Partition of Closely Related Proteins in Aqueous Two-Polymer Phase Systems. Human Hemoglobin Variants and Hemoglobins from Different Species*

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ABSTRACT: Mixtures of aqueous solutions of two different polymers at appropriate concentrations give rise to aqueous–aqueous two-phase systems. Partition coefficients, K, of cells and particles in such phases is determined in great part by their surface charge. In the present work the partition of proteins in dextran–polyethylene glycol phase systems was studied. As model proteins we chose hemoglobins which have well-known structures and properties. The K values of human hemoglobin A and variant hemoglobins S and C are the same at their respective isoelectric points while hemoglobin F differs significantly. Derivatives such as oxy-, deoxy-, carbon monoxide, carbamino-, met- or "stripped" hemoglobins all have similar K's reflecting, among other things, that certain conformational changes in hemoglobins are not detected by partition in these systems. Marked differences in partition behavior have been found, however, between hemoglobins from different mammalian species, with human having the highest and pig the lowest K values. Verification of the partition in single tube experiments was obtained by countercurrent distribution of different hemoglobins singly and in mixture.

The observed K's have no apparent correlation with the hemoglobins' relative electrophoretic mobilities. The basis for the greatly different partition coefficients of such a closely related molecular species as mammalian hemoglobins remains under investigation.

When aqueous solutions of different polymers are mixed in certain concentrations they form liquid two-phase systems (Albertsson, 1960). Such aqueous–aqueous phase systems are suitable for the partition of cells, cell particles, membranes, and macromolecules (Albertsson, 1960, 1969, 1970; Walter, 1969). Quite generally, the partition is greatly dependent on the ionic composition of the phase system and on the positive or negative charge of the partitioned substance (Albertsson, 1960; Walter *et al.*, 1968), a phenomenon that permits one to determine the isoelectric point of proteins by a series of partitions (Albertsson *et al.*, 1971). In the case of cells and particles one of the primary determinants of the partition coefficient is surface charge (Walter *et al.*, 1965, 1967, 1970). For some proteins a correlation between molecular weight and partition has been found (Walter and Sasakawa, 1969), an increase in molecular weight being associated with a lower partition.

Since so little is known about the factors involved and the possible influence of protein conformation on partition we have examined the behavior in two-polymer phases of some closely related proteins of known structure and chemical and physiological properties (human hemoglobin variants and hemoglobins from different species) under a number of conditions.

It was found that, while their molecular weights are essentially the same, significant differences in the partition of hemoglobins A and F as well as in that of mammalian hemoglobins from different species are evident in dextran–polyethylene glycol systems. Further, the K's have no apparent relation to the relative electrophoretic mobilities of the hemoglobins in question.

Methods

Preparation of Hemolysates. Fresh human or animal blood was collected in acid–citrate–dextrose (ACD) solution. The cells were washed five times with an aqueous isotonic salt solution. Cord blood was the source of hemoglobin F and contained 85% fetal hemoglobin as determined by alkali denaturation (Singer *et al.*, 1951; Miale, 1967). Hemoglobins S and C were obtained from patients who were homozygous for these hemoglobins. Red cells were lysed in four times their volume of water. Stroma were removed by centrifuging (12,800g for 20 min) at 4° (Rigas and Koler, 1961). The resulting hemolysates were dialyzed against water overnight in the cold room (5°). Hemoglobin concentration was determined by measuring the absorbance of the lysates at 540 nm.

Preparation of Hemoglobin Derivatives. DEOXYHEMOGLOBIN was obtained by adding a small quantity of solid sodium dithionite into a lysate which was subsequently dialyzed against distilled water overnight. Partition was carried out in nitrogen-saturated phase systems. The spectrum of deoxyhemoglobin (as well as that of all the hemoglobin derivatives) was checked on a Beckman DK-2 recording spectrophotometer. METHEMOGLOBIN was prepared by adding 1.2 moles of potassium ferricyanide/mole of iron in a lysate. The resulting solution was dialyzed overnight against water to remove any excess reagent.

