M. Sc. Experimental Medicine

Vijay Laxmi Sawh

STUDIES ON ASSOCIATION-DISSOCIATION PHENOMENA OF HEME-PROTEINS

ABSTRACT

The dissociation of two heme proteins, chick hemoglobin and bovine liver catalase has been studied in presence of ionic (LiCl, NaCl, KCl and $CaCl_2$) and non-ionic solutes (formamide, urea, guanidine-hydrochloride and 2-chloroethanol), and in the alkaline pH range of pH 8.0 - pH 11.6. The extent of dissociation depends on the nature of the solvent media. At a low protein concentration bovine liver catalase dissociates. The gross conformation of chick hemoglobin in the ionic and non-ionic solutes is not affected as indicated by the viscosity measurements. The conformation of bovine liver catalase, however, appears to be affected. The Soret band intensity of both the proteins remains unaffected in presence of ionic solutes except $CaCl_2$. Non-ionic solutes decrease the Soret band intensity. Both types of solutes affect the enzymatic activity of catalase.

The alkaline dissociation of chick hemoglobin appears to begin at pH 10.3, and that of bovine liver catalase at pH 9.3.

A comparison of the dissociation of chick hemoglobin and bovine liver catalase points out that the over-all forces holding the subunits of chick hemoglobin in the polymeric form are greater than those in bovine liver catalase. Preliminary studies on avian (duck and chick) hemoglobins indicate that the avian hemoglobins are more resistant to dissociation than the mammalian hemoglobins (human adult, human fetal, equine and bovine).

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CHAPTER I

INTRODUCTION

The physicochemical studies on protein structure have provided a better understanding of the relationship between their macromolecular structure and their biological function. Each protein reflects a unique sequential arrangement of amino acids folded into a compact shape. Studies of various kinds show that proteins may be comprised of a single polypeptide chain, or multi-chains. The multi-chains reflect an association of single polypeptide chains, like or unlike, with or without covalent linkages between the constituting chains. In some cases proteins, like enzymes, need prosthetic groups to perform their specific biological function. Hemeproteins are involved in various biological functions, e.g. myoglobins and hemoglobins (1, 2) act as oxygen carriers, cytochrome-c (3) functions as a reversible electronic oxidation-reduction carrier, catalase and peroxidase (4) as oxido-reductases.

The molecular weight of hemoglobin is approximately integral multiples of the minimum molecular weight obtained from chemical analysis of the heme. The iron content in hemoglobin, first accurately reported by Engelhart (5), was 0.35%. Based on this value the minimum molecular weight per heme was calculated as 16,700. However, Adair (6) reported an approximate molecular weight of hemoglobin as 60,000 - 68,000, employing osmotic pressure measurements. In 1926 a similar value was obtained by Svedberg and Fahraeus (7) during the first ultracentrifugal analysis of proteins. Later on in 1934, Svedberg and Hedenius (8) reported the molecular weight of vertebrate hemoglobin to be of the order of 64,000 - 68,000; the only exception being that of the cyclostomes which was 17,000. In 1933, Pedersen (9) observed

the dependence of the molecular weight of horse hemoglobin on the protein concentration. The 'S' value of horse hemoglobin showed the maximum value only within a definite concentration range. Tiselius and Gross (10) reported that the value of the diffusion constant ($D_{20,w}$) increased markedly with increasing dilution implying thereby that the hemoglobin molecule dissociated at a low protein concentration. That the osmotic pressure increased with the decrease in the concentration of fetal sheep hemoglobin, as observed by McCarthy and Popják (11) could be explained by the fact that the hemoglobin dissociates on dilution. Based on the experimental results revealing the dependence of S values on concentration of horse and sheep hemoglobins, Gutfreund (12, 13) concluded that at a very low protein concentration the hemoglobin molecule first dissociates into halves and further on to quarters. Recently this observation has been confirmed by Schachman and Edelstein (14) for human oxyhemoglobin. The stability of hemoglobin under varying pH conditions was studied by several authors. According to Gralén (15) horse HbCO did not dissociate within the range of pH 6.0 - pH 9.5. However, Field and O'Brien (16) demonstrated that human hemoglobin dissociated into dimers in the acidic pH's, pH 6.0 - pH 3.5, exhibiting a single Schlieren peak with a broadening of the sedimentation boundary. The broad peak was attributed to a rapid association-dissociation equilibrium. Using light-scattering and osmotic pressure measurements Reichman and Colvin (17) reported a mean molecular weight of 20,000 for horse hemoglobin in the pH range of pH 1.8 - pH 2.0. Data in the early literature indicate that the hemoglobin molecule dissociates at an alkaline pH as well as at an acid pH condition (18). In 1959, Hasserodt and Vinograd (19) observed a

reversible and symmetric dissociation of human carbonmonoxyhemoglobin to half molecules between pH 10.0 - pH 11.0. Results reported by Kurihara and Shibata (20) were not in total agreement as they showed a decrease in S value which was not accompanied by a decrease in molecular weight. The dissociation of bovine, human and fetal carbonmonoxyhemoglobins to dimers at alkaline pH (the range studied pH 5.7 - pH 11.0) was observed by Gottlieb et al (21).

Concentrated salt solutions were also able to dissociate hemoglobins, although the hemoglobins of different animal species differ considerably in their behavior. Burk and Greenberg (22) obtained a molecular weight of 34,000 for horse hemoglobin in 6.66 M urea. The effect of urea and other amide derivatives on the sedimentation and diffusion constants as well as other properties of horse carbonmonoxyhemoglobin was studied by Steinhardt (23). The S value decreased from 4.5 S in water to 3.2 S in 4 M urea, and the $D_{20,w}$ value increased from 6.9 to 7.8 x 10^{-7} cm^2 sec^{-1}. The value of 'S' was independent of the time of exposure to urea, and of urea concentration between 4 M and 8 M urea (23). Formamide and acetamide produced an effect similar to that of urea (23). In contrast Gutter et al (24) obtained only a slight decrease in molecular size of horse hemoglobin in 4 M urea and no decrease for human hemoglobin although the decrease in the S value was nearly the same. Kurihara and Shibata (20) showed that the dissociation of horse hemoglobin began at 1.5 M urea and was complete at 8 M urea, in which the molecules exist as dimers. Unlike Steinhardt (23), these authors observed a further decrease in the S values in solutions containing 4 M urea or above. On the other hand, in 2.5 M guanidine hydro-

chloride hemoglobin dissociated to dimers and with an increase in solute concentration, monomers were produced, the dissociation being complete at 6 M guanidine hydrochloride. In 1965 Kawahara et al (25) studied the dissociation of human carbonmonoxyhemoglobin in presence of urea, guanidine hydrochloride, several organic solutes and $CaCl_2$ by evaluating the weight-average sedimentation velocity coefficient as a function of reagent concentration. These investigators demonstrated that in the presence of the aforementioned reagents dissociation proceeded to dimers without any appreciable conformational change of the subunits. Guanidine hydrochloride at high concentration dissociated hemoglobin to monomers with complete unfolding of the polypeptide chains and the release of the heme group from the protein moiety.

The disruptive effect of concentrated NaCl solution on human and horse hemoglobin has long been known. The earlier ultracentrifugal analysis by Anderson (26) and the osmotic pressure measurements by Gutfreund (13) indicated a decrease in molecular weight of hemoglobin in NaCl solutions at a concentration above 0.5 M. That the S value of human oxyhemoglobin decreased from 4.4 S to about 3.6 S in 1 M NaCl and to 3.3 S in 2 M NaCl was reported by Rossi-Fanelli et al (27), Cecil and Snow (28), Grossman and Tanford (29), Benesch and Benesch (30), and Benesch et al (31). These investigators also noted the dissociation of hemoglobin in concentrated KCl solution. In 1964 Kirshner and Tanford (32) stated that the observed decrease in the S values in salt solutions reflected the dissociation of hemoglobins into dimers, the solutes used in their studies being NaCl, $CaCl_2$, $MgCl_2$ and ammonium sulfate.

The molecular weight of bovine globin and horse globin was estimated

to be 37,000 and 29,000 respectively employing osmotic pressure measurements (33). These data were substantiated by the ultracentrifugal analysis of Gralén (15). Smith and his collaborators (34) demonstrated two chemically distinct components each of molecular weight 16,000. The molecular weight of adult and fetal human globins evaluated in a salt-free medium by Rossi-Fanelli et al (35, 36) was 18,000 and 41,000 to 42,000 at a moderate ionic strength. These authors were of the opinion that native globin near the iso-ionic point displays a reversible association-dissociation phenomenon in which are molecular species, monomers (α- and β-), dimers (αβ) and tetramers $(\alpha\beta)_2$ participate. At an acidic pH, dissociation reaches a maximum and two electrophoretic components (molecular weight 18,000) appear (17). These experimental results suggested that in certain conditions of pH the hemoglobin molecule may undergo a reversible dissociation producing dimers and monomers. The hydrodynamic studies on hemoglobin indicated that in solution the molecules are symmetrical and spherical in shape. The frictional ratio was evaluated as 1.1.to 1.3 (2, 18), the divergence from 1.0 being attributed to hydration of the protein molecule.

Studies of viscosity increment (37), dielectric dispersion (38), and low angle x-ray scattering of hemoglobin (39) are in good agreement with the size and shape as revealed by x-ray crystallographic analysis. Studies on the atomic model of hemoglobins showed that the hemoglobin molecule is spheroidal with dimensions, 64 x 55 x 50 Å (40, 41) and with four interlocking electron-dense strands, two of one type (α chains) and two of another (β chains), arranged in a pseudo-tetrahedral array to form two subunits each consisting of two non-identical α and β units. Geometrically, the α-chain is quite similar to the β-chain (42). The four heme groups are located at

the surface of the molecule in four widely separated pockets embedded in the polypeptide chains. The iron atoms lie at the corners of an irregular tetrahedron. The distance between these prosthetic groups is far too great to permit any direct interaction between them (43). The surface area of the α-chains exactly fit those of the β-chain, and since the α-chain is inverted over the β-chain, there is a large area of contact between them. Van der Waals interactions between a multitude of side chains as well as several hydrogen bonds possibly contribute to the structural complementarity which exists between α and β chains. Recent studies by Perutz and his collaborators based on 2.8 Å resolution (44) revealed that $\alpha_1\beta_1$ contact is more extensive and made up thirty-four residues while that of $\alpha_1\beta_2$ is nineteen residues. No contacts between like chains were visible, however this does not preclude the existence of salt linkages involving α-amino and carboxyl terminal residues. The possible polar links are few. There is a large central cavity, represented by two boxes 20 x 8-10 x 25 Å through the core of the molecule (44). Nearly all the polar side chains are in contact with water or in the internal cavity, while the non-polar residues lie in the interior of the individual subunits or in superficial crevices to minimize contact with water or else at point of contacts between the unlike subunits. This characteristic symmetrical organization of the four subunits is essentially identical with hemoglobins with molecular weight near 64,000. The general construction of the native tetrameric hemoglobin molecule in aqueous environment appears to be such as to lead to the least free energy and the greatest possible entropy of the protein (45).

Although the hemoglobins of mammals and other vertebrate species

show great uniformity in size and shape, the tendency towards depolymerization varied widely from one animal species to the other, even among the same species. For example, human fetal hemoglobin is resistant to alkaline denaturation while human adult hemoglobin is alkali-labile (46). The variability in alkali-resistance characteristics of avian hemoglobins has been reported by Saha and his collaborators (47, 48). An avian hemoglobin of maximum alkali resistance, chick hemoglobin, was chosen for the present study. Physiological environmental conditions of chick hemoglobin differ from those of mammalian hemoglobins in electrolyte concentration, body temperature and rate of circulation. The presence of multiple hemoglobins in avian species has been reported by Saha and his co-workers (49-51). The situation is different in some other animals, e.g. hemoglobins of sheep (52), deer (52) and goat (53), where the occurrence of different (two or more) types seems to be controlled by allelomorphic genes. Normal human hemoglobin is made up of one major component, Hb A, and minor components. Mutants of human hemoglobin A (54) are most numerous and widely studied. α- and β-chain substitutions may occur with one amino acid or two amino acids (55, 56).

The 'S' value of the major component of chick hemoglobin was found to be 4.2 S (18) in agreement with values reported for other vertebrate hemoglobins. The intrinsic viscosity of chick hemoglobin was found to be 3.5 - 3.6 cc/gm (57) which is essentially identical to that reported for carbonmonoxyhemoglobin A by Kawahara et al (25).

The chick hemoglobins differ from mammalian hemoglobins (human and horse) in amino acid composition. The $\alpha^1{}_2$-, $\beta^1{}_2$-, $\alpha^2{}_2$, and $\beta^2{}_2$ chains of

Hb 1 and Hb 2 contain 311, 310, 300 and 286 amino acid residues (58), adult human and horse hemoglobins contain 282 (59, 60) and 292 (61) residues only in the α- and β-chains. The number of hydrophobic residues is greater in chick hemoglobin than that in the mammalian hemoglobins. Table I presents a comparative amino acid composition of chick, duck, horse and human hemoglobins.

Another heme protein, bovine liver catalase, has been adopted as a model during the present investigation. The catalases are respiratory enzymes (H_2O_2:H_2O_2 oxido:reductases EC 1.11.1.6) involved in the biological oxidation: $2H_2O_2 \rightarrow 2H_2O + O_2$. Catalase activity is present in nearly all animal cells and organs: the liver, erythrocytes, and kidney are rich sources. The turnover rate for catalase is very high, for crystalline catalases the catalatic activity (Kat.f.) ranges from 30,000 to 60,000 depending on the source of the enzyme (4). The enzymic properties of catalases isolated from many sources has been extensively studied (62-65), but relatively little information on the structure of the protein moiety is known.

In 1933 Stern (66) determined the molecular weight of purified catalase, isolated from horse, pig and cow livers, horse kidney and cow erythrocytes as 68,900 and stated that the catalase molecule had a dimension similar to that of hemoglobin. Stern and Wyckoff (64) obtained 11 S for equine liver catalase and suggested the molecular weight to lie between 250,000 and 300,000. A molecular weight of 225,000 was reported by Agner (65) for his preparation of equine liver catalase. Agner observed that the catalase solution was not homogeneous, since a component (3.2 S) made up 15 to 20% of the preparation. In 1938 Sumner and Gralén (62) obtained 11.3 S

TABLE I

Amino Acid Composition of Mammalian, Horse and Human (59-61) and Avian, Chick and Duck (48) Hemoglobins.

	Horse	Human Adult	Chick Hb 1	Chick Hb 2	Duck Hb 1	Duck Hb 2
Lysine	44	44	47	44	47	47
Histidine	38	38	34	26	36	34
Arginine	14	12	19	19	18	20
Aspartic Acid	54	50	53	54	56	56
Threonine	24	32	30	28	30	22
Serine	38	32	24	27	26	35
Glutamic Acid	36	32	49	61	49	86
Proline	22	28	25	22	23	26
Glycine	48	40	38	34	39	35
Alanine	62	72	80	70	88	93
Cys/2	4	6	8	8	8	9
Valine	58	62	58	54	59	48
Methionine	4	6	7	12	6	13
Isoleucine	-	-	29	18	27	6
Leucine	80	72	74	68	68	74
Tyrosine	12	12	17	17	12	21
Phenylalanine	30	30	33	32	33	33
Tryptophan	6	6	8	6		

for their preparation of crystalline bovine liver catalase, and calculated the molecular weight to be 248,000 using 4.1 as the diffusion coefficient. In 1940 these authors reported a value of the diffusion coefficient for both equine and bovine liver catalase to be approximately 4.5. This gives a molecular weight value of 225,000. According to Deutsch (67) equine erythrocyte catalase has showed 11.8 S at a protein concentration of 1.0%, the diffusion constant 4.1 - 4.2 x 10^{-7} (Fick unit) and the calculated molecular weight to be 250,000. Thus it appears that the molecular weight of catalases is between 225,000 to 250,000 (4).

Catalase possesses a prosthetic heme group linked to a protein moiety. It is a typical globular protein, compact and sparingly solvated (62, 63). The latter conclusion was reached by Malmon (68) from the studies on the radius of gyration which is 39.8 Å measured for bovine liver catalase, and by Shirakawa from the intrinsic viscosity value of 3.9 cc/g of a fairly active sample (69). The intrinsic viscosity of .039 dl/gm at 4° reported by Samejima and Yang (70) also implies a compact molecule. They stated that the axial ratio of the molecule was approximately 5.0 assuming an ellipsoid, the major axis or length being 240 Å.

Tanford and Lovrien (71) suggested that crystalline bovine liver catalase has a sedimentation coefficient value of 11.6 S. The lyophilized catalase powder in solution produced three components, 11.3, 7.6 and 4.15 S which corresponded to the whole, half and quarter molecules respectively. In contrast, Samejima and Shibata (72) reported the presence of subunits one-sixth the whole molecule in urea and formamide solutions. Schroeder et al (73) reported from their studies of the partial amino acid sequences

that bovine liver catalase is composed of identical subunits, and Samejima and Yang (79) were also of the same opinion. Schroeder et al detected 460 residues per subunit which would account for about 85% of the total residues if four subunits are present, or 65% if only three subunits are present. The number of acetyl groups per molecule of 250,000 averaged 5.3. If the number of subunits were considered as four, an unequal distribution of acetyl groups is implied. Based on the findings of electron microscopy studies, Valentine (75) suggested in 1964 the presence of six similar subunits, four of which are identical, whereas Longley (76) inferred the presence of four subunits. From electron microscopy studies, Sund et al (77) indicated that the catalase molecule consisted of four subunits of equal size.

Reports in the earlier literature indicate the dissociation of catalase both in acidic and alkaline solutions. Sumner and Gralén (62) observed at pH 9.9 , 1.25 S and 4.5 S components, and at an acidic pH (pH 2.8), 1.6 S component. Since a single symmetrical peak of 4.6 S or lower was obtained with acid- or alkali-denatured catalase, Tanford and Lovrien (71) suggested that a complete dissociation to quarter molecules was achieved. On the alkaline side a progressive decrease in S value with increasing pH is observed which they suggested was due to possible unfolding of the molecule. In 1963 Samejima and Yang (70) observed the decrease in S value of catalase from 11.4 S to 4.5 S upon acid denaturation. As the acid-denatured protein showed a molecular weight of 120,000, Samejima and Yang concluded that the native catalase molecule is cleaved into two physically indistinguishable subunits. Recently, Samejima et al (74) reported that bovine liver

catalase dissociated into 4 indistinguishable subunits at pH 12.0 and molecular weight of 60,000, accompanied by conformational changes of the monomeric unit.

A complicated picture evolved from the studies on the dissociation of bovine liver catalase in presence of non-ionic solutes. In formamide (0 - 14 M) and urea (0 - 9 M) Samejima and Shibata (72) observed the presence of three components - 3.0 S, 6.0 S and 2.1 S, which they attributed to subunits of 1/3- and 2/3- and 1/6- size of the native molecule. In 3 M formamide the presence of 3.0 S component was detected and its proportion increased with increasing formamide concentration. At 8 M formamide the 6.0 S component first appeared and the concentration of 3 S component decreased. Above 10 M formamide, the 2.1 S component appeared. These investigators concluded that dissociation of catalase occurred in three stages: first to the subunit of size 1/3- whole molecule; secondly recombined to a size representing 2/3- whole molecule, and thirdly dissociated to the subunit of 1/6- whole molecule. In urea solutions the 3 S component appeared in the range 3 - 5 M, together with the 11 S component. Above 6 M urea only the 6 S component was observed. For urea, the dissociation occurred in two stages; first to the 1/3- molecule and then to the 2/3- molecule (72).

Although the catalases were isolated from many sources relatively little information on the primary structure of the protein moiety is available. In 1947 Bonnichsen (78) reported some of the amino acids present in equine liver catalase. Later Schnuchel (79) reported the amino acid composition of bovine liver catalase. In 1962 Schroeder et al (80) published

the amino acid composition of two mammalian (equine and bovine) liver catalases. An evaluation of the percentage difference in the number of residues and in the ratio of one amino acid to another suggest a difference in lysine, arginine, histidine, aspartic acid, glutamic acid, valine and phenylalanine content. They also observed a difference in the chromatographic behavior of the bovine and equine liver catalases on DEAE cellulose. While equine liver catalase emerged from the column with 0.1 M tris-HCl buffer, pH 7.5, 0.2 M concentration of buffer was required for bovine liver catalase. This implies a difference in the surface charge characteristics of these two liver catalases.

Immunochemical and chemical investigation undertaken by Saha et al (81) on the liver and erythrocyte catalases isolated from various mammalian species (horse, cow, rabbit and man) revealed the similarities and dissimilarities of the species-specific enzymes. Since the antigenic determinant sites reflect the amino acid sequence of the particular segment of the polypeptide chain, the immunochemical cross-reaction implies a strong similarity in the antigenic sites of these catalases. (Table II).

The conformation of macromolecules composed of varying proportions of helical and non-helical segments, are determined mainly by the intramolecular forces (82) even though polymer-solvent interactions may affect the conformation to various degrees. Analyses of protein conformation by x-ray diffraction studies in recent years has provided us with somewhat clearer understanding of the relationship that exists between the structure and function of the protein molecules. According to current crystallographic studies of proteins like chymotrypsin, ribonuclease, carboxypeptidase A, carbonic anhydrase, cytochrome-C, lysozyme, myoglobin, and hemoglobin (83, 84), the protein molecule is compact with a well defined interior and

TABLE II

Amino Acid Composition of Liver and Erythrocyte Catalases*

	RLC	RLC	REC-1
Lys	116	126	120
His	89	70	74
Arg	130	121	126
Asp	294	266	284
Thr	96	107	106
Ser	97	104	95
Glu	197	213	213
Pro	166	167	168
Gly	155	154	142
Ala	154	157	154
Cys/2	-	-	12
Val	141	156	147
Met	41	44	45
Ileu	79	85	89
Leu	154	162	152
Tyr	86	80	78
Phe	132	120	132
Cysteic Acid	16	26	25
Tryp	22	24	25

* Anil Saha, unpublished observation.

exterior; the interior distinctly non-polar, the exterior polar in character. The cooperative interaction between the solvent environment and the protein molecules is of vital importance. A subtle change in the solvent environment (aqueous medium) does not necessarily change the gross conformation of the protein molecule.

Liquid water, a dipolar molecule, exists as an association of more than one molecule (85-89) which stems from the formation of hydrogen bonds between adjacent water molecules. At any instant, water molecules may be tetrabonded (icelike), tribonded, dibonded, monobonded and non-hydrogen bonded (free water), the mole fraction of each species being a function of temperature (88). The formation of hydrogen bonds reduces the freedom of molecular motions and results in a more ordered structure. When an electrolyte is added to water, it produced dissociation, the degree of dissociation being dependent on the concentration and the nature of the electrolyte. Since simple ions have dimensions and charges comparable to those of the water molecule, they can be expected to change the charge separation of the water molecule thereby affecting the water structure. Thus the net effect of the ionic solute is to promote increasing disorder, in spite of the fact that the water molecules orient themselves around the ion. On the other hand, the addition of non-ionic solutes to water results in an increasing order of water molecules around the non-polar solute resulting in an increasing degree of hydrogen bonding (89). Concentrated solutions containing both ionic and non-ionic solutes have been observed to affect the polymeric status (90). The hydrodynamic properties of the protein molecule in a solvent offers an understanding of any change in size

and shape that may occur when a protein is subjected to the influence of structure-disruptive ions (91, 92).

The monomer association and polymer dissociation in the case of proteins have been extensively studied employing sedimentation velocity and equilibrium analyses. The rate of sedimentation (S value) of a protein molecule in a solvent environment is a function of its size and shape. A reduction in 'S' value implies a dimunition in size and/or a change in the shape (91). The sedimentation-velocity method requires a determination of the diffusion coefficient for calculation of the molecular weight. The sedimentation equilibrium method provides rather a direct approach to the evaluation of molecular weight. In previous years, despite the long duration required to attain an equilibrium state which extended over a few days, the sedimentation equilibrium experiments were undertaken (93-95) and the results provided the convincing evidence that the phenomenon of association-dissociation occurred in proteins. The operational difficulties encountered with the sedimentation equilibrium runs made the sedimentation velocity method an acceptable and popular technique for the study of the association-dissociation phenomena. A break-through was provided by the approach to equilibrium method as suggested by Archibald (96) and a recent adaptation by Yphantis (97) for the attainment of equilibrium state within a relatively short period. At this end, equilibrium analysis has greatly been facilitated by the outstanding contribution of Schachman and his collaborators (98-100) in the commercial availability of the automatic photoelectric scanner (101).

The electronic absorption spectra of the chromophoric groups reflect the measure of the interaction with the solvent and also the level

of the excited state (102). The solvent perturbation technique of difference spectroscopy as developed by Laskowski (103) and Herskovitz and their collaborators (104-106) has provided valuable information in regard to the availability of the chromophoric groups, tyrosine and/or tryptophan, in globular proteins. The method can also be applied for the study of localized conformational changes in proteins with a prosthetic group. The proteins studied in this investigation as models contain the prosthetic group, heme (protoporphyrin IX) (107) and showed an absorption maximum at 415 mμ with native hemoglobins and at 407 mμ with native catalase. A change in the absorption maxima in the heme region (Soret band intensity) thus reflects the localized interaction between the heme groups, creviced in the polypeptide chain, and the particular solvent environment. Differential absorption spectra of tyrosine appears around 295 mμ and the relative intensity increases with the increase in alkalinity of the media. Spectrophotometric studies on the tyrosine ionization with or without the help of denaturants provide valuable information in regard to the degree of availability of all the tyrosine residues present (108-111). Similarly the accessibility to tryptophan by solvent molecules can be studied by the solvent perturbation difference spectroscopy (106). Studies on the fluorescence emission spectra of tryptophan provide a relatively direct approach to evaluate its availability (112-114). A shift in the fluorescence emission spectra either to higher wavelengths (free tryptophan) or lower wavelengths (more buried tryptophan) or changes in the quantum yield thus provide us a measure of localized conformational change with respect to the solvent environment of tryptophan residues.

CHAPTER II

MATERIALS AND METHODS

Section 1

A. Preparation of Hemoglobin

Erythrocytes were collected in heparinized isotonic saline at 3° by cutting the jugular vein of the adult chicks (49, 115). Avian erythrocytes were washed by suspending four times to ten times their volume of isotonic saline at 3° and spinning down each time in a refrigerated centrifuge at 600 x g for 15 min. The buffy coat was removed during the washing procedure by suction. Packed cell volume was determined by centrifuging the erythrocytes at 1000 x g for 15 min. The packed avian erythrocytes were then lysed with an equal volume of cold distilled water and 0.4 volume of toluene and kept overnight at 3°. The suspension was centrifuged at 10,000 x g for 15 min. at 3°, clear hemoglobin solution was withdrawn with a syringe and centrifuged again at 10,000 x g for 15 min. The hemoglobin solutions were stored in small vials at -20° and thawed prior to their use. The hemoglobin solutions were diluted with the appropriate buffers to a solution of desired protein concentration. Under normal conditions of experimentation the hemoglobin solutions were used only for two to three weeks when stored at cold room temperature.

Ion-exchange chromatography on IRC-50 XE-64 (Bio-Rex 70, 200-400 mesh, 10.2 meq/g (dry wt.), Bio-Rad Laboratories) was employed to separate the hemoglobins. Hemoglobin solution (2 ml, 50-100 mg) was applied to an

IRC-50 XE-64 column, 5 x 50 cm, which was previously equilibrated with 0.15 M Na citrate buffer, pH 6.5 (58). The column was developed with initial developer (500 ml) of 0.15 M Na citrate buffer, pH 6.5 and changed to Na citrate buffer, pH 6.5 of higher Na concentration. Buffer solutions used in the chromatographic development contained 20 mg KCN/liter of solution. Fractions (5 ml) were collected and absorbancy of each fraction was determined at 415mμ with a Beckman Quartz DU Spectrophotometer. The peak zones were pooled and concentrated by centrifugation of the dilute solution at 4° and 10,000 x g for 16 hr. The concentrated hemoglobin solution thus obtained was freed of buffer salt by dialyzing against several changes of appropriate buffer.

B. Preparation of Catalases

Bovine liver catalase was obtained from Boehringer and Sons, Mannheim, Germany, and also prepared in our laboratory according to the method published by Saha et al (81). Essentially the liver tissues were ground very fine with a meat grinder and lysed with two volumes of distilled water at room temperature. The solution was then centrifuged and the precipitate removed. The proteins in the supernates were removed by using ethanol: chloroform (3:1, v/v) mixture. The mixture was stirred for 30 min. and the coagulated proteins were first removed by filtration through a fine muslin cloth and the fine particles suspended in the filtrate were then removed by centrifugation at 20°. The solution containing ethanol:chloroform mixture was concentrated in an evaporator and the denatured proteins were centrifuged off. Ammonium sulfate fractionation (0.3 - 0.6 saturation at pH 7.0)

was carried out, the precipitate dissolved in distilled water and the residual ammonium sulfate removed by dialysis against water.

The dialyzed solution was treated with acetone at -15° and the fraction that was precipitated at 0.3 - 0.6 saturation was processed further. The centrifugation was carried out at -20° during the acetone fractionation. The resulting precipitate was dissolved in the minimum amount of cold water and dialyzed exhaustively at 3° to free the solution from acetone. Ammonium sulfate precipitation was carried out alternatively at pH 5.0 and at pH 7.0, the cycle being repeated thrice. The purity of the preparation was checked by using the absorbance ratio of 278 and 407 mμ. At the end of the three cycles of crystallization with ammonium sulfate at pH 5.0 and at pH 7.0, the quality of the material that crystallized first at pH 7.0 could not be improved by repeated crystallization as judged by the absorbance ratio of 278:407 mμ. Further purification was obtained by column chromatography.

Column chromatography of catalases was carried out on cellulosic ion-exchanger DEAE-cellulose (Cellex D, ion exchange capacity - 0.67 meq/g, Bio-Rad Laboratories). The column, 2.5 x 30 cm, was packed with DEAE-cellulose and equilibrated at 3° with 0.075 M tris-HCL buffer, pH 7.5. A sample of catalase solution (100 mg in 5 ml) was developed stepwise with tris-HCL buffer, pH 7.5: first with 0.075 M, 0.1 M and finally with 0.15 M. The peak areas exhibiting a maximum absorbance at 278:407 mμ were pooled and concentrated by centrifuging at 40,000 rpm at 4° in a Spinco Model L preparative ultracentrifuge for 15 hr. Catalase was found to settle at the bottom of the centrifuge tubes. The concentrate was removed and dialyzed against appropriate buffer used for the various experimental procedures.

Section 2

A. Amino Acid Analysis

Amino acid analysis was performed with a Beckman/Spinco Amino Acid Analyzer Model 120 B using an accelerated procedure of amino acid analysis with a buffer flowrate of 70 ml/hr and ninhydrin flowrate of 35 ml/hr at 55° (116). A short column, 0.9 x 5.0 cm for basic amino acids, and long columns 0.9 x 58 cm, for neutral and acidic amino acids were used. These columns contained Beckman Custom Spherical Resin PA-35 and PA-28 (Beckman Instruments, Inc.). The amino acid analyzer was fitted with high sensitivity cuvette, range expander and automatic regeneration system, the recorder chart speed being 6"/hr with printing dot every two seconds. The buffers contained n-propanol and benzyl alcohol as suggested by Hubbard (117). For the normal protein hydrolysis run, the protein solutions were dialyzed exhaustively against 0.1 M ammonium bicarbonate buffer, pH 8.6 to free the solutions from salt. An aliquot representing approximately 1-2 mg of protein was dried overnight at 108° in a forced circulation oven. The dried protein was hydrolyzed with glass distilled 6 N HCl at 108° for 22 and 70 hr. under vacuum. The hydrolysis tube containing protein and HCl was repeatedly purged with special grade N_2 flushed through alkaline pyrogallol solution to remove residual O_2. The hydrolysis loss was evaluated and the extrapolated values of the amino acid residues/mole were calculated using Fortran IV language and IBM 360 computer program.

