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Specific Interaction of Peptides with Nucleic Acids. Evidence for a "Selective Bookmark" Recognition Hypothesis†

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ABSTRACT: Additional studies of the interaction specificities of di- and tripeptide amides with various DNA of different A-T–G-C compositions are reported. The results of the temperature-dependent proton magnetic resonance (pmr), ultraviolet, circular dichroism, viscometric, equilibrium dialysis, and melting temperature studies are presented. It is found that peptides containing aromatic amino acids interact selectively with the nucleic acid helix to form a "sticky complex." An intercalation model, in which the aromatic residue of the peptide is partially inserted between base pairs, accounts for the observed pmr and viscometric results. It is also shown that bending of the DNA helix occurs upon complexation of peptides containing aromatic amino acid residues which is dependent on the primary structure of the latter. Moreover, the equilibrium dialysis studies show for the L-lysyl-L-aromatic and hydrophobic amino acid amides (*e.g.*, L-lysyl-L-tryptophanamide, L-lysyl-L-tyrosinamide, L-lysyl-L-phenylalaninamide, and L-lysyl-L-leucinamide) the following order of increasing A-T selectivity: leucine < tyrosine < phenylalanine < tryptophan. The possible role of the above types of interaction in (1) the packaging of DNA in nucleohistone complexes (*e.g.*, chromatin) and (2) protein–nucleic acid recognition process is discussed. In particular, it is concluded that the "bookmark" hypothesis proposed by Brown (Brown, P. E. (1970), *Biochim. Biophys. Acta 213*, 282) and Gabbay *et al.* ((1972a), *Biochemistry 11*, 3429) takes on added significance now that it is shown that the aromatic amino acid residues not only can intercalate, but do so with some degree of selectivity with respect to primary sequence of the peptide as well as base-pair specificity.

In the previous paper from this laboratory which dealt with the interaction of over 50 peptides with nucleic acids (Gabbay *et al.*, 1972a), it was shown by proton magnetic resonance (pmr) techniques that the peptides containing aromatic amino acids interact selectively with the nucleic acid *helix*. For example, the pmr data indicate that the aromatic amino acids, when present in dipeptides and/or dipeptide amides, experience an upfield chemical shift and line broadening which is indicative of a ring current anistropic effect and a "sticky complex," respectively (Pople *et al.*, 1959; Jardetsky and Jardetsky, 1962). In addition, viscometric studies show that the aromatic amino acid dipeptide amides, *e.g.*, L-lysyl-L-phenylalaninamide, L-lysyl-L-tyrosinamide, cause a dramatic decrease in the specific viscosity, η_{sp}, of DNA. A nonclassical intercalation model in which the aromatic residue of the peptide is *partially* inserted between base pairs seems to account for the pmr and viscosity results (Gabbay *et al.*, 1972a).

In this paper the interaction specificity of various di- and tripeptides (containing aromatic amino acids) with nucleic acids of different A-T–G-C compositions are examined by pmr, ultraviolet (uv), circular dichroism (CD), viscometric, and equilibrium dialysis. It is found that the hydrophobic amino acids have a higher affinity to A-T than to G-C sites and the selectivity for A-T sites increases according to the following order: leucine < tyrosine < phenylalanine < tryptophan. The possible significance of these observations to protein–nucleic acid recognition processes is discussed.

Experimental Section

Analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. Infrared spectra were recorded on either a Perkin-Elmer Model 337 or a Perkin-Elmer IR-10. Pmr spectra were recorded on either a Varian A-60A or a Varian XL-100 spectrometer equipped with a variable-temperature probe. Circular dichroism measurements were recorded on a Jasco J-20 spectropolarimeter at ambient temperature. Viscosity studies were performed with a low-shear Zimm viscometer from Beckman Instrument Co. Ultraviolet and visible absorption measurements were recorded on either a Cary 15 or a

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[1] Abbreviations used: n-DNA, native DNA; d-DNA, denatured DNA.

