Partition Behavior of Amino Acids and Small Peptides in Aqueous Dextran–Poly(ethylene glycol) Phase Systems†

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ABSTRACT: Partition coefficients of seven free amino acids, three dinitrophenylated amino acids, and ten peptides were examined at different pH's in aqueous dextran–poly(ethylene glycol) phase systems containing either sodium chloride or sodium sulfate. It has previously been shown that when, in the case of proteins, partition coefficients so obtained are plotted *vs.* pH, two curves are obtained (one for each salt system) which cross close to the isoelectric point of the respective proteins. In addition a relation between the partition coefficient (K) at the isoelectric point and the protein's molecular weight is in evidence for a number of non-hemoproteins with the smaller molecule having the higher K value. The K's of simple amino acids are close to 1 (0.9–1.1) at any pH and in phases containing either sodium chloride or sodium sulfate. There are no cross-partition curves or cross-points. Acidic and basic amino acids display some tendency toward pH-dependent partitions as do di- and tripeptides. Carnosine (β-alanylhistidine), a dipeptide, and the octapeptide oxytocin (molecular wieght about 1000) show clear cross-partition curves with a cross-point K of 0.87 at pH 7.6 for carnosine and a K of 1.7 at pH 6.7 for oxytocin. Thus, if cross points are obtained with small molecules they cross, as in the case of proteins, close to the material's isoelectric point. The partition coefficients obtained for amino acids and small peptides indicate that the previously described relation of decreasing partition coefficients (at the cross-point) with increasing molecular weight found for a number of non-hemoproteins does not hold below a certain molecular weight. Dinitrophenylation of amino acids affects partition markedly possibly due to the increased hydrophobicity of such derivatives. Higher partition coefficients are obtained in sodium sulfate phase systems for dinitrophenylated amino acids, a possible consequence of the unequal partition of sodium sulfate itself in these phases which, in turn, may result in the top phase being even more hydrophobic than the bottom phase. When comparing the partition of the basic amino acid arginine and of Dnp-arginine, it appears that the hydrophobicity of the latter outweighs its basic character in determining partition (arginine has a higher partition in the sodium chloride phase while the dinitrophenylated derivative has a higher partition in the sodium sulfate system).

Aqueous solutions of dextran and of poly(ethylene glycol) when mixed above certain concentrations yield liquid two-phase systems with a poly(ethylene glycol)-rich upper phase and a dextran-rich lower phase (Albertsson, 1971). Such systems can be buffered and made isotonic and have proved useful not only for the separation of cells, particles, membranes, and macromolecules (Albertsson, 1970, 1971; Walter, 1969; Miller and Walter, 1971; Brunette and Till, 1971; Walter and Sasakawa, 1971; Walter *et al.*, 1972a,b) but also in providing information on the surface properties of the materials being partitioned (Walter *et al.*, 1967, 1972a,c; Walter and Sasakawa, 1971; Walter and Selby, 1966).

Since some inorganic salts partition unequally between the phases (Johansson, 1970), an electrical potential arises between them (Albertsson, 1971; Reitherman *et al.*, 1973). Furthermore, a ζ potential between the two phases has been demonstrated by electrophoresis of droplets of one phase in the other (Seaman and Walter, 1971). The magnitude and sign of the potential depend greatly on the ionic composition of the phase system examined. Conversely, the partition of materials in these phases depends greatly on their charge (Walter *et al.*, 1967; Brooks *et al.*, 1971; Albertsson *et al.*, 1970). In the case of proteins, the considerable dependence of partition on salt composition of the phase is virtually eliminated at the isoelectric point (Albertsson *et al.*, 1970), a phenomenon that can be used to determine the isoelectric point of a protein by partition at different pH's in phases containing one of two different salts (Albertsson *et al.*, 1970; Walter and Sasakawa, 1971; Sasakawa and Walter, 1971, 1972). The curves obtained when plotting the partition coefficients obtained in the phases containing the two different salts *vs.* pH cross at the protein's isoelectric point (cross-point). The absolute K value obtained for protein depends on additional parameters including molecular weight (Sasakawa and Walter, 1972); perhaps conformational properties and hydrophobic–hydrophilic interactions between the protein and the phases are also involved (Sasakawa and Walter, 1972; Albertsson, 1971), since species-specific differences in the partition coefficients at the cross-point have been found in the case of hemoglobins (Walter and Sasakawa, 1971), hen and turkey egg-white lysozymes, and β-galactosidases from two different *Escherichia coli* strains (Sasakawa and Walter, 1972).

