Northrop, D. B., and Wood, H. G. (1969b), *J. Biol. Chem. 244*, 5820.
Peck, R. L., Wolf, D. E., and Folkers, K. (1952), *J. Amer. Chem. Soc. 74*, 1999.
Perrin, D. D., and Sharma, V. S. (1966), *J. Inorg. Nucl. Chem. 28*, 1271.
Schneider, P. W., Brintzinger, H., and Erlenmeyer, H. (1964), *Helv. Chim. Acta 47*, 992.
Sigel, H. (1967), *Chimia 21*, 489.
Sigel, H. (1968), *Eur. J. Biochem. 3*, 530.
Sigel, H. (1969), *Eng. Publ. Off., Univ. Ill. 67*, 55.
Sigel, H., Becker, K., and McCormick, D. B. (1967), *Biochim. Biophys. Acta 148*, 655.
Sigel, H., Griesser, R., Prijs, B., McCormick, D. B., and Joiner, M. G. (1969a), *Arch. Biochem. Biophys. 130*, 514.
Sigel, H., and McCormick, D. B. (1970), *Accounts Chem. Res. 3*, 201.
Sigel, H., McCormick, D. B., Griesser, R., Prijs, B., and Wright, L. D. (1969b), *Biochemistry 8*, 2687.
Sillén, L. G., and Martell, A. E., Ed. (1964), *in* Stability Constants of Metal Ion Complexes, Special Publication No. 17, London, The Chemical Society, Burlington House.
Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965), *J. Chem. Phys. 43*, 3123, 3133.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Weijlard, J., Purdue, G., and Tishler, M. (1954), *J. Amer. Chem. Soc. 76*, 2505.
Wright, L. D., Cresson, E. L., Skeggs, H. R., Wood, T. R., Peck, R. L., Wolf, D. E., and Folkers, K. (1950), *J. Amer. Chem. Soc. 72*, 1048.

Studies of Self-Association and Conformation of Peptides by Thin-Film Dialysis*

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ABSTRACT: The tyrocidine antibiotic polypeptides are known to exhibit self-association. They were therefore used as test substances to document the behavior to be expected by the method of thin-film dialysis. Self-association with these peptides is indicated by concentration dependence, a reverse curvature of the escape plot, and a retardation of the rate of diffusion by addition of salt but an acceleration by the addition of ethanol or other hydrophobic bond breaking solvent additives. A survey of dipeptides by thin-film dialysis has revealed that L-lysyl-L-lysine, L-lysyl-L-arginine, and L-histidyl-L-histidine exhibit a behavior characteristic of self-association in a pH region where all the acidic and basic groups are charged. L-Glutamyl-L-glutamic acid does likewise at a pH in the range where the carboxyl groups are fully charged. L-Arginyl-L-arginine does not appear to show this behavior.

Since thin-film dialysis rates are particularly sensitive to conformational changes, comparative data for a number of di-, tri-, and tetrapeptides in various solvent environments are presented. The results in many cases are those expected from the molecular size and shapes of Corey–Pauling–Koltun molecular models. In other cases the results suggest whether a compact conformation or an extended conformation is preferred.

The so-called quaternary structures of proteins and nucleotides are thought to result from the interplay of various binding forces such as hydrophobic bonds, coulombic forces, π bonds, and hydrogen bonds. The contribution of each individual interaction or small section of the molecule is considered to be relatively weak as compared with most covalent bonds but their sum in concerted effect can be strong. Such a theory agrees well with experience but leaves much to be understood about the detailed nature of each type of interaction and its contribution to the whole. A truly clear understanding is made uncertain by the many variables involved in the study of the larger molecules. There is also a corresponding vagueness about the relative contribution each of these forces makes in the determination of tertiary structure.

In the attempt to simplify the experimental approach to the development of more definite concepts it would seem logical to turn to the more simple problem presented by smaller molecules. Here, on the basis of present theory, it is to be expected that specificity would be much reduced and that the interactions would be much weaker. For example, no enzyme or antibody containing less than 100 or more amino acid residues has been discovered. Nonetheless, it could be worthwhile to study smaller molecules provided meaningful techniques could be found and applied to selected models of well-known structure.

Techniques available at present for this type of study include among others ultracentrifugation, nuclear magnetic

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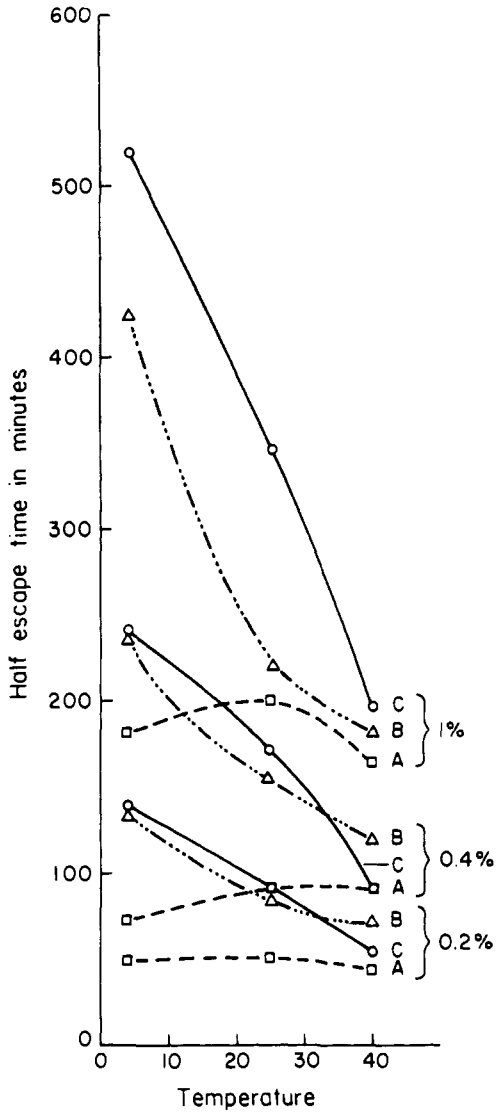