CARBON MONOXIDE HEMOGLOBIN AND CARBAMINOHEMOGLOBIN were obtained by bubbling carbon monoxide or carbon dioxide, respectively, into lysates for about 30 min. Phases used in the partition of these hemoglobin derivatives were similarly saturated with the respective gases.

"STRIPPED" HEMOGLOBIN is hemoglobin essentially free of

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adhering organic phosphate esters (especially 2,3-diphosphoglycerate) and was prepared following the procedure outlined by Benesch *et al.* (1966).

Preparation of Hemoglobin from Young and Old Red Blood Cells. Young and old red blood cells were obtained by centrifugation making use of the greater specific gravity of older cells when compared to younger ones. Lysates from young and old cells were prepared in a manner identical with that outlined by Fischer and Walter (1969) for unfractionated blood.

Preparation of Phase Systems. Aqueous dextran–polyethylene glycol phase systems, buffered and containing certain salts (see below) were used in this work (Albertsson, 1960). Dextran 500, batch 3202, was obtained from Pharmacia Fine Chemicals, N. J., and polyethylene glycol was obtained (Carbowax 6000) from Union Carbide, Los Angeles, Calif. In the present study stock solutions of appropriate concentration were prepared: dextran (20%, w/w), polyethylene glycol (40%, w/w), 0.4 M NaCl, 0.2 M Na_2SO_4, and a series of 0.04 M buffers (glycine or phosphate) spanning the pH range from 5.0 to 10.0. A mixture containing 14% (w/w) dextran and 8.8% (w/w) polyethylene glycol was prepared by weighing out appropriate quantities of the stock polymer solutions. Partition of proteins was carried out as follows: 2 g of the mixture containing 14% (w/w) dextran and 8.8% (w/w) polyethylene glycol, 1 g of 0.4 M NaCl solution *or* 0.2 M Na_2SO_4 solution containing a quantity of lysate adequate for absorbance measurements (approximately 3–6%), and 1 g of 0.04 M buffer were weighed into small centrifuge tubes. The entire mixture was well agitated. The final phase system prepared as described had in addition to lysate the following composition: 7% (w/w) dextran, 4.4% (w/w) polyethylene glycol, 0.1 M NaCl and 0.01 M glycine or sodium phosphate buffer (system I); 7% (w/w) dextran, 4.4% (w/w) polyethylene glycol, 0.05 M Na_2SO_4, and 0.01 M glycine or sodium phosphate buffer (system II). A phase system containing 7% (w/w) dextran, 4.4% (w/w) polyethylene glycol, and only 0.005 M glycine or sodium phosphate buffer (low salt) was also prepared (system III). The phase systems, at room temperature, were centrifuged at 1200*g* for 10 min to speed phase settling; 0.5 ml of top phase and 0.5 ml of bottom phase were carefully pipetted from the phase system in each tube, and each diluted by addition of 2.0 ml of water. The solution was mixed and the absorbance was measured at 540 nm or 410 nm on a Gilford spectrophotometer (Model 240). The data are expressed in terms of partition coefficients (K, ratio of absorbance of top phase to absorbance of bottom phase) of the hemoglobins in the phase systems. pH was measured directly on the remaining phase.

Countercurrent Distribution of Hemoglobins. An automatic thin-layer countercurrent distribution apparatus (with 120 cavities) as described by Albertsson (1965) and constructed by Stalprodukter, Uppsala, Sweden, was used in this work. Phase system I with a pH 7.2 sodium phosphate buffer was used in the countercurrent distribution experiments with human, pig, and human + pig hemoglobins. The phase system containing human, pig, or human + pig hemoglobins (about 38 mg of each hemoglobin) was adjusted so that the top to bottom phase volume ratio was 0.8/0.6. The phase was well shaken and 1.4 ml of the mixture pipetted into each of four cavities ("load cavities") when the hemoglobin mixture was subjected to countercurrent distribution, and into each of two cavities when one of the two species' hemoglobins was used alone. All other cavities of the extraction train were loaded with 0.6 ml of bottom phase and 0.8 ml of top phase. The apparatus was set for a cycle of 30-sec shaking followed by a 7-min settling time and a transfer. A total of 120 transfers was completed at room temperature (20–23°).