In the present paper we have extended our previous investigations on the partition of proteins (Walter and Sasakawa, 1971; Sasakawa and Walter, 1972; Walter *et al.*, 1972b) to the structural components of proteins (*i.e.*, to small peptides and amino acids). We hoped thereby to gain additional in-

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formation on the relation between (a) molecular weight and partition coefficient (Sasakawa and Walter, 1972), (b) molecular size and cross-partition, and (c) some indication of possible hydrophilic–hydrophobic interactions in the determination of the partition coefficient (Albertsson, 1971). An understanding of the nature of the interaction of the partitioned material with water, polymers, and salts constituting the phase system is useful since it may provide information on the (surface) properties of the partitioned substance.

Methods

Amino Acids. ^{14}C-Labeled amino acids such as glycine, serine, phenylalanine, tryptophan, tyrosine, glutamic acid, and arginine were obtained from CEA (Commissariat a l'Energie Atomique, France). All labeled amino acids were checked for purity by paper chromatography (*n*-butyl alcohol–acetic acid–water, 4:1:5) and radioassay.

Dinitrophenylated amino acids (Dnp-Gly, Dnp-L-Asp, and Dnp-L-Arg) were purchased from Sigma Chemical Co., St. Louis, Mo. Peptides Gly-Gly, Gly-Gly-Gly, Gly-Glu, Gly-Lys, Ac-Gly-Gly-NH_2, L-Ala-L-Ala-L-Ala, D-Ala-D-Ala-D-Ala, L-carnosine, oxidized glutathione, and oxytocin (synthetized) were obtained from Sigma Chemical Co., St. Louis, Mo.

Chemicals. All salts were of analytical grade.

Preparation of Phase Systems. Aqueous dextran–poly(ethylene glycol) phase systems, buffered and containing certain salts, were used in this work. Dextran T500 (lots 3202 and 5996) and dextran T40 (lot 2514) were obtained from Pharmacia Fine Chemicals, N. J., or Uppsala, Sweden. Poly(ethylene glycol) was obtained under the trade name Carbowax 4000 or 6000 from Union Carbide, New York, N. Y.

In the present study the methods of preparation were as described previously (Albertsson *et al.*, 1970; Walter and Sasakawa, 1971; Sasakawa and Walter, 1972). Stock solutions were made of dextran T500 (20%, w/w), poly(ethylene glycol) 6000 (40%, w/w), 0.4 M NaCl, 0.2 M Na_2SO_4, and a series of 0.04 M buffers (glycine or sodium phosphate) spanning the pH range from 3.5 to 11. A mixture containing 14% (w/w) dextran and 8.8% (w/w) poly(ethylene 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) poly(ethylene glycol), 1 g of 0.4 M sodium chloride solution *or* 0.2 M sodium sulfate solution containing certain amounts of amino acid or peptide and 1 g of 0.04 M buffer were weighed into small centrifuge tubes. The entire mixture was well agitated. The final phase systems had, in addition to amino acids or peptides, the following compositions: 7% (w/w) dextran, 4.4% (w/w) poly(ethylene glycol), 0.1 M sodium chloride and 0.01 M glycine or phosphate buffer (system I); 7% (w/w) dextran 4.4% (w/w) poly(ethylene glycol), 0.05 M sodium sulfate and 0.01 M glycine or phosphate buffer (system II). The phase systems were centrifuged at room temperature for 10 min at 1200*g* to speed phase settling.