FIGURE 1: Plots of half-escape times against temperature with tyrocidines A, B, and C at three different starting concentrations. The values given have been corrected for viscosity and temperature to 25° assuming direct proportionality to a change in absolute temperature and inverse proportionality to a change in viscosity.

resonance, optical rotatory dispersion, circular dichroism, ultraviolet spectra, infrared spectra, proton exchange, and thin-film dialysis. The use of various combinations of these techniques is being explored in this laboratory (Williams, 1968; Ruttenberg *et al.*, 1966; Stern *et al.*, 1968, 1969; Craig, 1968; Laiken *et al.*, 1969a,b). These preliminary studies and those in other laboratories indicate the need for study by widely differing techniques. Thus a spectroscopic technique such as nuclear magnetic resonance can give detailed information concerning the orientation of a specific covalent linkage in a specific solution environment (Stern *et al.*, 1968) while thin-film dialysis can give information about overall diffusional size and/or shape in a specific solvent environment (Craig, 1964). One of the advantages of this technique is that it permits critical study in very dilute solution.

The selection of model solutes for this type of investigation is an equally important aspect. The polypeptide antibiotics which are of the order of one-tenth the molecular size of the smaller proteins offer certain advantages over other peptides because of the conformational restrictions imposed by their covalent ring structures (Craig, 1964). This advantage has more recently been recognized by other workers in conformational studies (Vanderkooi *et al.*, 1966; Balasubramanian, 1967; Liquori and Conti, 1968; Quadrifoglio and Urry, 1967).

Still smaller models should also be of great interest if definite intermolecular interactions can be demonstrated. Here the thin-film dialysis technique is an excellent tool since membranes can be made easily which will either pass or exclude solutes as small as amino acids (Craig and Ansevin, 1963). A recent survey of available dipeptides has revealed a number which exhibit the characteristic deviation from ideal behavior which larger peptides and proteins, known to associate, exhibit (tyrocidine, Ruttenberg *et al.*, 1966; insulin, Craig *et al.*, 1960; glucagon, Craig *et al.*, 1965; hemoglobin, Guidotti and Craig, 1963). The behavior of some of these small peptides in thin-film dialysis will be described in this paper.

Methods and Materials

Tyrocidine B was obtained by countercurrent distribution (Williams and Craig, 1967) of a sample of crude tyrocidine supplied by the Wallerstein Co. We thank them for this material. L-Lysyl-L-α-lysine trihydrochloride was purchased from the Cyclo Chemical Co. Its purity and identity were studied by paper electrophoresis and by Sephadex chromatography. A single spot or effluent curve occurring in the expected region was obtained. Its identity was also conclusively established by nuclear magnetic resonance spectroscopy with a Varian 220-MHz spectrometer. Only the proton resonances expected on the basis of the dipeptide formula were found. According to information obtained from the Cyclo Chemical Co., it has been synthesized by coupling dicarbobenzoxylysine with mono-ϵ-carbobenzoxylysine methyl ester and subsequent removal of the protecting groups. The other dipeptides were also obtained from the Cyclo Chemical Co.

The alanine tetrapeptides in Table VI were gifts from Drs. M. Sela and I. Schecter of the Weisman Institute, Rehovoth, Israel. We wish to thank them for the samples.

The dialysis experiments were carried out as previously described (Craig and Konigsberg, 1961) in an acetylated membrane made from size 18 seamless cellulose Visking tubing. The acetylation was such as to provide a half-escape time of 150 min at 25° in 0.01 N acetic acid with L-leucyl-L-tyrosine or with a second membrane to provide a 50-min half-time. Analyses were made generally by optical density measurement at either 225 mμ or with L-leucyl-L-tyrosine at 280 mμ. In certain cases the ninhydrin reagent was used. In others the optical density results were checked by ninhydrin. No result was considered acceptable unless the recovery totaled 100 $\pm$ 5%. In certain cases absorption on the membrane was noted by an initial low recovery which could be corrected by further elution of the membrane. Adsorption can give an anomalous escape time (Craig and Ansevin, 1963). Where this occurred it is so designated in the tables. For each determination a sample of 0.5–10 mg of peptide was taken. Thus the initial concentration was in the range of 0.003–0.05 M since the retentate volume was 0.5 ml.

TABLE I: Fifty Per Cent Escape Times in Minutes of Tyrocidines A, B, and C at Various Temperatures and Starting Concentrations.