Total cys/2 content was determined as cysteic acid after performic acid oxidation of the protein according to Moore (118). Tryptophan content

was measured by hydrolyzing the protein (4-5 mg) in 2 M $Ba(OH)_2$ for 70 hr. at 108° in quartz tubes in an atmosphere of nitrogen (119). Ba was removed as $BaCO_3$ by bubbling CO_2 gas through the solution followed by centrifuging off $BaCO_3$ precipitate. The precipitate was washed with distilled water and the combined supernate was evaporated under vacuum on a rotary evaporator. The residue was dissolved in distilled water (10 ml) and filtered through a sintered glass funnel. The solution was lyophilized and the content was dissolved in 5 ml of 0.2 M citrate buffer, pH 2.2. 0.025 ml of 0.2 M Na_2SO_4 solution was added to ensure complete precipitation of Ba. The precipitate, if formed, was spun down and the solution was adjusted to pH 2.2 with a few drops of concentrated HCl. Tryptophan content was determined, using the amino acid analyzer, on a short column, 0.9 x 16 cm, packed with Beckman Spherical Custom Resin PA-35 (cross linking - 7.5%) developed at 55° with amino acid analyzer buffer, pH 5.1. The number of the tryptophan residues was computed by comparison with glycine, alanine, valine and leucine content of the proteins.

B. Analytical Ultracentrifugation

Sedimentation Velocity Analysis

The sedimentation velocity coefficient (S) values were determined using a Beckman/Spinco Analytical Ultracentrifuge Model E fitted with Schlieren optics, phase plate and electronic speed control unit. Generally the S values were determined at a rotor speed of 56,000 rpm at 20°. An-D rotors together with single sector cells, 12 mm and 2° fitted with Kel-F

center pieces, or double sector cells, 12 mm and 2.5° fitted with filled Epon center pieces were chilled to 20° before they were placed inside the rotor chamber. Schlieren plots were photographed by automatic camera exposure system at 16 min. intervals using Kodak plates 103F and with Wratten filter No. 29 for hemoglobin, and Spectroscopic plate IIG and filter 77A for catalase. The sedimentation velocity coefficient values ($S_{20,w}$) were corrected for the actual temperature of the centrifugal run, rotor stretching, viscosity and density of the solutions (18, 120, 121) using the Svedberg equation,

$$S_{20,w} = \frac{1}{\omega^2} \frac{d \ln r}{dt} \frac{\eta_t}{\eta_{20,w}} \frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{t,soln.}}$$

ω is the rotor speed in radians/sec, r the distance of the peak (the mid point) from the meniscus with respect to time, t, and the term η_t, the viscosity of the solvent at the temperature of the experiment, and $\eta_{20,w}$ the viscosity of water at 20°, $\bar{v}$ the partial specific volume of the solute, $\rho_{20,w}$ the density of the solution at 20° and the pressure of 1 atmosphere. The term,

$$\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{t,\ sol'n}}$$

was ignored because of its insignificant contribution towards the $S_{20,w}$ value of the globular proteins (18).

Relative viscosity measurements were carried out with Cannon-Ubbelohde semi-micro dilution viscometers (50 ml) at 20° ± 0.01° with water outflow time of 254 and 272 sec. The relative viscosity was determined according to the equation,

$$\eta_{rel} = \frac{t_{20,sol'n}}{t_{20,water}},$$

where $t_{20,sol'n}$ = outflow time for solution, $t_{20,water}$ = outflow time for water (120, 121). The solutions introduced into the viscometers were repeatedly filtered through Millipore filters (pore size 0.45 μ) fitted in 5 ml syringes. The intrinsic viscosity of the proteins was obtained according to the calculation (120, 121),

$$[\eta] = \lim_{c \to o} \frac{\eta_{sp}}{c}, \qquad \text{where} \quad \eta_{sp} = \frac{t - t_o}{t_o}$$

t = outflow time for solute, t_o = outflow time for solvent, c = g protein/ 100 ml solution. The concentration of the protein (mg protein/ml of solution) used for the ultracentrifugal analysis and viscometry was determined by dialyzing exhaustively a concentrated solution of protein against 0.2 M ammonium bicarbonate buffer, pH 8.6, followed by determining the dry weight which was obtained by drying an aliquot at 108° for 16-18 hr. in a forced circulation oven. Throughout these operations, precautions were taken to remove dust particles from the solutions by filtering them repeatedly through the Millipore filters and using the glassware washed with dust-free water.

In view of the varying degrees of unfolding that could be produced in the polypeptide chains under different experimental conditions, it was decided to mix the protein solution with the varying concentration of solutes at a fixed time interval (30 min) prior to the rotor attaining full speed. The total time elapsing between the mixing of the hemoglobin solution with various solutes and the last Schlieren photo exposure was approximately 100

min, whereas in the case of catalase the time was approximately 80 min. Solutions containing higher concentration of proteins were dialyzed against 0.1 M tris-HCl buffer, pH 8.6. The protein and concentrated solutions containing various solutes were mixed in such proportion that the desired concentration for both could be achieved simulataneously. For viscosity determination the total time elapsing between the mixing of the solutions and temperature equilibrium inside the bath was approximately 20 min prior to the first reading. The time between the mixing of the solutions and the last viscosity determination was approximately 90 min.

Apparent diffusion coefficient values ($D_{20,w}$) were determined at 20° by employing Schlieren optics, valve type and capillary type synthetic boundary cells, a rotor speed of 9000 rev/min. Diffusion coefficient was determined following the equation,

$$D = \frac{(A/H)^2}{4\pi t}$$

where A is area, H height and t time in sec. (122).

Sedimentation Equilibrium Analysis

Sedimentation equilibrium experiments were carried out with a Beckman/Spinco Analytical Ultracentrifuge Model E fitted with ultraviolet monochromator, automatic photoelectric scanner (98-100) and multiple cell scanning accessories (101). Both An-D (2 hole rotor) and An-F (4 hole rotor) rotors in conjunction with double sector cell, 12 mm, 2.5° filled-Epon center pieces were used at 20°. When An-D rotor was used, one of the rotor

holes contained the cell and the other the counterbalance (specific for scanner operation). In the case of the An-F rotor, three cells were used in conjunction with one counterbalance. A short liquid column, 0.115 ml of protein solution, was layered on 0.05 ml FC-43 fluorocarbon oil (Beckman Instruments, Inc., Spinco Division) in one sector of the double sector cells, while 0.2 ml buffer solution was placed in the other sector. Hamilton microliter syringes were used for the volume determination. The protein solution was mixed with solutions containing various solute concentrations prior to the initial speed (9000 rpm). High speed sedimentation equilibrium using a short liquid column as described by Yphantis (97) was followed during the present study. The initial concentration of the protein solution C_o, was determined at 280 mμ , 15 min after the rotor attained the initial speed of 9000 rpm. Occasionally an overspeed of 4000-6000 rpm was employed over the actual operational rotor speed to facilitate the cell meniscus clearance. During the solvent:solute interaction studies the rotor speed was so chosen that the total time elapsed for the attainment of equilibrium state was made approximately the same (15 hr) for the different solute systems used. At each calibration step, the optical density at 280 mμ recorded by the scanner was related to the protein solutions, the optical densities of which were determined simultaneously at 280 mμ by a Cary Recording Spectrophotometer Model 14. The operational parameters for the normal equilibrium runs were maintained as follows: operation - split beam; the optical slit width - 0.1 mm; mode - absorbance; optical density range - 2 O.D.; scanner recorder sensitivity - 500 mv/cm; scan speed - medium (4.75 x 1); and scanner chart speed - 5 mm/sec.

The density of the solutions was determined with 5 ml capillary vent pycnometers. The partial specific volume, $\bar{v}$, was determined by following the equation,

$$\bar{v}_{app} = \frac{1}{\rho_o} - \frac{1}{c}\left(\frac{\rho - \rho_o}{\rho_o}\right)$$

where ρ_o and ρ were the densities of solvent and solution, and c the protein concentration in g/ml of solution (91).

During the studies on solvent:solute interaction, the partial specific volume, $\bar{v}$, was assumed to be constant for both the whole molecule and the subunits unless mentioned specifically. Analysis of sedimentation equilibrium data was carried out according to the equation of Svedberg and Pedersen (18),

$$M_{app} = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \cdot \frac{d\ln c}{dr^2}$$

where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume of the protein, ρ the density of the solution, ω the angular velocity in radians/sec, c the concentration evaluated at a radial distance, r. The evaluation of the sedimentation equilibrium data will be discussed in the appendix.

Alkaline Dissociation Studies

The protein solutions were dialyzed at 3° against the appropriate buffer for 16 - 18 hr prior to the ultracentrifugal analysis. The buffers were prepared by mixing varying proportions of K_2HPO_4, K_3PO_4 and KCl to

achieve the desired pH and ionic strength 0.25. The pH of the solution was determined by a Beckman Research Model pH meter. For the sedimentation equilibrium experiments the required dilution of the protein was made prior to the start of the run.

C. Spectrophotometric Studies

Direct and differential spectra under solvent perturbation systems were recorded with a Cary Double Beam Recording Spectrophotometer Model 14. The differential spectra measurements were carried out in rectangular tandem double cells of path length 0.4375 cm, the cells (matched pair sets, Suprasil Quartz) were obtained from Hellma, GMBH, Germany. The direct absorption spectra were obtained with 1 cm matched pair, Suprasil Quartz, single compartment cells (Hellma GMBH, Germany). For reasons described earlier, the time interval of mixing the solute with the solvent containing different ionic and non-ionic solutes and the spectral determination was maintained identical. In most cases, the spectra were obtained from 450 mμ to 250 mμ or to 230 mμ using a hydrogen lamp. For the sake of operational convenience, the hydrogen lamp was used for the spectral shifts which lie between 350 and 450 mμ . For the evaluation of the shift in the absorption maxima the solvent base lines were determined separately with the matched pair cells. For both direct and differential spectra special care was taken with solvent and solution to avoid contamination by the dust particles. A wavelength scan speed of 25 Å/sec was used unless otherwise mentioned.

Differential spectrophotometric titrations of phenolic groups were carried out using KOH of varying molarities as titrant. Both the protein

solution and KOH solutions contained 0.2 M KCl. To a 3 ml volume of protein solution, varying amounts of titrant weredelivered by a micrometer syringe burette. An identical volume of 0.2 M KCl was added to the reference protein solution at neutral pH to negate the dilution effect resulted from the repeated addition of titrant to the sample cell. Beckman Research Model pH meter was used for the determination of pH of the protein solution before and after each spectral determination. Precautions were taken to keep the solutions free from atmospheric CO_2.

Fluorescence Spectra

Fluorescence excitation and emission spectra measurements were performed on a Hitachi-Perkin Elmer Fluorescence Spectrophotometer Model MPF 2A fitted with a recorder which was coupled to emission monochromator wavelength drive. A xenon lamp was used. Measurements were made in 1 cm path length cells of low fluorescence quarts (Pyrocell Corp.) the cell compartment being thermostated at 22°. The time of mixing for solvent:solute interaction was maintained constant as mentioned earlier. The concentration of protein used was 0.2 O.D., at 280 mμ, operational parameters were: emission slit width - 4 mμ; excitation slit width - 10 mμ; excitation wavelength - 278 mμ; filter - 310 mμ; recorder sensitivity - 4.

Enzymic Activity of Catalase

The enzymic activity was determined by measuring the rate of disappearance of H_2O_2 in presence of catalase. The procedure followed herein was that of Bonnichsen, Chance and Theorell (123) except that the concentration of H_2O_2 was determined iodometrically according to Sumner and Dounce (4).

Catalase solution was diluted with ice-cold 0.01 M Na phosphate buffer, pH 7.0 and 1.0 ml of the dilute catalase solution was added to 50 ml of hydrogen peroxide solution (1 ml of 30% H_2O_2 in 750 ml of 0.01 M Na phosphate buffer, pH 7.0). Iodometric titration was performed to evaluate the rate of disappearance of H_2O_2 and the reaction constant K.

$$K = \frac{1}{t} \log^{10} \frac{A}{A-x}$$

where A = ml $Na_2S_2O_3$ used at 0 min, A-x = ml $Na_2S_2O_3$ used at 2, 4, 6 and 8 min, t = 0, 2, 4, 6 and 8 min. Results were expressed as the decrease in enzymic activity on the basis of Kat.f value obtained with the control catalase solution.

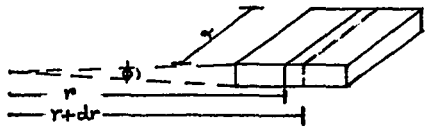
$$\text{Kat.f} = \frac{K}{\text{g catalase/ml}}$$

During the evaluation of the effect of the solutes on the enzymic activity, the concentration of catalase was maintained the same (4 mg/ml) as described during the sedimentation velocity experiments. The dilution of the catalase solution in presence of the perturbants to the desired protein concentration suitable for the determination of enzymic activity was made by using the same solvent:solute system.

Appendix - Sedimentation Equilibrium

Sedimentation equilibrium is attained when the material migrating across a given surface in a centrifugal direction is exactly balanced by the transport centripetally due to diffusion, i.e. the net transport per unit time, dm/dt, across the surface in the cell is zero (18, 120).

$$\frac{dm}{dt} = \phi r \alpha \, (cs\omega^2 r) - D\frac{\partial c}{\partial r} = 0 \qquad [1]$$



where ϕ is the sector angle in radians, α the thickness of the cell along the optical path, r the distance from the center of rotation to a surface within the cell, ω the angular velocity of the cell in radians/sec, c the concentration of the protein solution, S the sedimentation coefficient and D the diffusion coefficient.

$$\therefore \; cs\omega^2 r = D\frac{\partial c}{\partial r} \qquad [2]$$

On rearranging, $$\frac{S}{D} = \frac{1}{\omega^2 c r}\frac{\partial c}{\partial r} = \frac{2}{\omega^2}\frac{d\ln c}{dr} \qquad [3]$$

Svedberg (18) proposed the following equation,

$$M = \frac{RTS}{(1-\bar{v}\rho)D} \qquad [4]$$

for the determination of the molecular weight of a solute. Substituting equation [3] in the Svedberg equation [4] one obtains,

$$M = \frac{2\,RT}{(1-\bar{v}\rho)\omega^2}\,\frac{d\ln c}{dr^2} \qquad [5]$$

Alternatively, in the equilibrium state, the total potential (chemical and gravitational potentials) is assumed to be uniform throughout the centrifuge cell, i.e. the derivative of the total potential is set equal to zero. This contention assumes an ideal solution and thereby neglects variation in density due to concentration and pressure changes inside the cell (120). According to Goldberg (124) the system at equilibrium could be described in

terms of a continuous sequence of phases of fixed volume and infinitesimal depth in the direction of the centrifugal field. Assuming that the total potential is uniform for this heterogeneous equilibrium he proposed an equation,

$$M_i\left(1-\bar{V}^{(r)}\rho^{(r)}\right)\omega^2 r\,dr=\sum_{k=1}^{s}\left(\frac{\partial\mu_i}{\partial c_k}\right)_{T^{(r)}pc_j} dc_k^{(r)} \qquad [6]$$

where i = 0, 1, 2, ... , s.

Because of the pressure gradient in the centrifuge cell and the compressibility of the solute and solution, many of the terms are dependent on r. Thus the sedimentation equilibrium equation is written with (r) to denote that these terms correspond to the surface at a distance (r) from the axis of rotation. The molecular weight of any component depends on all components in the system as expressed by the terms $(\partial\mu_i/\partial c_k)$. In the case of the non-ideal systems the chemical potential μ is defined (125) by $\mu_i=\mu^\circ+RT\ln a_i$ [7] where μ° is the chemical potential in the standard state, R the universal gas constant, T the absolute temperature in ° Kelvin, a_i the activity of the i^{th} component.

In a system consisting of a single charged macromolecular component, component 2 and a salt component 3 the activities a_2 and a_3 of the solute components may be expressed in the Scatchard formalism (126),

$$\ln a_2 = \ln m^2 + \sum \nu_{2i} \ln m_i + \beta_2 \qquad [8]$$

$$\ln a_3 = \sum \nu_{3i} \ln m_i + \beta_3 \qquad [9]$$

where m = concentration in molalities. The subscripts i refer to the ion species comprising component 3, and ν_{2i} and ν_{3i} are no. of moles of these species included in one mole of electrically neutral components 2 and 3. It follows that $m_i = \nu_{3i}\, m_3 + \nu_{2i}\, m_2$. Differentiating the equations

[8] and [9],

$$a_{22} = \left(\frac{\partial \ln a_2}{\partial m_2}\right)_{P,T,m_3} = \frac{1}{m_2} + \sum_i \left(\nu_{2i}^2/m_i\right) + \beta_{22} \qquad [10]$$

$$a_{23} = \left(\frac{\partial \ln a_2}{\partial m_3}\right)_{P,T,m_2} = \left(\frac{\partial \ln a_3}{\partial m_2}\right)_{P,T,m_3} = \sum_i \frac{\nu_{2i}\nu_{3i}}{m_i} + \beta_{23} \qquad [11]$$

$$a_{33} = \left(\frac{\partial \ln a_3}{\partial m_3}\right)_{P,T,m_2} = \sum_i \left(\frac{\nu_{3i}^2}{m_i}\right) + \beta_{33} \qquad [12]$$

Thus the equation [7] could be expressed as,

$$M_2 L_2 \, dr^2 = a_{22}^{(r)} \, dm_2 + a_{23}^{(r)} \, dm_3 \qquad [13]$$

$$M_3 L_3 \, dr^2 = a_{23}^{(r)} \, dm_2 + a_{33}^{(r)} \, dm_3 \qquad [14]$$

where

$$L_i = \frac{(1-\bar{v}_i\rho)\omega^2}{2RT} \quad , \quad i = 2,3.$$

The partial specific volumes $\bar{v}_2$ and $\bar{v}_3$ and the solution density have been assumed to be independent of T and angular velocity ω (124). Eliminating dm_3 from the equations [13] and [14],

$$\left(M_2 L_2 - M_3 L_3 \frac{a_{23}^{(r)}}{a_{33}^{(r)}}\right) \frac{dr^2}{dm_2} = a_{22}^{(r)} - \frac{a_{23}^{(r)2}}{a_{33}^{(r)}} \qquad [15]$$

If we assume no interaction between the salt, component 3 and the macromolecule, component 2, i.e. $a_{23} = 0$ (127, 128), equation [15] is reduced to,

$$\frac{M_2^* L_2^* \, dm_2}{dr^2} = a_{22}^{*(r)} \qquad [16]$$

provided that the dependence of m_2 and m_3 on r does not require a variation in ν_{2i}. The redistribution of salt alone should be very slight in the centrifugal fields required for the sedimentation equilibrium and the effect of variation of m_3 with r can be ignored if the components are defined so that there is no interaction between salt and component 2 (127, 128).

Combining equations [10] and [16],

$$M_2^* L_2^* \left(\frac{d\ln m_2}{dr^2}\right)^{-1} = 1 + m_2 \left(\sum_i \nu_{2i}^{*2}/m_i + \beta_{22}^*\right) \qquad [17]$$

For dilute solutions the assumption $\frac{\nu_{2i} m_i}{m_3} << 1$ can be made.

Under these conditions the molecular weight obtained in a sedimentation equilibrium experiment is that of component 2 as defined by letting $m_3 = m_3'$ in dialysis (127, 128). The molecular weight then depends only on measurement of concentration or a property proportional to concentration at a position within the cell.

$$M_2^* = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \frac{d\ln c}{dr^2} \qquad [18]$$

Then the concentration distribution of the mass reflects the molecular weight of the macromolecule. This is the basic sedimentation equilibrium equation described for self association systems (129). For non-ideal systems of more than one component, the basic sedimentation equilibrium equation has been described as,

$$\frac{d\ln c}{dr^2} = \frac{(1-\bar{v}\rho)\omega^2}{2RT} M_{w,app} \qquad [19]$$

where $1/M_{w,app} = 1/M_{w(c)} + Bc$. $M_{w,app}$, apparent weight average molecular weight, and $M_{w,(c)}$, weight average molecular weight at concentration (c) (130). For ideal dilute solutions $B = 0$ at all concentrations.

In 1964 Yphantis (97) described a technique of determining molecular weight of macromolecular components in dilute solutions which is applicable

to paucidisperse solutions. The suggested technique was: (a) operational rotor sppeds about three times higher than usual so that meniscus concentration (Cm) could be neglected; (b) a short column height (approx. 3 mm); and (c) dilute solutions (0.05% and below). Assuming the conservation of mass in a sectorial cell, the distribution of the macromolecular components(s) can be described by the familiar exponential expression, $C = A \exp (\sigma r^2/2)$ [20] where the constant A has the value $0.5\ C_0\sigma(r_b^2-r_m^2) / \exp (\sigma r_b^2/2)-\exp (\sigma r_m^2/2)$, σ, the effective reduced molecular weight,

$$\frac{\omega^2 M (1-\bar{v} \rho)^o}{RT}$$

with $(1-\bar{v}p)^o$ evaluated at infinite dilution and pressure of 1 atm., C_o the initial concentration of the solute, r_b and r_m the radial distance to the cell bottom and cell meniscus from the axis of rotation. Using equation [20] one could obtain an idea of the theoretical distribution of a macromolecule in the ultracentrifuge cell for given operational parameters and M.

The theoretical distribution of the mass in the ultracentrifuge cell or in other words the slope of the plot (lnc vs r^2) was determined for the different proteins and their subunits at a particular angular velocity, ω, using computer (IBM 360) program with Fortran IV language. For the hemoglobins, the molecular weight of monomer, dimer and tetramer were assumed to be 18,000, 34,000 and 64,000 while the values for catalase monomer and dimer were 64,000 and 125,000. The partial specific volume of the hemoglobin subunits were assumed to be equal and 0.749, and that of the catalases monomer and dimer as 0.730. These values were assumed to be constant under all the experimental conditions of solvent:solute interaction system used.

The raw experimental data was fitted by the least square curve fitting method to obtain the plot lnc vs r^2. An IBM 360 computer program with Fortran IV language was used.

CHAPTER III

STUDIES ON CHICK HEMOGLOBIN

Introduction

The heme-proteins, like hemoglobins and catalases, consist of identical and/or near-identical subunits, for example, human adult hemoglobin (Hb A) is composed of α and β chains (for review, 1, 2). Though the amino acid sequence of α and β chains differs markedly, two α chains and two β chains constitute a tetrameric molecule - human adult hemoglobin, while two α chains and two γ chains make human fetal hemoglobin (Hb F). Although the over-all shape of these proteins is globular, the subunits are constituted of interspersed helical and non-helical segments. The sequential arrangement of amino acid residues influences the formation of helical and non-helical segments and close packing of the polypeptide chains into a compact shape as observed with hemoglobins (45). The way in which the various side-chain groups, polar and non-polar in character, are distributed on the subunits determines the nature of interaction between the subunits (44). The complementarity in structure between any two contact points of the neighbouring subunit polypeptide chains is important in maintaining the polymeric status of native proteins (131). The equilibrium in which tetrameric hemoglobin molecules maintain their polymeric status is largely dependent on the condition of the medium since the monomers are held together in a tetrameric state by the interaction of short range forces, especially in the concentrated protein solution. An extreme change in the environmental condition in

hydrogen ion concentration, concentration of solutes, dielectric properties of the media, and concentration of the protein, etc. produced the depolymerization of hemoglobin (for review 2, 90).

The present investigation describes the behavior of an avian heme-oriented polymer protein under varying conditions of solvent:solute interaction. Solutes of ionic and non-ionic types in an aqueous medium were employed to evaluate the stability of the polymer under different solvent perturbation systems. Dissociation of chick hemoglobin in alkaline buffers of pH 7.0 - 11.6 was also studied.

Experimental Results

Sedimentation velocity coefficient values of chick hemoglobin at various protein concentrations were found to be 4.4 S ($S^{o}_{20,w}$) in 0.1 M tris-HCl buffer, pH 8.6, and independent of protein concentration. The sedimentation velocity coefficient value of chick hemoglobin, reported by Svedberg (18) for Gallus gallus, is 4.2 S ($S^{o}_{20,w}$) which is within the range of results obtained during the present investigation. $S^{o}_{20,w}$ value of chick globin (α and β mixture) was found to be 3.2 S in 0.1 M Na acetate buffer, pH 5.0 (Fig. 1). Fig. 1 also presents the behavior of reduced and aminoethylated globin, $S_{20,w}$ value being 3.1 S. The S values indicate the presence of dimer, in which some aggregation appears to occur during normal preparation.

Fig. 2 (a,b,c, and d) shows the ultracentrifugal behavior of chick hemoglobin in the presence of increasing concentration of several ionic type solutes (NaCl, KCl, $CaCl_2$ and LiCl). The Schlieren plots revealed a single symmetrical peak. $S^{o}_{20,w}$ values of chick hemoglobin in 2.0 M NaCl:0.1 M

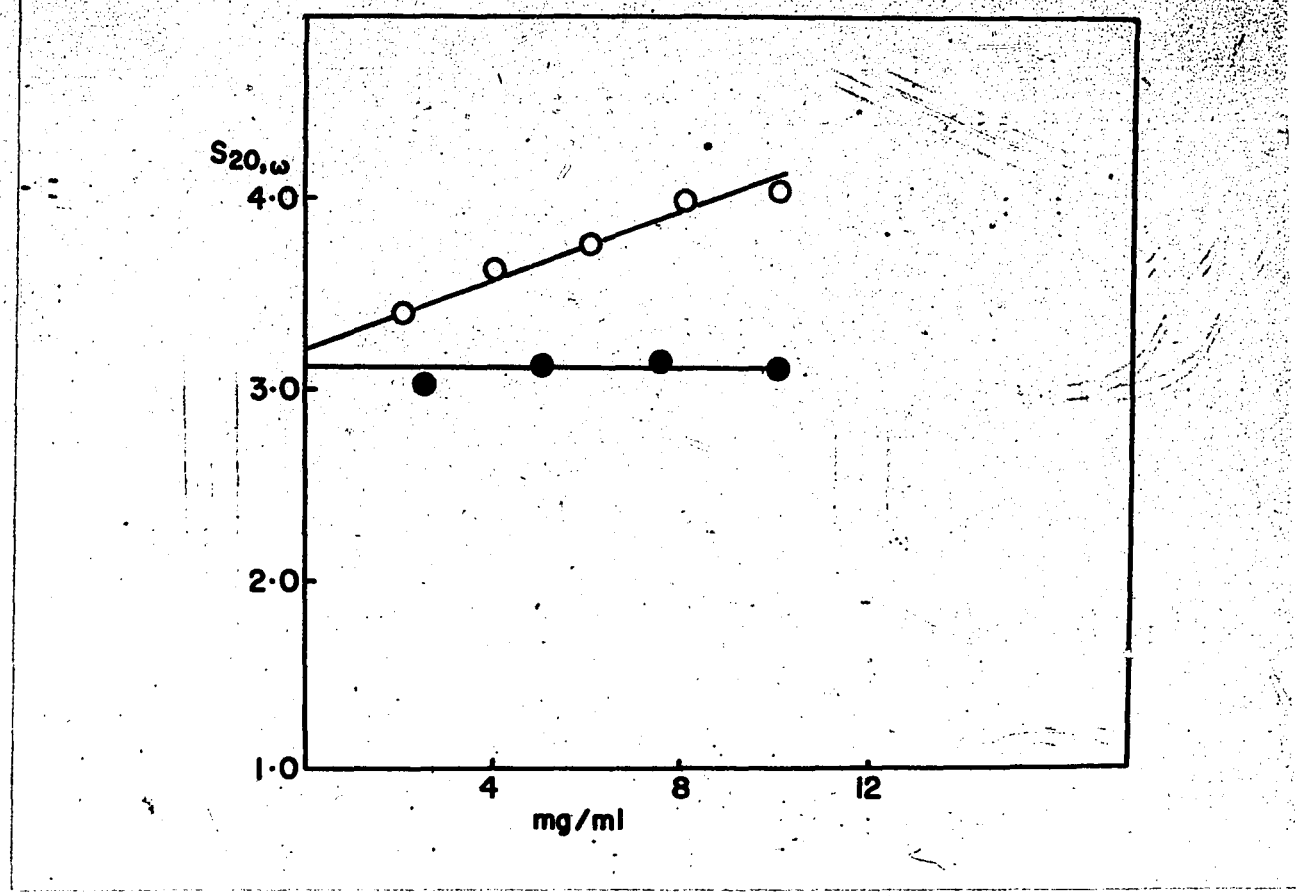


Fig. 1 The concentration dependent S value of chick globin ○—○ and amino-ethylated chick globin ●—● in 0.1 M Na acetate buffer, pH 5.0 at 20° at a rotor speed of 56,000 rpm.

tris-HCl buffer, pH 8.6, and 4.0 M NaCl:0.1 M tris-HCl buffer, pH 8.6, were 2.81 S and 2.17 S respectively. Thus, it appears that chick hemoglobin in 2.0 M NaCl:0.1 M tris-HCl buffer, pH 8.6, was dissociated from tetramer to dimer size. The limiting value of $S^{o}_{20,w}$ obtained in 4.0 M NaCl:0.1 M tris-HCl buffer, pH 8.6, was 2.17 S, indicating that the dissociation of chick hemoglobin molecules proceeded to monomer state. Myoglobin (mol. wt. 18,000) was found to be 1.85 S (2, 14). The $S_{20,w}$ value was dependent on protein concentration in both 2.0 M and 4.0 M NaCl. $S^{o}_{20,w}$ values in 2.0 M and 3.0 M KCl:0.1 M tris-HCl buffer, pH 8.6 were 3.14 S and 2.68 S respectively. Although the S value was independent of protein concentration at 2.0 M KCl, the S value in 3.0 M KCl increased with the increase in protein concentration. The disruptive effect of $CaCl_2$ was more pronounced than that observed with the other ionic solutes, NaCl, KCl and LiCl (Fig. 2a). $CaCl_2$ solution of $\rho_{20,w}$ - 1.088 produced monomers while the other ionic solutes of the same density dissociated chick hemoglobin to the dimer stage (Table I). $S^{o}_{20,w}$ value of chick hemoglobin in 1.0 M $CaCl_2$ was 1.85 S. The limiting S values in LiCl imply the dissociation of tetrameric hemoglobin molecules although the extent of depolymerization was small. The S values in 4.0 M LiCl decreased with an increase in protein concentration. $S^{o}_{20,w}$ value in 4.0 M LiCl was 3.40 S which is markedly different from that observed with other ionic solutes (Fig. 2).

The equilibrium constant K for the tetramer-dimer equilibrium at a particular solute concentration (moles/liter) and 20°, and at a varying concentration of protein (mg/ml) may be evaluated from the equation,

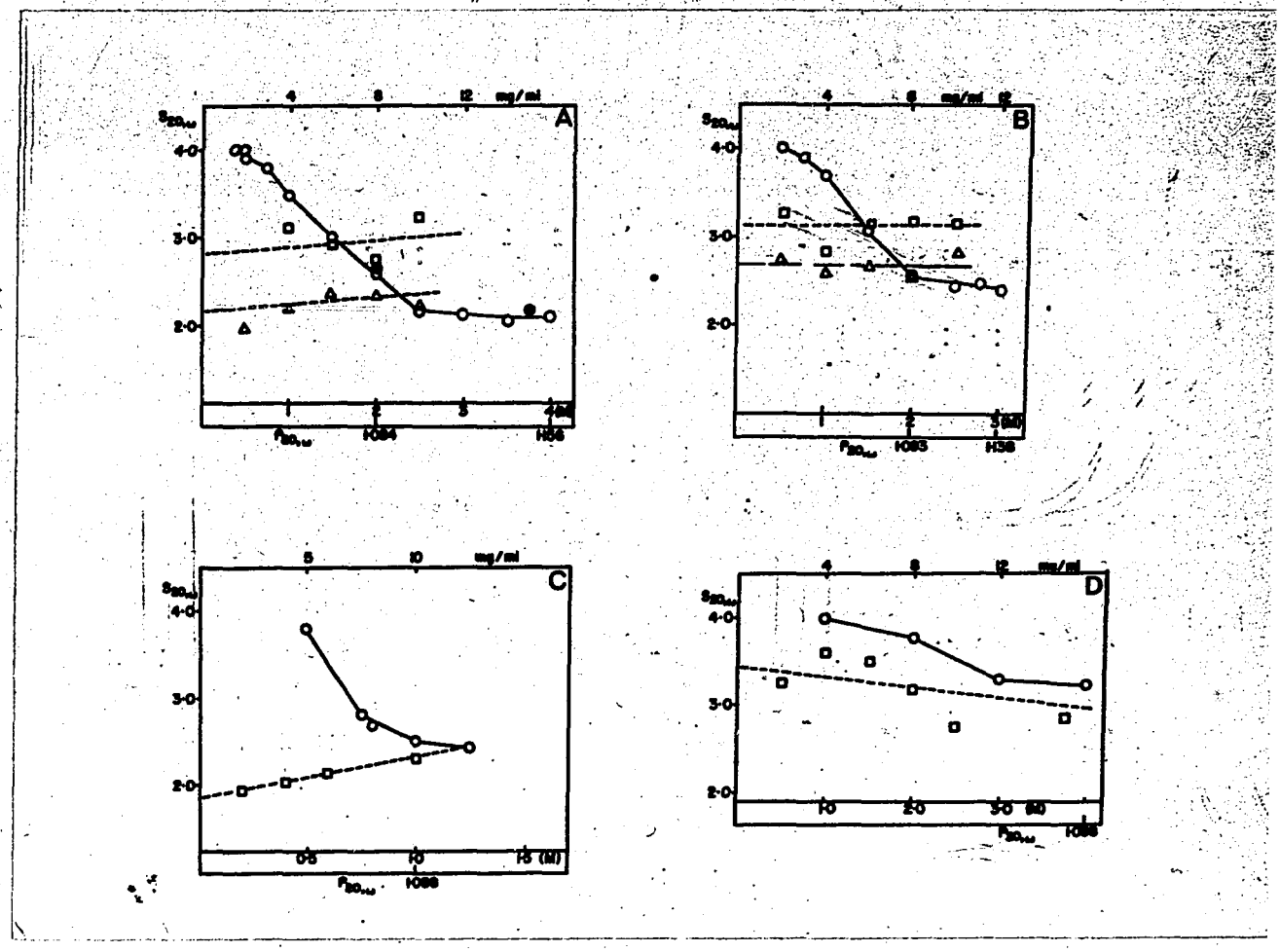


Fig. 2 Ultracentrifugal behavior of chick hemoglobin in presence of increasing concentration of ionic solutes.
The experimental parameters were: protein concentration - 4 mg/ml; buffer - 0.1 M tris-HCl, pH 8.6; temperature - 20°; rotor speed - 56,000 rpm. For $CaCl_2$ system, the buffer used was 0.1 M tris-HCl, pH 7.0.