In an analogous manner phase systems containing 13% (w/w) dextran T40 and 9% (w/w) poly(ethylene glycol) 4000 were made. Phase system III contained the same salt and buffer composition as phase system I. Phase system IV corresponded in salt and buffer composition to phase system II.

Determination of Partition Coefficients of Peptides. The partition coefficient, *K*, is defined as the ratio of sample concentration (or absorbance) in top phase to sample concentration in the bottom phase. Top phase (0.5 ml) and bottom phase (0.5 ml) were carefully pipetted from the phase system in each tube, and each was diluted by addition of 2.0 ml of water. The solution was mixed and the absorbance was measured at 220 nm against a top or bottom phase blank on a Gilford (Model 240) or Zeiss PMQ II spectrophotometer.

Determination of Amino Acid Partition Coefficients. Partition coefficients (*K*) of amino acids were determined by counting of ^{14}C activity in a Packard Tri-Carb liquid scintillation counter; 0.1 μCi of ^{14}C in 0.01 ml of amino acid was used in each case. After settling of the phases, 0.1 ml of top or bottom phase was carefully put on paper disks in vials, then dried at 75° overnight. Scintillation fluid (10 ml) (Walter *et al.*, 1972a) was added to each vial. Counts in top or bottom phase were corrected by using standard curves obtained with amino acids containing known amounts of ^{14}C and counted with or without top or bottom phase. The ratio of counts (after correction) in top–bottom phase gives the partition coefficient (*K* value) of the amino acid in the polymer two-phase system.

Determination of Partition Coefficients of Dinitrophenylated Amino Acids. *K* values of dinitrophenylated amino acids were determined by absorbance at 360 nm. That is, 0.5 ml of top or bottom phase was put into 2.0 ml of water, the solution was mixed, and absorbance was measured against a top or bottom phase blank. The purity of dinitrophenylated amino acids was checked by measuring the ratio of their absorbance (360/340 nm).

Results and Discussion

Partition of Amino Acids. The partition coefficient of amino acids was obtained by use of [^{14}C]amino acids and determining the distribution of the radioactivity in the top and bottom phase of selected two-polymer aqueous systems. The ratio of radioactivity per unit volume in the top phase/radioactivity per unit volume in the bottom phase gives the partition coefficient (*K*). Counting efficiency was found to be affected by the presence of the polymers constituting the phases: top phase [poly(ethylene glycol) rich)] affected the count less than bottom phase (dextran rich). Correction factors (see Methods section) were, hence, applied to the counts obtained in the presence of phase polymers.

Amino acids selected for study were glycine, as a representative of neutral, simple amino acids; serine, a hydroxylamino acid; glutamic acid, an acidic amino acid; arginine, a basic amino acid; and phenylalanine, tyrosine, and tryptophan as aromatic amino acids. In no case was a concentration dependence (between 0.025 and 0.5%) of amino acids (or peptides) on the partition coefficient in evidence.

Most of the observed *K* values for amino acids (except tyrosine) are close to 1.0 (range 0.9–1.1) between pH 3 and 11 in both phase system I (sodium chloride) and phase system II (sodium sulfate). While amino acids are charged materials their partitions appear only slightly affected by ionic composition and pH as depicted in Figures 1–3. Negatively charged proteins have higher partition coefficients in phases containing sodium sulfate rather than sodium chloride while the reverse holds for positively charged proteins (Albertsson *et al.*, 1970; Walter and Sasakawa, 1971; Sasakawa and Walter, 1972). When the partition coefficients, *K*'s, of proteins obtained in phases containing either sodium chloride or sulfate are plotted as a function of pH one obtains two curves which cross close to the protein's isoelectric point (Albertsson *et al.*, 1970). While amino acids also have their characteristic