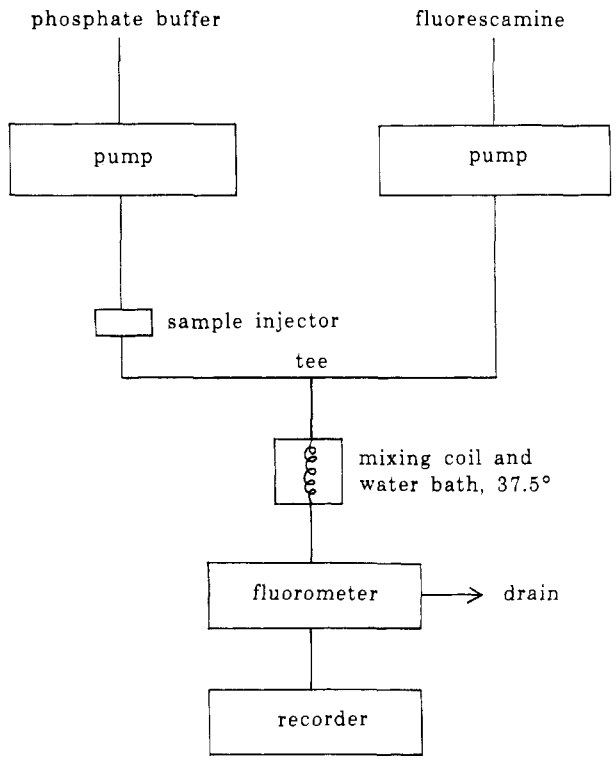


FIGURE 1: Flow system schematic for peptide analysis, The buffer and reagent were mixed in 1:1 proportions with a flow rate of 18 drops/min. The pumps were Milton-Roy mini-pumps. All tubing, connections, and injection port were Teflon (Chromatronix). The mixing coil was submerged in a Haake constant-temperature water bath maintained at 37.5°.

Gilford 240 spectrophotometer. Quantitative assay for peptide concentrations was made with fluorescamine (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione, kindly supplied by Dr. Weigele, Hoffmann-La Roche) using a Farrand ratio fluorometer with primary filter and secondary filters designed for excitation at 390 nm and emission at 490 nm.

All amino acid and peptide amides were either synthesized or purchased from Cyclo Chemical Corp. The authenticity of the purchased samples was checked by pmr, uv, CD, and in some cases by paper chromatography. The synthesized samples (indicated by asterisk in the tables) employed carbobenzoxy-, *tert*-butyloxy-, and other amino acid derivatives as intermediates (Cyclo Chemical Corp.) and were used without further purification. The mixed-anhydride procedure (Anderson *et al.*, 1967) was utilized in the synthesis of the peptides and the products were checked for authenticity by pmr, CD, uv, infrared (ir), and elemental analysis.

All solutions containing DNA and peptide systems were prepared in buffers made with deionized water. Salmon sperm DNA (8BA, ϵ_p 6500, Worthington Biochemicals), poly[d(A-T)], poly[d(A-T)] (lot no. 32), and *Micrococcus luteus* DNA were purchased from Miles Laboratories. The *M. luteus* DNA was further purified to remove the 2–3% residual protein by phenol extraction (Smith, 1970).

Equilibrium dialysis studies employed Visking dialysis tubing ($^{26}/_{100}$ Nojax casings) cut into 20-cm strips and carefully cleaned by boiling in a 50% aqueous ethanol solution containing 5×10^{-3} M EDTA and 5×10^{-2} M sodium bicarbonate for 1 hr and repeated three times. The membranes were then boiled in deionized water for 5 hr and repeated six times. The clean membranes were stored in deionized water containing 2% chloroform and stored at 0°. The dialysis experiments were conducted in a pair of Plexiglass blocks with ten shallow cylindrical depressions cut into each (3 × 0.25 mm). A clean membrane was placed between the cells to form two chambers. Each half of the Plexiglass block, prior to placement of the membrane, was lightly coated with silicon grease to prevent leakage. The blocks were securely fastened together by means of twelve screws and four C clamps. The top of the cells was sealed by placing a strip of lightly greased parafilm over the top of the Plexiglass block. Equilibration was allowed to take place for at least 20 hr at 7° during which time the Plexiglass blocks were gently shaken. Blank determinations showed that at a polynucleotide concentration of 1.0×10^{-3} mol of DNA-P/l. the optical density at 260 nm across the dialysis membrane was never greater than 0.030. It was also demonstrated that complete equilibration of the small molecules, *i.e.*, peptides, was complete in less than 10 hr.