A. S value in NaCl ○—○ and comparative points for Hb A, ● , the concentration dependent S values in 2.0 M NaCl, □---□; and in 4.0 M NaCl, △—△.

B. S value in KCl, ○—○. The concentration dependent S value in 2.0 M KCl, □---□ and 3.0 M KCl, △—△.

C. S value in $CaCl_2$, ○—○. The concentration dependent S value in 1.0 M $CaCl_2$, □---□.

D. S value in LiCl, ○—○. The concentration dependent S value in 4.0 M LiCl, □---□.

$$K = \frac{4\alpha^2 C_o \times 10^3}{(1-\alpha)M}$$

where C_o is the initial protein concentration (mg/ml) and M the molecular weight of undissociated hemoglobin. The weight fraction of hemoglobin in the dissociated form, α, may be calculated from the equation,

$$\alpha = \frac{S_T - S_{obs}}{S_T - S_D}$$

where S_T and S_D are the $S_{20,w}$ values of tetramer and dimer, and S_{obs} is the observed weight-average $S_{20,w}$ value at a particular solute concentration (moles/liter). These calculations were based on the assumptions that (i) no extensive conformational changes occurred during and after dissociation; (ii) no appreciable increase in ion-binding characteristics and hydration of the subunits took place; (iii) the S value is proportional to the molecular weight ($M^{0.66}$) (92); and (iv) no monomers formed. The limiting value of $S_{20,w}$ in 0.1 M tris-HCl buffer, pH 8.6, was considered as S_T - 4.4 S ($S^o_{20,w}$) and S_D as 2.8 S. That there is a difference in the disruptive effects of the three ionic solutes used (NaCl, KCl and LiCl) is apparent from the shape of the curve shown in Fig. 3; the curves representing 4.0 M LiCl and 2.0 M KCl follow each other closely whereas the curve representing 2.0 M NaCl diverges markedly.

Non-ionic solutes in the experimental media have long been employed to produce dissociation of polymer proteins (90). During the present investigation, four such non-ionic solutes (formamide, urea, guanidine-HCl, and 2-chloroethanol) in increasing concentration were employed to study depolymerization of chick hemoglobin (Fig. 4). These non-ionic solutes could

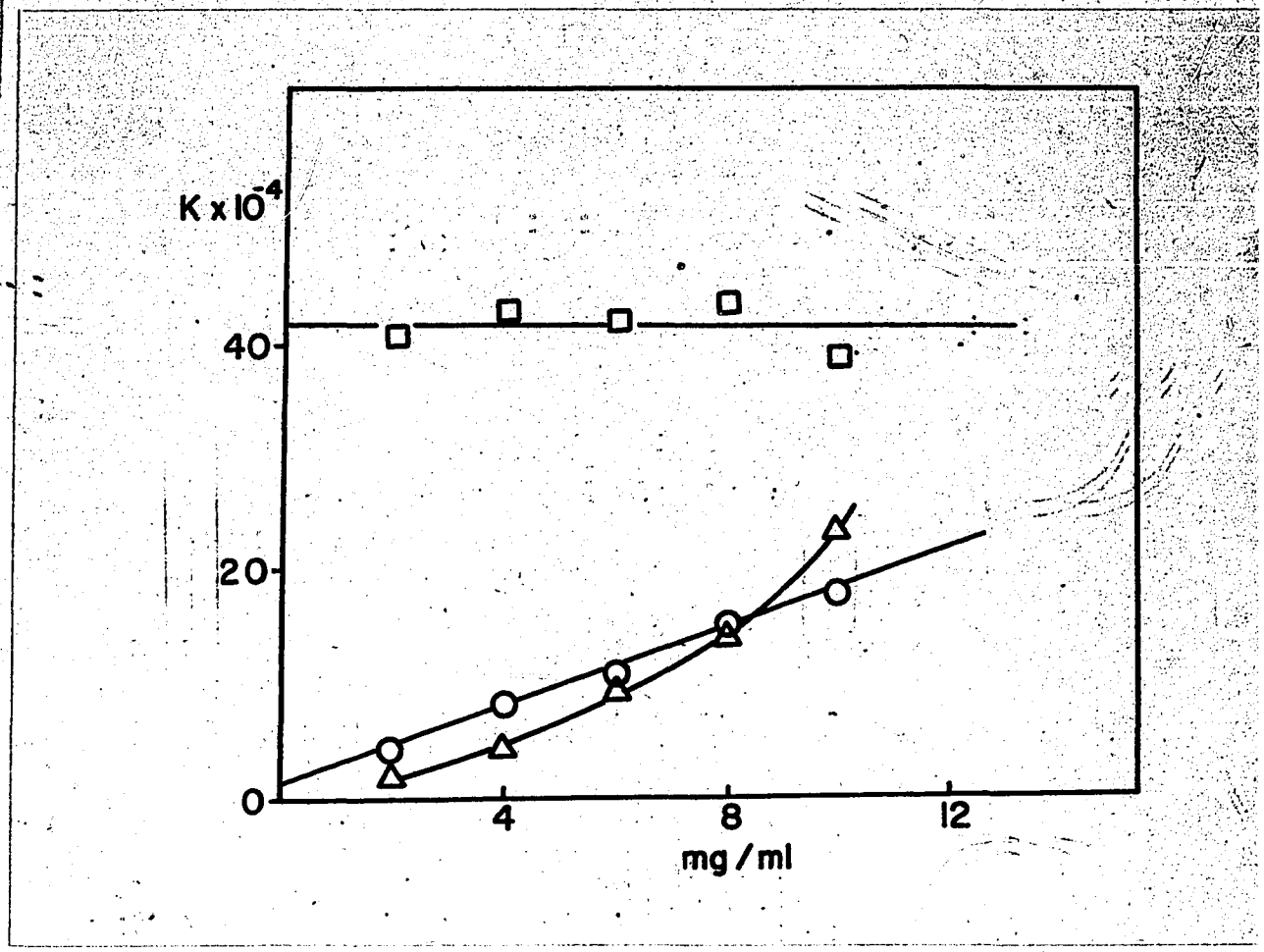


Fig. 3 The effect of protein concentration on K values (moles/liter) for the equilibrium reactions (tetramer-dimer) of chick hemoglobin in 2.0 M NaCl, □—□; 2.0 M KCl, ○—○; and 4.0 M LiCl, △—△.

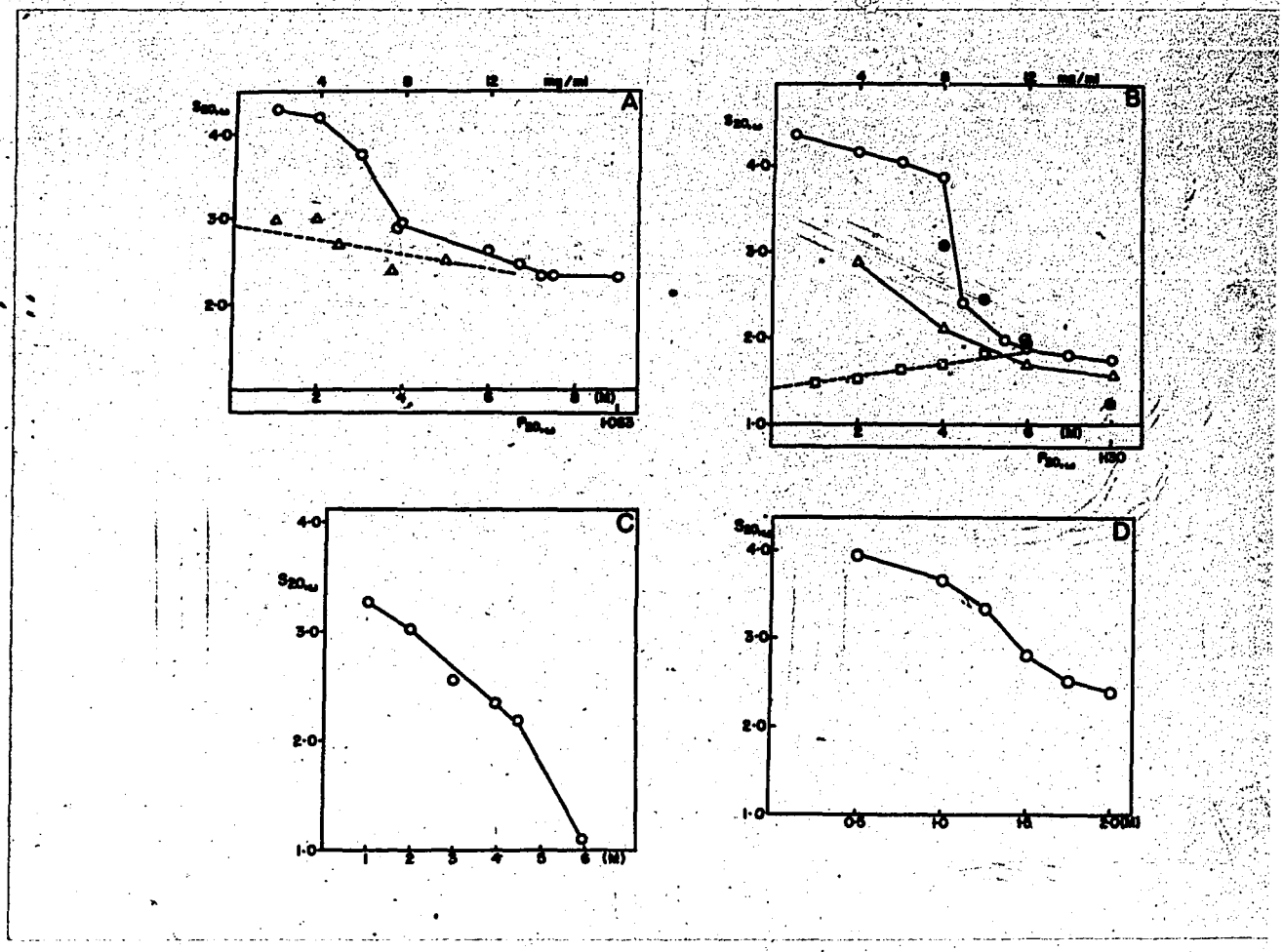


Fig. 4 Dissociation of chick hemoglobin in presence of non-ionic solutes. The experimental parameters are: protein concentration - 4 mg/ml; buffer - 0.1 M tris-HCl, pH 8.6; temperature - 20°; rotor speed - 56,000 rpm.

A. S value of chick hemoglobin in formamide, O—O; the concentration dependent S value in 9.0 M formamide, △—△.
B. S value of chick hemoglobin in urea, O—O; comparative values for Hb A, ● ; amino-ethylated globin, △—△; the concentration dependent S value in 8.0 M urea, □—□.
C. S value of chick hemoglobin in guanidine-HCl, O—O.
D. S value in 2-chloroethanol, O—O.

produce subunits of chick hemoglobin, dimer and monomer, depending on the solute concentration. A single symmetrical Schlieren peak was observed over the solute concentration range. At 9.0 M formamide solution the S value decreased with the increase in protein concentration. No precipitation of chick hemoglobin was observed with guanidine-HCl, although a change in color from red to reddish-brown was noticed at solute concentrations higher than 4.0 M guanidine-HCl. Kawahara et al (25) reported the denaturation of human carboxy hemoglobin in 6 M guanidine-HCl. These authors used β-mercaptoethanol at a concentration of 0.1 M to human carbonmonoxy-hemoglobin:6 M guanidine-HCl to keep the protein in solution. Extensive unfolding of the polypeptide chains seems to occur at 6.0 M guanidine-HCl in addition to dissociation. Table III presents some of the concentration-dependent $S_{20,w}$ values at a particular solute concentration.

Studies on the alkaline dissociation of chick hemoglobin were carried out in the pH range of 8.6 - 11.6 (Fig. 5). Fig. 5 also includes some of the $S_{20,w}$ values of Hb A and equine hemoglobin. The Schlieren plots displayed symmetrical peaks during the alkaline dissociation studies. The limiting value of $S_{20,w}$ was found to be 2.96 S at pH 11.6 indicating the presence of dimer subunits as a major component. The limiting value of $S_{20,w}$ reported for human carbonmonoxy Hb A was 2.0 S (19), for human carbonmonoxy Hb F 2.32 S, and for bovine carbonmonoxy Hb 2.41 S (21). Although the resistance of avian hemoglobins to alkaline denaturation varies considerably, the avian hemoglobins are generally considered to be an alkali-resistant type (47). It may be mentioned that chick hemoglobins belong to the group of avian hemoglobins exhibiting maximum resistance to alkaline denaturation (47, 48).

TABLE III

$S^{o}_{20,w}$ Values of Chick Hemoglobin*

Solute	Solute Conc. (M)	$\rho_{20,w}$	$S_{20,w}$
NaCl	2	1.084	2.81
	4	1.156	2.17
KCl	2	1.093	3.14
	3	1.138	2.68
$CaCl_2$**	1	1.088	1.85
LiCl	4	1.098	3.40
Formamide	9	1.063	2.92
Urea	8	1.130	1.40

* The values represent at a particular solute concentration and 0.1 M tris-HCl buffer, pH 8.6.

** Buffer used 0.1 M tris-HCl, pH 7.0.

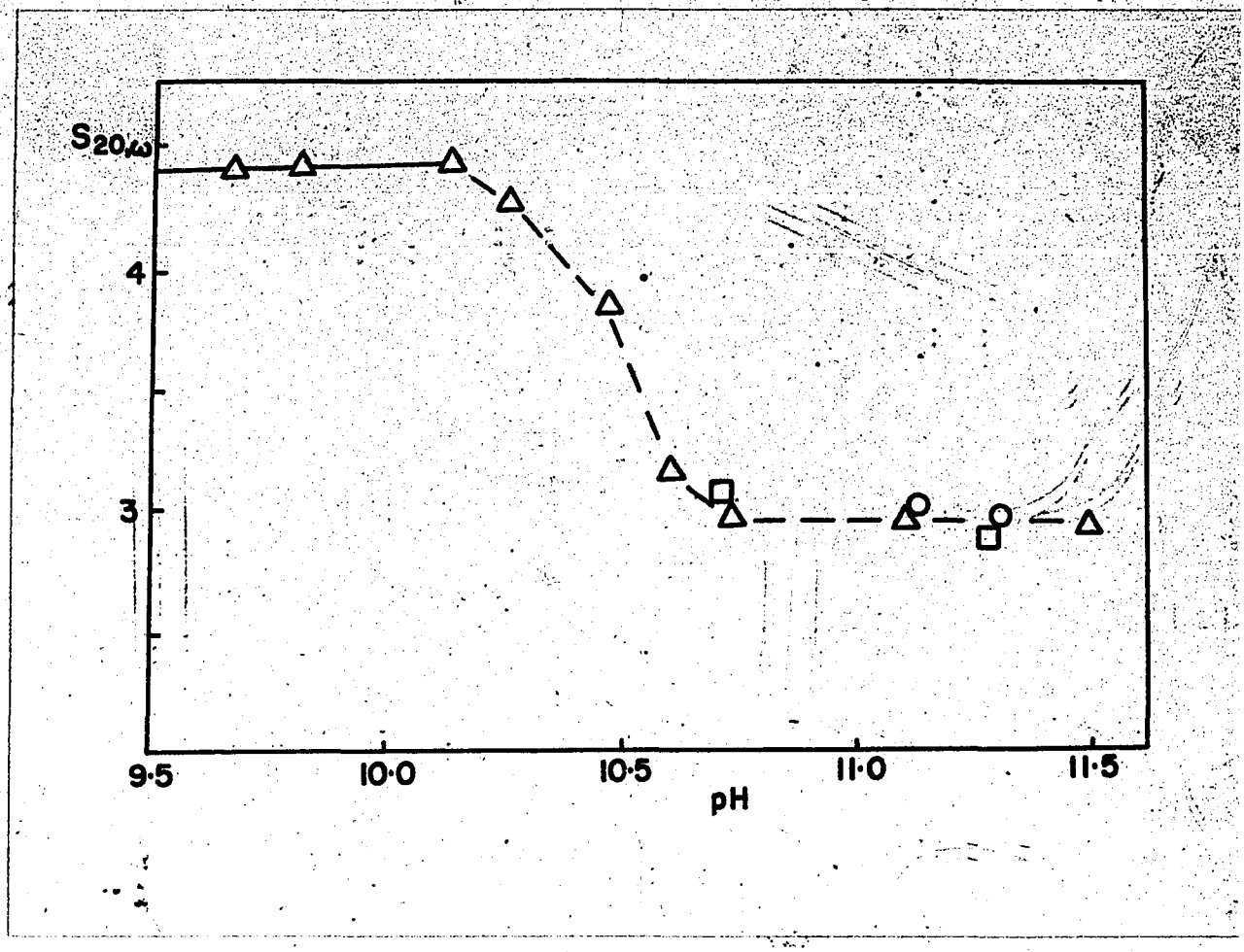


Fig. 5 S value of chick hemoglobin in alkaline pH, △—△ ; comparative points for HbA, □ , bovine Hb, ○ , The experimental parameters were: protein concentration - 4 mg/ml; buffer - phosphate-KCl, μ - 0.25, dialysed 15 hrs; temperature - 20°; rotor speed - 56,000 rpm.

The sedimentation equilibrium method of studying the dissociation of polymer proteins in the presence of a third component (a strong dissociating agent), involved operational difficulties in determining the concentration of the protein at the cell meniscus. The availability of automatic photoelectric scanner (101) has facilitated studies of depolymerization, especially with a double sector ultracentrifuge cell where the optical density contribution of the dissociating agent and the buffer present in the protein solution may be ignored. This contribution is automatically deducted by the scanner circuit. The precision of comparative studies has greatly been improved with the electronic scanning device and the optical density chart recorder both operating at variable speeds to meet different requirements.

Further studies were carried out to evaluate the data on the dissociation of chick hemoglobin obtained by the sedimentation velocity methods as mentioned earlier. In these studies the solute concentrations were chosen to limit the dissociating systems either to a tetramer-dimer or a dimer-monomer equilibrium, the protein concentration being the same in both cases. Accordingly, several rotor speeds were employed to favor one subunit component; for example, the monomer distribution was favored in the monomer-dimer equilibrium or the tetramer distribution in dimer-tetramer equilibrium. In the presence of perturbants all the sedimentation equilibrium plots demonstrated the existence of an equilibrium condition between two kinds of subunits. A single component was observed only with chick hemoglobin in the native (tetramer) state. Schachman and Edelstein (19) reported the dissociation of human oxyhemoglobin into monomers at a very dilute concentration (0.002 mg/ml, 0.1 M Na phosphate buffer, pH 7.0). However, no dissociation of the chick hemoglobin molecule in 0.1 M tris-HCl buffer, pH 8.6 was observed

for the range of protein (0.1 - 0.3 mg protein/ml) concentration used during the present study (Fig. 6(i)). Figs. 6 [(ii), (iii)], depict the sedimentation equilibrium behavior of chick hemoglobin in the presence of ionic solutes (NaCl, KCl, $CaCl_2$ and LiCl). The shape of the plot, ln c vs r^2, indicates an equilibrium between the subunits of different sizes. No denaturation of chick hemoglobin in contact with FC 43 fluorocarbon oil was observed over a period of 18 hr.

Although the limiting value of S in 2.0 M NaCl indicates the presence of dimer predominantly, when these experiments were re-evaluated by the sedimentation equilibrium analysis, the presence of monomer was detected. The presence of monomer in 4 M NaCl is shown in Fig.6 (ii)B. The rotor speed was chosen to suit the monomer and is consequently an overspeed for the dimer component. This resulted in the heavier components being distributed at the bottom of the cell. The major species in 2.0 M NaCl is a dimer, although the characteristics of cell meniscus clearance indicates the presence of a very small amount of monomer. The predominating species in 3.5 M KCl is dimer, with a small amount of monomer; a situation comparable to that obtained with 2.0 M NaCl. It appears that the disruptive effect of KCl is weaker than that of NaCl. The major species in 1.0 M $CaCl_2$ is monomer. Although 4 M LiCl produced variable denaturation characteristics in dilute solution, the best results obtained indicated the presence of dimer as a major species. Besides the rotor speeds, the other operational differences between the two ultracentrifugal methods were the length of time and the protein concentration: 2 hr and 4 mg/ml in sedimentation velocity experiments, and 15 hr and 0.2 - 0.3 mg/ml in sedimentation

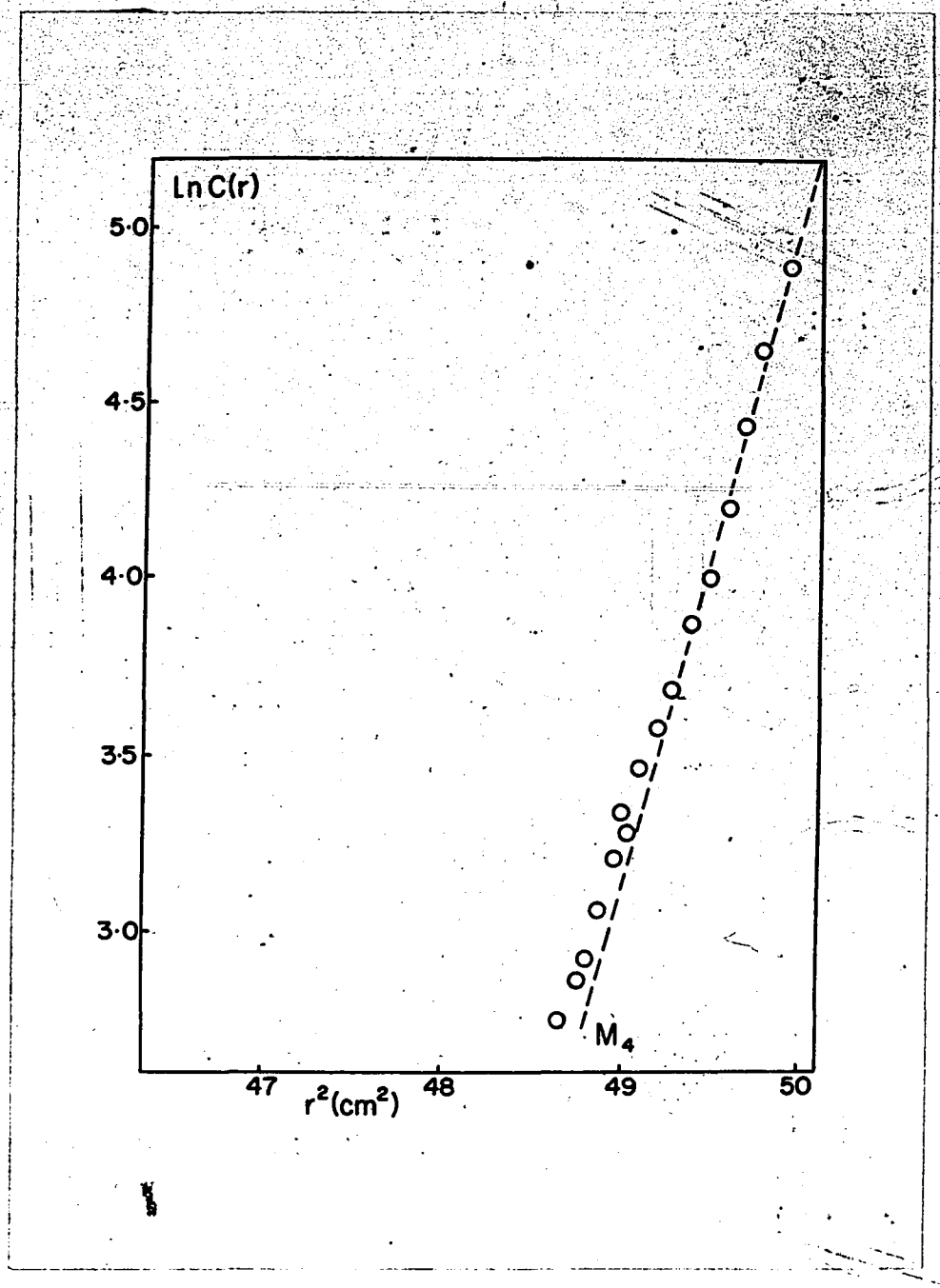


Fig. 6 Sedimentation equilibrium plots of chick hemoglobin in the presence of increasing molarities of ionic solutes. The plots represent the logarithm of concentration as a function of the square of the radial distance. The theoretical distribution of monomer is given by---M_1, dimer---M_2 and tetramer---M_4. The media employed in all cases contained 0.1 M tris-HCl buffer, pH 8.6. The initial protein concentration has been reported as absorbance at 280 mμ.

(1) Native chick hemoglobin, C_0 - .26; rotor speed - 24,000 rpm.

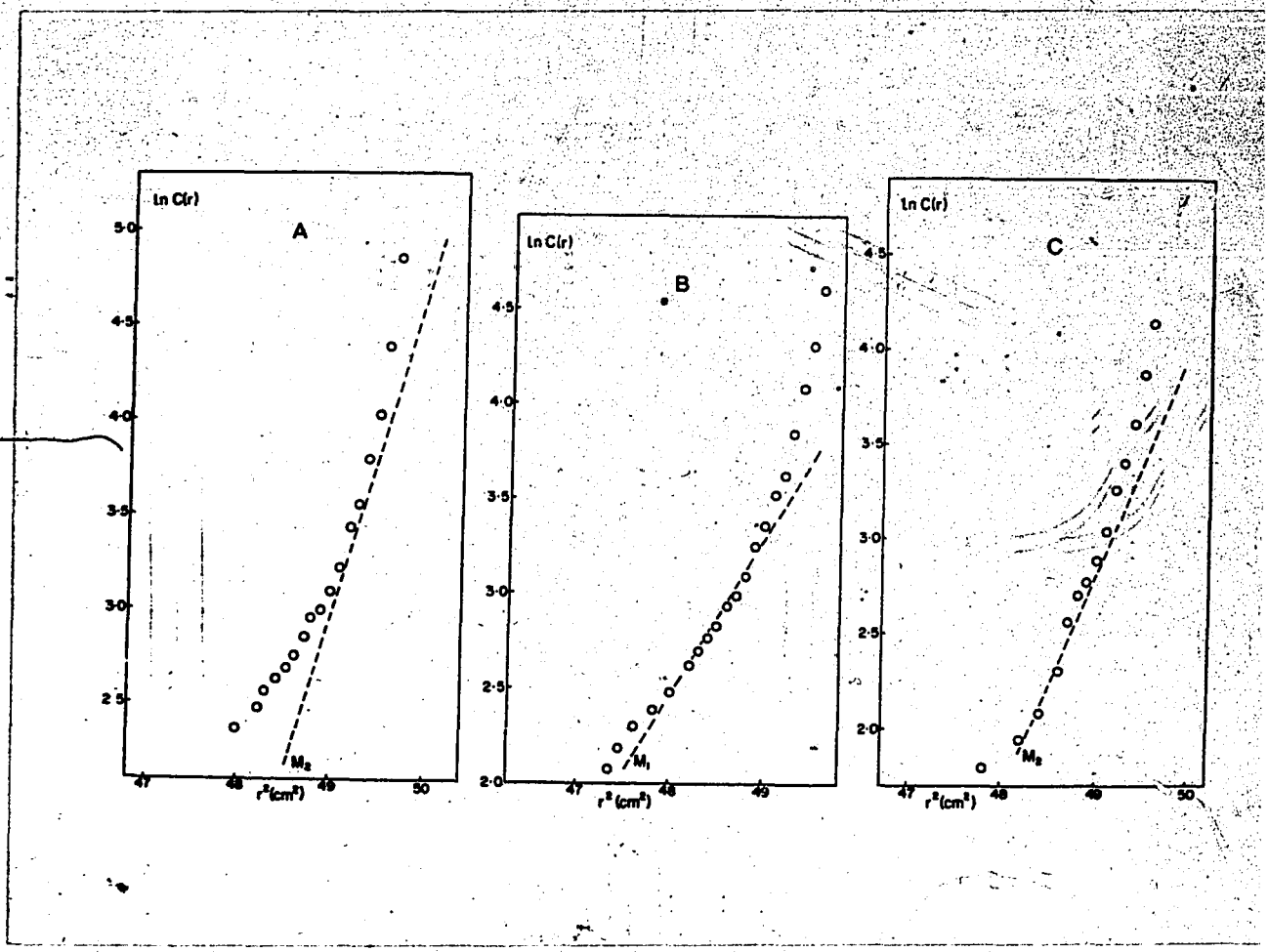


Fig. 6 Sedimentation equilibrium plots of chick hemoglobin in the presence of increasing molarities of ionic solutes. The plots represent the logarithm of concentration as a function of the square of the radial distance. The theoretical distribution of monomer is given by ---M_1, dimer---M_2, and tetramer---M_4. The media employed in all cases contained 0.1 M tris-HCl buffer, pH 8.6. The initial protein concentration has been reported as absorbance at 280 mμ.

(ii) A. Chick hemoglobin in 2.0 M NaCl, C_0 - .25; rotor speed - 36,000 rpm.
B. Chick hemoglobin in 4.0 M NaCl, C_0 - .24; rotor speed - 36,000 rpm.
C. Chick hemoglobin in 3.5 M KCl, C_0 - .24; rotor speed - 36,000 rpm.