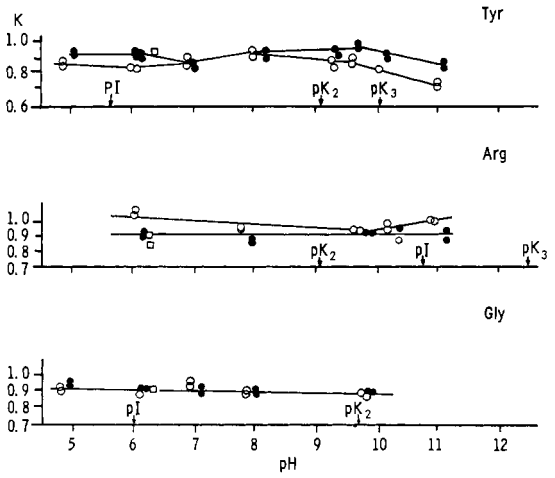

FIGURE 1: Partition curves of amino acids. Partition coefficients, K's, in phase system I (○, containing sodium chloride) and in phase system II (●, containing sodium sulfate) are plotted as a function of pH. □, indicates K's in a phase system devoid of salt.

isoelectric point(s) no cross-partition curves or cross-points are obtained. It is therefore likely that a minimum molecular size is required for the necessary interaction between partitioned material, water, polymers, and salts that gives rise to cross-partition curves.

The neutral amino acids, glycine and serine, have quite similar K values in phase systems I and II as well as in one containing no salt (Figures 1 and 2). There is some tendency for the partition of basic and acidic amino acids to be affected by the ionic composition and by pH (Figures 1 and 2). Arginine has a higher K value in phase system I (sodium chloride) at pH 6–11 than in phase system II. Glutamic acid has a higher K value in this phase system below pH 3.

There is some indication that amino acids with hydrophilic character give higher K values in phase system I (sodium chloride) and amino acids with hydrophobic properties have higher K's in phase system II (sodium sulfate) as shown in Figures 1 and 3 (tyrosine, tryptophan, phenylalanine) and discussed further below for dinitrophenylated amino acids.

Amino acids which have two pK's (pK_1, pK_2) seem to have simple linear partition curves when their partition coefficients are plotted *vs.* pH (see glycine, serine, phenyalanine). Amino acids which have three pK's appear to show greater differences in their partition coefficients in phase systems I and II at different pH's (see arginine, glutamic acid, tyrosine).

Partition of Dinitrophenylated Amino Acids. Dinitrophenylation confers considerable hydrophobicity on an amino acid. To probe the relation between hydrophobic character and

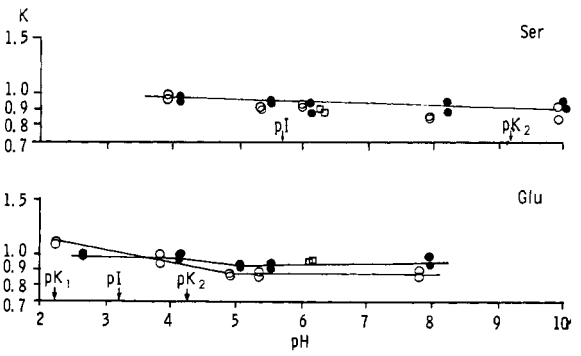

FIGURE 2: Partition curves of amino acids. See caption to Figure 1.

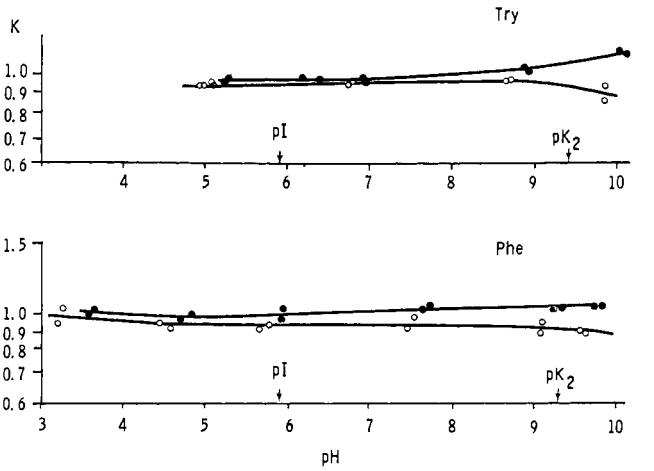

FIGURE 3: Partition curves of amino acids. See caption to Figure 1.