The quantitative determination of the peptide concentration utilized the fluorescent reagent fluorescamine (Weigele *et al.*, 1972). A flow system for the analysis was designed and the schematic diagram is shown in Figure 1. The buffer was 0.005 M sodium phosphate (pH 7.4) and the reagent concentration was 15 mg/100 ml of Spectrograde dioxane. The dioxane was dried prior to use by storage over sodium metal which also helps to remove peroxide contaminants. Sufficient Teflon capillary tubing was used to allow a 1-min mixing time at 37.5°. The concentration of the peptide was determined from the amplitude of the fluorescence signal. Standard peptide solutions were utilized to prepare a standard curve so that the concentration of the equilibrated peptide could be determined. This procedure was repeated each time a given peptide was analyzed. The analyses of the standard solutions were found to be reproducible to within ±2% in the range of 0.1–1.0 nmol of peptide.

Results and Discussion

Pmr Studies. Temperature-dependent pmr studies of the free and DNA-bound peptides in D_2O were conducted according to previous published procedure (Gabbay *et al.*, 1972a) using 0.02 and 0.16 mol of peptide and DNA-P per l., respectively, at pD of 7.0 ± 0.1. In all cases, no observable difference in the pmr spectra of free peptide and DNA–peptide complex is noted when the peptides *do not* contain an aromatic amino acid, *i.e.*, phenylalanine, tyrosine, and tryptophan. However, peptides which contain aromatic amino acids exhibit an upfield chemical shift and line-broadened pmr signals of the aromatic protons as well as the methylene protons ($-CH_2-$ group next to the aromatic ring) upon binding to DNA. It should be noted that the pmr spectra of free peptides are nearly identical at 37 and 90°. Moreover, the absence of a concentration dependence on the pmr spectra of *free* peptides is observed with all systems, suggesting that self-association of the peptides as the mechanism which leads to the line broadening and the upfield chemical shift of the aromatic protons in the presence of DNA is not occurring. However, the possibility exists that the peptides may bind cooperatively to DNA, resulting in a DNA-induced peptide self-association which may give rise to spurious pmr changes. Additional work along these lines is in progress.

The temperature dependent chemical shifts, $\Delta\delta_1$ and $\Delta\delta_2$, of the aromatic and CH_2 protons, respectively, of free and DNA-bound peptides are shown in Table I. Observations which were examined in detail in a previous paper (Gabbay *et al.*, 1972a) are briefly summarized. It is noted that the extent of interaction of the aromatic residue with DNA base pairs is

dependent on (1) the aromatic residue, *i.e.*, phenylalanine, tyrosine, and tryptophan, (2) the primary sequence of the peptide, and (3) the stereochemistry of the α-carbon atom of the amino acid residues, *i.e.*, L-lysyl-L-phenylalaninamide *vs.* L-lysyl-D-phenylalaninamide. Additional pmr studies show: the tripeptide amides containing two lysyl residues (**26–28**) do not show as large an upfield shift for the phenylalanine ring protons when bound to native DNA (n-DNA), as do the tripeptides containing two phenylalanine residues and one lysyl residue, **29–31**. Moreover, the extent of broadening of the aromatic protons in the n-DNA–**26–28** complexes is not as great as in the n-DNA–**29–31** complexes which in turn are not as broadened as the aromatic protons of L-lysyl-L-phenylalaninamide, **17**. In addition, the tripeptides, **26–31**, exhibit a greater upfield chemical shift in the denatured DNA (d-DNA) complexes than in the native DNA complexes. The following order of increasing upfield shifts of the aromatic ring protons of the two types of tripeptides with n- and d-DNA are observed.

for n-DNA: L-Lys-L-Phe-L-PheNH$_2$ (**30**) < L-Phe-L-Phe-L-LysNH$_2$ (**31**) < L-Phe-L-Lys-L-PheNH$_2$ (**29**)

for d-DNA: L-Lys-L-Phe-L-PheNH$_2$ (**30**) = L-Phe-L-Lys-L-PheNH$_2$ (**29**) < L-Phe-L-Phe-L-LysNH$_2$ (**31**)

for n-DNA: L-Phe-L-Lys-L-LysNH$_2$ (**28**) = L-Lys-L-Phe-L-LysNH$_2$ (**27**) < L-Lys-L-Lys-L-PheNH$_2$ (**26**)

for d-DNA: L-Lys-L-Phe-L-LysNH$_2$ (**27**) < L-Lys-L-Lys-L-Phe (**26**) < L-Phe-L-Lys-L-LysNH$_2$ (**28**)