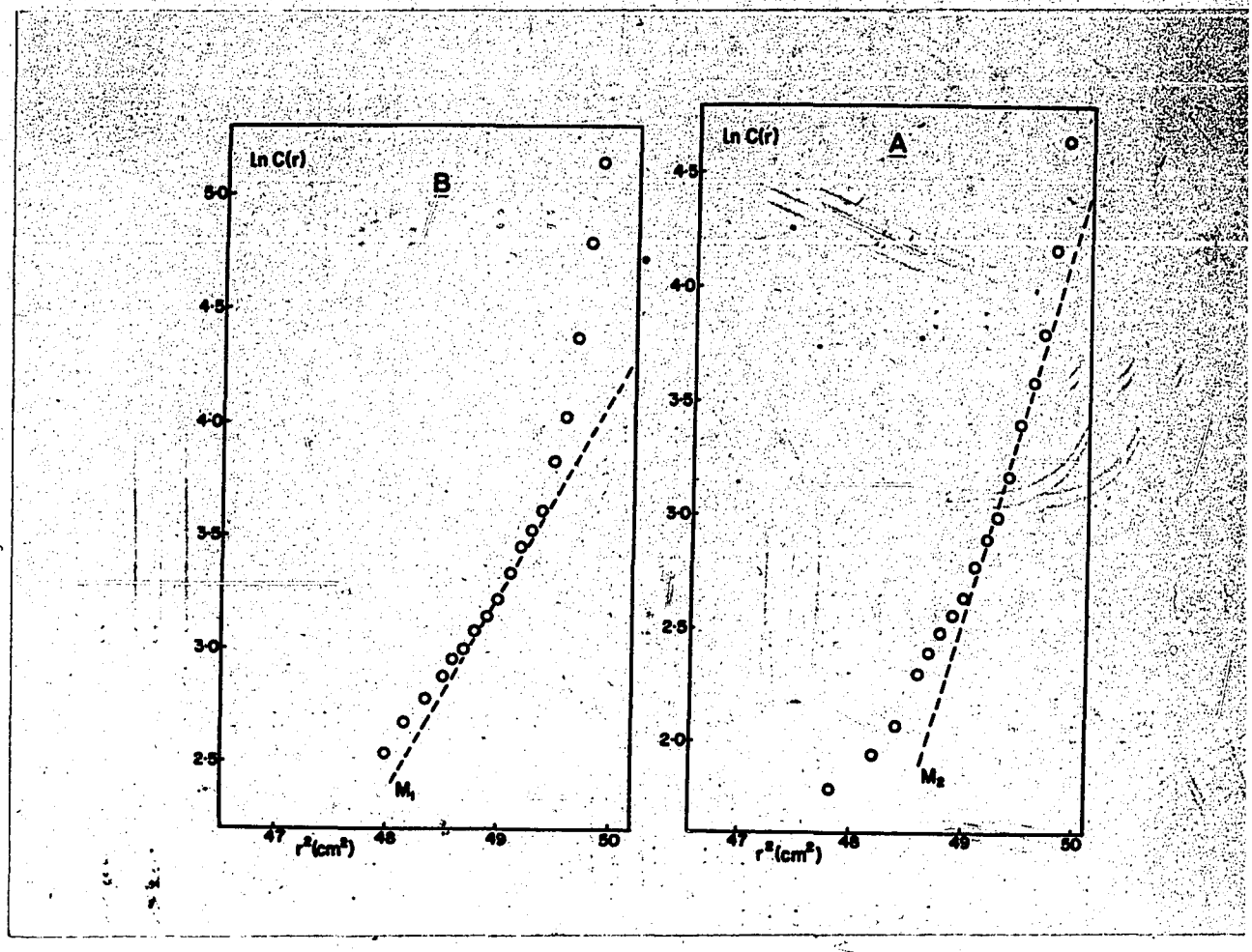


Fig. 6 Sedimentation equilibrium plots of chick hemoglobin in the presence of increasing molarities of ionic solutes. The plots represent the logarithm of concentration as a function of the square of the radial distance. The theoretical distribution of monomer is given by --- M_1, dimer--- M_2, and tetramer-- - M_4. The media employed in all cases contained 0.1 M tris-HCl buffer, pH 8.6. The initial protein concentration has been reported as absorbance at 280 mμ.

(iii) A. Chick hemoglobin in 4 M LiCl, C_0 - .22; rotor speed - 36,000 rpm.
B. Chick hemoglobin in 1.0 M $CaCl_2$, C_0 - .33; rotor speed - 36,000 rpm.

equilibrium experiments. The equilibrium plots shown in Figs. 7A, 7B and 7C were obtained with urea and formamide. In 4.0 M urea the major species is dimer with very small amount of monomer while in 8.0 M urea, more monomers were obtained. Fig. 8 (A and B) demonstrates that with the increase in alkaline pH, the dissociation progresses as in pH 10.6 and pH 11.6 the predominant species were dimer and monomer respectively. Fig. 9 presents the sedimentation equilibrium behavior of chick globin (normal preparation) and aminoethylated globin, both the preparations being ($\alpha\beta$) mixture in 0.1 M Na acetate buffer, pH 5.0, where the major component is a dimer with a small amount of monomer. In 2 M urea:0.1 M Na acetate buffer, pH 5.0, the major component is dimer with a small amount of monomer (Fig. 9 c).

Studies on the dissociation of chick hemoglobin necessitate evaluation of several factors, such as preferential ion-binding of macromolecular components, the partial specific volume of the subunits, and the conformational change. It is known that there is no appreciable binding of NaCl to isoionic hemoglobin molecule, and the interaction between NaCl and hemoglobin is smaller than that between NaCl and other common proteins (132, 133). Cox and Schumaker (134) have reported a value of 0.1 g/g protein in CaCl solution. Several authors found no appreciable differences in the partial specific volume (132, 133). Tanford and his collaborators (25, 32) reported that ionic and non-ionic solutes do not produce a significant change in the conformation of hemoglobin molecules under solvent perturbation system. The intrinsic viscosity (Fig. 10) of chick hemoglobin for both ionic and non-ionic solutes (2.0 M KCl, 1.0 M $CaCl_2$ and 8.0 M urea) was 2.6 cc/gm. Apparently these solutes did not affect the gross molecular

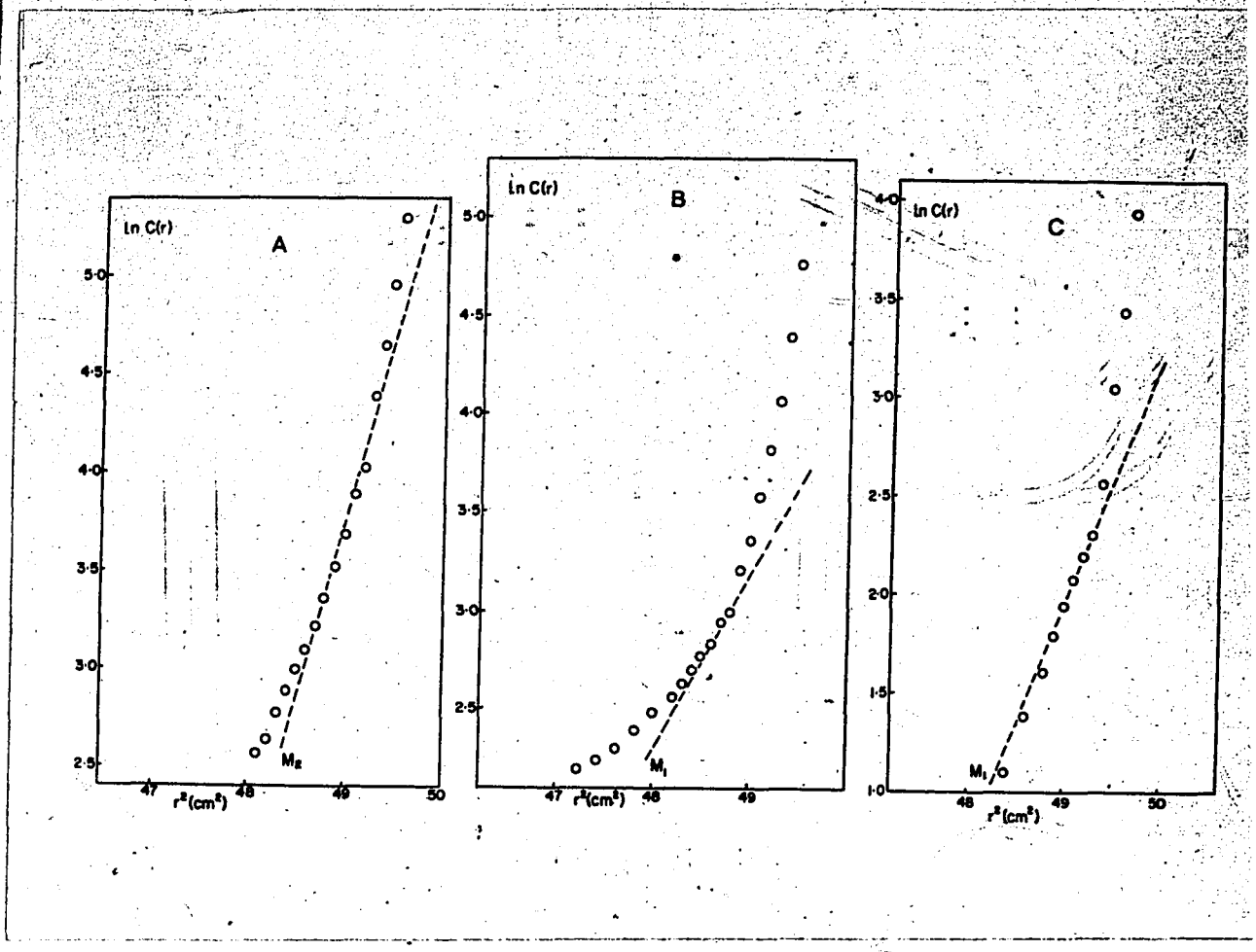


Fig. 7 Sedimentation equilibrium plots of chick hemoglobin in non-ionic solutes. The experimental conditions were maintained the same as mentioned in the case of ionic solutes.

A. Chick hemoglobin in 4.0 M urea; C_0 - .25; rotor speed - 36,000 rpm.
B. Chick hemoglobin in 8.0 M urea; C_0 - .23; rotor speed - 40,000 rpm.
C. Chick hemoglobin in 7.5 M formamide; C_0 - .33; rotor speed - 40,000 rpm.

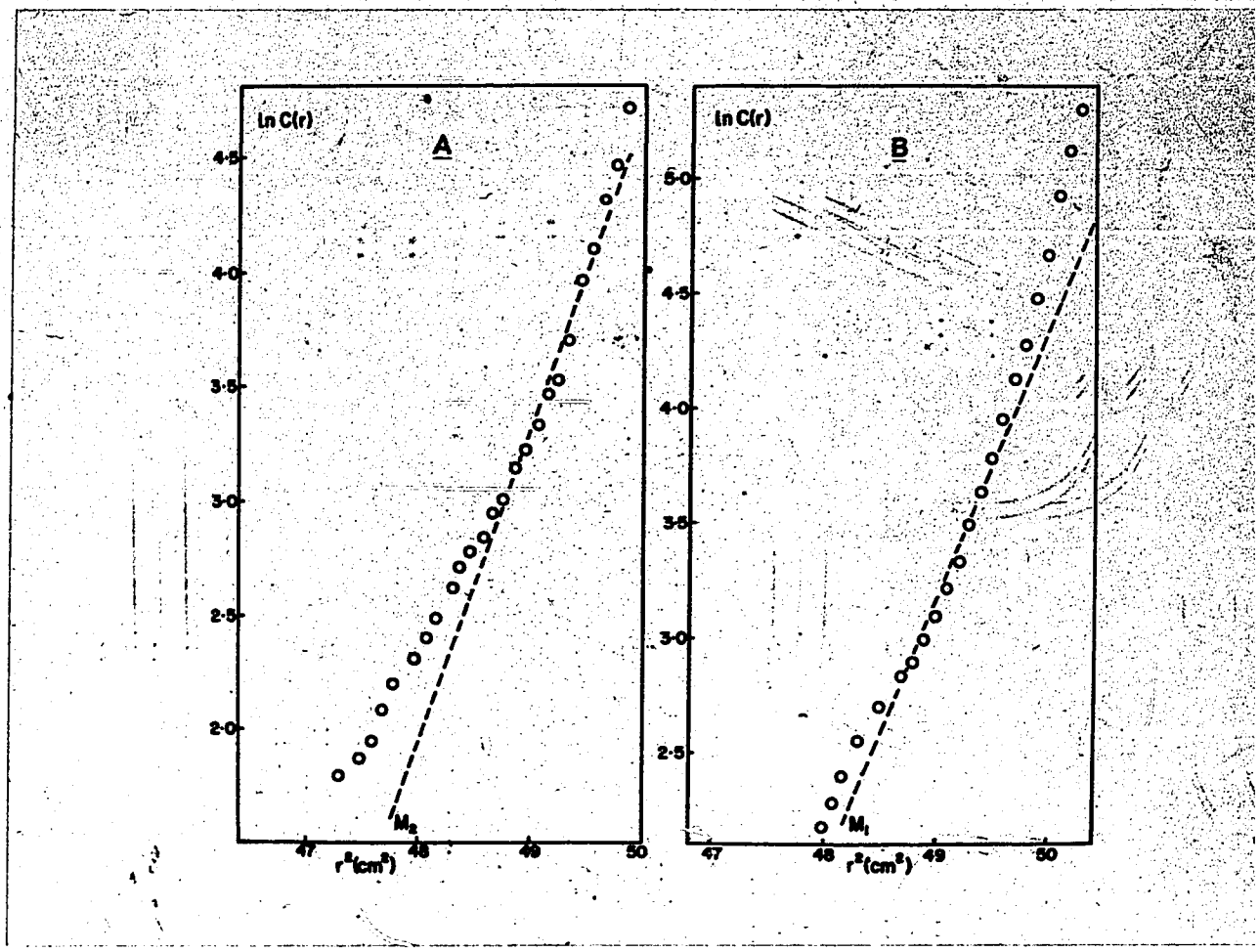


Fig. 8 Sedimentation equilibrium plots of chick hemoglobin in alkaline pH. The experimental parameters were: buffer - phosphate-KCl; μ (ionic strength) - 0.25; dialysis time - 15 hr; temperature - 20°.

A. Chick hemoglobin at pH 10.6, C_0 - .27; roter speed - 26,000 rpm.
B. Chick hemoglobin at pH 11.6, C_0 - .38; roter speed - 36,000 rpm.

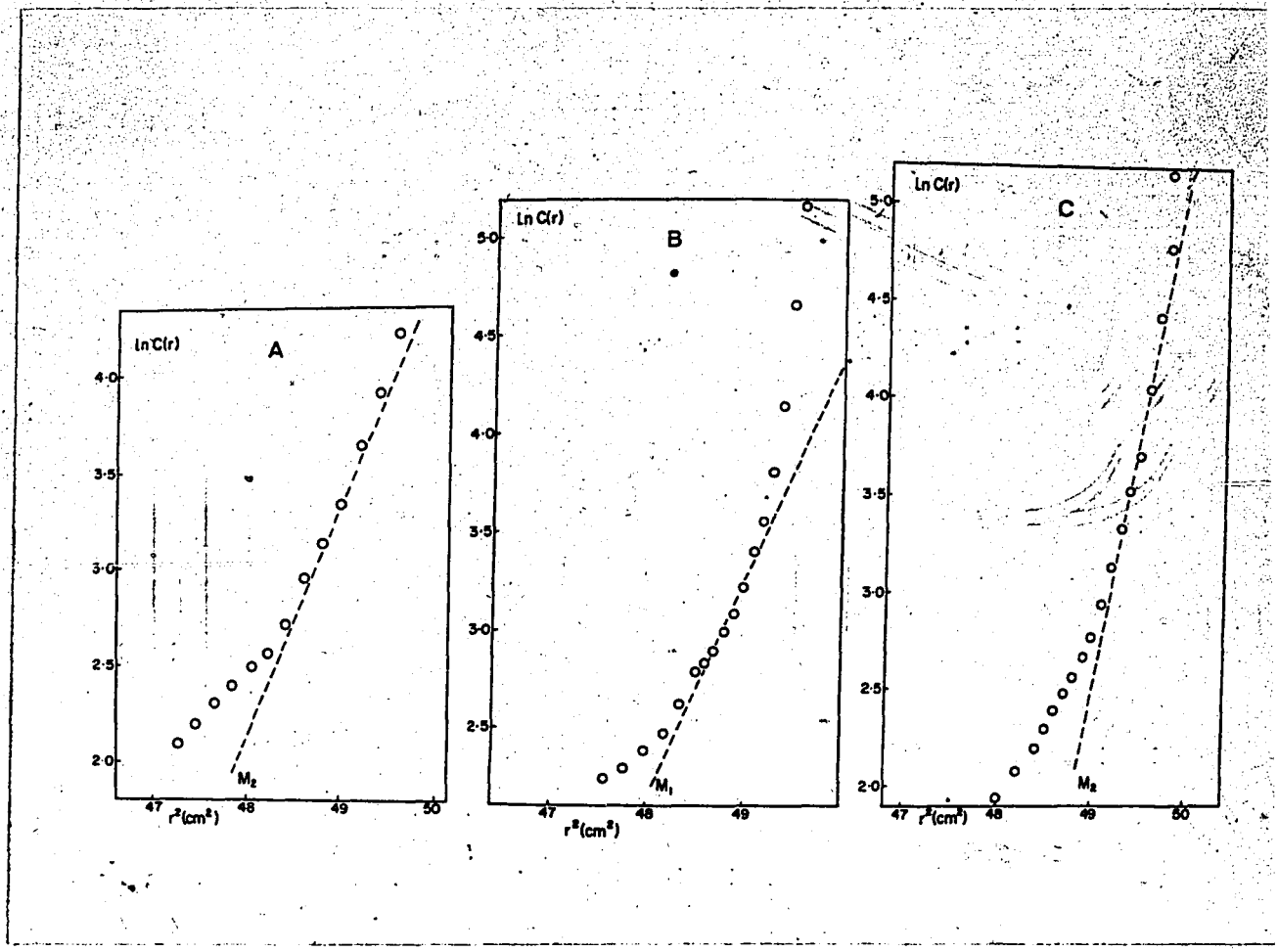


Fig. 9 Sedimentation equilibrium plots of chick globin.

A. Chick globin in 0.1 M Na acetate buffer, pH 5.0, C_0 - .36; rotor speed - 22,000 rpm.
B. Aminoethylated chick globin in 2 M urea; 0.1 M Na acetate buffer, pH 5.0, C_0 - .45; rotor speed - 36,000 rpm.
C. Aminoethylated chick globin in 0.1 M Na acetate buffer, pH 5.0, C_0 - .42; rotor speed - 36,000 rpm.

conformation of chick hemoglobin to an extent detectable by viscosity measurements (Fig. 10 a,b).

Despite the fact that no gross conformational change could be assessed under the solvent perturbation systems used during this investigation, attempts were made to evaluate the localized conformational changes, if any. Differential spectrophotometric studies on chick hemoglobin at the maximum solute concentration in ionic and non-ionic solute systems were undertaken at a wavelength range extending from 450 mμ to 250 mμ. No appreciable variation in the differential spectrophotometric curve of hemoglobin was observed with NaCl, KCl, LiCl and ethylene glycol. However, diminution in the Soret band intensity (E^{M}_{heme}) was observed with 1.0 M $CaCl_2$, 9.0 M formamide, 8.0 M urea, and 6.0 M guanidine-HCl, (Fig. 11 a,b). Although there was a decrease in the Soret band (415 mμ) intensity in other solutes, a shift in the Soret band from 415 to 425 mμ (Fig. 11b) was observed only in guanidine-HCl. Since the sedimentation velocity experiments required 2 hr and sedimentation equilibrium experiments 15 hr, the rate of denaturation, if there was any, was evaluated throughout this period under varying conditions of solvent:solute interaction.

A localized conformational change seems to occur with the increase in time for a particular solute concentration. Time-dependent solvent perturbation spectra of chick hemoglobin were studied with 9 M formamide, 8 M urea and 1 M $CaCl_2$ at 2 hr and 15 hr, a further decrease in ΔE^{M}_{heme} was noted; for example 8.6 and 23.0 (9 M formamide), 51.3 and 80.0 (8 M urea), 6.3 and 20.6 (1 M $CaCl_2$) x 10^3 were obtained at 2 hr and 15 hr respectively.

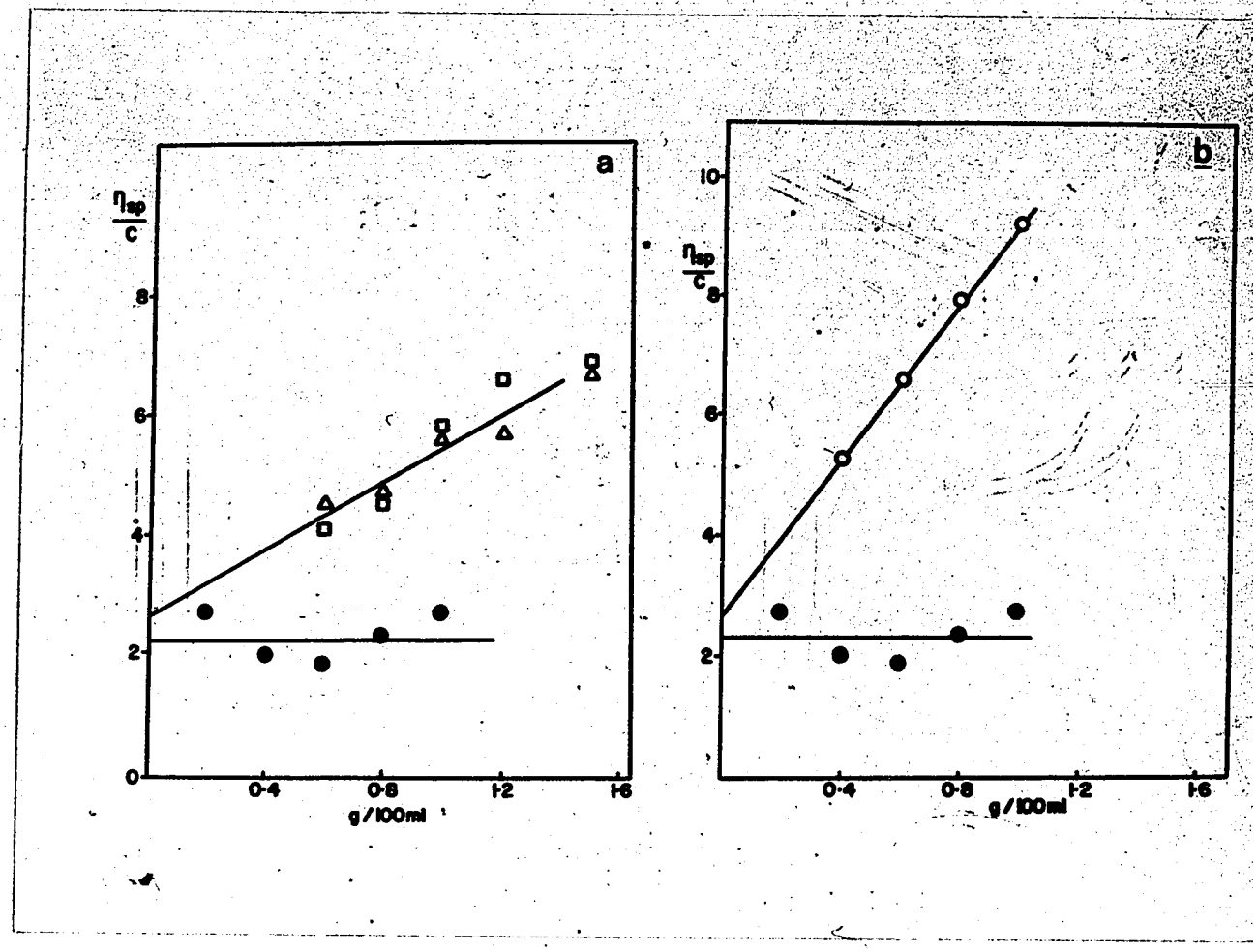


Fig. 10 a. Intrinsic viscosity of chick hemoglobin in 2.0 M KCl:0.1 M tris-HCl buffer, pH 8.6, □ ; 1.0 M $CaCl_2$:0.1 M tris-HCl buffer, pH 7.0, △ ; the intrinsic viscosity for native chick hemoglobin, ● ; buffer - 0.1 M tris-HCl, pH 8.6.

b. Intrinsic viscosity of chick hemoglobins in 8.0 M urea, O—O; native chick hemoglobin, ● ; buffer - 0.1 M tris-HCl, pH 8.6.

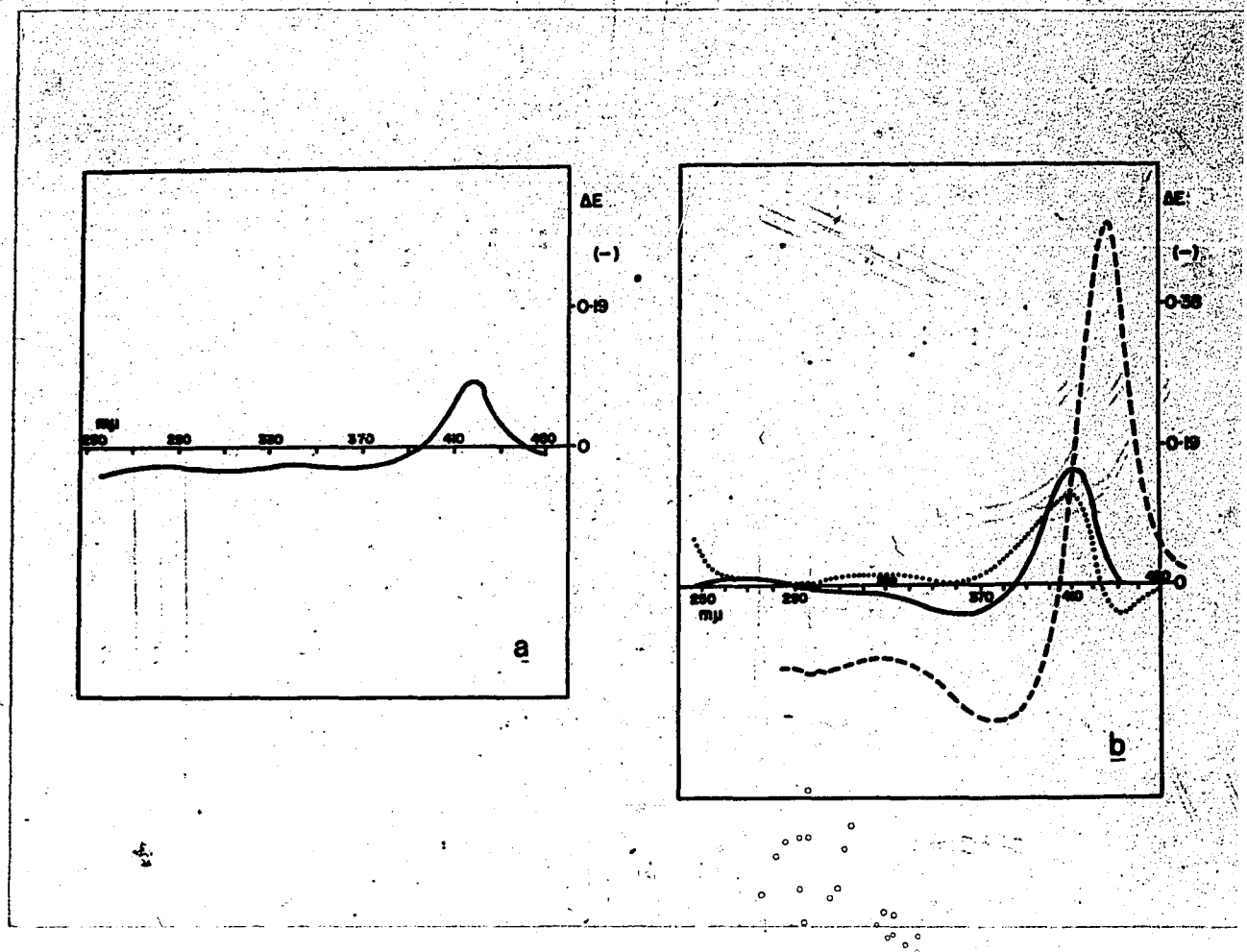


Fig. 11

A. Solvent perturbation difference spectra of chick hemoglobin in 1.0 M $CaCl_2$:0.1 M tris-HCl buffer, pH 7.0. The scan was taken five minutes after the mixing of the solutions.

B. Solvent perturbation difference spectra of chick hemoglobin in presence of non-ionic solutes; —— 8.0 M urea; ····· 9.0 M formamide; - - - - - 6.0 M guanidine-HCl. The scans were taken five minutes after the mixing of the solutions. The media contained 0.1 M tris-HCl buffer, pH 8.6.

Tyrosine ionization studies on chick hemoglobin in 0.2 M KCl (Fig. 12) revealed that, of the 17 tyrosine groups available, as evaluated by the amino acid analysis, 15 tyrosines were ionized between pH 12.5 - 12.75. Four tyrosine residues were ionized at pH 10.3 and five at pH 10.6. Thus, during the dissociation of chick hemoglobin from a tetrameric state to the dimeric state (till pH 10.6), approximately 5 tyrosine residues were ionized. Between pH region 10.3 and 11.3, 8 tyrosine residues were ionized, leaving 3 more tyrosine residues to be available at higher alkaline pH. At present, the total number of prototropic (sulfhydryl, lysyl, tyrosyl and guanidyl) groups besides tyrosines have not yet been evaluated. Studies on the hydrogen ion equilibria of chick hemoglobin are in progress.

Comparative Studies on Avian and Mammalian Hemoglobins

In the previous sections, it was reported that the dissociation of chick hemoglobin depends on the nature of solute and also on the solute concentration. Thus it appears that the extent of dissociation is dependent on the disruptive field induced in the suspending media provided the experimental conditions are identical. It was considered to be of interest to assess the relative strength of dimer:dimer association, and also monomer: monomer association, and to compare these relative equilibria with other hemoglobins. The following mammalian hemoglobins: human hemoglobin A, human hemoglobin F, equine hemoglobin and bovine hemoglobin were used, and compared with avian hemoglobins obtained from duck and chick. Preliminary studies have been reported herein. Fig. 13 shows the sedimentation equilibrium behavior of duck and chick hemoglobin in 2.0 M NaCl. The mass distribution as presented

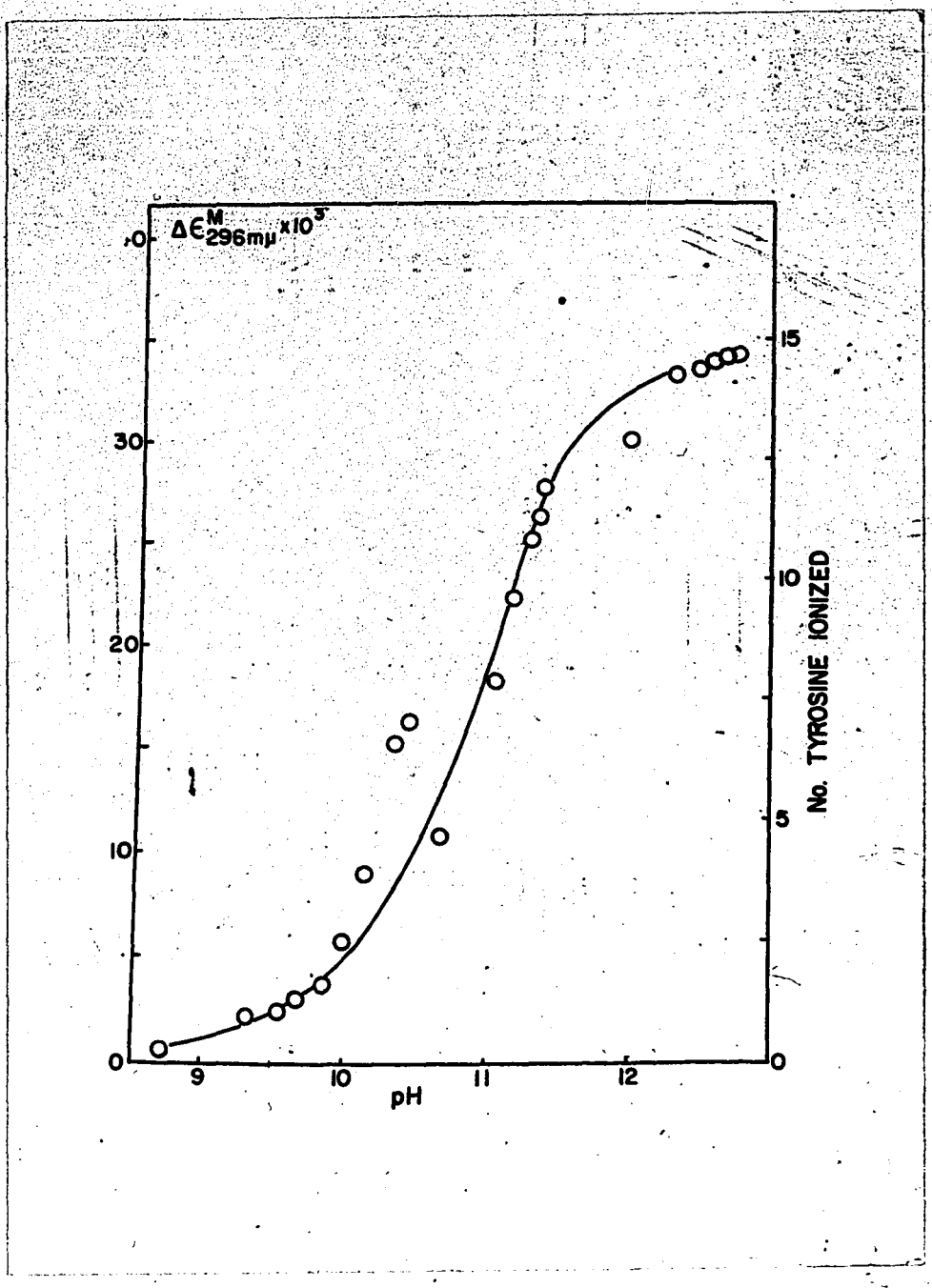


Fig. 12 Spectrophotometric titration of tyrosine residues of chick hemoglobin in 0.2 M KCl.

demonstrated their behavior as almost identical, the major species being a dimer. The solute concentration was increased further to 4.0 M NaCl and the sedimentation equilibrium behavior of equine hemoglobin and human hemoglobin F compared with that of chick hemoglobin. The main species in all cases was monomer although the rate of clearance in the upper part of the cell (meniscus region) was observed to be greater with chick hemoglobin. It implies that a larger proportion of dimer may be present with chick hemoglobin. This inference could be substantiated by a closer comparison of the intermediate scans obtained during the comparative runs (Fig.14).