behavior in two-polymer aqueous phases, a number of dinitrophenylated amino acids (glycine, aspartic acid, arginine) were partitioned in phase systems I and II at different pH's. The partition coefficients of dinitrophenylated amino acids are similar to those of the corresponding free amino acid in phase system I (sodium chloride). They are, however, markedly higher in phase system II (sodium sulfate) than the K's found in this phase for the corresponding amino acids. As shown in Figure 4, K's of Dnp-glycine are close to 1.0 in phase system II instead of 0.9 (see Figure 1). Hence, hydrophobic character appears to be reflected to a greater extent in the sodium sulfate than the sodium chloride system. This may be related to the fact that sodium chloride itself partitions almost equally between the phases while sodium sulfate favors the bottom (dextran-rich) phase (Johansson, 1970). Hence the relatively greater hydrophobic character of the upper phases (Albertsson, 1971) may be still further increased. Dnp-arginine and Dnp-aspartic acid partitions show no pH dependence at all (Figure 4). Free arginine and glutamic acid partitions do display some variation with pH (Figures 1 and 2).

Species-specific differences in the partition of some proteins have been described (Walter and Sasakawa, 1971; Sasakawa and Walter, 1972) even though the molecular weights of the corresponding proteins examined from different species were similar. The difference in partition may thus be, in part, a

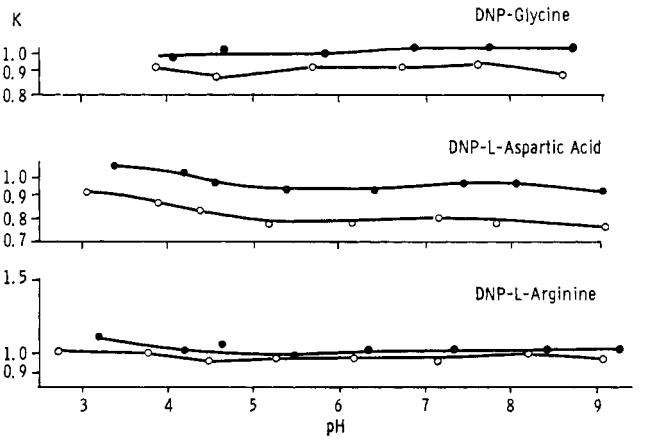

FIGURE 4: Partition curves of dinitrophenylated amino acids. Partition coefficients, K's, in phase system I (○) and in phase system II (●) are plotted as a function of pH.

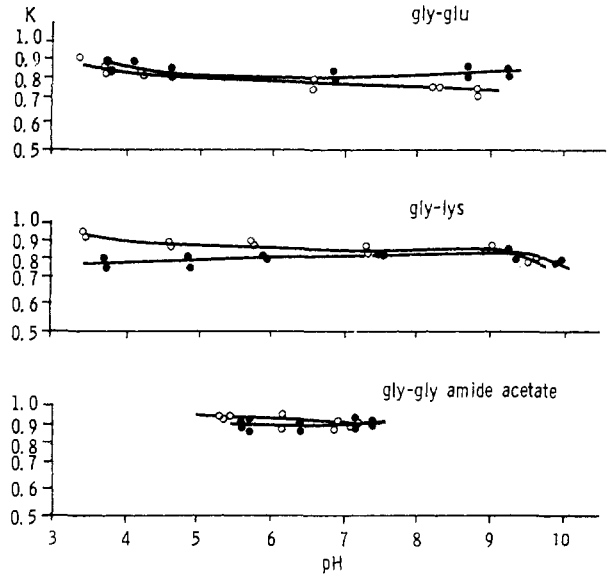

FIGURE 5: Partition curves of glycyl peptides. Partition coefficients, K's, in phase system I (○) and in phase system II (●) are plotted as a function of pH.