Conditions	1%			0.4%			0.2%		
	A	B	C	A	B	C	A	B	C
4°, 0.1 N HAc	182	425	520	73	236	241	50	133	140
15°, 0.1 N HAc					153				
25°, 0.1 N HAc	200	220	346	92	155	173	51	83	92
40°, 0.1 N HAc	165	182	198	90	119	92	44	71	55

Throughout this paper, except in Table I and column 2 of Table IV, where comparative half-escape times with different solvents and temperatures are given the values have been corrected for temperature and viscosity to 25° and the viscosity of water using the Stokes–Einstein equation as is done in free diffusion. It is recognized that this does introduce a measure of uncertainty as regards quantitative comparisons. As far as we have been able to draw conclusions from comparison of a large number of solutes of known conformational stability, this correction may tend to make the half-escape time somewhat too short in alcohol solutions by roughly 10–20%. Conclusions involving the use of alcohol are therefore made only when differences in comparative values are much larger than this.

Results and Discussion

Very early in our studies with membrane diffusion (Craig *et al.*, 1957) it was postulated that with solutes exhibiting known self-association behavior, a variety of escape patterns could be obtained depending on the type of association involved, the solvent system, the porosity of the membrane, the concentration, and the temperature. Most of the polypeptide solutes which showed the characteristic aggregation were proteins. Thus insulin (Craig *et al.*, 1960) showed definite nonideal behavior and hemoglobin (Guidotti and Craig, 1963) was shown to behave as a dissociable heteroaggregate.

The dialysis behavior characteristic of dissociating systems includes the following. (1) A dialysis rate markedly slower on the basis of its formula molecular weight than that expected, together with either a positive or negative deviation from a straight line in the escape plot as shown in Figures 1 and 2. (2) A half-escape time varying widely with different initial concentrations as shown in Table I and Figure 1. (3) Marked acceleration of the dialysis rate by addition of organic solutes known to repress hydrophobic bond formation, *e.g.*, alcohols, urea, dimethylformamide, etc., or any dissociating agent as shown in Table II. With linear polypeptides not cross-linked by covalent bonds these solvents usually give a marked decrease in the dialysis rate in the absence of association, an effect strikingly different from that observed with dissociable solutes. (4) A definite decrease in the dialysis rate when a salt is added to the solvent. (5) Unexpectedly large temperature coefficients.

Although the rules enumerated appear to hold for peptides where conformation and hydrophobic interaction play a dominant role they may not hold for more complicated systems such as proteins. Thus alcohol may cause certain proteins to associate and increasing the ionic strength may favor dissociation of an associating system.

TABLE II: Effect of Various Alcohols on the Dialysis Rate of Tyrocidine B at 25°.

Solvent	T/2 in min	T/2 Corrected for Viscosity
0.1 N Acetic acid	245	
10% Methanol–0.1 N acetic acid	120	97
10% Ethanol–0.1 N acetic acid	91	67
10% Propanol–0.1 N acetic acid	92	65
20% Methanol–0.1 N acetic acid	103	68
20% Ethanol–0.1 N acetic acid	59	33
20% Propanol–0.1 N acetic acid	53.3	28
30% Methanol–0.1 N acetic acid	96	55
30% Ethanol–0.1 N acetic acid	45	21
30% Propanol–0.1 N acetic acid	52	22
50% Methanol–0.1 N acetic acid	45	25

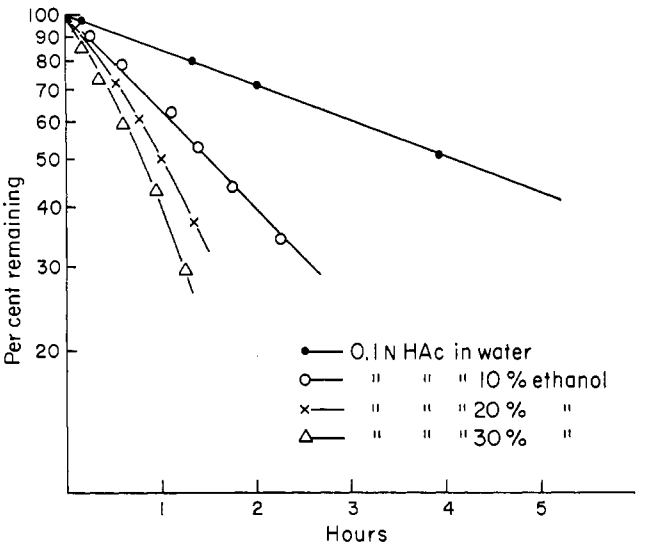

FIGURE 2: Escape plots of tyrocidine B in various alcohol solutions.

TABLE III: Fifty Per Cent Escape Times in Minutes for Peptides in Slower Membranes at 25° in Different Solvent Environments.

Solvent	L-Leu-L-Tyr	L-Tyr	L-Leu-L-Ser	L-Leu-L-Leu	L-Arg	L-Arg-L-Arg
H_2O	150		135	140	30	73
0.01 N HAc (pH 3.5)	138	29	108			
25% Methanol	125		123			
	L-Lys-L-Lys	L-His-L-His	L-Glu-L-Glu	L-Lys-L-Lys-L-Lys		
H_2O	>1320	147[a]	267	>900		
0.01 N HAc (pH 3.5)	1320	415	180			
0.1 M NaCl		270				
0.01 M Borate (pH 8.5)	110					
0.01 M Borate (pH 8.5)–0.1 M NaCl	870					
0.01 M PO_4 (pH 7.52)	540					
0.1 N Propionic acid	780					

[a] A certain amount of adsorption on the membrane was observed.

