meter cell and measure pH relative to standard.

(12) When a pair of electrodes with acceptable zero potential, develop anomalous zero potential in the course of their use, they should be well washed with water and placed in chloride solution of a particular concentration for a period of not less than one hour, or overnight if time permits, after which period zero potential may be rechecked. Experience in this work has shown that such a

pair of electrodes usually behave normally after such treatment.

On the average, every measurement of E.M.F. as described in the above section took about three hours, in addition to the period of three to four days needed for cleaning and preparing the electrodes.

R E S U L T S

Following the procedure described above for the preparation and use of silver/silver chloride electrodes, E.M.F. measurements were made under a variety of experimental conditions, and covering a range of protein concentration from 1.0×10^{-6}M to 1.0×10^{-3}M, potassium chloride concentration from 1.0×10^{-4}M to 1.0×10^{-1}M, and pH from about 6 to 11. The results of the measurements are outlined in this chapter. Unless otherwise specified, these results refer to the following general experimental conditions:

(i) Temperature was 25 ± 1°C (air-conditioned laboratory).

(ii) Ferrimyoglobin solutions were made by dissolving the appropriate weight of lyophilized solid in potassium chloride solution of the required concentration.

(iii) Cells were constructed with equal potassium chloride concentrations (c_1) in both electrode compartments; one compartment having protein (molar concentration m) dissolved in its potassium chloride solution, thus

$$Ag,AgCl(s)/KCl(aq.,c_1)/KCl(aq.c_1)+Protein(aq.m)/Ag,AgCl(s)$$

(iv) Cell E.M.F. values are the potentials of the electrode in the protein + chloride compartment with respect to the other. These are the values attained by the concentration cell after the lapse of sufficient time for equilibration, as detailed in the previous chapter.

(v) E.M.F. values as reported have a precision (reproducibility) of $\pm$ 0.05 mv or better.

Preliminary Data Using Cell With Salt Bridge

It was first necessary to test the effect of protein on the E.M.F. of the cell, and consequently to detect liquid junction potential, binding of ions, and possibly other effects. Preliminary experiments were performed with potassium chloride versus potassium chloride solutions to test polarity of the cell, and with myoglobin to test its effect on the E.M.F., as well as with albumin to compare results with those of Scatchard[11]. The experiments showed that the effect of myoglobin as well as albumin on the E.M.F. of potassium chloride cells is similar in magnitude to that reported by Scatchard[11], for his work on albumin, but in both cases the E.M.F.'s were of opposite sign to that reported by Scatchard. The results are outlined below:

(i) Effect of Myoglobin: Constant myoglobin concentration (1.8×10^{-4} M), varying chloride concentration, E.M.F. readings taken for 10 minutes (following Scatchard's technique [11,12]):

KCl(M)	1.00×10^{-3}	5.00×10^{-3}	9.00×10^{-3}	9.00×10^{-2}
E.M.F.(mv)	- 7.75	- 8.32	- 4.00	- 2.44

(ii) Polarity Test: The following cell was set up:

Ag,AgCl(s)/KCl(aq.0.01M)/KCl(aq.0.001M)/Ag,AgCl(s)

and it was found that

E.M.F. = E(0.001M)-E(0.01M) = +58 mv (as expected)

(iii) A repeat of an experiment by Scatchard[11] on human serum albumin (4.0×10^{-4}M) in potassium chloride (7.5×10^{-4}M) gave:

Measured E.M.F. (cell type 2, with salt bridge) =-9.05 mv

Measured E.M.F. (cell type 3, with capillary junction)=-9.00 mv

Scatchard's corresponding value[11] for 7.6×10^{-4}M albumin in 7.5×10^{-4}M sodium chloride was +7.9 mv. It seems probable, therefore, that the E.M.F.'s reported by Scatchard & co-workers[11] were erroneously reversed in sign.

A series of experiments was next performed using capillary junction cells and covering a wide range of pH and of chloride and myoglobin concentrations. The results of the measurements are outlined below.

Results Obtained Using Capillary Junction Cells

1. Effect of Myoglobin and Chloride Concentrations on E.M.F.

Table 1.A lists the results of measurements of E.M.F. of concentration cells with myoglobin/chloride solution versus chloride solution, using varying myoglobin and chloride concentrations. The concentration of chloride solutions increases down columns for constant myoglobin concentration, and the latter increases along horizontal rows for constant chloride concentration.

Along with every E.M.F. measured, the corresponding pH of the myoglobin/chloride solution was carefully measured with the appropriate calibration based on National Bureau Standards[41]. Table 1.B lists the pH values thus determined for the myoglobin/chloride systems included in Table 1.A.

Table 1.A

Measured E.M.F. for Cells Containing KCl (c_1) versus KCl(c_1)/Myoglobin at Varying Myoglobin and Chloride Concentration

Mb Concentration (M)	$1.0x10^{-6}$	$1.0x10^{-5}$	$1.0x10^{-4}$	$1.0x10^{-3}$
KCl Concentration (M)	E.M.F. (mv)	E.M.F. (mv)	E.M.F. (mv)	E.M.F. (mv)
$1.0x10^{-4}$	+5.42	+4.76	-5.37	-47.00
$5.0x10^{-4}$	-0.31	-2.19	-5.18	-15.00
$1.0x10^{-3}$	-0.40	-2.88	-4.44	- 6.49
$5.0x10^{-3}$	-1.70	-3.37	-4.16	- 4.02
$1.0x10^{-2}$	-1.39	-1.77	-4.47	- 1.24
$5.0x10^{-2}$	-0.97	-1.04	-1.68	+ 1.45
$1.0x10^{-1}$	-0.37	-0.54	-0.16	+ 1.36

Table 1.B

Measured pH of Solutions in Table 1.A

MB Concentration (M)	$1.0x10^{-6}$	$1.0x10^{-5}$	$1.0x10^{-4}$	$1.0x10^{-3}$
KCL Concentration (M)	pH	pH	pH	pH
$1.0x10^{-4}$	5.89	6.05	6.28	6.52
$5.0x10^{-4}$	6.00	6.14	6.30	6.47
$1.0x10^{-3}$	5.86	6.12	6.36	6.53
$5.0x10^{-3}$	5.79	6.02	6.35	6.65
$1.0x10^{-2}$	5.94	6.20	6.36	6.72
$5.0x10^{-2}$	5.67	5.96	6.41	6.70
$1.0x10^{-1}$	5.78	6.15	6.38	6.86

Conclusions:

(i) Since protein/chloride solution gives an E.M.F. which is not zero with respect to chloride solution of the same concentration but without protein, then the protein has an effect on the liquid junction potential, on the chloride ion activity, or on both.