The sedimentation equilibrium behavior of these hemoglobins in 8 M urea:0.1 M tris-HCl buffer, pH 8.6 has been illustrated in Fig.13c where scans from two comparative runs were superimposed. This difference in the behavior reflected in the plot, $\underline{\ln c(r) \text{ vs } r^2}$, where both avian hemoglobins cleared faster from the cell meniscus region than the mammalian hemoglobins, equine Hb and human hemoglobin F. In these runs the experimental conditions were maintained identical.

Sedimentation velocity coefficient of equine and human hemoglobin A showed the formation of the dimer as major component in the range pH 10.6 - pH 11.6. Sedimentation equilibrium analysis revealed the dimeric unit as the major species with small amounts of monomer. However, at pH 11.6 the major species is the monomer. In short, sedimentation velocity experiments revealed the presence of dimer as a major species (2.96 and 3.5 S), sedimentation equilibrium analysis showed the presence of monomer. The major difference between the two methods of analytical ultracentrifugation was the concentration of the protein, the time period of the subunits in a particular alkaline pH, and operational rotor speed. That the avian hemoglobins are comparatively resistant to dissociation is shown by the presence of heavier components (Fig. 1

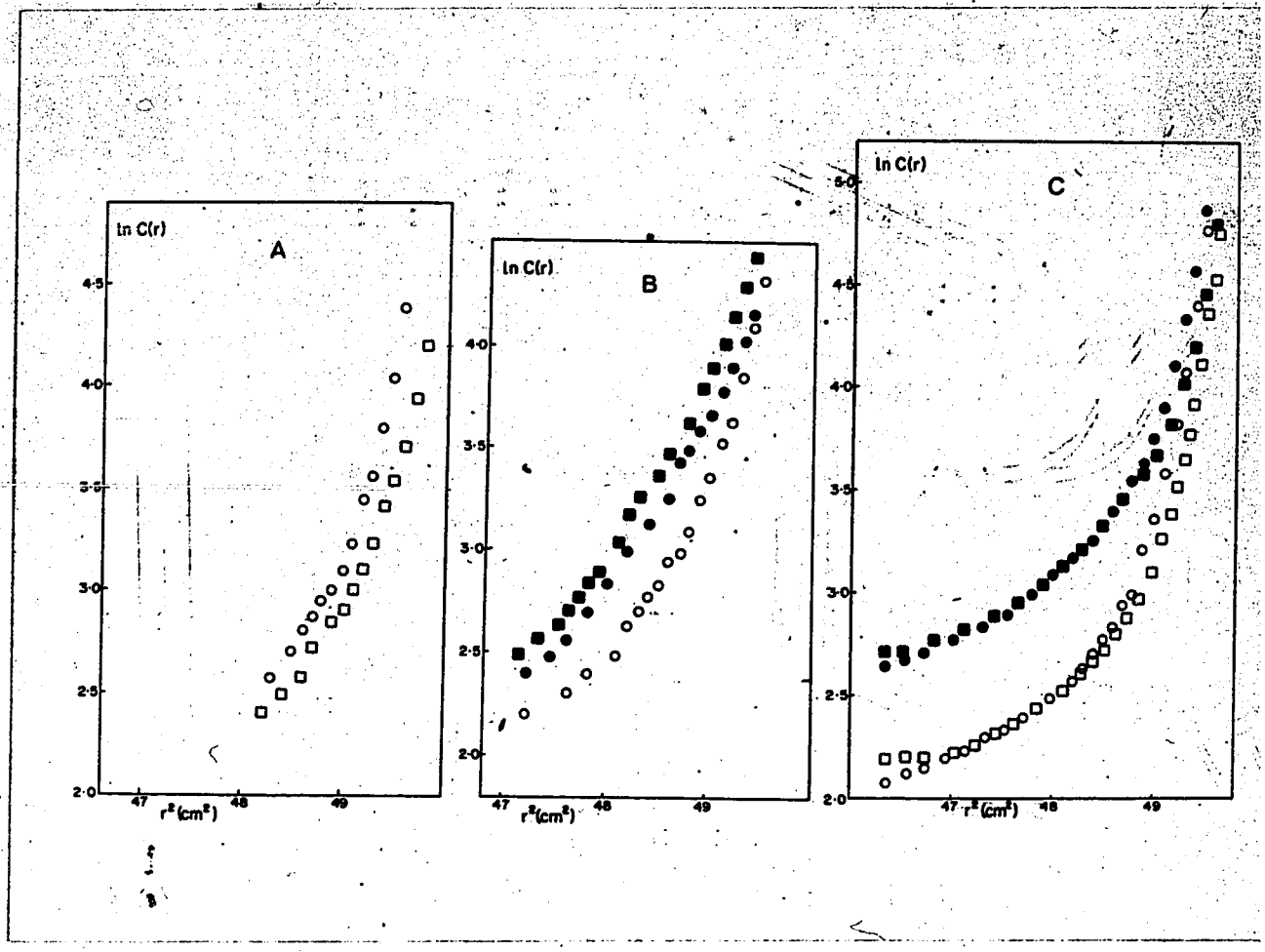


Fig. 13

A. Comparative sedimentation equilibrium plots in 2 M NaCl, C_0 - .24 O.D. chick hemoglobin, ○ and duck hemoglobin □ , rotor speed - 36,000; buffer 0.1 M tris-HCl, pH 8.6.

B. Comparative sedimentation equilibrium plots of chick hemoglobin, ○ , C_0 - .24 O.D.; human fetal hemoglobin, ● , C_0 - .22 O.D.; and equine hemoglobin, ■ , C_0 - .26 O.D.; in 4 M NaCl. Rotor speed - 36,000 rpm; buffer - 0.1 M tris-HCl, pH 8.6.

C. Comparative sedimentation equilibrium plots of chick, ○ , C_0 - .23; duck, □ , C_0 - .29; equine, ■ , C_0 - .26; and human fetal, ● , C_0 - .24; hemoglobins in 8.0 M urea:0.1 M tris-HCl, pH 8.6. The rotor speed is 40,000 rpm.

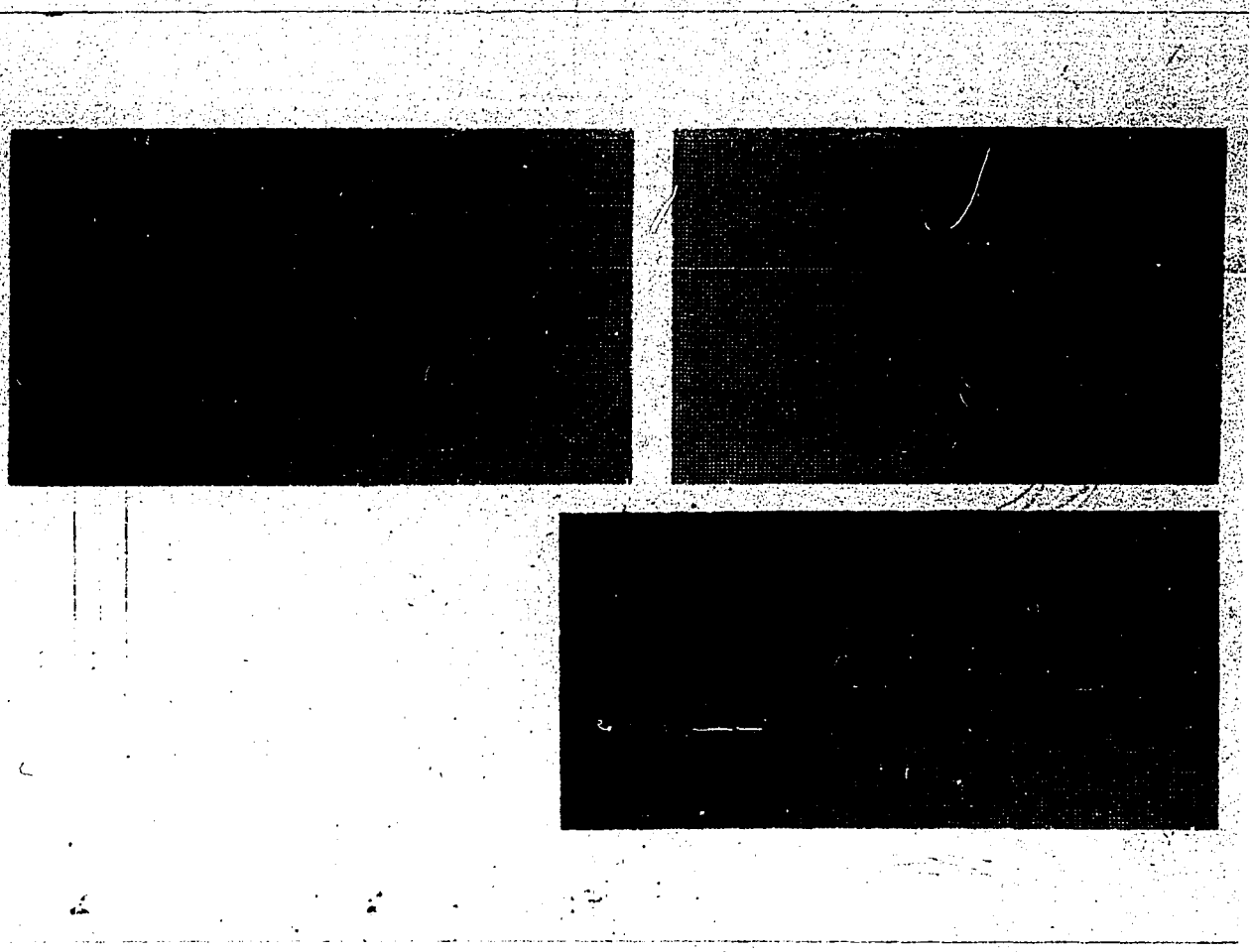


Fig. 14 Scanner equilibrium plots for (a) equine, (b) duck and (c) human fetal hemoglobins in 4 M NaCl. The experimental parameters were the same as in 13 B. The scan was taken 6 hours after the start of the run.

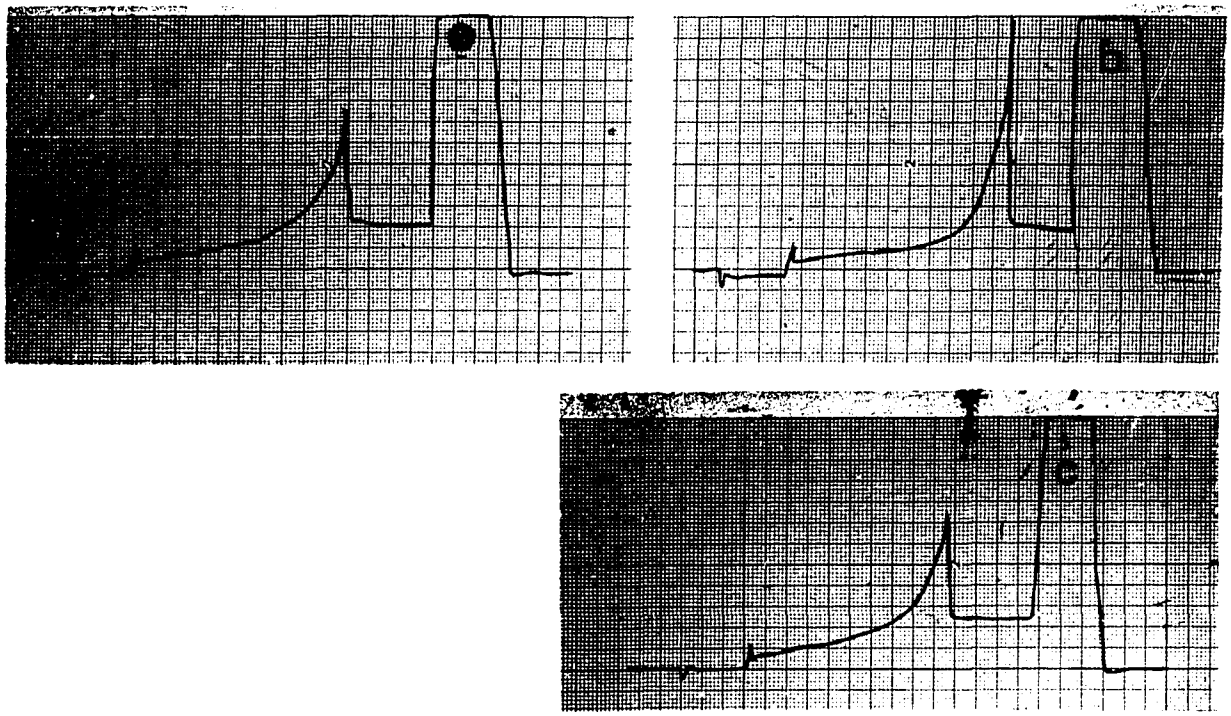


Fig. 14 Scanner equilibrium plots for (a) equine, (b) duck and (c) human fetal hemoglobins in 4 M NaCl. The experimental parameters were the same as in 13 B. The scan was taken 6 hours after the start of the run.

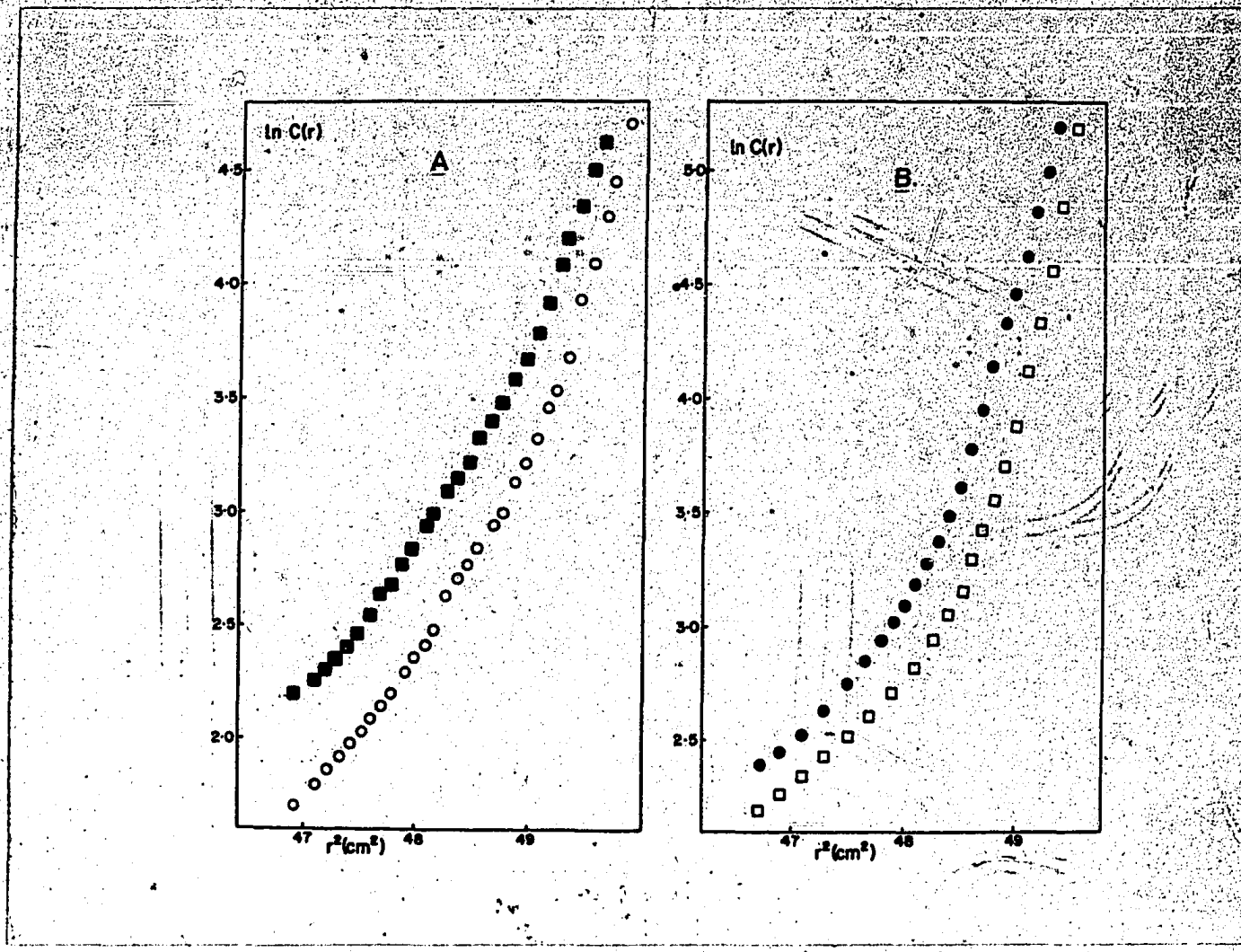


Fig. 15

A. Comparative sedimentation equilibrium plots: chick hemoglobin, ○ , C_0 - .27 O.D.; and equine hemoglobin, ■ , C_0 - .29 O.D.; at pH 10.6. Rotor speed - 26,000 rpm.

B. Comparative sedimentation equilibrium plots of duck hemoglobin, □ , C_0 - .40 O.D.; and human adult hemoglobin, ● , C_0 - .40 O.D. at pH 11.6. Rotor speed -36,000 rpm.

Tyrosine ionization studies of duck hemoglobin, similar to that reported earlier with chick hemoglobin, showed that in 0.2 M KCl, 15 ± 0.5 of the total 17 tyrosine residues become available for ionization studies between pH 12.5 and 12.75. Five residues were ionized till pH 10.6, where duck hemoglobin dissociates from a tetrameric state to a dimer unit. Between pH 10.3 and 11.3, 8 tyrosine residues were ionized and the rest were available at higher alkaline pH. The behavior of tyrosine residues in duck hemoglobin follows closely to that of chick hemoglobin.

Discussion

A better understanding of the problems involving the monomer association of hemoglobins has been greatly facilitated by the pioneering contributions of Kendrew (135) and Perutz (40-45) and their collaborators. The three dimensional atomic models of myoglobin and hemoglobins have displayed the proximal contacts between the monomers of two types (α and β). Since the polypeptide segments of these subunits are located within the polar and non-polar contacts between α and β residues, the dissociation of the $(\alpha\beta)_2$ tetramer could be achieved by both ionic and non-ionic type solutes. A study of the atomic model of horse and human hemoglobins projected to human hemoglobin mutants (131) indicated that the replacements of most of the amino acid residues on the surface of hemoglobin molecule is rather innocous but the protein molecule is extremely sensitive to mutation to cause alterations of the internal non-polar contacts especially those near the hemes. These amino acid mutations involving the polar and non-polar character of the side chain

near the heme groups affect the biological function of the hemoglobin molecules.

Avian hemoglobins offer another excellent model for the study of molecular mechanisms of monomer association since these molecules have more polar and non-polar groups than human hemoglobins, and also the interior milieu of chick erythrocytes is different from that of the human system. The avian hemoglobins, for example, are generally alkali-resistant and the body temperature of the birds is approximately 40°. The electrolyte concentration is also higher in the avian blood system. Comparative study on depolymerization employing solvent perturbants of the ionic and non-ionic type revealed that the mammalian hemoglobins like Hb A, Hb F, equine Hb and bovine Hb dissociate to a greater degree under identical conditions than the avian hemoglobins (chick and duck). It may be surmised that for a particular disruptive field induced in a solvent perturbation medium, the inter-monomer association is stronger with the chick and with the duck hemoglobins as compared to these mammalian hemoglobins. Addition of electrolytes to an aqueous medium produced ion-solvent interaction, contributed to by both cations and anions. The electrolytes, by virtue of their strong electrostatic fields, induce a strong orienting effect on neighbouring water molecules. In all probability the interaction of increasing number of ions with the side chain prototropic groups available on the surface of the protein molecule cause an electrostatic induction of polarizability which causes the relative dislocation of the subunits and ultimately dissociation. Studies on the dielectric properties of hydrated hemoglobin molecules indicate that the structure of water molecules bound to hemoglobin is intermediate, like those

of ice and normal water (137). In a 20% hemoglobin solution there are approximately 1075 water molecules oriented to a tetramer hemoglobin molecule (137).

The non-ionic solutes used during the present investigation to produce dissociation of chick hemoglobin were amide derivatives (formamide, urea and guanidine) and alcohol derivatives (2-chloroethanol). Addition of urea to water produced urea-water clusters (102, 138). These clusters provide the interstitial spaces in the bulk solution which accommodate the hydrocarbon side-chain. Thus, urea solubilized amide and peptide, and the hydrophobic groups (139, 140). The dislocation of the subunits from position and eventually the dissociation could thus be produced by these non-ionic solutes by acting on the contact sites and essentially changing the water structure surrounding the strategic groups. The mutual displacement of the polypeptide chains (dimer or monomer) may be a result of the solubilizing action of the non-ionic solutes on the available hydrophobic groups. The dimer and the monomers of chick hemoglobin were stable in the aqueous system containing both ionic and non-ionic solutes. When these subunits were recombined by restricted dialysis of solutes the recombined subunits showed oxygen equilibrium characteristics comparable to the native tetramer (167). Although the mode of disruption by non-ionic solutes is quite distinct from that of ionic solutes, the recombined molecules did not reveal any apparent loss of oxygen equilibrium characteristics.

During the solvent:solute interaction studies a broad relationship between the experimental results obtained with the two techniques of analytical ultracentrifugation could be recognized. Dilution had no appreciable effect on

the dissociation of tetramer in presence of solutes. However, high alkaline pH produced more dissociation at low protein concentration, such as those used in sedimentation equilibrium analyses. To be precise, the alkaline dissociation study should be considered as a charge-interaction phenomenon. The subunits acquire varying degrees of charges resulting from the ionization of various side chain prototropic groups and subsequently producing changes in surface-charge density; and at the same time they are accommodated within the charged solvent matrix. Avian (chick and duck) hemoglobins at high alkaline pH's produced less monomers than the mammalian hemoglobins implying a stronger α-β association in chick and duck hemoglobin.

CHAPTER IV

DISSOCIATION OF BOVINE LIVER CATALASE

INTRODUCTION

During the last decade, studies on the amino acid sequence of proteins especially enzymes in conjunction with the x-ray diffraction studies have provided us with a better perspective of the active sites and also the molecular conformation detailing the side chain distribution of the constituent amino acid residues (83, 84). The cooperative interaction that endows the protein molecules with the property for enzyme action is varied in nature: for example, the cooperation between two histidine residues (res. 119 and 20) located at extreme ends of ribonuclease (141) on one hand, and the allosteric interaction between two subunits of aspartic transcarbamylase, where individual subunits are inactive but collectively they manifest the enzyme action (142). In this regard molecules like hemoglobins where a single unit, a monomer of hemoglobin, comparable to myoglobin is biologically reactive. Although in these cases, the active center site is mainly involved with the enzymic activity, the other segments of the protein molecule are also essential in providing the active center with the particular attribute. The cooperative interaction of S-peptide (residue 1-20) with ribonuclease S (residue 21-124) is essential for the ribonuclease activity (143). Some of the enzymes are quite resistant to the effect of structure-disruptive ions and some of them revealed marked resiliency. The enzymes which are polymer proteins display wide variability as to the degree of recombination between the neighbouring subunits and restoration of the original enzymic activity vary to a great extent. Hemoglobins when dissociated from tetrameric state to dimer and subsequently

to monomer by reagents like urea or NaCl could regain the oxygen combining capacity on removal of structure-disruptive ions. This section describes the effect of various structure-disruptive ions on the polymeric status of a heme-oriented polymer protein, catalase, and also on the enzymic properties. The structural integrity of the polymer under solvent:solute interactions has been evaluated by analytical ultracentrifugation. Since the heme group is the active center, spectrophotometric studies were carried out to evaluate the effect of the different solvent perturbants on the Soret band intensity. Tyrosine ionization was studied to assess the degree of the availability of tyrosine residues in solvent perturbation systems. Attempts were made to evaluate localized conformational changes using fluorescence spectrophotometry.

EXPERIMENTAL RESULTS

The sedimentation velocity coefficient value of chromatographically pure bovine liver catalase at infinite dilution ($S^{o}_{20,w}$) was found to be 11.6 S which is in correspondence with the values reported by Sumner and Gralén (62). $S^{o}_{20,w}$ value of the apo-catalase was found to be 4.0 S in 0.1 M glycine-HCl buffer, pH 3.5 (Fig. 16.). Apo-catalase was found to be unstable between pH 4.5 - pH 7.0. The dependence of S values on the concentration of apo-catalase was greater than that observed with catalase. The value of $D_{20,w}$ in 0.1 M tris-HCl buffer, pH 8.6:0.1 M NaCl was 2.3×10^{-7} (Fick unit) which is in good agreement with the values reported by Sumner and Gralén (62) and by Samejima and Shibata (72).

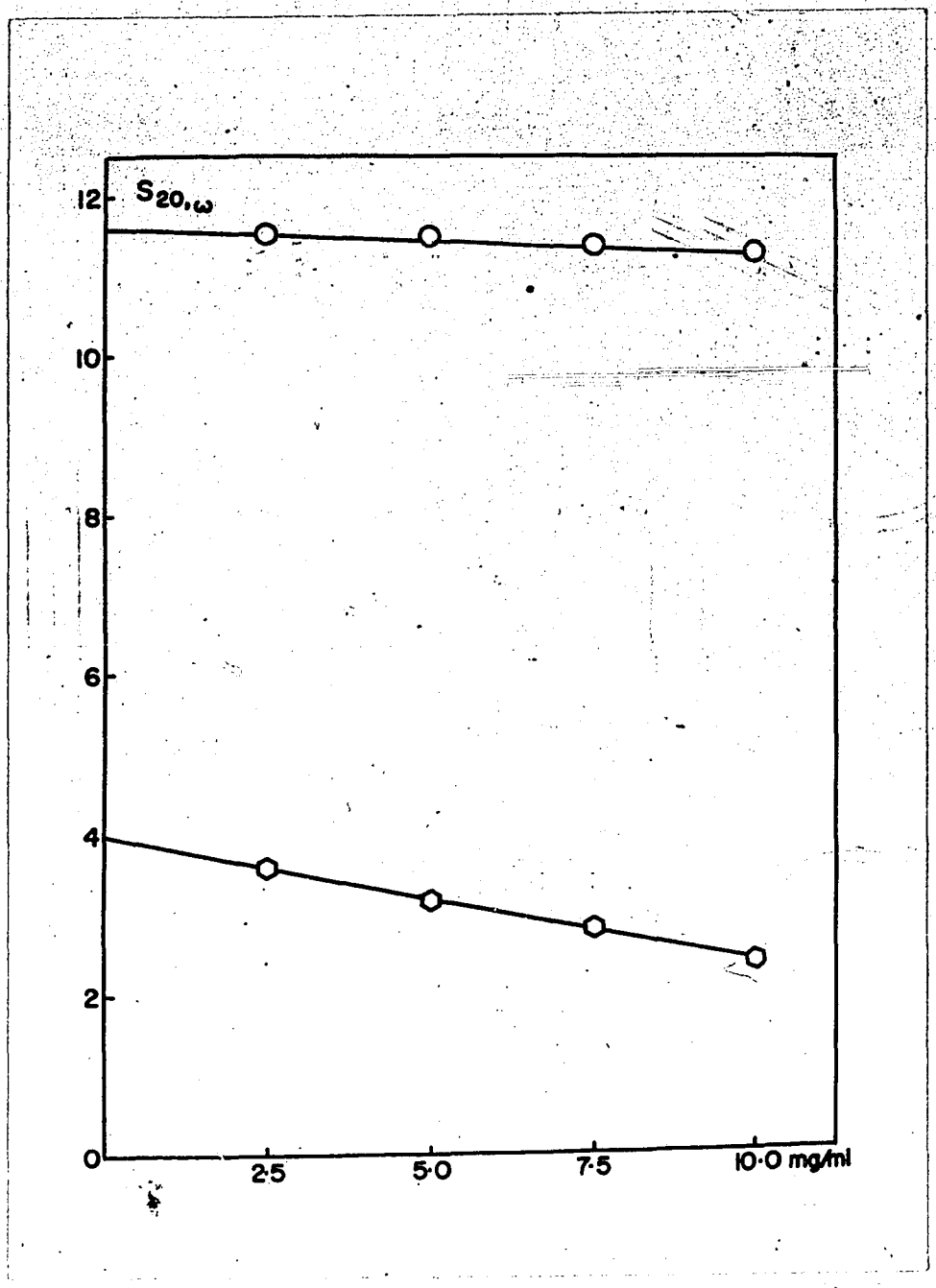


Fig. 16 The concentration dependent S values of bovine liver catalase in 0.1 M tris-HCl buffer, pH 8.6, ○—○; and of apo-catalase in 0.1 M glycine-HCl buffer, pH 3.5, ⬡—⬡.

Fig. 17 (A, B, C, and D) depicts the sedimentation behavior of bovine liver catalase in presence of various concentrations of ionic solutes (NaCl, KCl, LiCl and $CaCl_2$). If assumptions were made that no extensive conformational change occurred during dissociation, no appreciable increase in preferential ion-binding and hydration of the subunits took place, and the S value is proportional to the molecular weight, $(M)^{0.66}$ (92), the S values of half molecule and quarter molecule of bovine liver catalase would be 7.2 S and 4.6 S, respectively. A value of 4.6 S corresponds approximately to the S value obtained in the case of hemoglobins of mol. wt. 64,500. The reduced S value implies a diminution in the size of the molecule. However, in LiCl solution the S value had decreased but it did not reach the limiting S value as was obtained with NaCl and KCl. A single symmetrical peak was observed in the Schlieren plot with NaCl, KCl and LiCl; whereas in contrast, $CaCl_2$ system produced two peaks in the Schlieren plot as illustrated in Fig. 18. $CaCl_2$ was found to be more effective in producing depolymerization. Table IV presents the percent distribution of the fast and slow moving components in presence of varying concentrations of $CaCl_2$. Bovine liver catalase precipitated at a concentration higher than 1.25 M $CaCl_2$. A steady decrease in the S value of the fast-moving component was noted with $CaCl_2$ of concentration 0.75 M and higher.