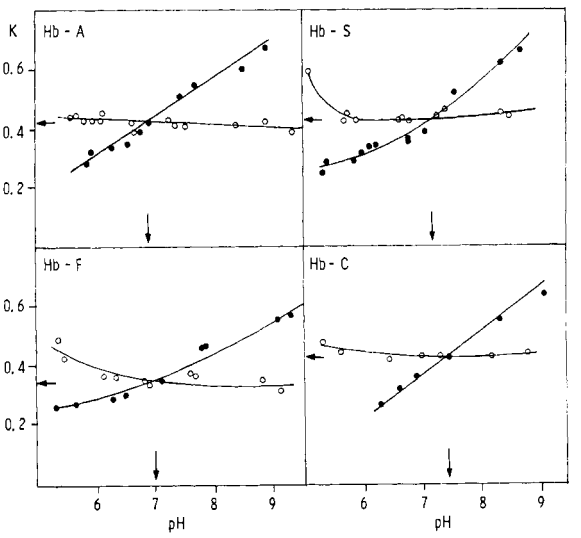

FIGURE 1: Partition coefficients, K, of human hemoglobins A, S, C, and F in phase system I (○-○, containing NaCl) and phase system II (●-●, containing Na_2SO_4) as a function of pH. The arrows indicate the position of the cross-point in each case (*i.e.*, K and pH). For details, see text.

Analysis of Countercurrent Distribution. After completion of 120 transfers the contents of the cavities were collected directly into plastic tubes. Water (2 ml) was added to every other tube to break the phase. Absorbance at 540 nm was then measured. The remaining tubes, which still contained two phases, were centrifuged to assure that the phases were completely settled; 0.1 ml of top and 0.1 ml of bottom phase were sampled from each tube and pipetted into 0.4 ml of water. Absorbance at 540 nm (or 410 nm) was measured and the partition coefficient, K, was calculated for each tube (*i.e.*, absorbance in top phase to absorbance in bottom phase).

Electrophoresis on Paper of Hemoglobins. The relative electrophoretic mobility of hemoglobins from different species and the purity of human hemoglobin variants were examined by paper electrophoresis. Barbital buffer (0.2 M) (pH 9) and S & S paper no. 2041 were used. The hemoglobins were electrophoresed for 20 hr at 4° with a constant 4 V/cm.

Results and Discussion

Partition of Different Human Hemoglobins: Hemoglobins A, F, S, C. The partition of proteins in aqueous, two-polymer phase systems is greatly influenced by the ionic composition (Albertsson, 1960). Further, the partition depends on the charge (whether positive or negative) of the protein (Albertsson, 1970; Albertsson *et al.*, 1971; Walter *et al.*, 1968). Thus the partition coefficients for proteins are different in different salt media and also vary as a function of pH. In Figure 1 the partition coefficients obtained in phase systems I and II at different pH's for normal human hemoglobins A and F and abnormal hemoglobins S and C are shown. In each case the curves obtained in phase system I and phase system II cross at a pH close to the isoelectric point of the investigated protein (Albertsson *et al.*, 1971). The arrows on the figure

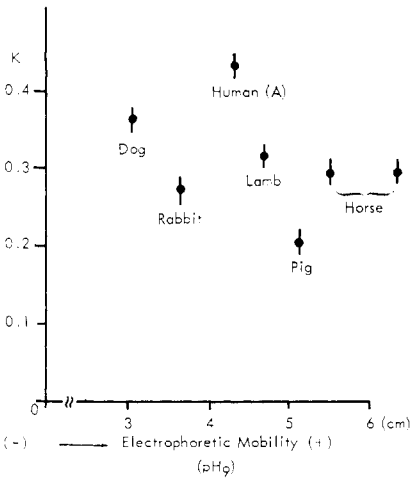

FIGURE 2: Partition coefficients, K, and relative electrophoretic mobilities on paper of hemoglobins from different species. Phase system I was used at pH 7. Sodium barbital buffer was used for electrophoresis at pH 9. Electrophoresis conditions: 4 V/cm, 20 hr, 4°.