(ii) If only liquid junction potential is affected, the E.M.F. has to tend to zero as myoglobin concentration tends to zero; but results show that this is not the case.

(iii) If only chloride ion activity is affected, through binding to the protein, then chloride ion activity should decrease in the protein/chloride compartment resulting in a positive E.M.F.; but results, again, show that this is not the case.

(iv) Hence, a more complicated effect is encountered, and the measured E.M.F.'s should be considered representing a combined effect of myoglobin on liquid junction potential, tending to make the E.M.F. more negative, and through binding, tending to make the E.M.F. more positive; together with any other unknown effect.

(v) E.M.F. measurements should be taken under conditions of minimum binding to get a measure of E_j (equation 8).

In view of the above argument, it was decided next to investigate the effect of pH on the measured E.M.F.

2. Effect of pH on The Measured E.M.F.

Preliminary measurements showed that pH, as expected, had a

large effect on the E.M.F. of the concentration cells used. Thus using 1.0×10^{-3}M myoglobin in 1.0×10^{-2}M potassium chloride, and adjusting the pH of both electrode solutions to the same pH with hydrochloric acid or sodium hydroxide, the following data were obtained:

pH of Myoglobin/KCl solution	3.56	6.72	9.50	10.40
E.M.F. (mv)	+31.00	+1.24	-10.50	-10.41

Similar measurements were made under conditions of varying potassium chloride and myoglobin concentration. The results are given in Table 2.A.

Table 2.A

Variation of E.M.F. with pH

Myoglobin Concentration (M)	1.0×10^{-4}			1.0×10^{-3}		
KCl Concentration (M)	pH 6.5 (mv)	pH 9.5 (mv)	pH 11.0 (mv)	pH 6.5 (mv)	pH 9.5 (mv)	pH 11.0 (mv)
5.0×10^{-4}	-5.18	-41.87	-41.55	-15.00	-54.70	-72.76
1.0×10^{-2}	-4.47	-	- 7.08	+ 1.24	-10.50	-10.41
5.0×10^{-2}	-1.68	- 3.38	- 2.35	+ 1.45	- 2.33	- 2.35

Conclusions:

It is necessary, for every system, to measure the E.M.F. at pH 11 (assuming minimum binding at this pH) and to apply this value to the corresponding E.M.F. obtained without pH adjustment (i.e. at pH 6-7), as a correction for the liquid junction potential to obtain the net E.M.F. under the given conditions. This net E.M.F. is assumed to be solely due to binding of chloride ions.

Table 2.B lists net E.M.F. values for different myoglobin/-chloride systems reported in the previous experiments, corrected for zero potentials and myoglobin liquid junction potential as explained in the previous paragraph.

Table 2.B

Net E.M.F. Values (mv) of
Myoglobin/chloride versus Chloride Cells

KCl Concentration (M)		0.0005	0.001	0.005	0.01	0.05	0.1
Myoglobin Concentration (M)							
1.0×10^{-6}	pH ca. 6	- 0.31	- 0.40	- 1.70	- 1.39	- 0.97	-
	pH ca. 11	-27.60	-19.45	- 4.41	- 3.23	- 1.23	-
	net E.M.F.	+27.29	+19.05	+ 2.71	+ 1.84	+ 0.26	-
1.0×10^{-5}	pH ca. 6	- 2.19	- 2.88	- 3.37	- 1.77	- 1.04	-
	pH ca. 11	-19.01	-16.78	- 7.68	- 3.26	- 1.55	-
	net E.M.F.	+16.82	+13.90	+ 4.31	+ 1.49	+ 0.51	-
1.0×10^{-4}	pH ca. 6.5	- 5.18	- 4.44	- 4.16	- 4.47	- 1.68	- 0.16
	pH ca. 11	-41.55	-22.20	- 7.98	- 7.08	- 2.35	- 0.66
	net E.M.F.	+36.37	+17.76	+ 3.82	+ 2.61	+ 0.67	+ 0.50
1.0×10^{-3}	pH ca. 6.5	-15.00	- 6.49	- 4.02	+ 1.24	+ 1.45	+ 1.36
	pH ca. 11	-72.76	-49.34	-18.80	-10.41	- 2.35	- 1.92
	net E.M.F.	+57.76	+42.85	+14.78	+11.65	+ 3.80	+ 3.28

3. Calculations of Chloride Binding to Myoglobin

Tables 3.A and 3.B list the total chloride concentration in moles/liter, pH values, net E.M.F. values (E) in millivolts, together with the calculated free chloride concentration (c) in moles/liter, average binding ($\bar{v}$), ionic activity coefficient (f), electrostatic interaction correction factor (w) and other related parameters necessary for plotting the data and testing binding equations; for 0.001M and 0.0001M myoglobin respectively.

The net E.M.F. values (E) reported represent, as previously explained, the contribution of chloride binding only to the measured E.M.F.

Free chloride concentrations (c) are calculated from equation (8) above and represent the concentration of the unbound chloride ions.

The average binding ($\bar{v}$) i.e. the number of chloride ions bound per myoglobin molecule, is calculated from equation (10) and from the knowledge of the free chloride concentration and total myoglobin concentration.

The ionic activity coefficient, (f), is calculated from equation (26) which is derived from Debye-Hückel theory. The ionic strength, (I), appearing in that equation is considered as equal to the total stoichiometric chloride concentration.

The electrostatic interaction factor, (w), is calculated from equation (24), which is derived from equation (23) by taking the radius (b) of myoglobin as 18 Å and the distance of closest approach (a) as 20 Å. The reported $\bar{w}$ is obtained by multiplying (w) by factors

Table 3.A

Calculation of Chloride Binding to Myoglobin (1.0×10^{-3} M)

Total Cl^- (moles/-liter)	pH	Net E.M.F. E (mv)	Free Cl^- c (moles/-liter)	1/c	Average Binding $\bar{v}$	$1/\bar{v}$	$\bar{v}/c$	Ionic activity coeffi-cient f	$\bar{w}$	$\frac{\bar{v}\exp(2\bar{w}\bar{v})}{cf}$
5.0×10^{-4}	6.47	57.67	0.55×10^{-4}	18000	0.46	2.17	8280	0.9754	0.070	9014
1.0×10^{-3}	6.53	42.85	1.93×10^{-4}	5180	0.81	1.23	4196	0.9663	0.067	4834
5.0×10^{-3}	6.65	14.78	2.16×10^{-3}	463	2.84	0.36	1315	0.9312	0.057	1945
1.0×10^{-2}	6.72	11.65	6.40×10^{-3}	156	3.60	0.28	562	0.9082	0.0511	896
5.0×10^{-2}	6.70	3.80	4.43×10^{-2}	23	6.8	0.15	154	0.8363	0.037	303
1.0×10^{-1}	6.86	3.28	8.82×10^{-2}	11	11.80	0.09	133	0.7999	0.030	345