Depolymerization of bovine liver catalase was also studied with varying molarities of non-ionic solutes (formamide, urea and 2-chloroethanol) (Fig. 19, A, B, & C). The non-ionic solutes produced two symmetrical peaks as observed in the Schlieren plots (Fig. 20a-c). The fast moving component exhibited a value of 9 S or higher whereas the slow sedimenting component

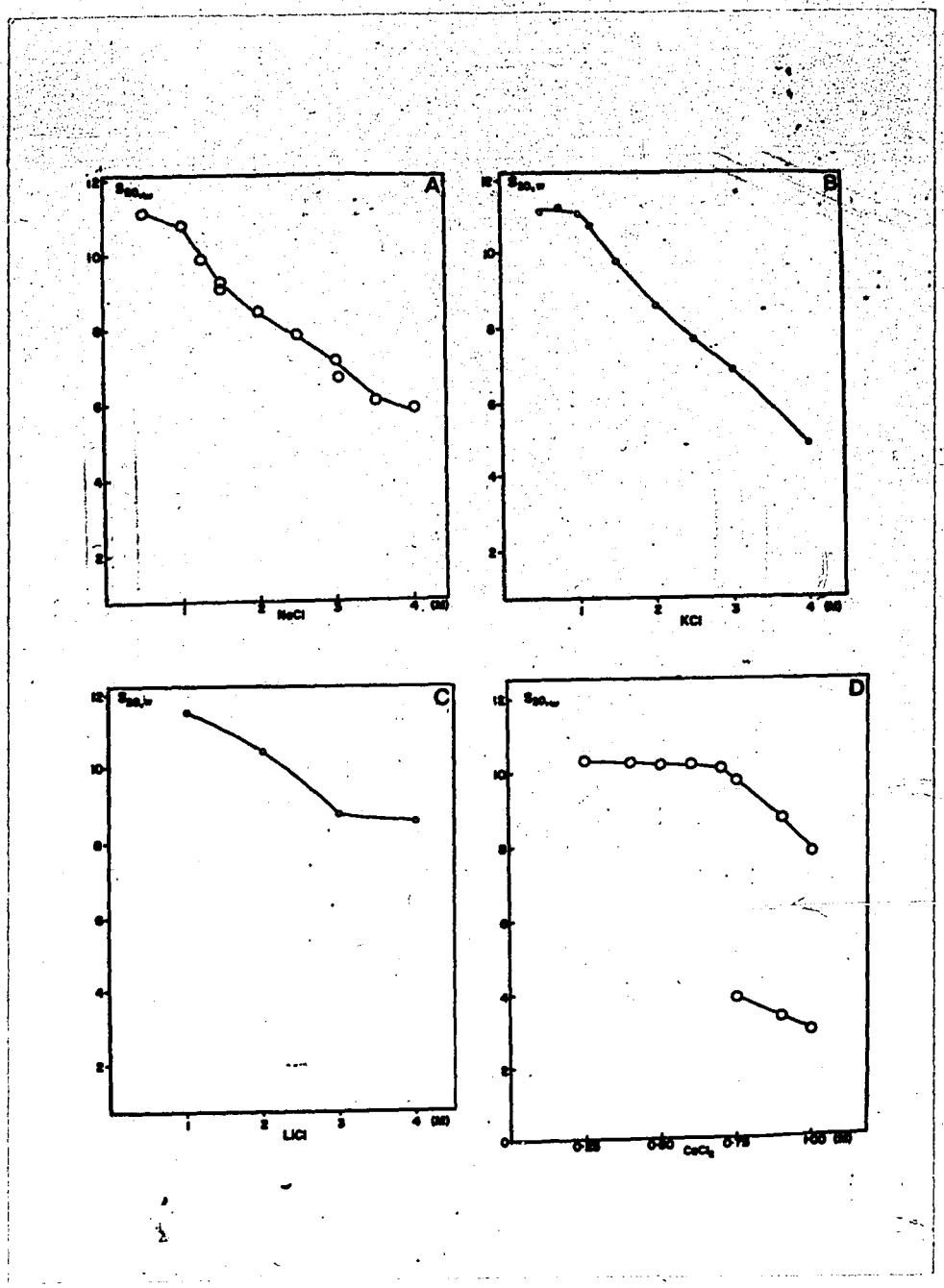


Fig. 17 The ultracentrifugal behavior of bovine liver catalase in ionic solutes. Protein concentrations - 4 mg/ml; buffer - 0.1 M tris-HCl, pH 7.0; temp. - 20°; rotor speed - 56,000 rpm.

A. S value in NaCl
B. S value in KCl
C. S value in LiCl
D. S value in $CaCl_2$

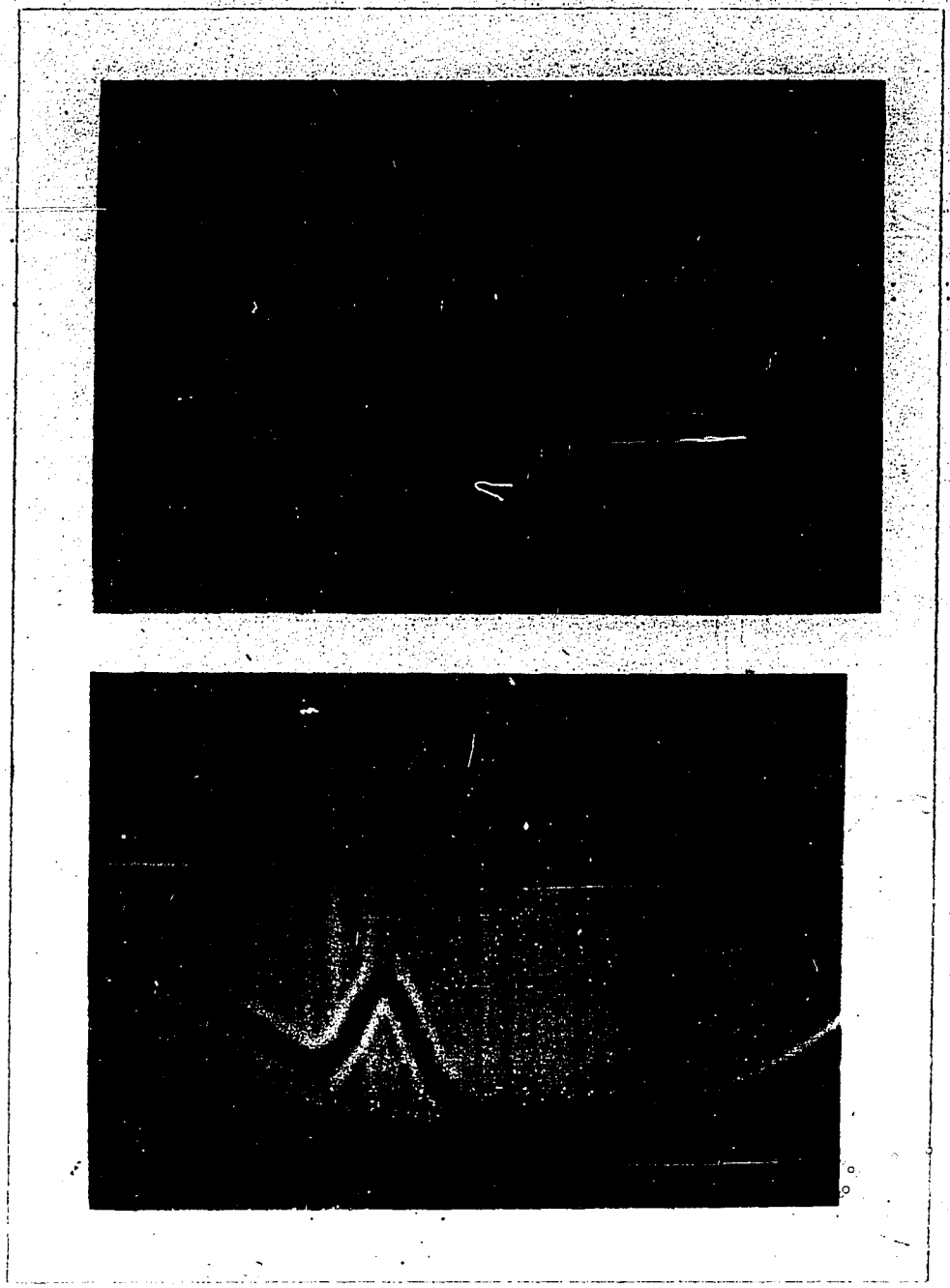


Fig. 18 Schlieren plots of bovine liver catalase in a) 2 % NaCl (lower plot) and 3 M NaCl (upper plot) and b) in 0.9 % $CaCl_2$. The protein concentration for the latter case was 14 mg/ml.

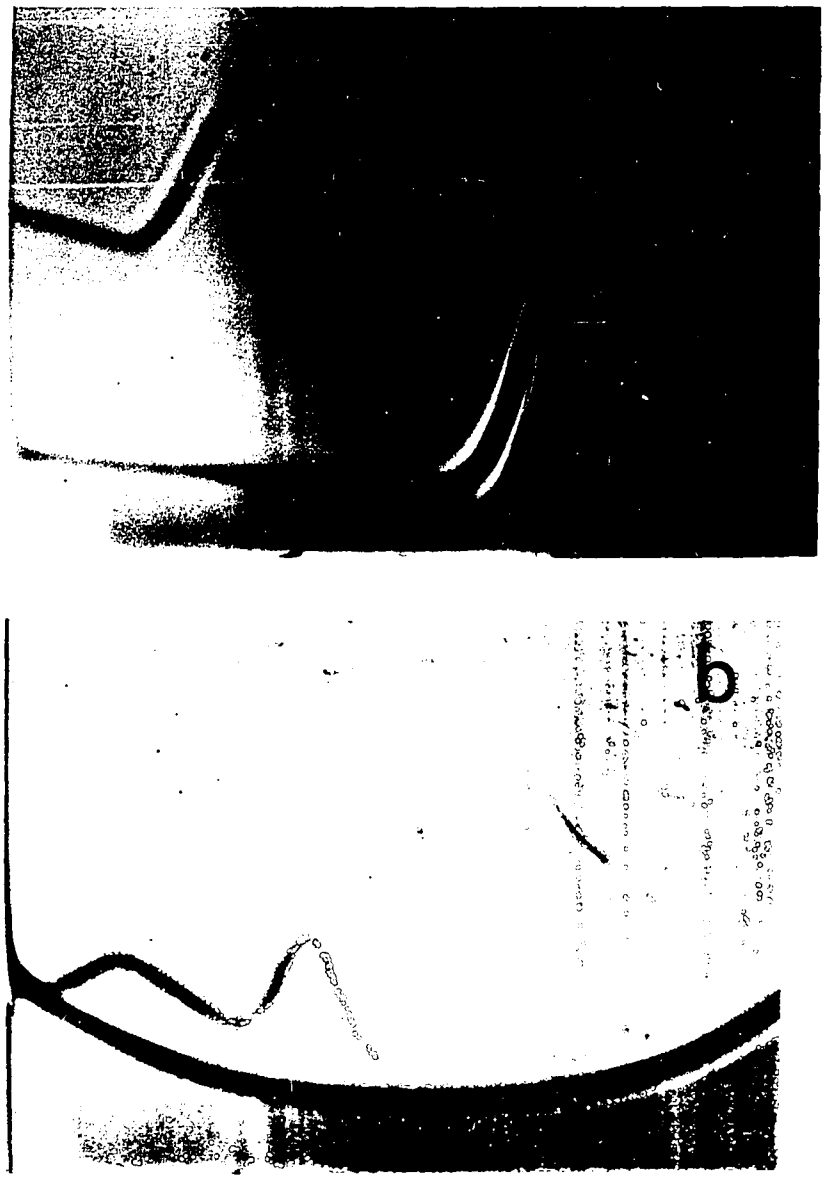


Fig. 18 Schlieren plots of bovine liver catalase in a) 2 M NaCl (lower plot) and 3 M NaCl (upper plot) and b) in 0.9 M $CaCl_2$. The protein concentration for the latter case was 14 mg/ml.

TABLE IV

Dissociation of Bovine Liver Catalase in $CaCl_2$

Molarity*	$S_{20,w}$		% Composition	
	Fast Comp	Slow Comp	Fast Comp	Slow Comp
0.25	10.30	-	100	-
0.40	10.25	-	100	-
0.50	10.20	-	100	traces
0.60	10.20	-	100	traces
0.70	10.10	-	100	traces
0.75	9.75	3.86	83	17
0.90	8.71	3.27	70	30
1.00	7.81	2.91	37	63

*contained 0.1 M tris-HCl buffer, pH 7.0.

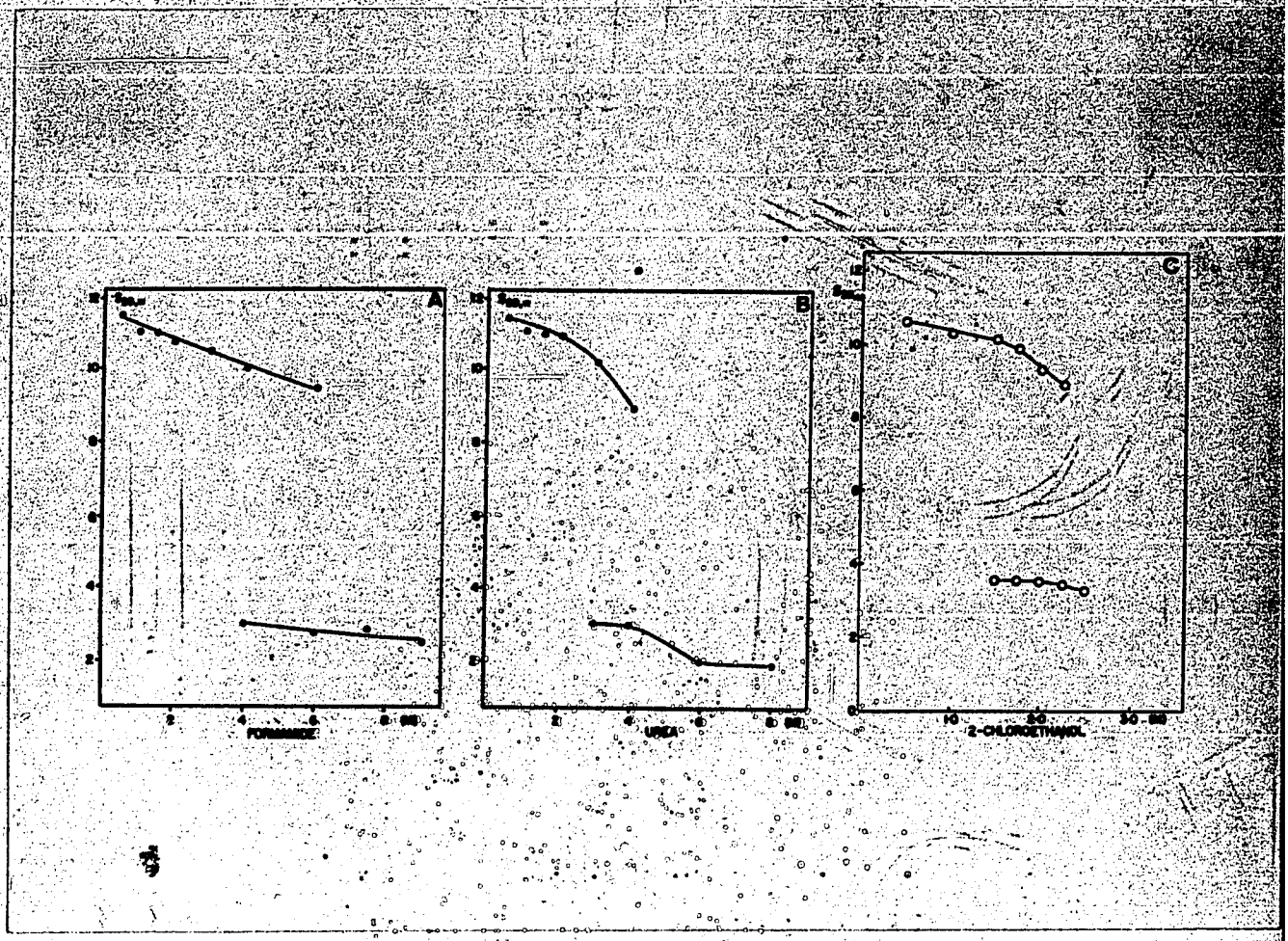


Fig. 19 The ultracentrifugal behavior of bovine liver catalase in non-ionic solutes. Protein concentration - 4 mg/ml; buffer - 0.1 M tris-HCl, pH 8.6; temp. 20°; rotor speed - 56,000 rpm.

A. S value in formamide
B. S value in urea
C. S value in 2-chloroethanol

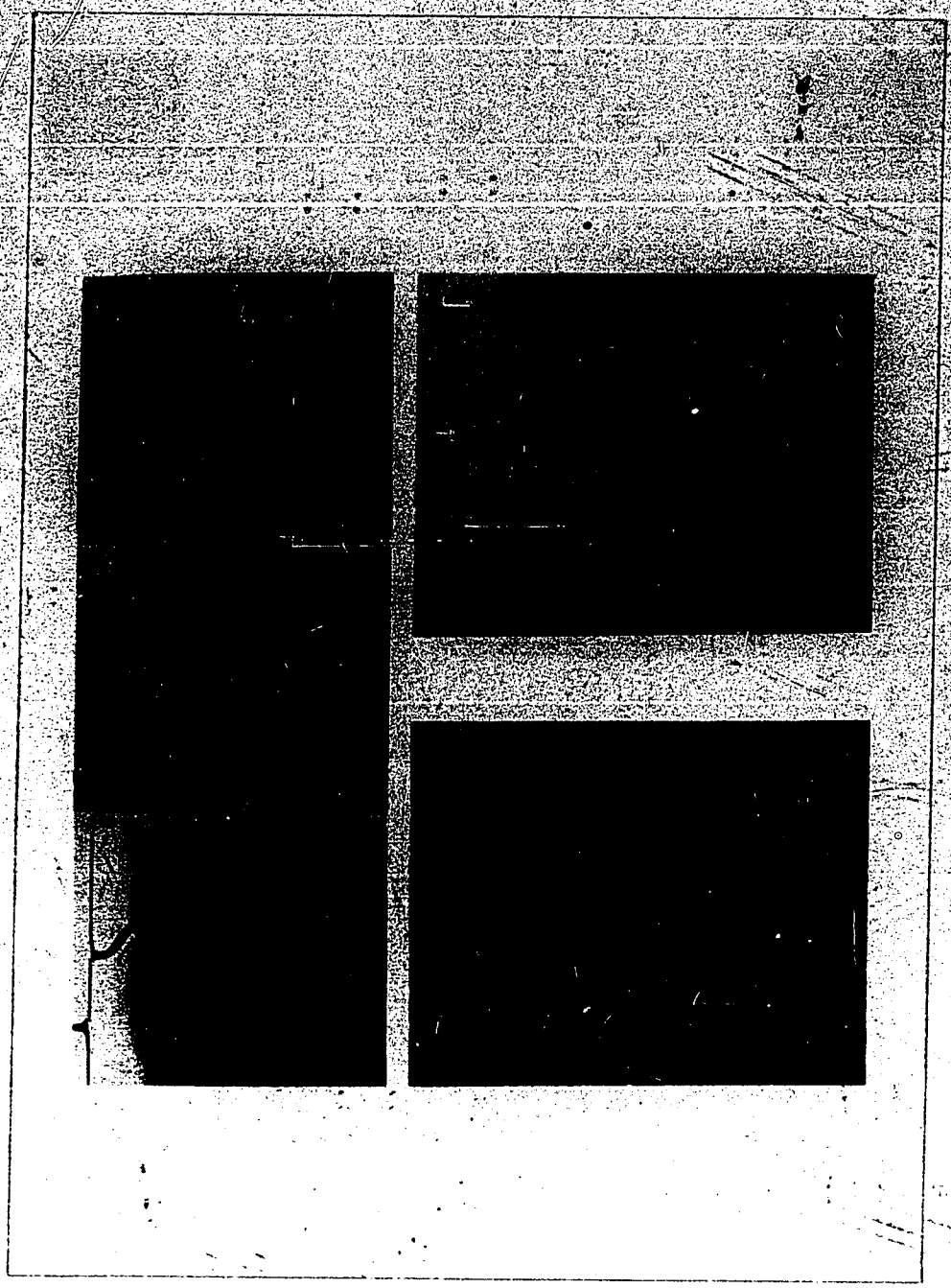


Fig. 20 Schlieren plot of bovine liver catalase in (a) formamide, (b) urea, (c) 2-chloroethanol, (d) pH 10.3, and (e) a mixture of bovine liver catalase:pooled normal human immunoglobulin G and bovine serum albumin.

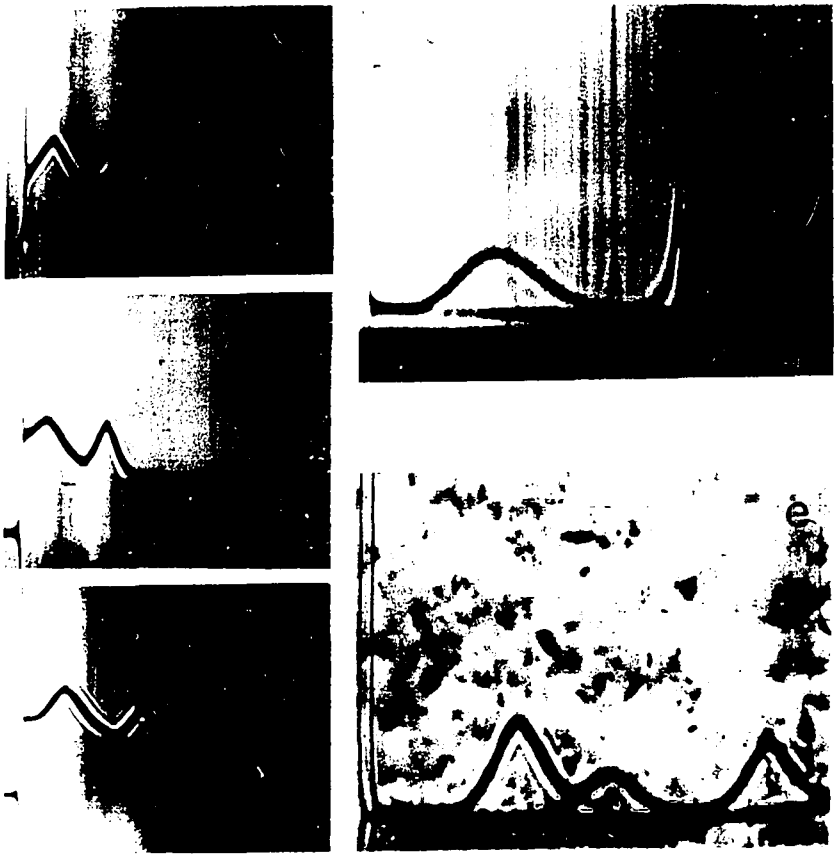


Fig. 20 Schlieren plot of bovine liver catalase in (a) formamide, (b) urea, (c) 2-chloroethanol, (d) pH 10.3, and (e) a mixture of bovine liver catalase:pooled normal human immunoglobulin G and bovine serum albumin.

showed S values between 3.0 and 3.5. The fast moving component showed a decrease in S values, a sharper decrease being noted in urea. Similar was the case with the slow moving component. Tables 5, 6, 7 show that during the dissociation of bovine liver catalase in presence of non-ionic solutes (formamide, urea and 2-chloroethanol), the relative proportion of slow component increased with the increase in solute concentration. The relative distribution of the polymer and the subunits as produced by the different perturbants was obtained by planimetric determination of the area under each Schlieren peak. The refractive index gradient with respect to concentration of protein does not follow precisely the relative change in area under the refractive index gradient curve. However, the stepwise variation in perturbant concentration did not produce an excessive aberration in the experimental data as observed in Tables 4-7, where the relative increment in the concentration of the perturbants was small.

Alkaline dissociation of bovine liver catalase starts at pH 9.3 and thereon the S values reflect the increasing production of quarter size molecule (Fig. 21). A progressive diminution in the S value of the dissociation subunit was noticed, the range being 4.96 to 3.12 S whereas the range of the fast moving component was 11.0 - 11.2 S. Two Schlieren peaks were observed during the alkaline dissociation as illustrated in Fig. 20 D and with the increase of the pH the monomers were formed at an increased rate (Table 8). A distinct difference in the dissociation was observed with catalase as compared to that of chick hemoglobin, for example, the dissociation of chick hemoglobin occurs between pH 10.3 and pH 11.6, whereas the dissociation of catalase ranges between pH 9.3 and pH 10.8.

TABLE V

Dissociation of Bovine Liver Catalase in Formamide

Molarity*	$S_{20,w}$		% Composition	
	Fast Comp	Slow Comp	Fast Comp	Slow Comp
3.36	9.68	3.21	82	18
3.84	9.56	3.00	78	22
4.32	9.61	3.14	78	22
4.80	9.60	3.09	48	52
5.28	9.87	3.17	48	52
5.76	9.18	3.07	40	60
6.24	9.02	3.01	24	76

* contained 0.1 M tris-HCl buffer, pH 8.6.

TABLE LVI

Dissociation of Bovine Liver Catalase in Urea

Molarity*	$S_{20,w}$		% Composition	
	Fast Comp	Slow Comp	Fast Comp	Slow Comp
2.88	10.03	3.06	85	15
3.36	9.87	3.03	81	19
3.84	9.84	3.11	60	40
4.32	9.36	3.01	36	64
4.80	9.05	3.21	16	84
5.28	-	3.39	-	100

* contained 0.1 M tris-HCl buffer, pH 8.6.

TABLE VII

Dissociation of Bovine Liver Catalase in 2-Chloroethanol

Molarity*	$S_{20,w}$		% Composition	
	Fast Comp	Slow Comp	Fast Comp	Slow Comp
0.50	10.61	-	100	-
1.00	10.34	-	100	-
1.50	10.11	3.58	73	27
1.75	9.89	3.56	35	65
2.00	9.32	3.54	28	72
2.25	8.87	3.43	12	88
2.50	-	3.29	-	100

* contained 0.1 M tris-HCl buffer, pH 8.6.

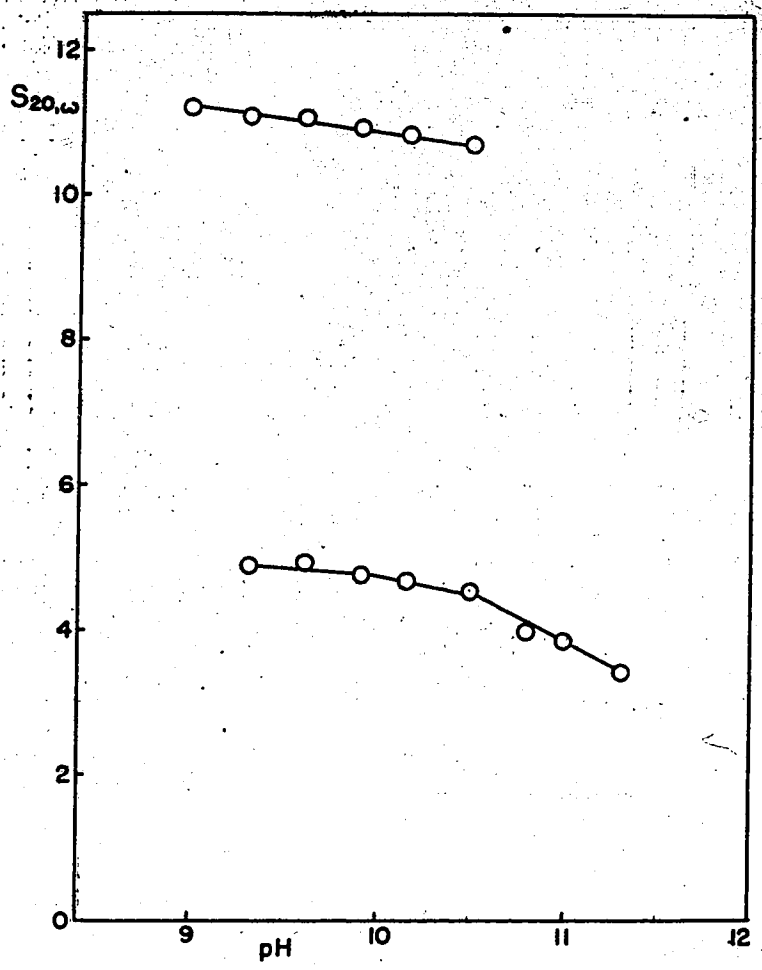


Fig. 21 The S value of bovine liver catalase in alkaline pH. The experimental parameters were: protein concentration - 4 mg/ml; buffer - phosphate-KCl; μ - 0.25; time of dialysis - 15 hrs; temperature - 20°; rotor speed - 56,000 rpm.

TABLE VII

Alkaline Dissociation of Bovine Liver Catalase

pH*	$S_{20,w}$		% Composition	
	Fast Comp	Slow Comp	Fast Comp	Slow Comp
9.0	11.21	-	100	-
9.3	11.08	-	100	traces
9.6	11.08	4.96	81	19
9.9	10.92	4.78	79	21
10.15	10.89	4.41	67	33
10.5	10.98	4.54	14	86
10.8	-	3.93	-	100
11.0	-	3.82	-	100
11.3	-	3.12	-	100

* PO_4-KCl buffer $\mu = 0.25$

The Johnston-Ogston effect (144) was studied by using a non-interacting system employing varying proportions of bovine liver catalase with bovine serum albumin (mol. wt. 70,000) (Fig. 22) and human immunoglobulin G (mol. wt. 150,000). The S value of native bovine liver catalase was not appreciably influenced by the presence of the non-interacting proteins, although their molecular size closely parallel those of half molecule and quarter molecule size subunits of catalase. Fig. 20 E. depicts the Schlieren pattern obtained for a mixture of catalase:immunoglobulin G: albumin in 0.1 M tris-HCl, pH 8.6. Three peaks were obtained for the non-interacting system, in contrast to the two peaks obtained with the interacting system involving the subunits of catalase molecule. The reduced S values as observed may be affected to a certain extent by the conformational changes which occurred during the whole procedure of dissociation. The frictional ratio f/f_o, was obtained from the equation (120),

$$f/f_o = \left(\frac{(1-\bar{v}\rho)}{(D^o_{20,w})^2 \times S^o_{20,w} \times \bar{v}} \right)^{1/3}$$

where D is the diffusion coefficient, $\bar{v}$ the partial specific volume and ρ the density of the solution. The value of f/f_o in the case of polymeric bovine liver catalase was found to be 1.85, the partial specific volume, $\bar{v}$, being 0.715. $S^o_{20,w}$ value of apo-catalase was 4.0 S and the partial specific volume was 0.730. Using these values together with $D_{20,w}$ as 6.8 obtained with 6 M and 8 M urea, the frictional ratio f/f_o, was found to be 1.26. Using the S value of catalase quarter molecules as calculated theoretically (4.6 S) and as observed experimentally (3.5 S), the frictional ratio was

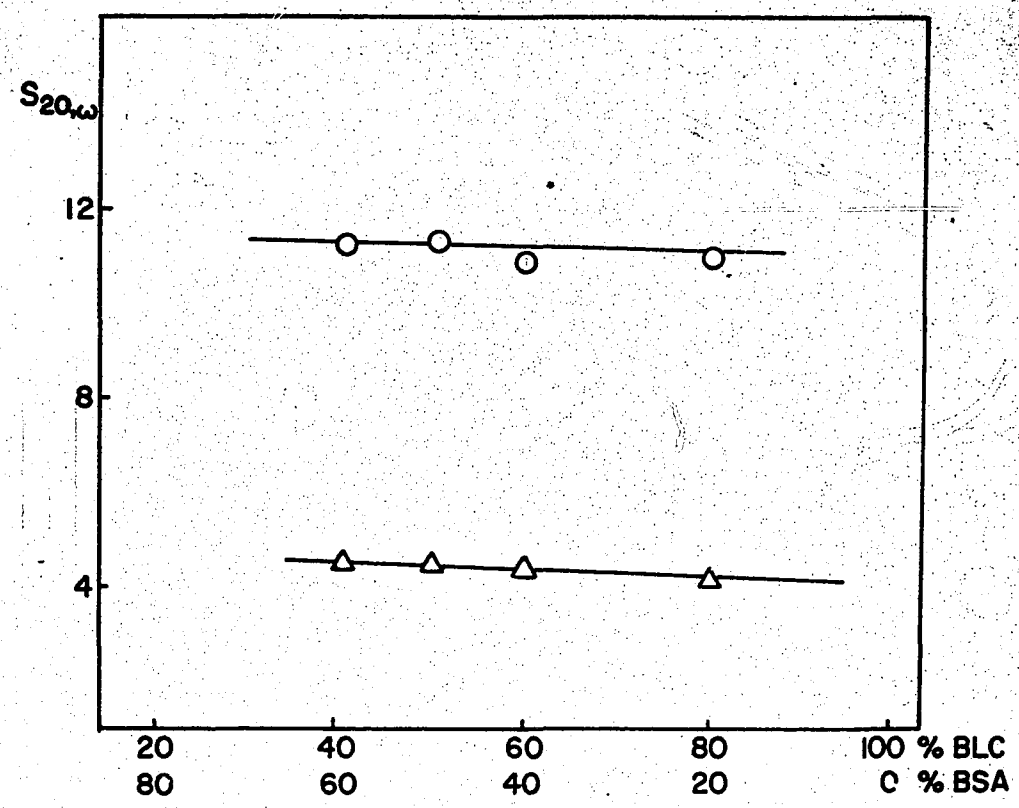


Fig. 22 Johnston-Ogston effect for varying ratio of bovine liver catalase, O—O: bovine serum albumin, Δ—Δ, in 0.1 M tris-HCl, pH 8.6; total protein concentration - 8 mg/ml; rotor speed - 56,000 rpm; temp. - 20°.

found to be 1.21 and 1.32 respectively. These values strongly suggest that conformational changes occur when tetrameric bovine liver catalase dissociates into quarter molecules.