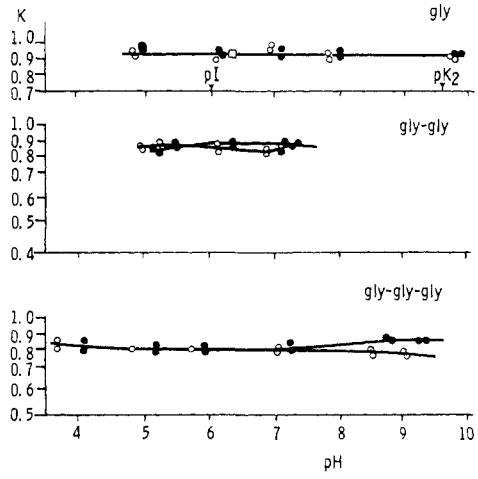

FIGURE 6: Partition curves of glycyl peptides. See caption to Figure 5. Open square indicates the K value in a phase system devoid of salt.

reflection of a species-specific difference in the hydrophobic–hydrophilic amino acid content at the molecular surface exposed to the phases. For example, the quantity of tyrosine,

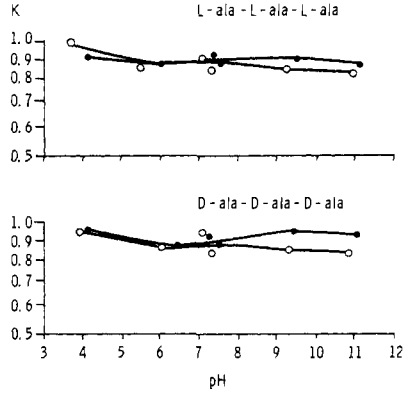

FIGURE 7: Partition curves of L- or D-alanylalanylalanine, Partition coefficients, K's, in phase system I (○) and phase system II (●) are plotted as a function of pH.

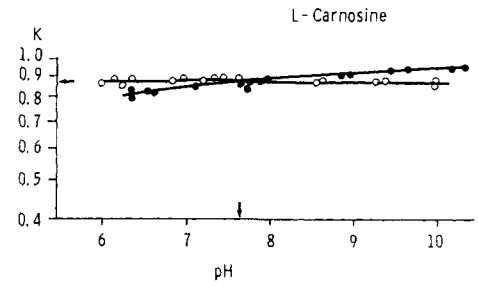

FIGURE 8: Partition curves of carnosine (β-alanylhistidine). Partition coefficients, K's, in phase system I (○) and in phase system II (●) are plotted as a function of pH. Arrows indicate K and pH at the cross-point.

tryptophan, phenylalanine, valine, and leucine on the outside of native hemoglobin α-helical regions A, B, F, G, and H is larger in human than in pig hemoglobin (Braunitzer *et al.*, 1964). K value of human adult hemoglobin is appreciably greater than that of pig (Walter and Sasawaka, 1971). The factors that affect partition are probably many but hydrophobic character of the partitioned molecular species does appear to contribute to it.

Partition of Small Peptides. To gain additional insights into the factors that affect partition of proteins especially with regard to the smallest required molecular weight that will yield cross-partition curves, a number of small peptides were studied. These included glycylglutamic acid, glycyllysine, glycylglycine, glycylglycylglycine, glycylglycinamide, L-carnosine (β-alanylhistidine), oxidized glutathione, D- or L-alanylalanylalanine, and (synthetic) oxytocin.

The K values of glycyl peptides in phase systems I and II are 0.9 for glycylglycine and 0.83 for glycylglycylglycine. These are slightly lower than the K of free glycine (0.93) shown in Figure 5. K's of glycyl peptides are fairly similar in phase systems I and II, although a slight pH dependence is discernible (Figures 5 and 6), particularly at the lower pH range in the case of the basic peptide glycyllysine and at the upper pH range for glycylglycylglycine and the acidic peptide glycylglutamic acid. No clear indication of a cross-point is present, however, in any of these partition diagrams (Figures 5–7).