Table 3.B

Calculation of Chloride Binding to Myoglobin (1.0×10^{-4} M)

Total Cl^- (moles/-liter)	pH	Net E.M.F. E (mv)	Free Cl^- c (moles/-liter)	1/c	Average Binding $\bar{v}$	$1/\bar{v}$	$\bar{v}/c$	Ionic activity coeffi-cient f	$\bar{w}$	$\frac{\bar{v}\exp(2\bar{w}\bar{v})}{cf}$
5.0×10^{-4}	6.30	36.37	1.24×10^{-4}	8060	3.76	0.27	30300	0.9754	0.018	35.360
1.0×10^{-3}	6.36	17.76	3.73×10^{-4}	2000	5.00	0.20	10000	0.9663	0.017	12.266
5.0×10^{-3}	6.35	3.82	4.3×10^{-3}	231	6.80	0.15	1571	0.9312	0.014	2.040
1.0×10^{-2}	6.36	2.61	9.03×10^{-3}	111	9.70	0.10	1077	0.9082	0.013	1.522
5.0×10^{-2}	6.41	0.67	4.87×10^{-2}	21	13.00	0.08	267	0.8363	0.009	360
1.0×10^{-1}	6.38	0.50	9.81×10^{-2}	10	19.00	0.05	192	0.7999	0.008	324

of 0.4 and 0.1 for 1.0×10^{-3}M and 1.0×10^{-4}M myoglobin respectively. The introduction of these factors is necessary for making experimental plots symmetrical[7] and for compensating for the myoglobin concentration effect.

Figure 8 is the classical plot of the average binding, $(\bar{v})$, as a function of log (free chloride concentration)[11,27], i.e. log c; for two myoglobin concentrations. No plots are shown for myoglobin concentrations of 10^{-5}M and 10^{-6}M, because these solutions are too dilute to give reliable results. The curves, are smooth and non-linear indicating that more than a single class of sites are encountered in chloride binding to myoglobin.

Figures 9.A and 9.B show the relationship of $1/\bar{v}$ to $1/c$ for 1.0×10^{-3}M and 1.0×10^{-4}M myoglobin. The curves in Figure 9.A are in conformity with equations (29) extended for many classes of sites. A portion of each curve was therefore expanded in Figure 9.B to give approximately linear plot. The common intercept of both curves thus plotted, on the $1/\bar{v}$ axis, is a measure of the total number of sites. This was found, from Figure 9.B, to be 20.

It should be noted, however, that several investigators have criticized the use of such linear plots on the grounds that "they have the disadvantage of concealing deviations from the ideal laws, and of tempting straight lines where there should be curvature"[1]. An alternative plot recommended by Scatchard[1] is to plot $\bar{v}/c$ against $\bar{v}$. This is shown by the points in Figure 10 for the two myoglobin concentrations. The intercepts of the curves on the $\bar{v}$ axis give a total number of sites of 20 for both concentrations; and the intercept

on the ordinate gives first intrinsic association constants (k_1) of 15,000 and 50,000 for 1.0×10^{-3}M and 1.0×10^{-4}M myoglobin respectively. The curves shown in the same figure correspond to hypothetical myoglobin solutions of three classes of sites each comprising 1, 6, and 13 sites respectively with respective intrinsic binding constants of 15,000, 100 and 10; and 50,000, 5,000 and 20 for 1.0×10^{-3}M and 1.0×10^{-4}M myoglobin respectively. It is seen that the experimental points for 1.0×10^{-3}M myoglobin fit exactly the first theoretical curve, while those for 1.0×10^{-4}M myoglobin fit the second curve. The values used for plotting these theoretical curves were calculated from equation (27) by trying different values for the number of classes, the number of sites per class and the intrinsic constants for each site. The intercepts and slopes of various experimental curves obtained so far served as a guide for guessing the right values for the sought parameters.

In Figure 11 the average binding ($\bar{v}$) is plotted as a function of $\frac{v \exp(2\bar{w}\bar{v})}{cf}$ for the two myoglobin concentrations, together with the theoretical curves calculated from equation (28) corresponding to three classes of sites of 1, 6 and 13 sites per class and respective intrinsic binding constants of 15,000, 100 and 10 for 1.0×10^{-3}M myoglobin and 50,000, 5,000, and 20 for 1.0×10^{-4}M myoglobin. The agreement between theory and experiment is remarkable especially for 1.0×10^{-3}M myoglobin.

Figure 12 shows the variation of pH with the average binding corrected for the electrostatic interaction. The relationship is linear for both myoglobin concentrations, with a slope of about unity.

The intercepts of the lines on the pH axis give the isoionic pH values[4]. These are 6.47 and 6.25 for 0.001M and 0.0001M myoglobin respectively.

The results of the above calculations and plots are summarized in Table 3.C.

Table 3.C

Summary of Results at 25°C

Myoglobin (mole/l)	Isoionic pH	Chloride Binding Sites and their Constants		
		n_1 (k_1)	n_2 (k_2)	n_3 (k_3)
0.001	6.47	1 (15,000)	6 (100)	13 (10)
0.0001	6.25	1 (50,000)	6 (5,000)	13 (20)

4. Effect of Temperature

An attempt has been made at detecting the effect of temperature on chloride binding. E.M.F. was measured at two different temperatures, namely 5.0°C and 25.0°C; the following results were obtained for two myoglobin concentrations in 0.10M potassium chloride.

Myoglobin (mole/l)	Net E.M.F. at 5.0°C (mv)	Net E.M.F. at 25.0°C (mv)
0.001	3.40	3.28
0.0001	0.54	0.50

The results indicate that temperature has only a slight effect on the net E.M.F. and consequently on binding. This is in conformity with earlier findings[9,11]. The effect has thus not been further investigated.