Depolymerization of bovine liver catalase was investigated further by employing sedimentation equilibrium methods. Sedimentation equilibrium runs of native bovine liver catalase at a concentration of 0.4 mg/ml, showed that a considerable amount of protein was left throughout the upper part of the cell. A comparative run conducted with human immunoglobin G, which is a linked-chain polymer protein of apparent mol. wt. 150,000, did not show any residual protein in the same region of the cell (Fig.23A). The distribution of catalase at the base of the cell paralleled closely that of human immunoglobulin G. These experiments indicated that at this protein concentration, catalase molecule dissociated. With the operational rotor speed being identical, a decrease in the protein concentration results in an increase in monomer formation (Fig. 23B) (quarter molecule of catalase-assumed mol. wt. being 64,000). It was also observed that with the protein concentration being constant, higher rotor speeds favored the mass distribution of the heavier components (Fig. 23.C. ..).

In view of the fact that sedimentation equilibrium analysis revealed the presence of half molecule and quarter molecule of catalase, dissociation of catalase on dilution was studied employing sedimentation velocity methods with the help of the photoelectric scanner. At a range of protein concentration, $A_{280_{m\mu}}$ 1.8 - 0.1, and a rotor speed range of 56,000 - 40,000 rpm, varying one operational condition at a time, the cell meniscus clearance

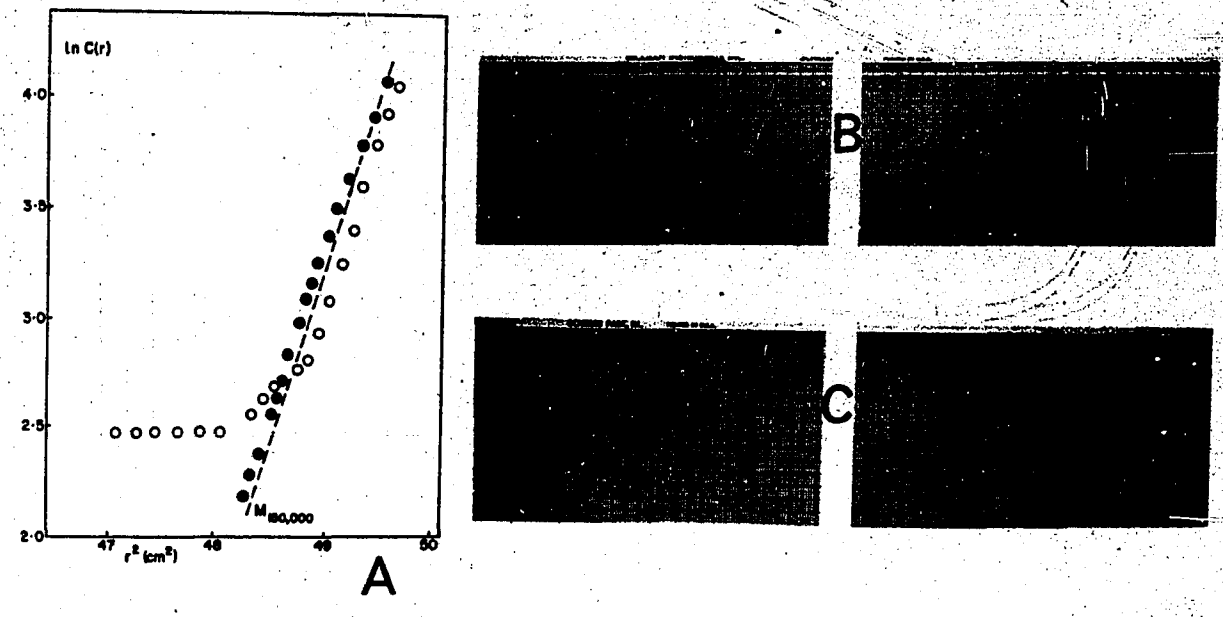


Fig. 23 A. Comparative sedimentation equilibrium plot of bovine liver catalase, ○ , C_o - .40 O.D., and human immunoglobulin G, ● , C_o - .42 O.D. in 0.1 M tris-HCl, pH 8.6; rotor speed - 14,000 rpm; temp. -20°. The theoretical distribution of human immunoglobulin G is shown by -------- $M_{150,000}$

B. Scanner equilibrium plots of bovine liver catalase at two concentrations: (a) C_o - .25 (b) C_o - .66. The rotor speed - 16,000 rpm; buffer - 0.1 M tris-HCl, pH 8.6. These scans were taken 5 hr. after start of the run.

C. Scanner equilibrium plots of bovine liver catalase, (C_o - .25 O.D. in 0.1 M tris-HCl, pH 8.6) at two rotor speeds (a) 12,000 rpm; (b) 20,000 rpm. These scans were taken 5 hrs. after start of run.

had an S value of approximately 10. Further decrease in protein concentration (below ~0.1 $A_{280\ m\mu}$) revealed the presence of monomers (3.5 S). Table IX shows the sedimentation behavior of bovine liver catalase at varying rotor speeds, and protein concentration.

Sedimentation equilibrium analyses were also carried out at three different temperatures, 15°, 20° and 25°, and at various low concentrations. It appears that the dissociation is greater at higher temperature and at lower concentration, while association is higher at lower temperature and higher concentration .

The sedimentation equilibrium plots of bovine liver catalase at different NaCl concentrations have been presented in Fig. 24 A . The protein concentration in all these comparative runs were maintained at 0.4 mg/ml. The comparative study showed that with the increase in the perturbant concentration, the extent of dissociation increased, or in other words, the extent of monomer formation increased. Figs.24B&C show the effect of other ionic solutes on the depolymerization of bovine liver catalase. The major species in 3.5 M KCl, is dimer unit with a small amount of monomer: on the other hand, in 1.0 M $CaCl_2$ it is mainly monomer. Similar sedimentation equilibrium plots of catalase in presence of non-ionic solutes were shown in Fig. 25 which demonstrates that mainly monomer species could be detected in 4 M urea, whereas sedimentation velocity analysis indicated the presence of 9.36 S and 3.01 S in approximately equal proportion. A similar result was obtained with 7.5 M formamide.

Solvent perturbation experiments were extended to study the chromophoric groups of catalase. The Soret band intensity is readily affected

TABLE IX

Sedimentation velocity characteristics of Bovine Liver Catalase at a low protein concentration and varying rotor speeds

Concentration* A_{280} mμ	Rotor speed	$S_{20,w}$
1.0	56,000	11.6
1.0	32,000	11.4
0.5	44,000	10.6
0.3	56,000	10.6
0.14	56,000	10.6
0.08	32,000	3.1
0.04	40,000	4.2
0.032	52,000	3.4

* contained 0.1 M tris-HCl buffer, pH 8.6.

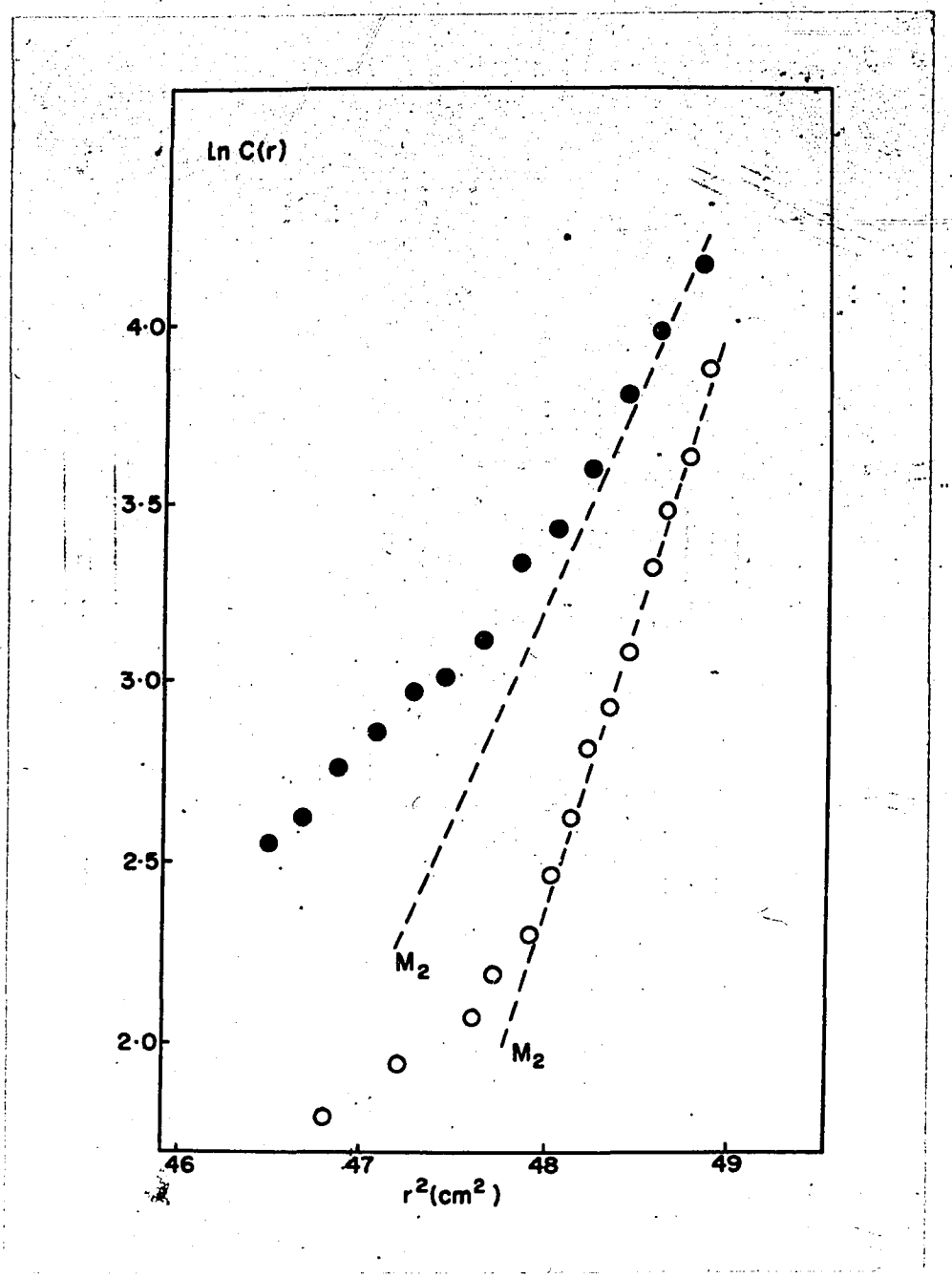


Fig. 24 Sedimentation equilibrium plot for bovine liver catalase in ionic solutes. The plots are presented as logarithm of concentration versus square of radial distance. The theoretical distribution of monomer is shown by, - - - - M_1 and dimer - - - - M_2.
The buffer is 0.1 M tris-HCl, pH 8.6, unless otherwise stated.
A. Bovine liver catalase in 2 M NaCl, ○ , C_o - .42; and 4 M NaCl, ● , C_o .43; rotor speed - 16,000 rpm.

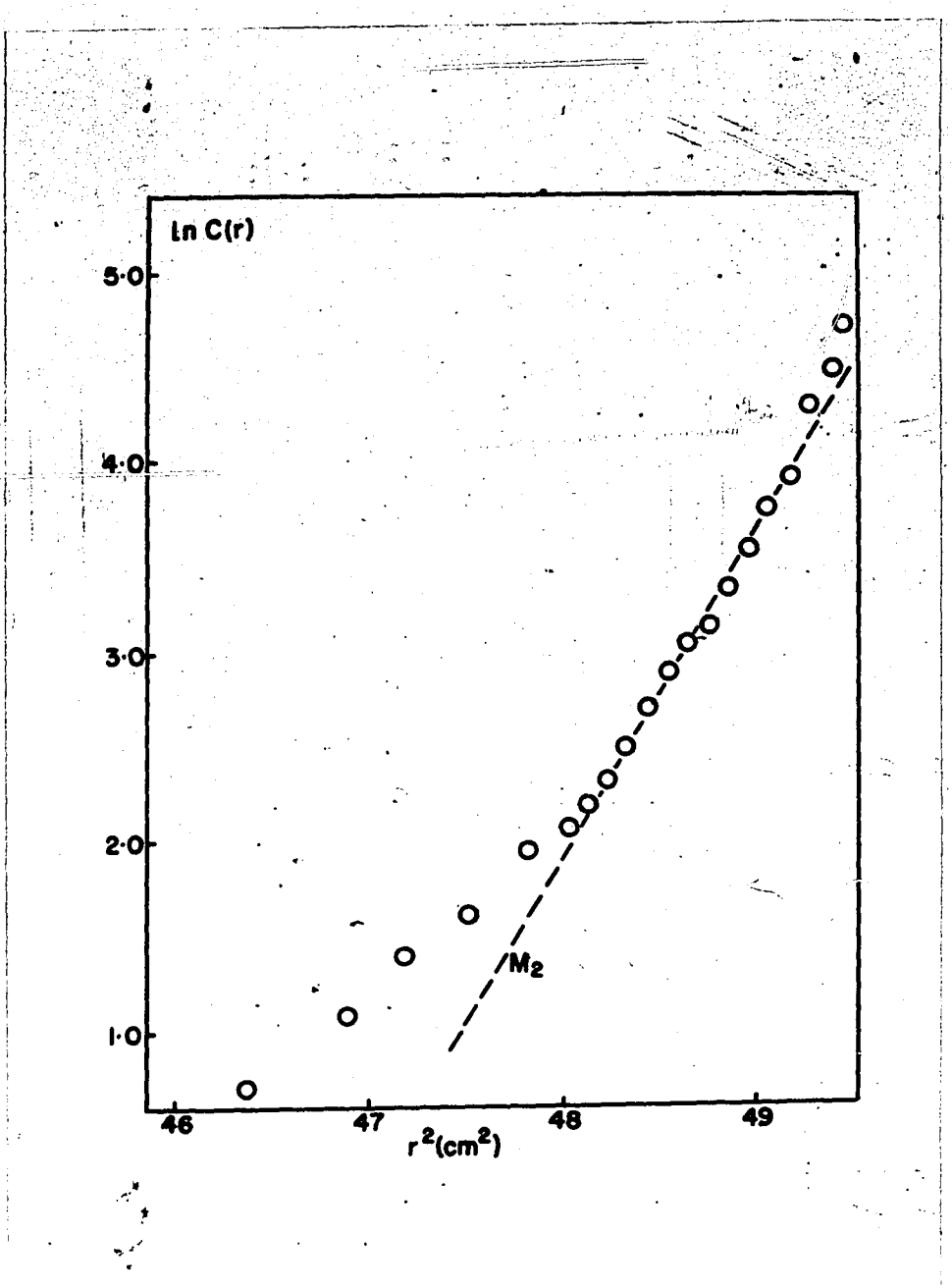


Fig. 24

B. Bovine liver catalase in 3.5 M KCl; C_o - .33; rotor speed - 20,000 rpm.

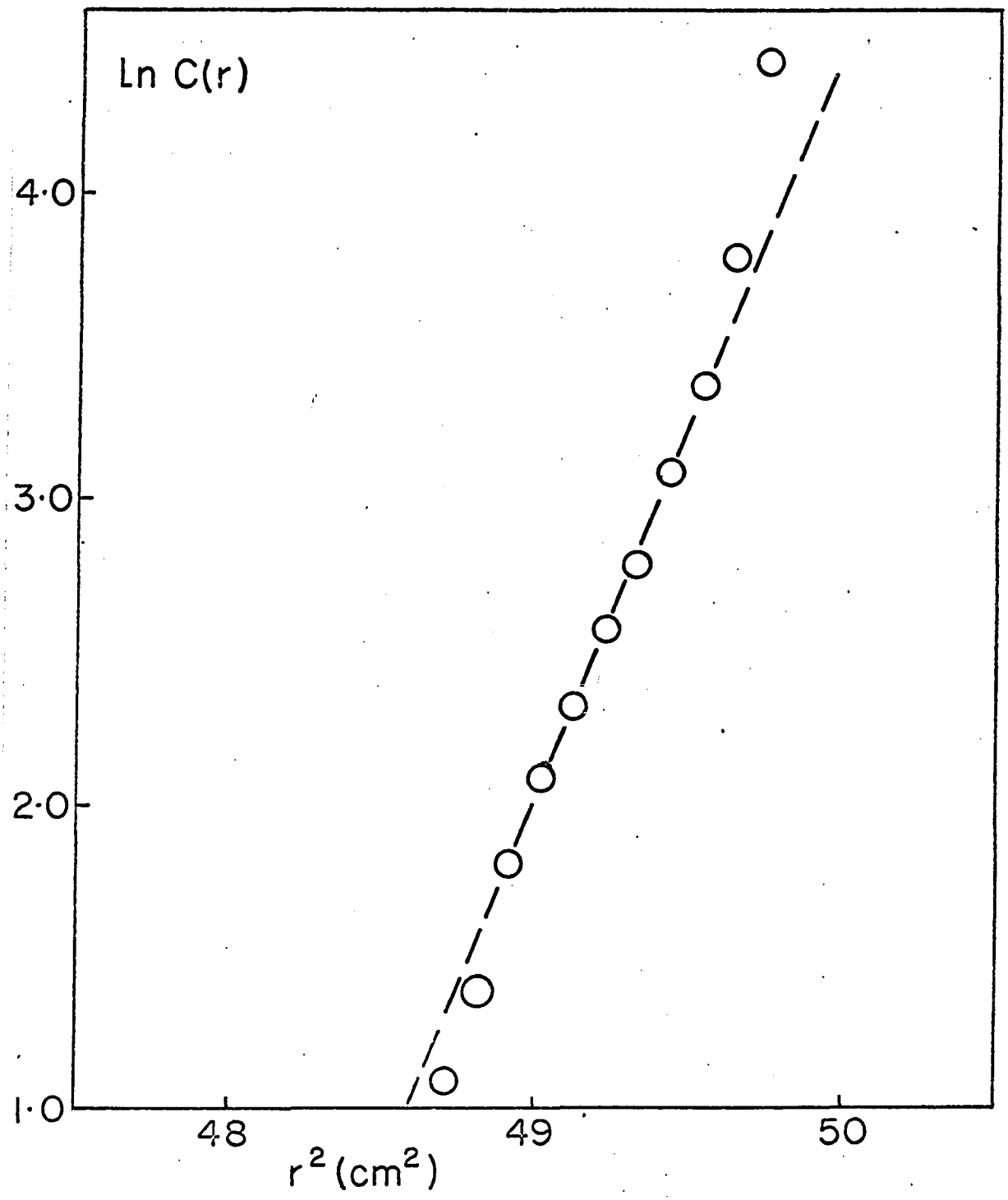


Fig. 24 C. Bovine liver catalase in $CaCl_2$: 0.1 M tris-HCl, pH 7.0; C_o - .23; rotor speed - 20,000 rpm.

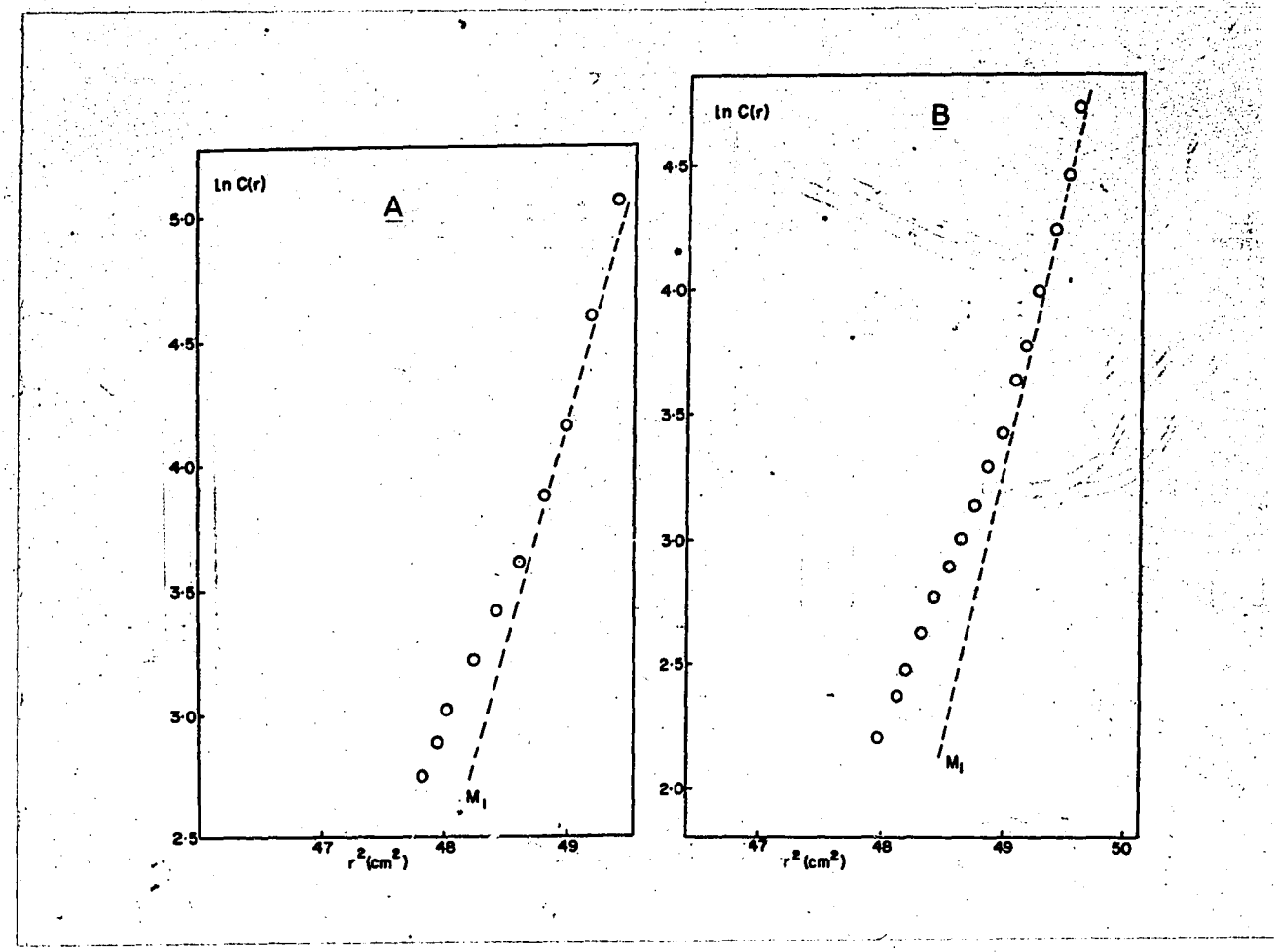


Fig. 25 Sedimentation equilibrium plots of bovine liver catalase in non-ionic solutes. The plots are represented as the logarithm of concentration versus the square of the radial distance. The theoretical distribution of monomer is shown by - - - M_1, and dimer - - - M_2. The buffer is 0.1 M tris-HCl, pH 8.6.

A. Bovine liver catalase in 7.5 M formamide, O , C_o - .34; rotor speed - 26,000 rpm.

B. Bovine liver catalase in 4 M urea, O , C_o - .36; rotor speed - 24,000 rpm.

by the non-ionic solutes rather than by the ionic solutes. Fig. 26[10] presents the ultraviolet absorption spectra of catalase in urea and 2-chloroethanol. The diminution of the Soret band intensity was observed with both urea and formamide. Solvent perturbation difference spectra in presence of 1.0 M $CaCl_2$, and 8.0 M urea and 9.0 M formamide were presented in Fig. 27 . $CaCl_2$ is the only ionic solute which affected the Soret band intensity of catalase. It is well known that the heme moiety of hemoglobin and also of cytochrome c and myoglobin is located in a hydrophobic region. The spectrophotometric studies show that the non-ionic solutes and $CaCl_2$ were capable of interacting with the chromophoric heme group.

The fluorescence emission spectra of proteins have provided a sensitive means to evaluate localized conformational changes. Fluorescence emission spectra of catalase in presence of ionic solutes and non-ionic solutes are presented in Fig. 28 . Native catalase showed the maximum emission spectra at 326 mμ. NaCl and KCl did not shift the fluorescence emission spectra of catalase, the maximum emission wavelength being 327-327 mμ in both cases. A spectral shift to 336 mμ was observed with 1.0 M $CaCl_2$. The fluorescence emission spectra in non-ionic solutes displayed a red shift and the values of the relative fluorescence intensity were increased markedly. The maximum fluorescence emission wavelength was 330 mμ in 1.0 M 2-chloroethanol, 345mμ in 9.0 M formamide, 348 mμ in 8.0 M urea, and 349 mμ in 6.0 M guanidine-hydrochloride.

The spectrophotometric titrations of the tyrosine residues were carried out in 0.2 M KCl, and in presence of varying concentrations of urea to gather information in regard to the availability of the tyrosine residues.

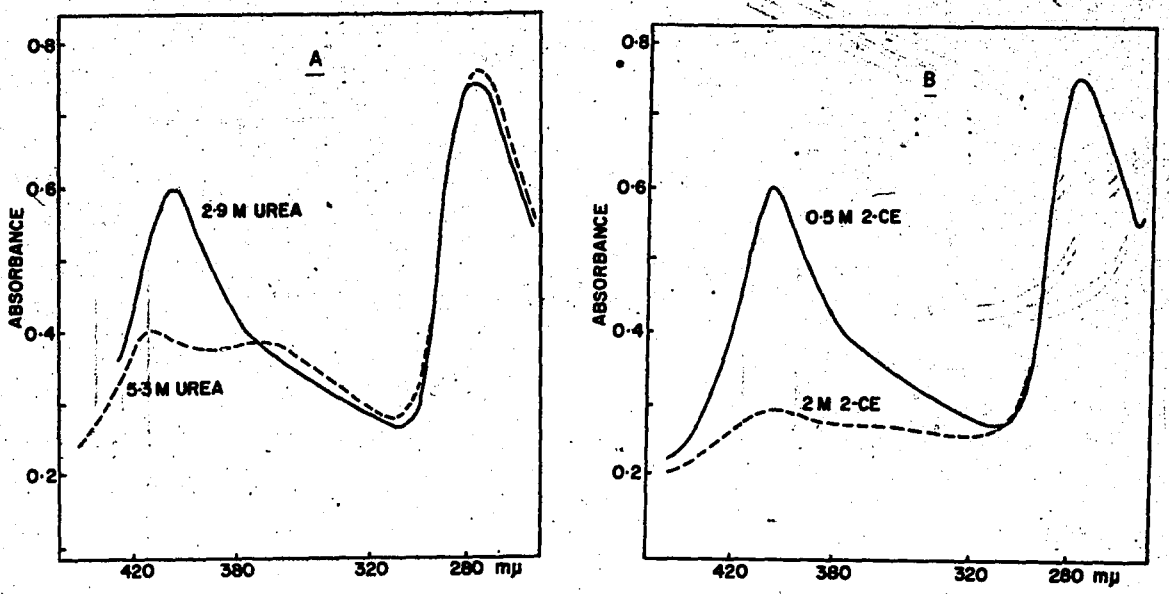


Fig. 26 Solvent perturbation spectra of bovine liver catalase in non-ionic solutes.

A. —— 2.9 M urea; ---- 5.3 M urea.

B. —— 0.5 M 2-chloroethanol; ---- 2.0 M 2-chloroethanol.

Scans were taken five minutes after solutions were mixed. The media contained 0.1 M tris-HCl buffer, pH 8.6.

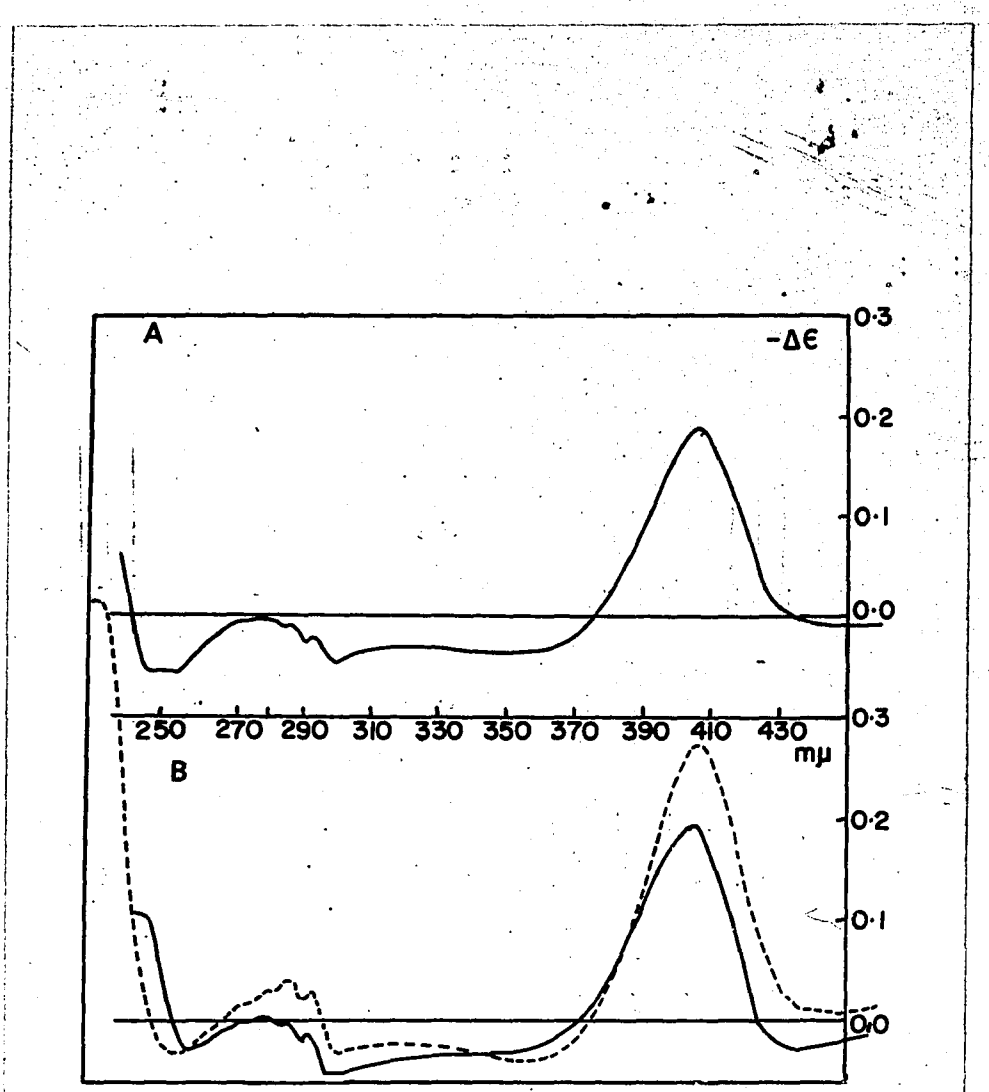


Fig. 27 A. Solvent perturbation difference spectra of bovine liver catalase in 0.1 M $CaCl_2$:0.1 M tris-HCl buffer, pH 7.0. The scan was taken five minutes after the mixing of the solutions.

B. Solvent perturbation difference spectra of bovine liver catalase in non-ionic solutes; ——— 9.0 M formamide; - - - - 8.0 M urea. The scans were taken five minutes after the mixing of the solutions. The media contained 0.1 M tris-HCl buffer, pH 8.6.