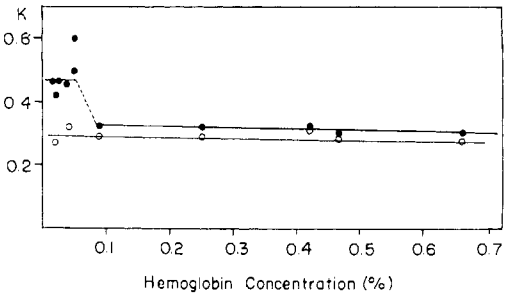

FIGURE 3: Partition coefficient, K, of rabbit oxyhemoglobin as a function of hemoglobin concentration. Phase system I was used at pH 7.0 (○-○) and at pH 9.8 (●-●).

indicate the K value and the pH associated with these cross-points. All adult hemoglobins (A, S, and C) have the same K value at the cross-point (0.43), while slight differences in the K values are in evidence at other pH's. The fetal hemoglobin (hemoglobin F) which differs in about 36 amino acid residues from the other human hemoglobins, has a significantly different K at the cross-point. It thereby behaves like one of the mammalian hemoglobins from other species (Figure 2, see discussion below). The four human hemoglobins investigated differ in their charge. The glutamic acid of hemoglobin A is replaced by valine in hemoglobin S and by lysine in hemoglobin C (Ingram, 1961). While hemoglobin S has a one charge unit difference when compared to hemoglobin A or C, there is no correlation between the observed partition and the charge change at any pH.

Hemoglobin S is known to undergo conformational changes when deoxygenated (Murayama, 1967). However, the K values determined for hemoglobin S under both oxygenated and deoxygenated states are identical (between pH 5 and 9.5). Thus the changes in tertiary and quaternary structures of hemoglobin S appear to have no bearing on its partition in these phase systems.

It is known that hemoglobin dissociates below pH 6 (Sasakawa *et al.*, 1963). Whether hemoglobin dissociates under acidic conditions in two-polymer aqueous phase systems is not clear. The change in K below pH 6 found in the system containing NaCl (Figure 1) for both hemoglobins F and S suggests that dissociation or denaturation (in any case some conformational change) may be taking place.

Partition of Hemoglobins from a Number of Different Species. In Figure 2 we have plotted the partition coefficients in phase system I (at pH 7) of a number of different mammalian hemoglobins against their relative electrophoretic mobilities (at pH 9). The comparison at the two different pH's is permissible because the K values for these hemoglobins in phase system I (with NaCl) is constant over this entire pH range. It is clear that there is no correlation between the partition of these hemoglobins and their electrophoretic mobilities. These results are thus quite different from the correlation established between partition in aqueous two-polymer phase systems and relative electrophoretic mobilities for (red blood) cells and (bacteriophage) particles (Walter *et al.*, 1965, 1970).

An unexpected result of our work with hemoglobins is the significantly different K values obtained for these closely related molecular species. Human hemoglobin A has the highest K, 0.43; dog hemoglobin 0.36; lamb 0.31; horse 0.29 (for both horse hemoglobins as established in a separate experiment by countercurrent distribution); rabbit 0.27 and pig hemoglobin the lowest K, 0.20. While there is some correlation between the partition coefficients at the isoelectric point of nonhemoglobin proteins and their molecular weights, with proteins of larger molecular weight having lower K values (Walter and Sasakawa, 1969), it is apparent that in the case of these mammalian hemoglobins, all of which have quite similar molecular weights, other factors must be involved in determining the different K values observed. Among other parameters we are currently investigating the effects of different degrees of hydrophobicity on the partition of proteins in these phases.