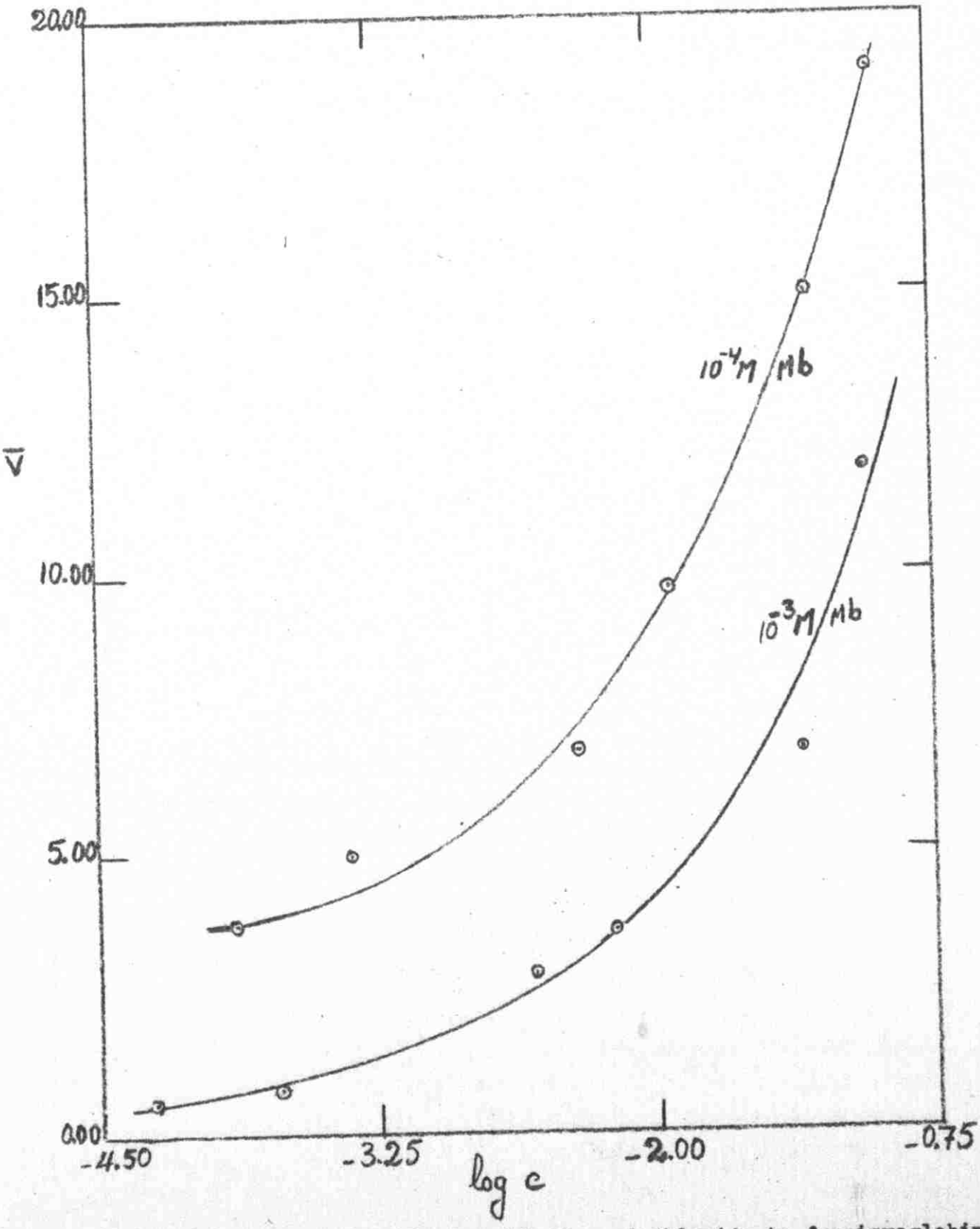

Figure 8. Variation of average binding of chloride to ferrimyoglobin ($\bar{v}$ as defined by equation 10) with log c, where c is the concentration of free (unbound) chloride; at 10^{-3}M and 10^{-4}M myoglobin concentrations.

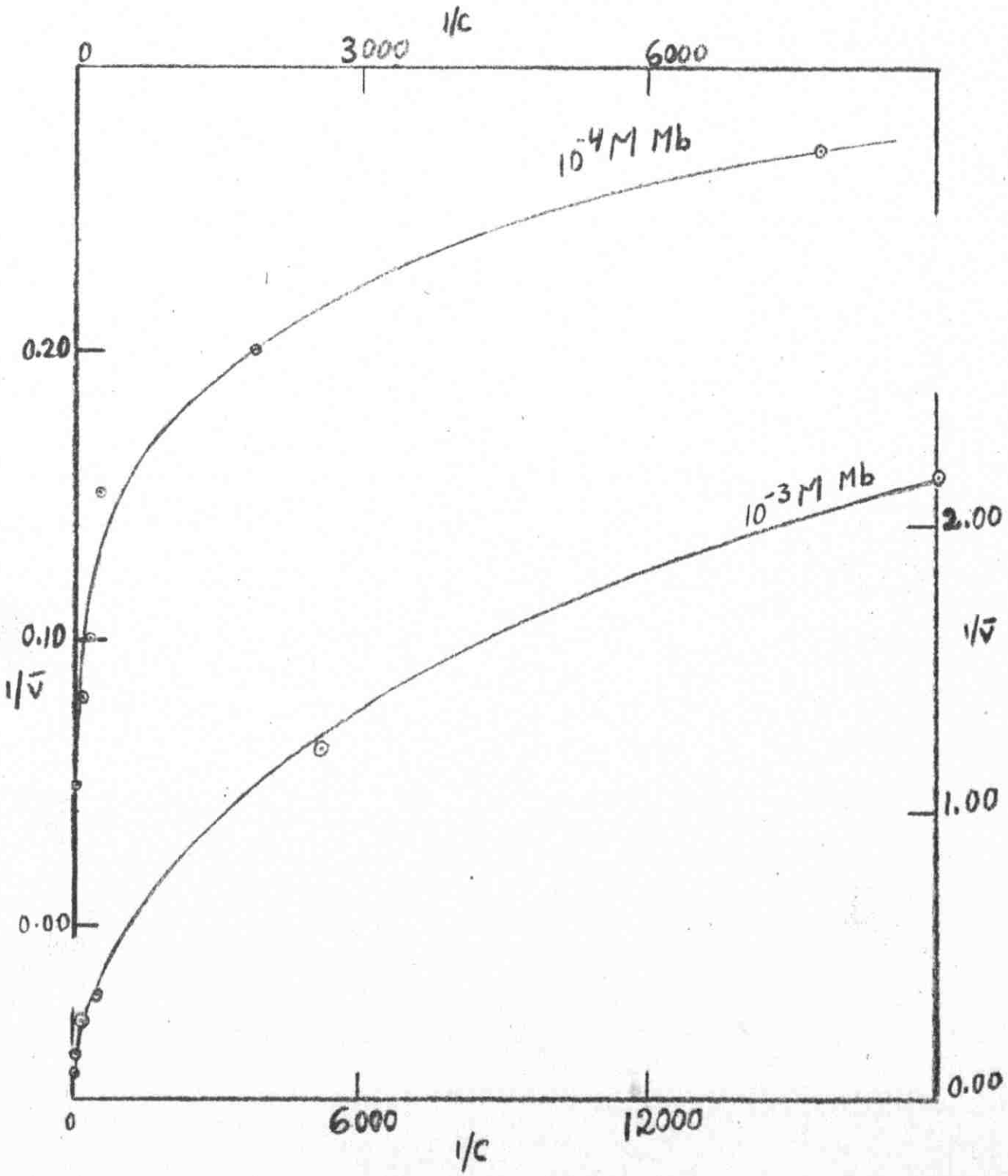

Figure 9A. Reciprocal plot of $1/\bar{v}$, $\bar{v}$ being the average binding as defined by equation 10, against 1/c, where c is the molar concentration of free (unbound) chloride. The scales for 10^{-3}M and 10^{-4}M myoglobin are shown separately.