The problem of interpreting dialysis data in the case of nonideality can be more complicated with a linear peptide capable of undergoing a marked conformational change in the different solvent environments and self-association as well. Here one effect may tend to offset the other. An estimation of the molecular weight by the ultracentrifuge in this case is particularly helpful if it can be made.

The smaller polypeptide antibiotics, gramicidin S-A, bacitracin A, polymyxin B_1, actinomycin D, and viomycin all showed near-ideal behavior in aqueous solution and gave straight-line escape plots consistent with their known molecular size. In this group the tyrocidines were outstanding exceptions. In fact, membranes of porosity suitable for studying the substances listed above would prevent diffusion of the tyrocidines almost completely except in strongly dissociating solvents (Ruttenberg *et al.*, 1966). This was not surprising since in the original characterization work (Battersby and Craig, 1952) the true monomeric molecular weight of a tyrocidine could be clearly established only by the method of partial substitution. Diffusion studies (Pedersen and Synge, 1948) and the ultracentrifuge also indicated definite association.

In any case the addition of ethanol markedly accelerates dialysis of the tyrocidines and gives the expected reverse curvature typical of a dissociating system as shown in Figure 2 and Table II. The concentrations of ethanol used, however, do not completely dissociate the aggregate. It is interesting that the escape pattern in the absence of ethanol is a reasonably straight line which does not indicate concentration dependance. Yet when concentration dependence was studied by varying the initial concentration of the peptide, the strong dependance noted in Figure 1 was observed. An explanation of this paradox could be that if the rate of dissociation is of the same order as or slower than the rate of dialysis the escape rate then may be controlled by the rate of dissociation as well as by the diffusional size. This was supported by the observation that a considerably more porous membrane in the absence of alcohol did not give a markedly faster dialysis rate. A time dependency was noted by Williams (1968) in his ultracentrifugal study. The relative effectiveness of methanol, ethanol, and propanol in dissociating tyrocidine B is shown in Table II. Ethanol and propanol are the most effective as would be expected from the theory that they inhibit hydrophobic bonding.

The tyrocidines are rather sparingly soluble in water. They adsorb to cellulosic materials rather strongly from an aqueous solution. However, lowering of the pH and the addition of acetic acid depresses the adsorption. A suitable solvent for thin-film dialysis is 0.1 N acetic acid. This gives satisfactory recoveries in a typical experiment. As shown in Figure 2, a straight-line escape pattern is given at 25° for tyrocidine B.

The membrane in this case was Visking 20 casing. It allowed insulin in 0.1 N acetic acid to dialyze at about the same rate as the tyrocidines under the same conditions. Insulin, however, under these conditions (Craig *et al.*, 1960) is known to exist as a partially dissociated dimer of mol wt 12,000. Gramicidin S-A mol wt 1142, which does not associate (Ruttenberg *et al.*, 1966; Stern *et al.*, 1968), gave a half-escape time of about 10 min. It would seem, therefore, that tyrocidine B in Figure 2 in 0.1 N acetic acid at 25° is dialyzing as if it were an aggregate containing in the range of five or six monomers.

Table I and Figure 1 give data showing the effect of changes of temperature and of starting concentrations in the range of 0.001–0.0075 M for tyrocidines A, B, and C. The order of the tendency to aggregate is C > B > A. This shows the effect of the indole nucleus in the side chain of a residue as compared with a benzene ring. Tyrocidine B differs from A by the replacement of a phenylalanine residue by tryptophan. In C two phenylalanine residues are replaced by tryptophans. In Table I the half-times have been corrected for viscosity and temperature to the viscosity of 0.1 N acetic acid at 25°. The temperature coefficients are of some interest in that for tyrocidine A they are those expected for an ideal solute or perhaps slightly less while with B and C they are consistently much too high.

Much remains to be learned about structures which favor

TABLE IV: Fifty Per Cent Escape Times in Minutes for Peptides in Faster Membranes at 25° and in Different Solvent Environments.

Solvent	L-Leu-L-Tyr		L-Tyr-L-Leu	L-Lys-L-Lys	L-His-L-His	L-Arg-L-Lys	L-Glu-L-Glu	L-Lys	L-Leu-L-Ser	L-Ala-L-Ala
		(40°)								
H_2O	50	22	48	100[a]	53	124	52	20		43
0.01 N HAc (pH 3.5)	53	21	48	381	110	174	40		41	
20% Ethanol–0.01 N HAc	43	32	51	125	98	185	75			
20% Ethanol	44	42	44	93	44	107	105			
0.1 M NaCl	58	42		570	60	275	200			
0.01 N HAc–0.1 M NaCl	58	40	48	590	150	170	110			
0.01 M PO_4 (pH 8.5)	100	53		206	68	120	430		104	
0.01 M Borate (pH 8.5)	101	40		95	73[b]	78[b]	520			
0.01 M Borate (pH 8.5)–0.1 M NaCl	78	34		190	65	110	540			
0.01 M Tris (pH 8.5)	115	94		109	138	76	420			
0.01 M PO_4 (pH 8.5)–20% ethanol	95	93		115	110	148	220			

[a] Reverse curve. [b] Positive curve.

self-association as well as the specific forces involved. The tyrocidines are especially interesting models for this type of study because of their rigidity and comparatively small size. Such a study has been under way in this laboratory for some time and sufficient data have been assembled (Ruttenberg *et al.*, 1966; Williams, 1968; Stern *et al.*, 1969) to indicate that a rigid conformational structure and hydrophobic bonding are probably the most important parameters involved.