Effect of Hemoglobin Concentration on Its Partition. According to Bonaventura and Riggs (1967), human normal hemoglobin dissociates at a concentration below 0.068%. We measured the partition coefficient of rabbit hemoglobin at concentrations ranging from 0.66% down to 0.01%. Figure 3 depicts our findings that the K of hemoglobin is constant at neutral pH but that in alkaline pH (9.8) the K increases from 0.3 to 0.5 at hemoglobin concentrations below 0.09%. It is likely that this change in K is a consequence of rabbit hemoglobin dissociation, a phenomenon apparently helped by high pH in these phase systems.

Partition of Different Rabbit Hemoglobin Derivatives. Figure 4 indicates the K of rabbit oxy- and methemoglobins as a function of pH in phase systems I and II. The partition coefficients at the cross-point are essentially identical for these two hemoglobin derivatives. The pH's at the cross-point are, however, different being 7.0 for oxy- and 7.2 for methemoglobin. Since the cross-point pH is indicative of the proteins' isoelectric points (Albertsson *et al.*, 1971) this latter finding is expected. Carbonmonoxyhemoglobin gives a partition pattern identical with oxyhemoglobin in these phases. Forster *et al.* (1968) have reported that carbon dioxide reacts with the N-terminal amino acid of hemoglobin, a reaction that often has appreciable effects on the solubility of the hemoglobin (Muir *et al.*, 1952). Carbaminohemoglobin, however, also partitions no differently than oxyhemoglobin.

Enzymes often undergo conformational changes as a function of reaction or interaction with cofactor and/or substrate (Markus *et al.*, 1968; Harte and Rupley, 1968; Imoto *et al.*, 1968). Similarly, hemoglobin undergoes a con-

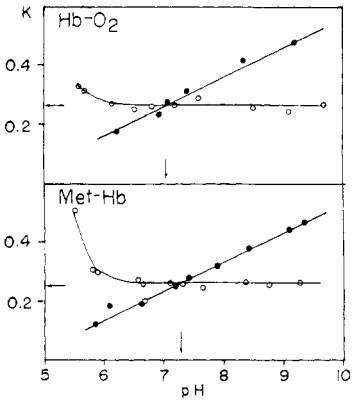

FIGURE 4: Partition coefficients, K, of rabbit oxy- and methemoglobins in phase systems I (○-○) and II (●-●) as a function of pH.

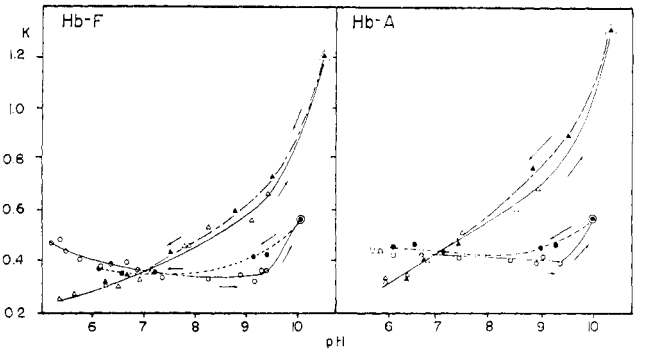

FIGURE 5: Back-partition of human hemoglobin A and F. Phase systems I (○-○, ●-●) and II (△-△, ▲-▲) were used. Open symbols and solid lines indicate the usual manner of partition and solid symbols and broken lines indicate partition coefficients of hemoglobin in highest alkaline phase system brought to indicated pH's. For details, see text.

formational change when converted from oxy- into deoxyhemoglobin (Muirhead and Perutz, 1963). Partition of oxy- and deoxyhemoglobins (just as in the case of human hemoglobin S, see above) reveals no difference in the cross-partition patterns.