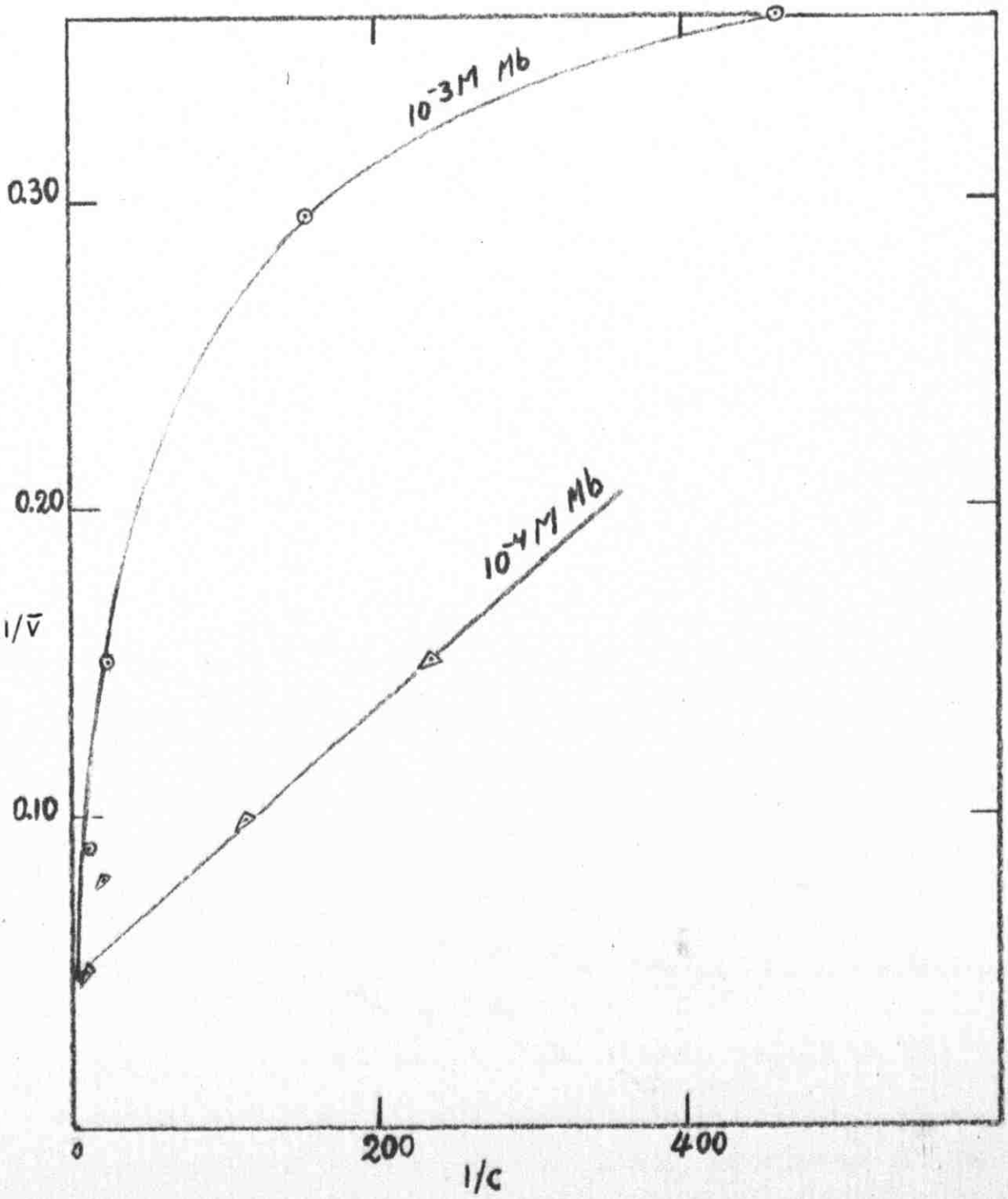

Figure 9B. Part of Figure 9A with expanded scale to show, at the high concentration limit, the near linearity of the $1/\bar{v}$ versus $1/c$ plot.