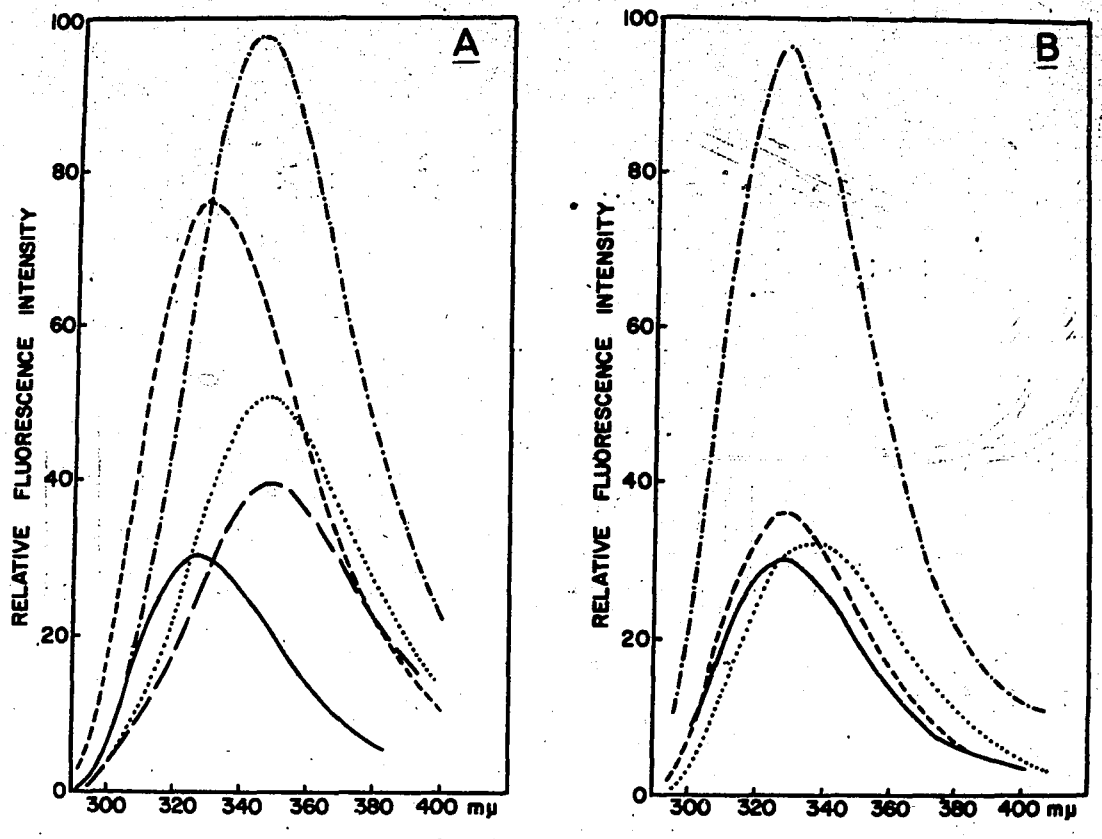


Fig. 28 A. Fluorescence emission spectra of bovine liver catalase in non-ionic solutes: buffer - 0.1 M tris-HCl, pH 8.6.
..... 8.0 M urea; -·-·- 9.0 M formamide; — — — 6.0 M guanidine hydrochloride; ----- 1.0 M 2-chloroethanol; ——— native bovine liver catalase.

B. Fluorescence emission spectra of bovine liver catalase in ionic solutes: buffer - 0.1 M tris-HCl, pH 8.6; except for $CaCl_2$, buffer - 0.1 M tris-HCl, pH 7.0.
..... 1.0 M $CaCl_2$; - - - - 4.0 M NaCl; -·-·-·- 3.5 M KCl; ——— native bovine liver catalase.
The scans were taken five minutes after solutions were mixed.

Although the total number of tyrosine residues as obtained from the amino acid analyses was 86, the number of available tyrosine residues at the maximum pH in 0.2 M KCl was 56; whereas with 8.0 M urea, 64 tyrosines were ionized. Fig. 29B shows that the increase in the urea concentration facilitated the ionization of tyrosines of abnormal pk values. It may be surmised that these tyrosine residues are relatively unavailable in normal solvent environment.

Enzymic Activity

Since bovine liver catalase is an enzyme exhibiting the property of an oxido-reductase, the effect of the solvent perturbation system on the molecular conformation has been studied by evaluating the enzymic activity for the time intervals used during the sedimentation velocity experiments (150 min. being the maximum time elapsing between the mixing and the termination of ultracentrifuge run). Figs. 30 A&B show that when catalase was subjected to ionic solutes of increasing molarity, the enzymic activity decreased with the increase in the solute concentration in the media and also with the increase in the time interval. With the ionic solutes the inhibition in the enzymic activity was quite rapid, between 0 - 15 min, and thereon the rate of loss in enzymic activity was slow. The enzymic activity was inhibited maximally by $CaCl_2$ and least by LiCl (Fig. 30). NaCl appeared to be more effective than KCl.

Fig. 31 shows the effect on the enzymic activity when bovine liver catalase is subjected to non-ionic solutes like formamide, urea and 2-chloroethanol. The concentration range studied with formamide and urea

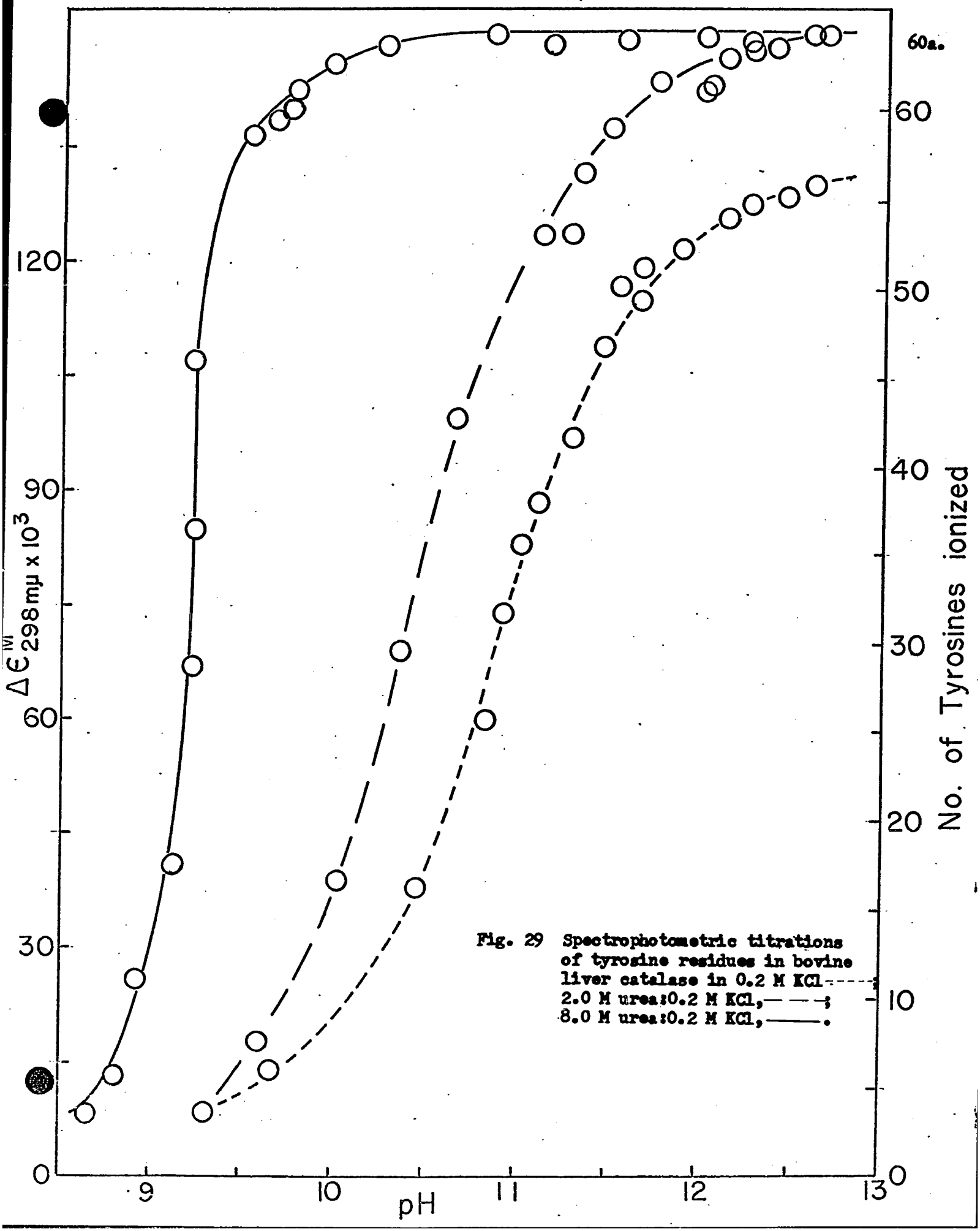


Fig. 29 Spectrophotometric titrations of tyrosine residues in bovine liver catalase in 0.2 M KCl ·····; 2.0 M urea:0.2 M KCl, — — —; 8.0 M urea:0.2 M KCl, ———.

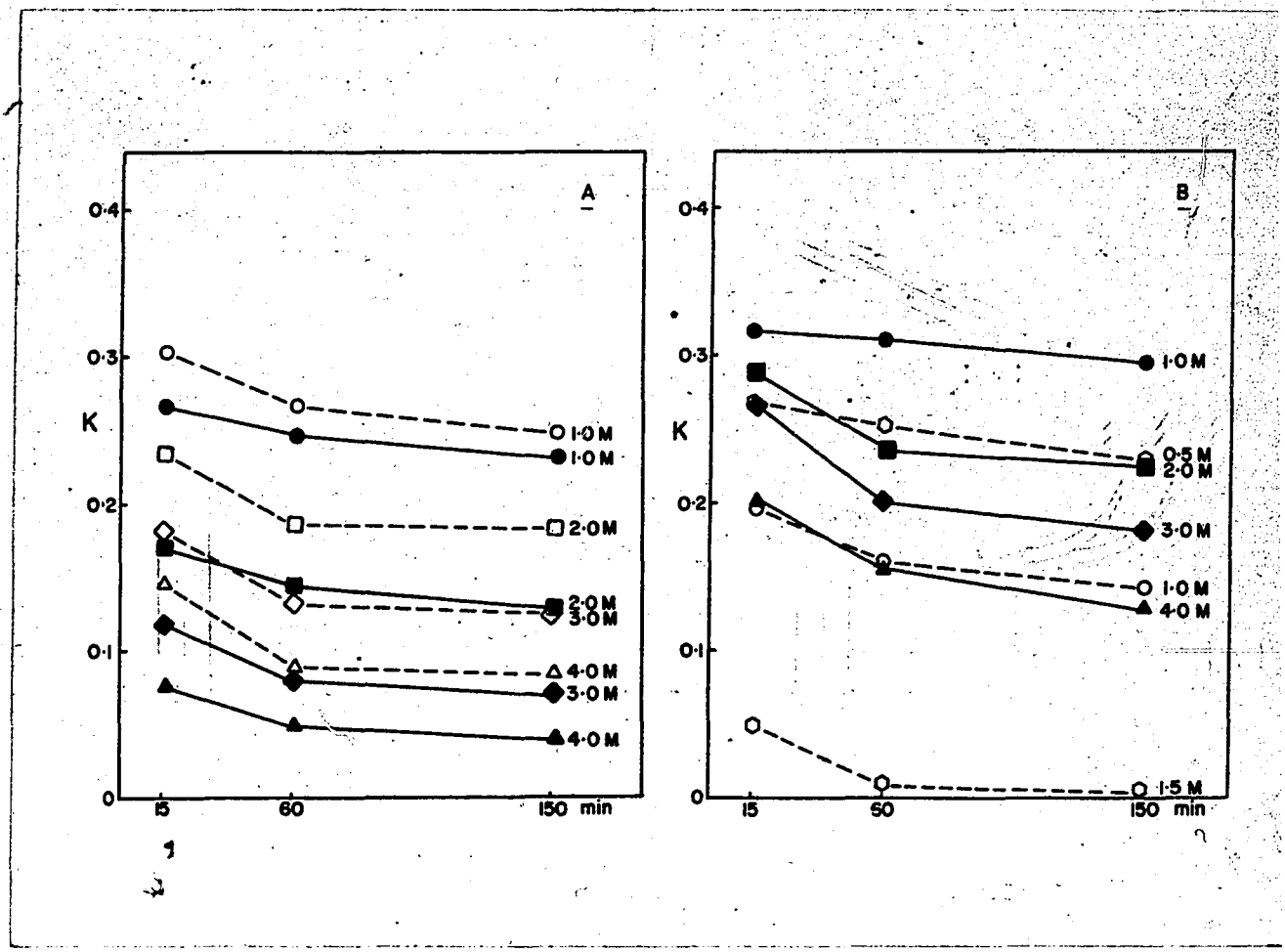


Fig. 30 Enzymic activity of bovine liver catalase in ionic solutes.

A. NaCl, —— and KCl- - - - -.

B. LiCl, —— and $CaCl_2$- - - - -.

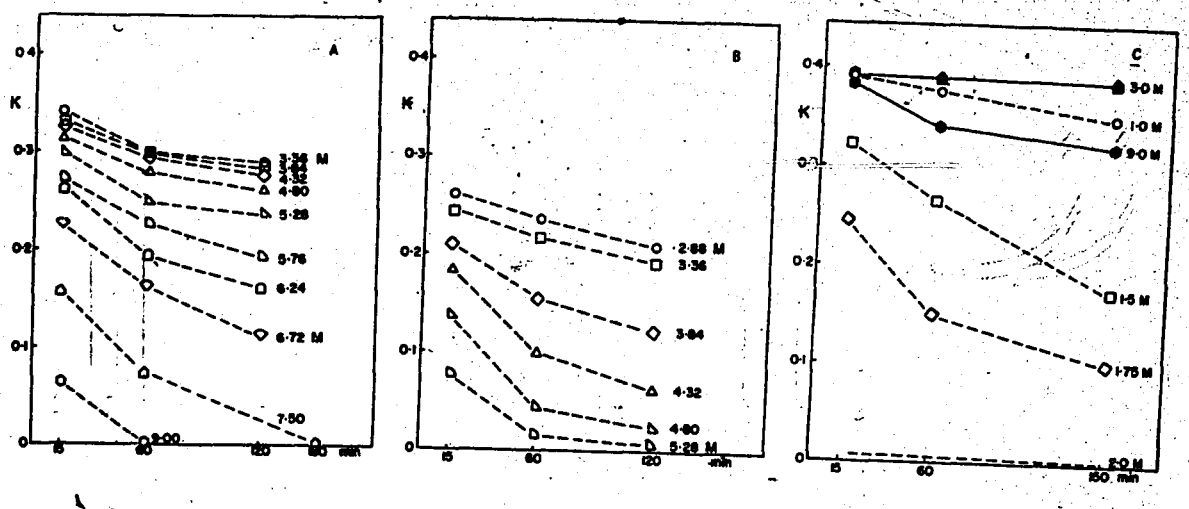


Fig. 31 Enzymic activity of bovine liver catalase in non-ionic solutes.

A. formamide

B. urea

C. 2-chloroethanol,---- and ethylene glycol, ——— .

systems showed the dissociation of catalase to monomer subunits (Figs. 2.3 a,b). The decrease in the enzymic activity of catalase was not appreciable in 3 M ethylene glycol. However, in 9 M ethylene glycol solution some loss in enzymic activity was noticed. No dissociation of catalase was observed in ethylene glycol system employing sedimentation velocity methods. Appreciable loss in enzymic activity was not observed with 3.0 M formamide. No change in enzymic activity of catalase has been observed with 0.5 M 2-chloroethanol solution over the specified time period. A control run using the reagent blank was conducted under all solute:solvent interaction systems under identical experimental conditions. All the solutes used during these investigations had very little effect as such. While both types of solutes were capable of inhibiting the enzymic activity, the loss of enzymic activity in non-ionic solutes in non-ionic solutes was progressive in comparison with that in ionic solutes. It may be mentioned here that the loss in enzymic activity over the time period for sedimentation equilibrium runs (14 hr) were not carried out during this investigation.

DISCUSSION

Analytical ultracentrifugation of bovine liver catalase under solvent:solute interaction has revealed very interesting features in regard to the monomer association. All the solutes, ionic and non-ionic, have been able to dissociate catalase with varying degrees of success. However, a distinct difference was observed between the sedimentation velocity characteristics with NaCl, KCl and LiCl on one hand, and $CaCl_2$, formamide, urea and 2-chloroethanol on the other hand. While the latter group produced

a Schlieren peak of approximately 3.5 S and another peak lower than 11.6 S, the former group showed only one broad Schlieren peak with progressively decreasing S values. When these experiments were compared with a non-interacting system (Catalase:human immunoglobulin G: bovine serum albumin), all three peaks could be observed. Catalase presents an interacting system, where a 4 S component may be considered as the monomer species, and a component of higher S value as a mixture of tetramer and dimer in varying proportion. Under similar solvent:solute interaction system the dissociation of tetrameric chick hemoglobin molecules proceeded to dimer and to monomer state but did not produce two Schlieren peaks under any conditions. The restricted removal of the perturbants from the solution would tend to produce intact tetrameric catalase molecule. These results imply that the subunits of catalase are in dynamic equilibrium. The sedimentation equilibrium studies revealed that the monomer association is dependent on the protein concentration, the presence of monomer-dimer equilibrium being observed in native condition (Fig. 23 B.). The presence of monomer could even be shown from sedimentation velocity experiments at a dilution of $A_{280\ mp}$ - 0.05, an increase in protein concentration resulted in an increased S value (9 S or higher). The S value also seems to be dependent on the rotor speed. The presence of a component of 6 - 7 S, i.e. pure dimer species, was not detected under the present experimental conditions. During the sedimentation equilibrium studies, the extent of monomer in the solution was dependent on the solute concentration. The sedimentation velocity experiments showed that the ionic solutes like NaCl, KCl and LiCl were not as efficient as $CaCl_2$, formamide and urea; whereas in accordance with the sedimentation equilibrium data these solutes were capable

of dissociating catalase to monomeric state, the extent of monomer formation depending on the perturbant concentration. This apparent disparity arises from the fact that the dimer-monomer equilibrium of the catalase is dependent on the protein concentration. Furthermore, the greater forces as obtained with the higher rotor speeds tend to favor the polymerization. It is interesting to note that the removal of heme produced monomer subunit. Apo-catalase was relatively unstable and could only be kept in solution at pH 4.0, a situation comparable to those observed with individual chains of chick hemoglobin (Chapter III).

The Soret band maxima (407 mμ) were not affected by dilution at a protein concentration range of $A_{280\ m\mu}$ - 0.2 - 1.6. The Soret band maxima remained unaffected by ionic solutes (NaCl, KCl and LiCl) although the intensity decreased with non-ionic solutes. Non-ionic solutes at lower concentration like 0.5 M 2-chloroethanol or 2.9 M urea, solvent perturbants like ethylene glycol and sucrose at a concentration of 20% produced very little change. These studies show that these solutes which are capable of inducing hydrophobic interaction are also capable of decreasing the Soret band intensity and also a shift in the maxima. As regards the ΔE^{M}_{heme}, the heme groups of catalase are more affected by the solutes than those of hemoglobin.

Studies on the fluorescence emission spectra pointed out that the tryptophan residues of catalase are strongly bonded in hydrophobic regions, and non-ionic solutes like formamide and urea, could produce the red shift. NaCl and KCl were ineffective in contrast to the effect of $CaCl_2$ where a red shift to 345 mμ was observed. At this end, $CaCl_2$ was less effective

compared to guanidine hydrochloride, urea or formamide. The tyrosine ionization spectra also revealed the strong hydrophobic interaction in the catalase molecule: for example, while the amino acid analysis revealed the presence of 86 tyrosine residues, 64 tyrosines were obtained in 8 M urea and 56 in 0.2 M KCl. It indicates that quite a large number of tyrosine residues are strongly hydrogen bonded and are not easily available to the solvent environment.

Both ionic and non-ionic solutes produced inhibition of the catalatic activity at a sufficient solute concentration. Since catalase exists mainly as a monomer at a protein concentration generally employed for the determination of its enzymic activity, the catalase monomer appears biologically to be the active unit. The solutes produce both gross and localized conformational changes in catalase which reflect on the enzymic activity to different degrees. The recombination experiments which are in progress in our laboratory conform to this fact.

CHAPTER V

DISCUSSION

Studies on polymers proteins have provided us with an interesting phenomenon of molecular association. This association is rather specific: for example, the association of α- and β-subunits of hemoglobins. These associations stand quite distinct from molecular aggregation which could be produced by physical and chemical means. Three dimensional atomic models of multi-chain proteins like hemoglobins (44, 45), α-chymotrypsin (145) and single chain proteins like myoglobin (135, 136) lysozyme (146-148) carboxypeptidase A (149-150) and ribonuclease A (151, 152) indicated that the polar side chain groups are in contact with the solvent molecules, and the non-polar groups are away from the solvent interaction being placed in the crevices or inside the molecule. The polar groups could stabilize the protein molecule via electrostatic interactions located within the same subunit or its neighbour. Hydrophobic interaction may also occur among inter- and intra-polypeptide chains (102). It appears that the polymer integrity is dependent on both the electrostatic and hydrophobic interactions. The gross conformation of these proteins in aqueous medium of neutral pH and low salt concentration is believed to be essentially the same as the molecular models derived from the crystals. Most of the proteins are compact molecules which stem from the specific folding of the polypeptide chains and which in turn was facilitated by disulphide bridges. There are several forces influencing the gross conformation of the protein: short range forces, e.g. Van der Waals forces, coulombic forces, etc., hydrogen bonds, hydrophobic

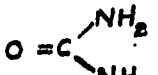
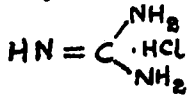
bonds (102, 153). These interactions are dependent on the sequential arrangement of the amino acid residues. Solvent environment is one of the major deciding factors towards the stabilization of polymer molecules. A suitable change in the solvent environment will counteract the inter-subunit stabilizing forces and eventually induce a depolymerization. It may, however, be mentioned that in very dilute solution, hemoglobin dissociates (10). Schachman and Edelstein reported the dissociation of human oxyhemoglobin A to monomer at a concentration of 0.002 mg/ml and to dimer, at a slightly higher protein concentration of 0.017 mg/ml (14). Bonaventura and Riggs observed that human oxyhemoglobin Kansas (β-102 thr → asn) dissociated to dimer form at protein concentration (0.1 mg/ml) higher than that required for human oxyhemoglobin A (154).

Solvent:solute interaction studies as presented herein were carried out in an aqueous medium. According to the current concept, liquid water is in equilibrium states varying from tetra-hydrogen bonded (ice-like) to non-hydrogen bonded structure (free water) (88, 153). The mole-fraction of each of the four species present depends on the temperature of the solution (0° - 80°). Addition of electrolytes to solvent (water) produced ion-ion, ion-dipole and dipole-dipole interaction in solvated condition, and alters the dielectric properties of the medium. Variation in the dielectric property of the medium depends on the characteristics of the solutes. During the present study the chlorides of alkali metals, Li, Na, K and alkaline earth metal, Ca, were used; while the former group represents uni-univalent electrolytes, the latter one di-univalent electrolyte. Comparing the disruptive effect of these electrolytes, LiCl was found to be least effective

with no significant differences being observed between NaCl and KCl. $CaCl_2$ was the most effective of the ionic solutes used. In all cases an increase in solute concentration produced an increasing disruptive effect. The ionic hydration values of NaCl, KCl and $CaCl_2$ are 3.5, 1.9 and 12.0 respectively (155). The dielectric constant of these electrolytes in water ($\epsilon_w = 78.54$) at 25° are 22.54, 34.54 and 43.54 for 4 M LiCl, 4 M NaCl, and 3.5 M KCl. These values were calculated from the equation $\epsilon = \epsilon_w + 2\delta c$, where ϵ, dielectric constant of the electrolyte at a concentration c; ϵ_w, dielectric constant of water; δ, half the molar depression; c, the concentration of solute in moles/liter (85). The mean ionic activity coefficients at 1.0, 2.0 and 3.0 molal solution are 0.492, 1.785 and 4.588 for NaCl and 0.365, 1.313 and 2.914 for KCl (156).

The non-ionic solutes employed were amide derivatives (urea, formamide and guanidine hydrochloride) and an alochol derivative (2-chloroethanol). According to Frank and Evans (89), introduction of a non-polar molecule induces an "iceberg" structure in bulk water. Schellman (157) reported that in dilute urea solution there is an equilibrium, $2(urea)_{aq} \rightleftharpoons (urea_2)_{aq}$, and that at higher urea concentrations further polymerization gives trimers, tetramers, etc. The addition of urea to water produced urea-water clusters. These clusters provide the interstitial space in the solution which accommodates the hydrocarbon moieties. Thus, urea appears to solubilize amide, peptide and hydrophobic groups (139, 140). Of the three amide derivatives used, the relative order of effectiveness was, guanidine-hydrochloride, urea and formamide.

The dual properties, ionic and non-ionic, of guanidine hydrochloride could account for the greater disruptive effect. It is interesting to note that although 2-chloroethanol has widely been used as a heliogenic solute (158), it was capable of depolymerizing both chick hemoglobin and bovine liver catalase; the disruptive effect is not as great as observed with the other amide derivatives.

Studies on the hydrodynamic properties based on analytical ultracentrifugation and viscosity determinations indicate that no gross conformational change accompanied the dissociation of chick hemoglobin in both ionic and non-ionic solutes. However, localized conformational changes as reflected in the diminution of Soret band intensity was observed with all non-ionic solutes,  (formamide);  (urea);  (guanidine hydrochloride); $CaCl_2$. Binding of these solutes to protein molecules has been reported by several investigators as insignificant. However, divalent ions like Zn^{++} and Cd^{++}, have been reported to react with the imidazole groups (159). Sperm whale myoglobin has been shown to bind both Cu and Zn (160, 161). Metalloenzymes like carboxypeptidase bind divalent cations (162). The accessibility of chromophoric side chains of tyrosine and tryptophan, is facilitated by the solvent perturbants (105). Solvent perturbation differential spectrophotometric studies has also revealed localized conformational changes in ribonuclease (163), bovine serum albumin (104). Furthermore it has been established that the stronger denaturants cause a helix-random coil transition. (164).

The existence of hemoglobins as a tetramer in an aqueous medium

(neutral pH, low salt concentration) is such that the free energy of contact between the four subunits is less than that between a subunit and the solvent environment. Removal of the heme moiety from chick hemoglobin produced a mixture of α and β chains, which exists in a dimeric form (αβ) (Figs. 1 & 9). The same observation was made with aminoethylated globin. A smaller disruptive force (4 M) is required to dissociate globin (αβ) dimers to monomers than for the dissociation of hemoglobin $(\alpha\beta)_2$ tetramers to monomers (8 M urea). It was also observed that in the absence of β-chains, the α-chains are unstable in nature and have to be kept in solution in presence of urea (2 M - 4 M urea, pH 3.5). Thus it appears that unlike chains stabilize each other. This in turn reflects a lower free energy of contact between the dissimilar subunits. It has been mentioned previously that the addition of a solute to the protein solution causes the dissociation of the protein molecule. To do so it must decrease the free energy of contact of the subunits with the solvent (165). The addition of strong electrolyte solutions is known to weaken polar interactions and strengthen non-polar ones. They would therefore be expected to weaken the forces between the like subunits and strengthen those between the unlike subunits. This explains why symmetrical dissociation occurs. The change in free energy, when the protein molecule dissociates would be a reflection of the binding energy between the like subunits. Kirshner and Tanford have reported a change in free energy of 5.1 kcal/mole for human carbonmonoxyhemoglobin and bovine ferrihemoglobin in 2 M NaCl (32). For chick hemoglobin under similar conditions, the changes in free energy was approximately 2.3 kcal/mole. It appears from these data that chick hemoglobin is more resistant to dissociation than the mammalian hemoglobins. A similar result was obtained from preliminary data comparing

the behavior of mammalian and avian hemoglobins (Chapt. 3, section 2).

Sedimentation velocity analysis of chick hemoglobin in presence of increasing concentration of ionic and non-ionic solutes showed a single peak of decreasing S value. In an interacting system, where the components are in equilibrium and the S values close (4.4 S and 2.8 S for tetramer and dimer, respectively), the refractive index gradient curve failed to resolve the rate of movement of individual components into peaks. The degree of dissociation, α, from the tetramer to dimeric state, could be assessed by relating the observed S values to the assumed values of tetramer and dimer. At a high solute concentration, the presence of monomer imposes a restriction on the evaluation of dissociation constant (tetramer $\rightleftharpoons$ dimer). Furthermore, the detection of monomer present in such a system is limited by the mode of operation employing Schlieren optics. This has, however, been overcome by the automatic photoelectric scanner operation in conjunction with sedimentation equilibrium method. Thus, traces of monomer could be detected in 2 M NaCl and so also in 3.0 M KCl, the corresponding S values being 2.81 S and 3.0 S. Similarly, traces of tetramer could be detected in 4 M urea. Whether or not a lower protein concentration for a particular solute concentration produced further dissociation could not be ascertained under present experimental conditions.

The monomer association as observed with bovine liver catalase has provided us with a very interesting but complicated phenomenon. Both ionic and non-ionic solutes are effective in dissociating catalase molecule. However, a distinctive feature was observed where the S value in NaCl, KCl and LiCl decreased with increase in solute concentration and a single Schlieren peak was observed. In contrast, the Schlieren plots revealed two

components in non-ionic solutes, the fast component with 9 S or higher, and a slow one with 3.5 S or lower. A component with an intermediate S did not appear. A marked decrease in S value compared to native catalase (11.6 S - $S^{o}_{20,w}$) implies a reduction in molecular size. Following a similar argument as used with chick hemoglobin, the presence of dimer is indicated in these solutes. If the decrease in the S values were a reflection of the degree of dissociation, the presence of monomer was detected at a stage wherein approximately 50% of the tetramer species had been dissociated. The range of solute concentration where the simultaneous presence of fast and slow components were observed, was rather narrow. $CaCl_2$ was the only ionic solute which produced two components, and produced the lowest S value for the fast moving component (Fig.17 D). It seems worthwhile to point out that a smooth S vs solute concentration plot was observed in NaCl and KCl without any break and the limiting S values were lower than those observed with the fast components in presence of non-ionic solutes, implying a smooth shift in the equilibrium states (tetramer $\rightleftharpoons$ dimer $\rightleftharpoons$ monomer). A similar departure in the behavior of $CaCl_2$ was observed in fluorescence emission spectra and differential spectrophotometric studies. A more pronounced change in the Soret band region was observed with the non-ionic solutes. Tryptophan residues were made available by the solvent environment by these solutes. In general, studies on bovine liver catalase pointed out a marked distinction in the behavior of non-ionic and ionic solutes.

The dissociation of hemoglobin from tetramer to dimer and monomer at a low protein concentration is an established fact (10, 14). At a protein concentration where chick hemoglobin exists in solution as tetramer, the

sedimentation equilibrium analysis showed the dissociation of bovine liver catalase from a tetrameric state to a mixed species of dimer and monomer. In comparison to chick hemoglobin, solutes at a lower concentration produced increasing amounts of monomer species. The experimental results with the ionic and non-ionic solutes imply that the stabilizing forces between the subunits of tetramer in catalase are weaken than those between the subunits of chick hemoglobin, and that both electrostatic and hydrophobic interactions play an important role in the polymer stabilization.

The theories of low speed sedimentation equilibrium assume that the chemical potential is independent of pressure. For a typical sedimentation equilibrium experiment at low angular velocity and with a short fluid column, the effect of pressure has been considered to be small (18). Since the ultracentrifuge is operated at moderate to high rotor speeds for analytical sedimentation velocity experiments, high pressure of the order of 100 - 500 atmosphere may be generated at the cell bottom. Kegeles, Rhodes and Bethune (166) have pointed out that because of the overwhelming effects of high pressure gradients on macromolecular reactions, molecular weights cannot be determined with sufficient accuracy from the sedimentation coefficient until it is shown to be independent of speed and pressure. During present studies sedimentation equilibrium analysis showed that bovine liver catalase dissociated to a mixed species of monomer and dimer. A higher speed (Fig. 23) favored the formation of a heavier component. For a similar concentration as used in the equilibrium analysis (56,000 rpm) a species of 10.6 S were detected in the scanner velocity experiments, a value which indicates a mixture of tetramer and dimer. In the sedimentation velocity

experiments the presence of monomers could only be detected at very low protein concentrations. It appears that pressure generated in the ultracentrifuge cell influences the monomer-dimer association of bovine liver catalase, and that the dimer-dimer association is more rapid than the monomer association.

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