Back-Partition. K values of hemoglobins always show a steep increase at and above pH 10 (see Figure 5). Since hemoglobin F is resistant to alkaline denaturation while hemoglobin A is not, we were interested in determining whether hemoglobin A was irreversibly and hemoglobin F either not or reversibly denatured at high pH. To test this we developed a back-partition procedure (analogous to back-titration). In Figure 5 the solid lines show the cross-partitions of hemoglobins A and F in phase systems I and II obtained as previously described. The broken lines indicate the partition of these hemoglobins obtained when mixing in different ratios the hemoglobin in the phase of highest pH with hemoglobin-free phase of lower pH. The K's in such a back-partition are, within experimental error, superimposable on the partitions shown in the solid line (Figure 5) for both hemoglobins A and F. Hence no irreversible changes occur in hemoglobin A in the phase system at higher pH's. It should be noted that this method has discovered unexpected denaturation at high pH's in the case of some lysozyme derivatives, results of which experiments will be reported in full elsewhere. The rapid increase of hemoglobin K's at and above pH 10 may well be a function of pH-dependent dissociation of the hemoglobin molecule into dimers (Vinograd and Hutchinson, 1960).

Partition of Human Hemoglobins from Young and from Old Red Blood Cells and of "Stripped" Hemoglobin. An increased oxygen affinity to hemoglobin derived from old erythrocytes as compared to that from young red cells have been reported (Edwards and Rigas, 1967). This decreased oxyhemoglobin dissociation of older hemoglobin has found at least a partial explanation in the changed intracellular environment that accompanies erythrocyte aging. The quantity of 2,3-diphosphoglycerate present has a marked effect on the oxyhemoglobin dissociation (Benesch and Benesch, 1967) and the observed change in oxygen affinity to hemoglobin probably reflects a diminished level of 2,3-diphosphoglycerate in older cells. It has been shown that 2,3-diphosphoglycerate cannot be removed by simple dialysis against water but can be removed (almost completely) by treating the lysate with higher ionic strength solutions followed by passage through Sephadex (Benesch *et al.*, 1966). Hemoglobin that has been put through such a procedure is dubbed stripped. Young and old red blood cells were separated by ultracentrifugation (Fischer and Walter, 1969), making use of their different specific gravities. Hemoglobin from young or old cells, stripped hemoglobin and hemoglobin to which 2,3-diphosphoglycerate had been added were all partitioned in phase systems I, II, and III. No changes in partition pattern were apparent in any of these preparations. Since the differences, if any, between these hemoglobins may be small we are continuing to study their partition in other phase systems and by countercurrent distribution.

Countercurrent Distribution of Different Hemoglobins. In order to test the validity of partition experiments done in single tubes, we carried out some countercurrent distribution experiments. In Figure 6B,C we show the curves obtained, after 120 transfers, for pig and normal human hemoglobins in phase system I. The position of the respective peaks for these two hemoglobins clearly reflects the lower K of pig hemoglobin as compared to human hemoglobin. The partition coefficients determined in different tubes of the extraction train for each of these hemoglobins is constant, except for the left part of the pig hemoglobin distribution curve. This indicates that the human hemoglobin is reasonably homogeneous as is the pig hemoglobin under the bulk of the curve. The bump in the left end of the pig distribution curve, which is reproducible, is indicative of a minor hemoglobin component which has a lower K than the major part of pig hemoglobin.

In Figure 6A a countercurrent distribution of a mixture of human and pig hemoglobins is depicted. Again 120 transfers in phase system I were completed. Although the two components are not completely separated after this number of transfers on our countercurrent plates, the existence of two major components are indicated by the peak of the curve at the 29th cavity and the shoulder between cavities 31 and 35. Further, the K values increase continuously from left to right, from 0.10 to 0.36, reflecting the heterogeneity of the mixture. Again the lower K's associated with the pig hemoglobin at the very left of the pig hemoglobin distribution (see Figure 6B) are also apparent here. It is clear that the different K's for human and pig hemoglobin determined in single-tube experiments are borne out by countercurrent distribution.