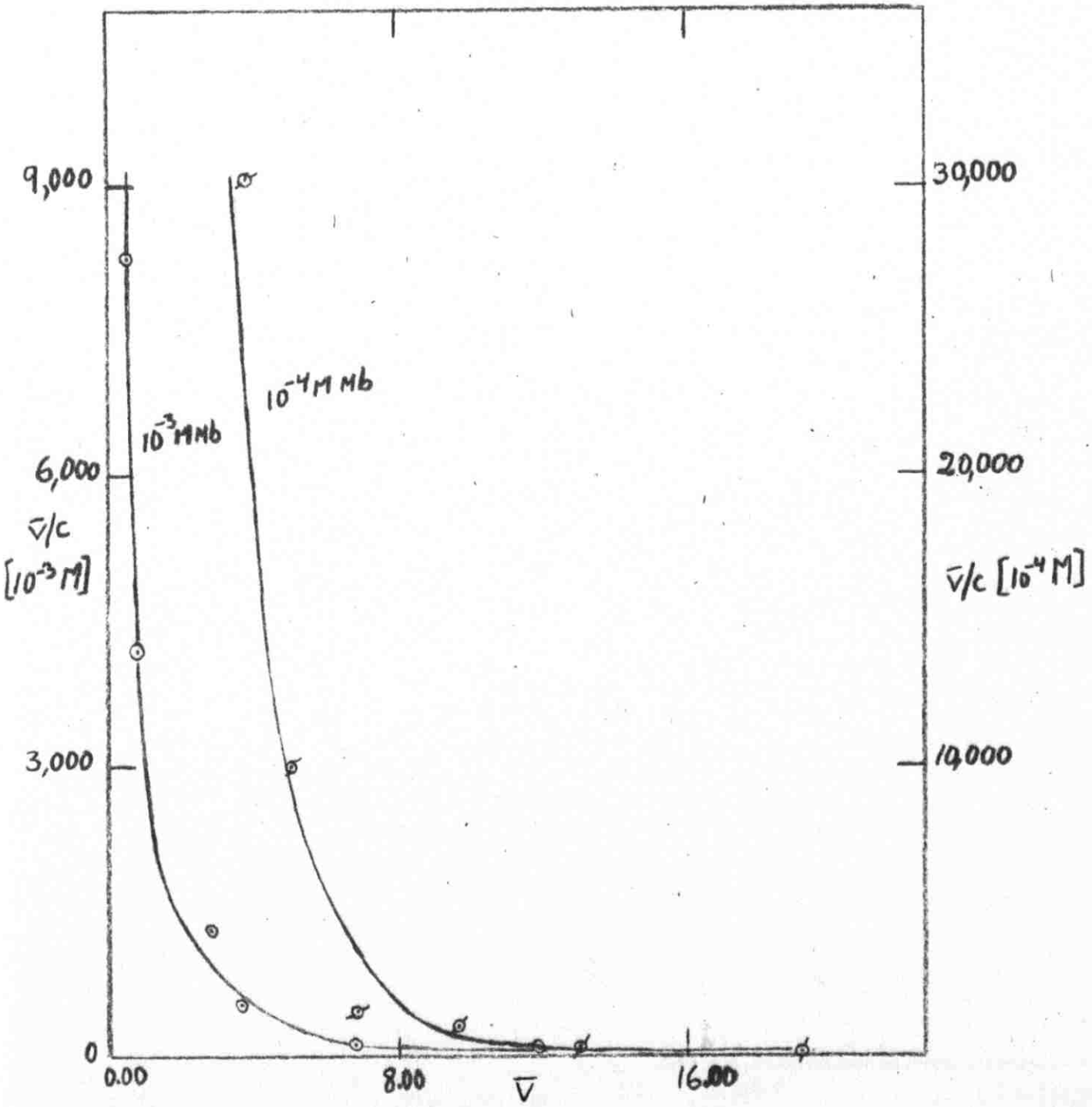

Figure 10. Plot of $\bar{v}/c$ versus $\bar{v}$ where $\bar{v}$ is the average binding as defined by equation 10, and c is the molar concentration of free chloride, for 10^{-3}M and 10^{-4}M myoglobin. Experimental results are shown by points while the curves are theoretical corresponding to calculations based equation 27 assuming three classes of sites as in the text.

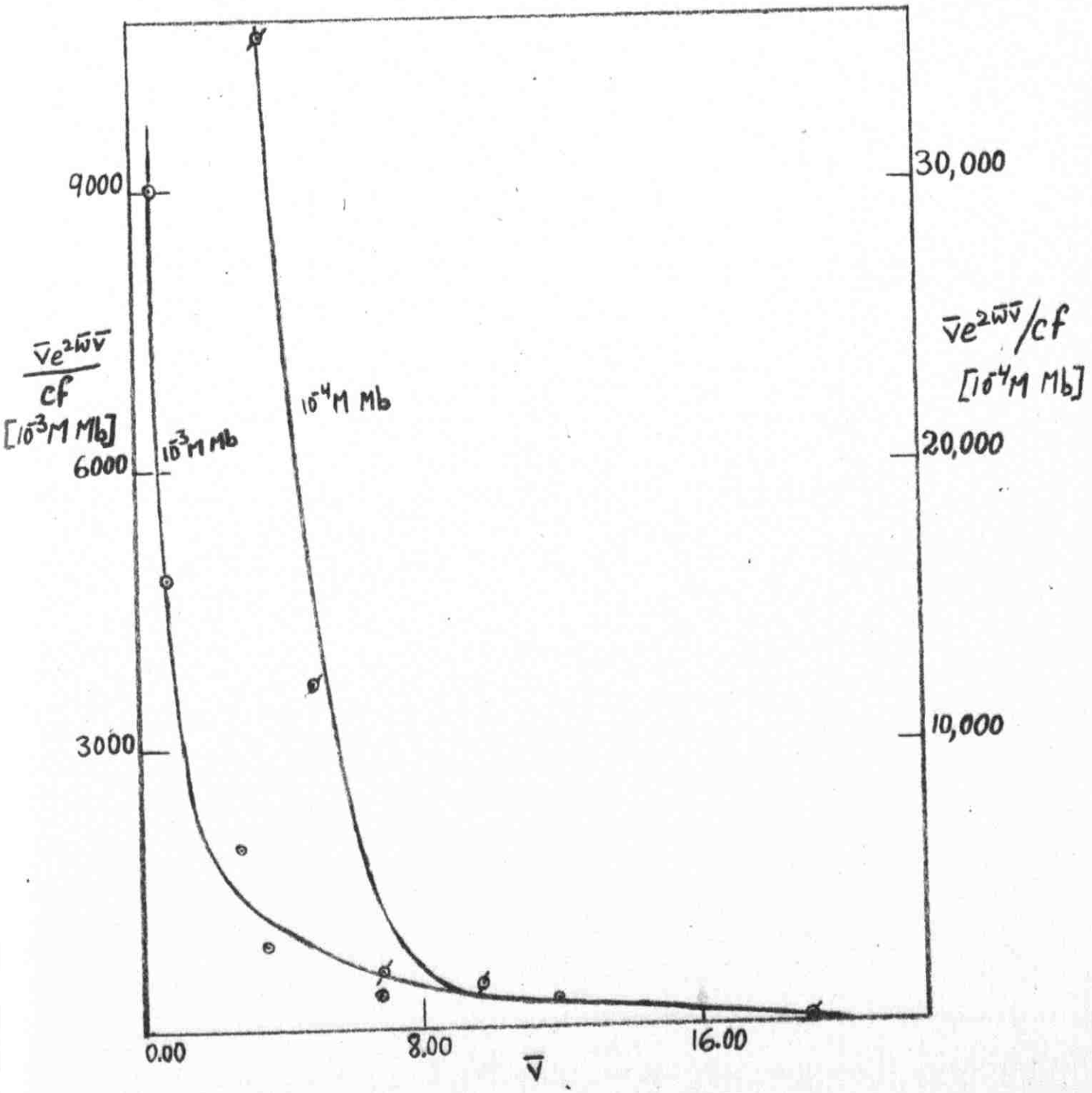

Figure 11. Plot of $(\bar{\nu}/cf)\exp(2\bar{w}\bar{\nu})$ versus $\bar{\nu}$ where $\bar{\nu}$ is the average binding as defined by equation 10, c is the molar concentration of free chloride, f the ionic activity coefficient, $\bar{w}$ being the corrected electrostatic interaction factor taken as 0.4 w and 0.1w for 10^{-3}M and 10^{-4}M myoblogin respectively. w is defined by equation 23. Experimental results are shown by points while the curves are theoretical corresponding to calculations based on equation 28 assuming three classes of sites as in the text.