If this is true, could there be smaller peptides providing a conformation favorable for the expression of hydrophobic bonding of sufficient strength to be recognized and therefore having possible quaternary structure? Recently we have been studying a series of dipeptides by thin-film dialysis to ascertain if conformational differences caused by hydrophobic bond breaking solvents can be detected. In the course of this work it was noted that the trihydrochloride of L-lysyl-L-α-lysine was completely anomalous in its dialytic behavior as shown in Tables III, IV, and V. Nonlinear escape plots were concentration dependent with reverse curvature in certain cases. The rate of diffusion was much too slow for a dipeptide and was accelerated by addition of ethanol or propionic acid but slowed by salt. This behavior coincides with that noted for the tyrocidines. In addition Sephadex chromatography indicated a diffusional size considerably larger than that expected for a dipeptide as is shown in Figure 3. Under the conditions used L-lysyl-L-lysine emerged at approximately the position found for bacitracin, mol wt 1422.

This behavior was first noted in a membrane of high diffusional selectivity (Craig, 1964) which gave a half-escape time for L-leucyl-L-tyrosine of 150 min at 25° in water, 138 min in 0.01 N acetic acid, and 125 min in 25% methanol. The selectivity of the membrane is shown in Table III by the fact that tyrosine and arginine gave 50% escape times of 29 and 30 min; L-leucyl-L-serine and L-leucyl-L-leucine gave half-escape times slightly but detectably lower than L-leucyl-L-tyrosine. All of these solutes and other neutral dipeptides studies in other membranes gave straight-line escape plots indicating purity and ideal behavior at these concentrations which at the start of the dialysis were in the range of 0.1–0.5% (0.003–0.02 M).

In contrast to this behavior (Table III), L-lysyl-L-lysine, L-glutamyl-L-glutamic acid, and L-lysyl-L-lysyl-L-lysine all gave half-escape times in H_2O much too high for the molecular weights indicated by their formulas. L-Lysyl-L-lysine and L-histidyl-L-histidine did likewise in 0.01 N acetic acid but L-glutamyl-L-glutamic acid diffused more rapidly than in H_2O. Moreover, depending on the concentration and solvent environment the escape plot had either a positive or reverse curvature and was markedly influenced by a change of pH, salt, and buffer.

These effects were all confirmed and more extensively investigated in calibrated membranes providing a threefold faster rate. The results are summarized in Table IV. In this table it can be seen that the half-escape time of L-leucyl-L-tyrosine or L-tyrosyl-L-leucine in water is little influenced by addition of acetic acid, sodium chloride, or alcohol. The rate is retarded by various buffers at pH 8.5 which could be a reasonable result caused by the discharge of the ionized amino group.

In contrast to these results there is the striking difference in the behavior of L-lysyl-L-lysine. Here the rate of dialysis is very slow and is retarded even further by sodium chloride, but accelerated by ethanol. At pH 8.5 in buffer the rate is accelerated, most strikingly so in borate buffer as will be discussed later. This buffer showed a much stronger effect than phosphate at the same concentration.

L-Histidyl-L-histidine, at the concentration studied, dialyzed normally in water (pH about 6) but too slowly at a lower pH. It also showed a salt effect but only at the lower pH. This indicates self-association when the imidazoles of the histidines are fully ionized. Tris buffer seemed to associate with the peptide at the higher pH. Ethanol had little effect even at the lower pH.

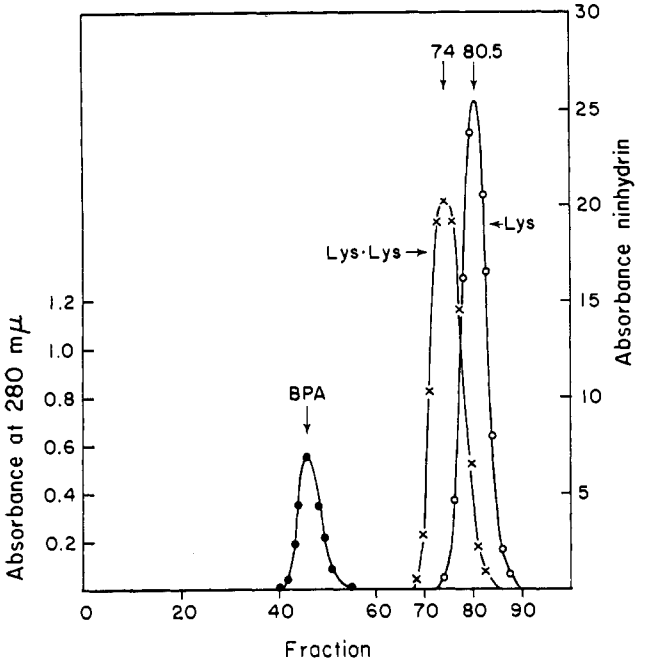

FIGURE 3: Comparative elution patterns of bovine plasma albumin, L-lysyl-L-lysine, and lysine in a column packed with Sephadex G-25.