Horse blood contains two hemoglobins which can be separated by chromatography or electrophoresis (Rodnan

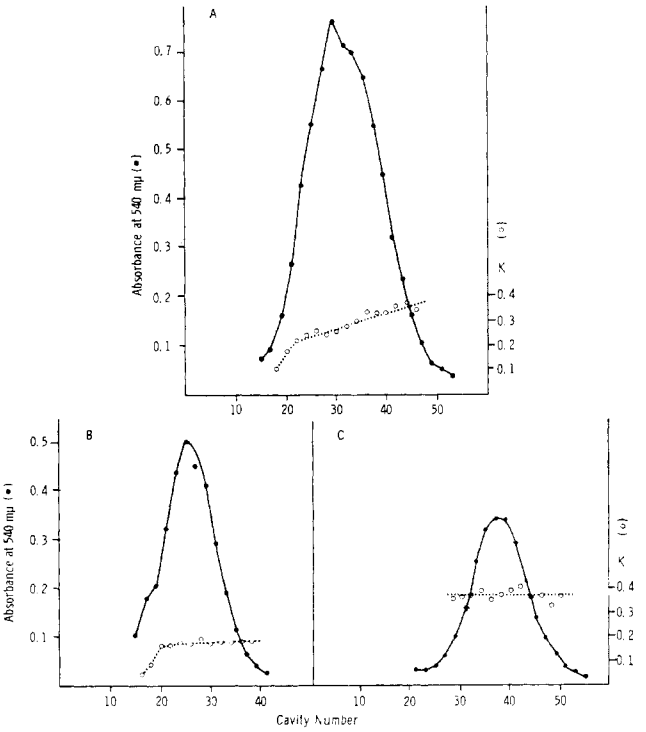

FIGURE 6: Countercurrent distribution curves of human + pig hemoglobins (A), pig hemoglobin (B), and human hemoglobin (C). (●-●) Distribution curve (absorbance at 540 nm). (O-O) Partition coefficient, K, of hemoglobin(s) in different cavities. 120 transfers were completed in phase system I, pH 7.2 (sodium phosphate buffer), at room temperature.

and Ebaugh, 1957; Perutz *et al.*, 1959). Countercurrent distribution of horse hemoglobin yields, however, only a single distribution curve with constant K values in all the cavities. We conclude that horse hemoglobins I and II have identical partition coefficients in these phases.

Conclusion

The basis for the large differences in partition of hemoglobins from various sources remains unresolved, but it is clear that factors other than molecular weight are involved. While the primary structures of human and rabbit hemoglobins differ to the greatest extent among the proteins studied (*i.e.*, at least 16% of their amino acid sequence (Naughton and Dintzis, 1962; Braunitzer *et al.*, 1964)), the greatest difference in partition is between human and pig hemoglobins (which differ by at least 11%). Further, certain conformational changes (*i.e.*, oxy- and deoxyhemoglobins A and S) are shown to be without effect on the partition as are changes produced in a number of hemoglobin derivatives.

The partition of materials in two-polymer phase systems depends on the interaction of water, the partitioned substance, the polymers and the ions present. This interaction is not understood at present. Thus subtle differences between the proteins in primary, secondary or higher dimensional structures as well as molecular shape in the phase system, hydrophobic character, etc., may have a bearing on the partition obtained. An investigation of factors involved in the partition of cells and macromolecules in two-polymer phases is being continued.