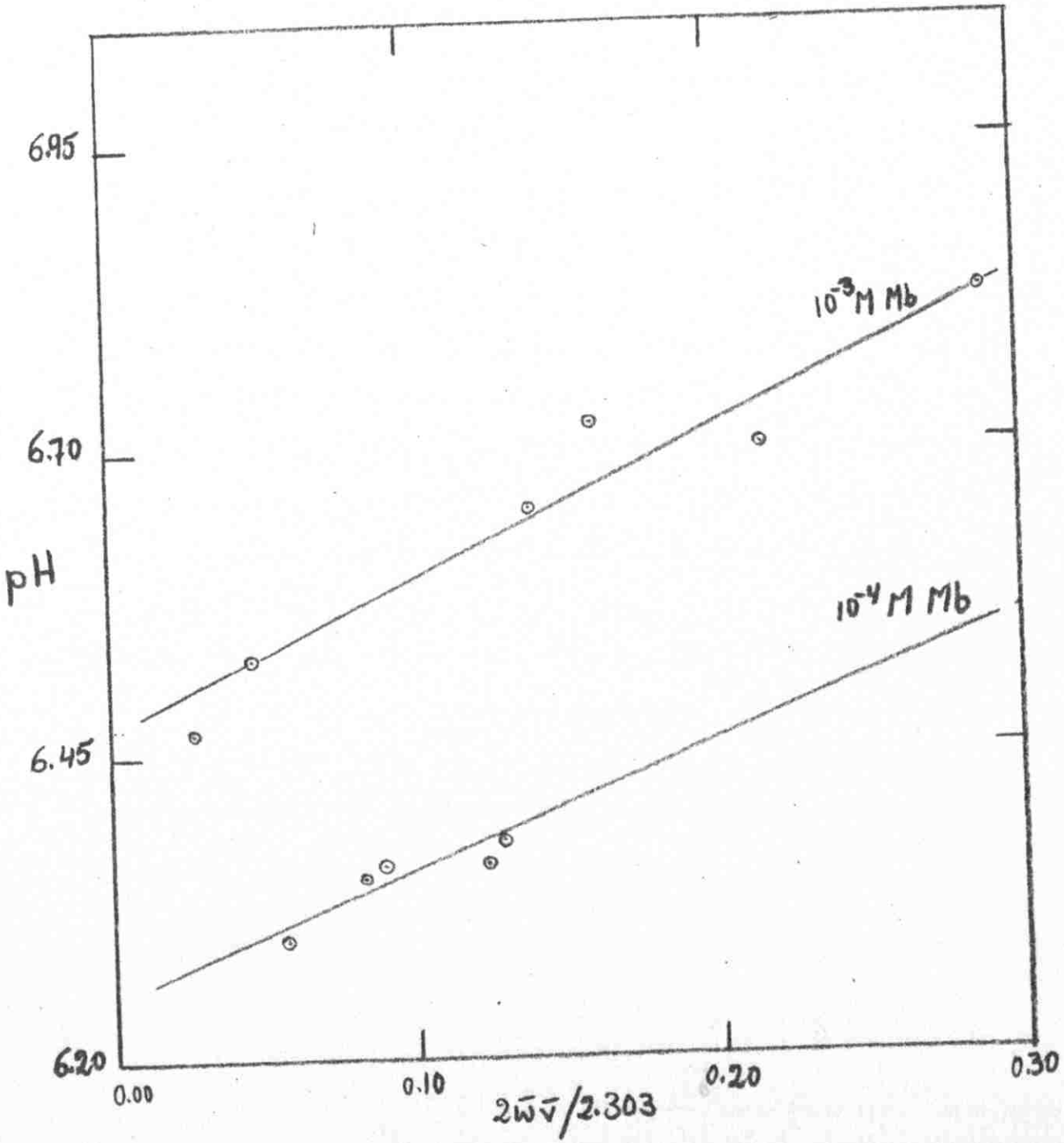

Figure 12. Variation of $2\bar{w}\bar{v}/2.303$ with pH of myoglobin/potassium chloride systems for 10^{-3}M and 10^{-4}M myoglobin. $\bar{w}$ is the corrected electrostatic interaction factor taken as 0.4w and 0.1w for 10^{-3}M and 10^{-4}M myoglobin concentration respectively; w being calculated from equation 23. $\bar{v}$ is the average chloride binding as defined by equation 10. The straight line have a slope of about unity.

D I S C U S S I O N

In the preceding experiments an attempt was made at detecting and evaluating the binding of chloride ions to ferrimyoglobin. E.M.F. measurements were carried out using concentration cells with liquid junction, and the calculations were based on the assumption that the cell E.M.F. was due to the combined effects of binding (which decreases the activity of chloride ions in the protein/chloride compartment), and liquid junction potential (which produces a negative potential varying with temperature, concentration and pH). It was further assumed that this latter effect could be estimated from duplicate measurements of E.M.F. in strongly alkaline solution where binding of anions is minimal[40]. It was then found by graphical and numerical analysis that the results are in closest agreement with the theoretical equations if three classes of binding sites on the protein are assumed with different numbers of sites and different binding constants as summarized in Table 3.C.

Reliability of Results:

The apparatus used for the E.M.F. measurements had a sensitivity as high as 5 μv, and the E.M.F. readings had a precision of 10 μv. Duplicate runs gave values well within 50 μv. It therefore follows that the uncertainty in the reported E.M.F. values ranges from about 0.1 to a maximum of 10%, a precision which is considerably better than that of similar work hitherto reported in literature.

The concentration of potassium chloride solutions was varied over the widest range possible. However, there are two limits to this range: At the lower limit (less than 5×10^{-4}M), where E.M.F. measurements are very important in determining the first association constant, the chloride to myoglobin concentration ratio is too low to give reliable results. With relatively concentrated chloride solutions (more than 0.1M) on the other hand, the relative decrease in chloride ion activity due to binding is too small to produce detectable E.M.F. Measurements at such high concentrations would otherwise have been extremely valuable in determining the total number of binding sites.

The total number of sites and their intrinsic association constants were determined graphically by non-linear extrapolation. Although results thus obtained involve some approximation, the validity of the extrapolations is supported by the fact that calculations were made for two myoglobin concentrations, and that the graphs corresponding to both concentrations gave a common intercept, indicating a total number of sites of 20 (see Figure 9B). The very steep initial part of the $\bar{\nu}/c$ versus $\bar{\nu}$ plots (Figure 10) required the assumption that one site on the protein binds chloride very tightly. Any assumption of larger number of sites in this class results in a less steep curve. Similarly, the very flat end of the curve called for the assignment of very low binding constant to a large part of the binding sites. The smooth curvature in the middle portion of the plot could be theoretically represented only by the assumption of a third class of sites with intermediate number

of sites and association constant.