L-Arginyl-L-lysine behaved like L-lysyl-L-lysine at the lower pH but to a somewhat weaker degree. This is interesting because as can be seen from Table III L-arginyl-L-arginine behaved normally and gave an escape rate somewhat faster even than expected. This result was confirmed by experiments in the membranes used for Table IV.

L-Glutamyl-L-glutamic acid behaved normally at low pH but was strongly influenced by salt. The pH in water was 3.8. It gave escape rates at a higher pH in several buffers indicative of self-association. Here the rate was increased by addition of ethanol. Apparently association requires ionization of the side chains.

Three different buffers, (phosphate, Tris, and borate) all at pH 8.5 and 0.01 M concentration were studied. There was little indication of phosphate binding except possibly with L-histidyl-L-histidine. On the other hand, there appeared to be definite evidence of an effect ascribed to binding with Tris and especially with borate. With Tris this effect was most apparent with L-histidyl-L-histidine.

Borate binding, Tables III and IV, was the most striking in the case of L-lysyl-L-lysine and in the case of tri-L-lysine, not shown in a table. Borate is known to bind with sugars containing *vic-cis*-dihydroxyl groups. It was found by T. Hettinger (unpublished results, 1968) in this laboratory, during structural studies with the antibiotic edeine, to bind with the *vic-cis*-hydroxyamino groups of a complex hydroxydiaminedicarboxylic acid. Paper electrophoresis clearly showed that in this case the positive charge of an amino group was removed. When the mobilities of the di- and tri-L-lysines were studied by paper electrophoresis both in the presence and absence of borate, it was found that without borate in a comparative experiment tri-L-lysine migrated toward the cathode faster than di-L-lysine and di-L-lysine considerably faster than lysine. Borate reversed this order showing

TABLE V: Effect of Load, Solvent, and Temperature on the Half-Escape Times of L-Lysyl-L-lysine and L-Argininyl-L-lysine.

	Range (mg)	L-Lys-L-Lys Trihydrochloride	L-Arg-L-Lys Diacetate
H_2O, 25°	1		71
	3		74
	9		124
20% Ethanol, 25°	1		57
	3		
	9		107
0.01 N HAc, pH 3.5, 25°	1		110
	3	251	110[a]
	9	381	174
0.01 N HAc–20% ethanol, 25°	1	96	97[a]
	3	96	185
	9	125	
0.01 N HAc, pH 3.5, 40°, 9-mg range		330	
0.01 N HAc–20% ethanol, 9-mg range 40°		134	

[a] Noticeably curved upward.

that the complex involved removal of the charge on amino groups. It is not indicated by the data with L-histidyl-L-histidine that there is complex formation. With L-arginyl-L-lysine the effect is much weaker than with L-lysyl-L-lysine and not sufficient to be certain there is real complexation.

An attempt was made to substantiate the postulated association of L-lysyl-L-lysine by gel filtration, a technique considered to separate solutes largely on the basis of their diffusional size. In Figure 3 are shown the elution curves of lysine and L-lysyl-L-lysine on Sephadex G-25. This grade of cross-linked dextran is considered to show exclusion of solutes only above molecular weight of a few thousand. From Figure 3 it is obvious that L-lysyl-L-lysine in salt solution has a diffusional volume much larger than would be expected from a dipeptide. Tri-L-lysine behaved the same way. When L-lysyl-L-lysine was studied again in the same Sephadex column but in Tris buffer at pH 8.5 it emerged at fraction 80.5, the exact position where lysine was found to be eluted. This result is consistent with the view derived from Table IV that L-lysyl-L-lysine is considerably less associated at the higher pH.

The dissociating effect of ethanol (Table V) is consistently shown wherever there is evidence of association. With L-lysyl-L-lysine the effect of alcohol is greater at the higher pH of water than in 0.01 M acetic acid. This also is the case with L-histidyl-L-histidine but the latter might be expected because of the lower ionization constant of the imidazole

groups. It is not as apparent with L-arginyl-L-lysine. At the higher pH, 8.5, with L-glutamyl-L-glutamic acid, alcohol again accelerates dialysis but not at the lower pH where association is not indicated. In fact in this case alcohol seems to slow dialysis, a result which could arise from a conformational effect resulting in a larger diffusional volume.

An attempt was made to confirm the postulated association behavior of the trihydrochloride of L-lysyl-L-lysine with the ultracentrifuge. A clear-cut result could not be obtained because of the high charge–charge interaction. The average value obtained was too low even for a monomeric L-lysyl-L-lysine and addition of NaCl did not give a higher value. A more convincing result was obtained with L-glutamyl-L-glutamate in 0.2 M NaCl at pH 8. Here the average molecular size was approximately double that expected for the monomer and appeared to decrease at a higher concentration. This could be due to the increased charge effect at the higher concentration. It thus appears that quaternary structure can exist with certain small highly charged peptides and that thin-film dialysis can reveal interactions at high dilution when they do exist. Similar observations have been made with nucleotides but here the solvent effects appear more complicated. This line of investigation is being continued with the objective of trying to explain the basis of such solute–solute interactions.