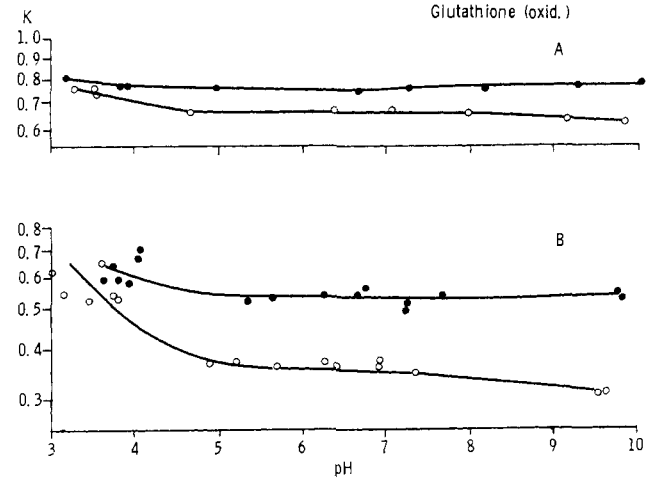

FIGURE 9: Partition curves of oxidized glutathione. Partition coefficients in part A show K's in phase system I (○) and in phase system II (●) plotted as a function of pH. Part B indicates the corresponding partitions in phase systems III and IV, respectively, For details, see text.

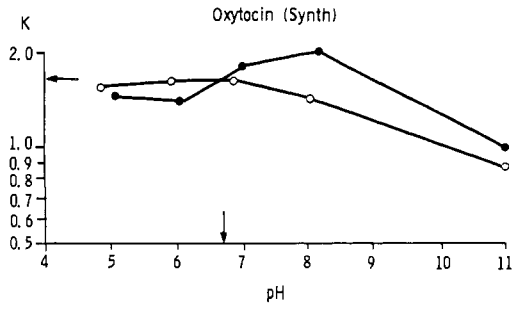

FIGURE 10: Partition curves of oxytocin. Partition coefficients, K's, in phase system I (○) and in phase system II (●) are plotted as a function of pH. Arrows indicate K and pH at the cross-point.

Since the phases contain D-dextran a difference in partition behavior of D and L substances is conceivable. As shown in Figure 7 the K values obtained with D- and L-Ala-Ala-Ala are the same.

Carnosine (β-alanylhistidine) is the smallest peptide studied (mol wt 226) that has a cross-partition point (see Figure 8). The cross-point pH of 7.6 is reasonable for carnosine's isoelectric point (see previous papers by Albertsson *et al.*, 1970; Walter and Sasakawa, 1971; Sasakawa and Walter, 1972; Walter *et al.*, 1972b). The presence of a cross-point for the small carnosine molecule may be due to the presence of histidine or may, in some manner, be related to the structure of biologically active molecules. Glutathione also appears to have a cross-point when partitioned in phase systems I and II (Figure 9A). However, oxidized glutathione is quite acidic and we were not able to obtain phase systems with a pH below 3. In Figure 9B, just for illustration, we have also partitioned glutathione in phase systems III and IV. Quite generally the differences apparent for substances partitioned in phase systems I and II are increased when they are partitioned in phases containing the same ionic composition but polymer(s) of lower molecular weight. In the latter case the cross-partition curves often intersect at a sharper angle (experiments on this will be presented at greater length in a subsequent communication).

The physiologically active peptide oxytocin, molecular weight approximately 1000, gives a cross-partition point at pH 6.7. K value at the cross-point is 1.7 (Figure 10). We have previously described a relation between partition coefficients at the respective isoelectric points of non-hemoproteins and the molecular weights (above mol wt 6000) of proteins (Sasakawa and Walter, 1972). Increasing molecular weights were found, for a number of proteins, to result in decreasing partition coefficients. In the case of oxytocin, the other investigated small peptides and the amino acids, the partition coefficients are uniformly lower than would be expected if the indicated relation between K and molecular weight previously described were extrapolated linearly to zero molecular weight. Hence, molecular weight appears not to be a significant factor in determining the partition coefficient of materials below a certain molecular weight.

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