It was on the above basis that the conclusion was arrived at regarding the chloride binding sites of myoglobin and their association constants (Table 3.C). Although the values of these constants are only approximate, they do, nevertheless, indicate their order of magnitude and the relative affinities of the various classes of binding sites for chloride ions.

It is to be noted that the results for the higher myoglobin concentration (10^{-3}M) give a closer measure of the total binding capacity, since at high concentration the cationic binding sites, being weak acids, are more highly protonated. Nevertheless, intrinsic binding constants have been assigned to the various binding sites at 10^{-4}M myoglobin, and the results are in qualitative agreement with the findings of Klotz[40], namely that the association constants increase substantially with decreasing protein concentration.

Influence of Protein:

It is well known that proteins interact with ions in solution, thus affecting pH and ionic strength simultaneously. Furthermore, the binding of ions to protein alters the charge distribution on the protein surface and affects the liquid junction potential. The complications introduced by the interdependence of such effects will now be considered.

Scatchard and Black[4] and Alberty[42] have pointed out that anion binding increases pH and cation binding decreases it; pH, in turn, affects the extent of binding. Similarly binding reduces the small

anion contribution to the ionic strength of the medium; it, likewise, affects the contribution of protein to the ionic strength, but in a way dependent on the effective charge of the protein. The ionic strength, in turn, may affect the extent of binding.

It was found in the course of this work that the control of pH and/or ionic strength through the introduction of buffer or other ions into the protein solution causes complications, presumably as a result of competitive binding of the ions in solution. In general, it has been the practice not to adjust pH or ionic strength. In this connection it is interesting to note that Klotz[40] has observed no significant difference in the competitive binding of methyl orange by albumin in phosphate buffer at ionic strengths of 0.05 and 0.13.

In the present work, ionic strength was computed, after Scatchard[11], on the basis of the total stoichiometric salt concentration and neglecting protein contribution. Likewise, the ionic activity coefficient, f, was calculated, after Scatchard[11], as being independent of the protein. Although changes in ionic strength and ionic activity coefficient are expected as a result of binding, calculations show that, in the range of the present experimental conditions, the relative changes are minor. In fact, no appreciable difference in the results obtained for binding sites was observed by recalculating the data on the basis of equating the ionic strength to the concentration of free (unbound) chloride ions.

The electrostatic effect of ionic strength is usually corrected for by the introduction of the electrostatic factor, w, which is

defined by equation (23), as a function of ionic strength, temperature, and dielectric constant of the solvent. The effective dielectric constant of the medium may be different from that of the pure solvent. Moreover, equation (23) does not allow for the possible effect of protein concentration on w. An accurate value of w should make experimental results (known to be correct by fitting equations not involving w) fit equations (22) and (31). This value of w may be different from that calculated using equation (23). Scatchard[23] suggested the use of equation (31) as a check on the soundness of the corrected values of w, say $\bar{w}$. Cannan, Kibrick and Palmer[7] applied a fractional factor (0.8) to the values of w as determined by equation (23) for albumin, to make them fit the experimentally determined values. w has also been described as an "empirical factor"[5] which is dependent on ionic strength, dielectric constant and temperature. For the purpose of this work the factors of 0.4 and 0.1 have been applied to w for 10^{-3}M and 10^{-4}M myoglobin concentrations respectively. This treatment takes into consideration the effect of protein concentration on w and, at the same time, keeps w as a function of dielectric constant, temperature, and ionic strength as governed by equation (23).

The present work has shown that myoglobin contributes to the measured E.M.F. of the concentration cell in which it occurs, other than through binding chloride ions. This contribution has been attributed to the effect of the protein on the liquid junction potential, a phenomenon which has not hitherto been adequately recognized by previous investigators. The use of the usual technique of a saturated potassium chloride bridge did not eliminate this effect,

contrary to what was assumed by Scatchard and co-workers[11,12]. No attempt has been made to calculate the theoretical liquid junction potential of the myoglobin solutions since, in addition to its involving inexact equations, the treatment calls for knowledge of the effective charge on the protein, its transport numbers, etc. These parameters have not yet been determined. Qualitatively, at any rate, the negative liquid junction potential of the protein seems reasonable on the grounds that in the experiments performed the pH was higher than the isoionic point of the protein, and any chloride binding would further reduce the positive charge on the protein.

Structural Considerations:

One of the most interesting aspects of the present work is that the results can be correlated with the detailed structure of myoglobin. It is known from the work of Edmundson and Hirs[3] that the cationic groups on the myoglobin molecule include 4 arginines, 12 histidines and 19 lysines. It is also known from the work of Breslow and Gurd[43] that six of the histidine groups are masked or inaccessible to interacting ions. The technique applied in the present investigations excludes the counting of the four arginine groups (normal pK greater than 12)[20]. The cationic groups most likely to be available to chloride binding are therefore the six accessible histidines and as many as available of the lysines. It is known from the amino acid sequence of myoglobin[3] that some lysines lie adjacent to negatively charged side groups; these may either neutralize the positive charges of the neighbouring lysines

or repel the negatively charged chloride ions, thus preventing binding to some lysine residues on myoglobin.

It therefore seems reasonable to identify the three classes of binding sites with 13 lysine amino ($-\overset{+}{N}H_3$) groups, 6 histidine imidazolium ($\overset{+}{N}H$) groups, and one group which is in a special position with exceptionally high binding affinity. The normal acid pK values are 9-10 for lysine and about 7 for histidine[20]; these groups could well be the cationic sites responsible for chloride binding, since the E.M.F. measurements were carried out at pH lower than 7 for normal binding and at pH about 11 for minimal binding. The single-site binding class is more difficult to account for. One possibility is to identify it with the special sulfate ion binding site observed by Stryer, Kendrew, and Watson[44] in their X-ray investigation of the azide complex of sperm whale myoglobin.

In view of its structural similarity to myoglobin, hemoglobin would be expected to show similar behaviour towards chloride ions, with substantially greater binding because of its larger amino acid content. Scatchard and co-workers have concluded from recent osmotic pressure measurements[14] that carbonylhemoglobin does not bind chloride ions. However, Haurowitz and Hardin[45] have reported evidence for the binding by hemoglobin of phosphate ions. Clearly the facts need re-investigation in the light of the present findings. Moreover, if chloride binding to myoglobin is further substantiated by other studies it would be necessary to re-evaluate the kinetic and thermodynamic data previously obtained on dilute solutions of proteins and chlorides where ion association could have a significant effect.

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