It seems of some interest that L-lysyl-L-lysine shows a strong tendency for self-association while L-arginyl-L-arginine does not. This could be explained by the assumption that the ionized NH_2—C(=NH)—NH— basic group shows a stronger tendency for ion pairing or interaction of some sort than does the NH_3^+ group with the result that L-arginyl-L-arginine can exist in solution as a collapsed structure similar to L-leucyl-L-tyrosine.

An attempt to reach a probable basis for the dialysis behavior found for dipeptides has been made by studying the possibilities and restrictions imposed by Corey–Pauling–Koltun models. With these a model of L-leucyl-L-tyrosine can be made and its conformation arranged so that the single carboxyl and amino groups are on the same side of the peptide bond and as close together as possible. The leucyl and tyrosyl side chains are then as close together as possible. This arrangement gives the most compact conformation possible with a maximum diameter approximating 11 Å. It would appear to be the conformation allowing maximum hydrophobic interaction of the side chains and minimal distance between the amino and carboxyl groups. It has been found through an nuclear magnetic resonance study of the phenylalanylvalines (Morlino and Martin, 1968) that this conformation is preferred when both ionizable groups are charged. Discharge of the ammonium ion causes shifts of the α-CH and the methyl protons of the valine residue consistent with their being moved further from the shielding effect of the benzene ring.

With L-lysyl-L-lysine a different conformation would be expected at a pH where all the amino groups and the carboxyl group are charged. Here the α-amino group and the carboxyl group would form a zwitterion but the two ϵ-amino groups would be expected to repel each other and give an extended conformation. If fully extended the models indicate that a maximum cross section approximately 17 Å in length would result. When the α-amino group is discharged as it would be at pH 8.5 a partial collapse would be expected because the carboxyl group would then attract one of the ϵ-amino groups. The data in Table IV are consistent with this hypothesis. An analogous behavior might be expected for L-arginyl-L-lysine, L-histidyl-L-histidine, L-glutamyl-L-glutamic acid, and L-arginyl-L-arginine. However, L-arginyl-L-arginine was found to behave like L-leucyl-L-tyrosine at different concentrations and higher pH or when salt or ethanol was added.

TABLE VI: Comparative Dialysis Rates for a Series of Alanine and Glycine Polypeptides at 25°.

Peptide	Mol Wt	Half-Escape Times (min)	Longest Axis (Å)	
			Extended	Collapsed
L-Ala	89	14	6.4	6.4
L-Ala-L-Ala	170	43	10.4	9.2
D-Ala-D-Ala	170	44		
L-Ala-L-Ala-L-Ala	231	87	14	11.5
L-Ala-L-Ala-L-Ala-L-Ala	302	163	17	11.5
D-Ala-L-Ala-L-Ala-L-Ala	302	145		
L-Ala-D-Ala-L-Ala-L-Ala	302	130		
L-Ala-L-Ala-D-Ala-L-Ala	302	140		
L-Ala-L-Ala-L-Ala-D-Ala	302	159		
Gly	75	8	6.0	6.0
Gly-Gly	132	19	10.4	8.0
Gly-Gly-Gly	189	28	14	8.5
Gly-Gly-Gly-Gly	246	40	17	9.0

A change of conformation alone cannot explain the dialysis behavior of L-lysyl-L-lysine, L-lysyl-L-arginine, L-histidyl-L-histidine, or L-glutamyl-L-glutamic acid with respect to change of concentration, effect of salt or ethanol. The behavior found is typical of self-association as was noted with the tyrocidines. If an extended conformation alone were postulated, salt would be expected to shield the repelling like charges and tend to allow the conformation to collapse. On the other hand, ethanol would tend to have the opposite effect because of a reduction of the dielectric constant of the solvent. In both cases the opposite was found.

It therefore can be postulated that self-association tends to occur when the most extended conformation is promoted. It is hoped to gain further information on this point by a study of the conformation by high-resolution nuclear magnetic resonance spectrometry. Such a study is under way.

In certain cases in Table IV there seems to be evidence of Tris binding but the binding of borate to L-lysyl-L-lysine is more striking in Table III as well as IV. At this concentration of borate the complex would probably be partially dissociated. A cyclic complex could well have a smaller diffusional volume than the free ionized peptide.

The data obtained in 0.01 N acetic acid in Table VI permit interesting comparisons regarding diffusional size when the longest axis is taken as the effective diffusional diameter. This should be the case since from nuclear magnetic resonance data the rate of tumbling (Carrington and McLachlan, 1967) is indicated to be in the range of 10^7–10^{11} times a second. Models of the peptides in Table VI were constructed from the Corey–Pauling–Koltun models and the approximate diam-

eters measured for the most collapsed and extended conformations. The lengths in Ångstroms for the longest axes are given in Table VI.