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Circular Dichroism Studies of the Flavin Chromophore and of the Relation between Redox Properties and Flavin Environment in Oxidases and Dehydrogenases*

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ABSTRACT: Flavin electronic transitions in the 300–500-nm region have been investigated using absorption and circular dichroism spectroscopy. Data from model flavin compounds in polar and nonpolar solvents, as well as from flavin bound in dehydrogenase-type flavoproteins, indicate the occurrence of a minimum of six vibronic bands corresponding to three $\pi \rightarrow \pi^*$ transitions. No evidence was obtained for $n \rightarrow \pi^*$ transitions. The intensities of the flavin circular dichroism bands are increased by acetylating the side-chain hydroxyl groups and by the environment provided by the binding site of the flavoprotein. The magnitude and sign of the rotational strength of the long-wavelength vibronic bands is particularly sensitive to the interaction of the ribityl hydroxyl groups with the isoalloxazine ring. This interaction is greatly increased when flavin is bound to protein. An analysis of the band positions and rotational strengths of the vibronic transitions in the visible circular dichroism spectra of the oxidized, semiquinone, and hydroquinone forms of several flavoprotein dehydrogenases indicates that the flavin environments in these proteins are quite similar. Such a correlation is also found to exist for some flavoprotein oxidases, although the nature of the flavin–protein contacts in these enzymes are clearly different from those in the dehydrogenases. Thus, circular dichroism spectroscopy provides a useful probe of the relations between flavoenzyme redox properties and the interactions which occur within the coenzyme binding site.

The spectral properties of the flavin chromophore (isoalloxazine) have been the subject of several theoretical and experimental studies (Fox *et al.*, 1967; Weber, 1966; Tollin, 1968; Kurtin and Song, 1968; Song, 1969). The information gained from this work is relevant to an understanding of protein–flavin interactions and to the relation between flavin environment and enzymic properties in flavoenzymes.

In most cases, the binding of flavin to a protein moiety results in wavelength shifts in the 300–500-nm region of the absorption spectrum. A partial resolution of the 450-nm band into three bands is also observed in many flavoproteins (Penzer and Radda, 1967). Similar spectral shifts and increases in resolution are found upon dissolving unbound flavins in nonpolar solvents (Harbury *et al.*, 1959; Kotaki *et al.*, 1967).

The shoulder which appears on the long-wavelength side of the 450-nm band has been attributed to an $n \rightarrow \pi^*$ transition (Kotaki *et al.*, 1966). However, since the polarization of the flavin fluorescence is constant across this absorption region, the resolved bands have alternatively been ascribed to vibronic structure (Weber, 1966). Fluorescence polarization is also constant across the 365-nm band (Weber, 1966), suggesting that only a single transition occurs in this spectral region. On the other hand, molecular orbital calculations (Fox *et al.*, 1967) and circular dichroism and magnetic circular dichroism spectra (Tollin, 1968) indicate the presence of a third $\pi \rightarrow \pi^*$ transition at approximately 340 nm. Recent fluorescence polarization measurements (Kurtin and Song, 1968) and circular dichroism studies (Miles and Urry, 1968) have provided evidence for the presence of still another transition (possibly $n \rightarrow \pi^*$) at 300 nm.

The present work represents an extension of the analysis of the circular dichroism and absorption spectra of free flavin (Tollin, 1968) and of a number of flavoenzymes. Inasmuch as the isoalloxazine ring is optically inactive, any circular dichroism bands observed are due to the asymmetric environment provided by the optically active ribityl side chain and, in the case of flavoenzymes, also to the asymmetric environment of the protein moiety. Due to a high degree of conformational mobility of the side chain, the circular dichroism spectra observed with unbound FMN and riboflavin reflect an average of the various interactions which can occur between the side chain and the isoalloxazine ring. This permits the simultaneous occurrence of both positive and negative dichroic bands for the same transition, and is probably responsible for the weak circular dichroism bands which are observed (Tollin, 1968). The low rotational strength (particularly in the 450-nm region) makes definitive interpretation quite difficult. An increase in rotational strength (5–20-fold) is observed upon binding the flavin to its site on a protein[1] or by increasing

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[1] Similar effects are observed in the circular dichroism spectra of tryptophan analogs and the tryptophyl residues of chymotrypsinogen (Strickland *et al.*, 1969).