It might be expected that the tetraalanines would behave as if they were more or less random in their conformation although limitations would be expected from the extensive calculations of Ramachandram, Scheraga, and others (Ramachandran and Sasisekharan, 1968). The striking difference in their rate of dialysis as compared with tri-L-alanine, however, is more easily explained if their conformation is more or less extended and perhaps quite rigid. There also appears to be a detectable although small difference caused by the configuration of the α-carbon atom.

Indeed, this conformation has been indicated by a study of the dipole moments of these peptides (Beacham *et al.*, 1965). Moreover, the distance between the charged groups was also shown to be less when the single D-amino acid residue was in a midportion of the chain rather than terminal. The results in Table VI are consistent with the earlier results of Beacham *et al.*

In comparing the differences shown in Table VI between the mono- and di-, the di- and tri-, and the tri- and tetrapeptides it must be remembered that the selectivity of the membrane becomes greater with the larger solute since the point of total exclusion is more closely approached.

Since the data in Table VI were obtained with the same calibrated membranes used in Tables IV, comparisons of dialysis rates and postulated diffusional diameters obtained from the Corey–Pauling–Koltun models are permissible. Thus the collapsed diameter postulated for L-leucyl-L-tyrosine in water approximates 11 Å. Its $T/2$ is 53 min, indicating a rate slightly slower than that of the dialanines but faster than that of tri-L-alanine. This value is consistent with the molecular dimensions of the alanine peptides. The extended model for L-lysyl-L-lysine measures 17 Å, only slightly larger than the extended form of tetra-L-alanine. The dialysis rates of the two are consistent if that for L-lysyl-L-lysine at pH 8.5 is taken for comparison. The tendency for self-association of L-lysyl-L-lysine at the lower pH precludes comparison.

As a further check on the postulated basis for the extension of the L-lysyl-L-lysine conformation the polybasic substances spermidine and spermine were studied. These contain no carboxyl groups and a fully extended conformation due to the repulsion of like charges should be favored. If so, their lengths derived from the models approximate 13.6 and 18.8 Å, respectively. In the calibrated membranes used in Table VI the half-escape times were 68 and 148 min, respectively. These values are not far from those expected from the data of Tables IV and VI. Self-association was not indicated.

The striking difference between the dialysis rates of the glycine and alanine polypeptides is worthy of comment. The Corey–Pauling–Koltun models clearly indicate much more steric hindrance in rotation about the bonds of the α-carbon atom for the alanines as compared to the glycines. This is consistent with earlier views (Beacham *et al.*, 1965) that the polyglycines more nearly approach a random and completely mobile conformation than the polyalanines and with the greater proportion of allowed conformations of the glycines calculated by Ramachandran and Sasisekharan (1968).

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References

Balasubramanian, D. (1967), *J. Amer. Chem. Soc. 89*, 5445.
Battersby, A. R., and Craig, L. C. (1952), *J. Amer. Chem. Soc. 74*, 4023.
Beacham, J., Ivanov, V. T., Kenner, G. W., and Sheppard, R. C. (1965), *Chem. Commun. 16*, 386.
Carrington, A., and McLachlan, A. D. (1967), Introduction to Magnetic Resonance, New York, N. Y., Harper & Row, p 187.
Craig, L. C. (1964), *Science 144*, 1093.
Craig, L. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 152.
Craig, L. C., and Ansevin, A. (1963), *Biochemistry 2*, 1268.
Craig, L. C., Fisher, J. D., and King, T. P. (1965), *Biochemistry 4*, 311.
Craig, L. C., King, T. P., and Konigsberg, Wm. (1960), *Ann. N. Y. Acad. Sci. 88*, 571.
Craig, L. C., King, T. P., and Stracher, A. (1957), *J. Amer. Chem. Soc. 79*, 3729.
Craig, L. C., and Konigsberg, Wm. (1961), *J. Phys. Chem. 65*, 166.
Guidotti, G., and Craig, L. C. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 46.
Laiken, S. L., Printz, M. P., and Craig, L. C. (1969a), *Biochemistry 8*, 519.
Laiken, S. L., Printz, M. P., and Craig, L. C. (1969b), *J. Biol. Chem. 244*, 4454.
Liquori, A. M., and Conti, O. O. (1968), *Nature 217*, 635.
Morlino, V. J., and Martin, R. B. (1968), *J. Phys. Chem. 72*, 2661.
Pedersen, K. O., and Synge, R. L. M. (1948), *Acta Chem. Scand. 2*, 408.
Quadrifoglio, F., and Urry, D. W. (1967), *Biochem. Biophys. Res. Commun. 29*, 785.
Ramachandran, G. N., and Sasisekharan, V. (1968), *Advan. Protein Chem. 23*, 329.
Ruttenberg, M. A., King, T. P., and Craig, L. C. (1966), *Biochemistry 5*, 2857.
Stern, A., Gibbons, W. A., and Craig, L. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 734.
Stern, A., Gibbons, W. A., and Craig, L. C. (1969), *J. Amer. Chem. Soc. 91*, 2794.
Vanderkooi, G., Leach, S. J., Nemethy, G., Scott, R. A., and Scheraga, H. A. (1966), *Biochemistry 5*, 2991.
Williams, R. C., Jr. (1968), Ph.D. Thesis, Rockefeller University, New York, N. Y.
Williams, R. C., Jr., and Craig, L. C. (1967), *Separ. Sci. 2*, 487.