These results are somewhat surprising and difficult to interpret at present especially since it is found by equilibrium dialysis (see below) that the tripeptide amides **26–28** bind more strongly to DNA than both the tripeptide amides **29–31** or the dipeptide amide, L-lysyl-L-phenylalaninamide (**17**). The upfield chemical shift data for n-DNA–peptide complexes, however, indicate that the phenyl protons of **26–28** experience less shielding from the n-DNA bases and a much lower restriction in tumbling rate than **29–31** or the dipeptide amide, **17**, *i.e.*, smaller upfield shift and sharper resonance signals. The explanation for these observations is not known at present. Conceivably, the geometry of the peptide–n-DNA complexes for dilysyl-containing tripeptide amides, **26–28**, is not appropriate for the intercalation of the phenyl ring. Molecular model studies fail to show why this is the case. For denatured DNA–peptide complexes, the peptides, **26–28**, exhibit a marked increase in the upfield chemical shift of the phenyl protons relative to n-DNA indicating that in the more flexible random-coil DNA–peptide complex an increased stacking of the phenyl ring of the phenylalanine residue with DNA bases occurs. It is also found that the extent of broadening of the aromatic signal is increased in the denatured DNA–peptide complexes, especially for d-DNA–L-lysyl-L-phenylalanine-L-lysinamide. Thus, the assumption that intercalation of the phenylalanine residue of **26–28** is dependent on the geometry of the complex is tenable, for when the rigid double helix is melted out and a greater flexibility is available to the complex, increased interaction is observed between the phenyl group and the DNA bases. Apparently, the geometrical requirements for the interactions of the phenylalanine residues of tripeptide amides, **29–31**, and L-lysyl-L-phenylalaninamide (**17**) to n-DNA is not as great as for **26–28**. This phenomenon could be due to the fact that there are only two positive charges on these amides as opposed to three positive charges for **26–28**. Thus, the increased flexibility in binding of **29–31** and the dipeptide amide, **17**, could allow for a more intimate association of the phenyl group with the base pairs of n-DNA (*i.e.*, a larger upfield shift and more broadened aromatic proton signals).

In summary, the pmr data indicate that the aromatic amino acids, when present in dipeptides and/or dipeptide amides, experience an upfield chemical shift and line broadening which is indicative of a ring current anisotropic effect and a "sticky complex," respectively. These effects are noted to a much smaller extent for the phenylalanine-containing lysyl and dilysyl tripeptide amides. It is found that an influencing factor in the magnitude of these effects is the primary structure of the dipeptide, dipeptide amide and tripeptide amides of phenylalanine- and tyrosine-containing systems. Tryptophan-containing peptides show a greater upfield chemical shift of the aromatic protons on binding to n-DNA than phenylalanine and tyrosine peptides, and, in addition, the magnitude of the upfield shift is independent of the sequence. Also, apolar amino acid residues, when present in the dipeptide, enhance the upfield chemical shift of the aromatic protons of phenylalanine and tyrosine. A similar effect is noted in the presence of amino acid residues containing an extra positive charge, *e.g.*, lysine. However, addition of two lysyl residues, *i.e.*, tripeptide amides, **26–28**, leads to a decrease in the interaction of the phenylalanine residue with native DNA. Presumably, this effect is due to a restricted binding geometry necessitated by the electrostatic interaction of the three positive charges with the phosphate anions on DNA which does not allow for intimate contact between the phenylalanine residue and the base pairs. However, in the d-DNA complex the increased flexibility in the DNA chain allows for a greater interaction of the phenylalanine residue with the bases of DNA.

Uv and CD Spectral Studies. The interactions of the peptides and peptide amides listed in Table I with salmon sperm n-DNA were also studied by uv absorption and CD techniques. Little or no difference is detected in the absorption spectrum of DNA in the presence of the dipeptide amide systems, *i.e.*, intensity at 260 nm varied by $\pm 3\%$. Similarly, little or no change is observed in the peak at 275 nm and the trough at 246 nm of the CD spectrum of DNA in the presence of most of the above systems. The results suggest that no gross alteration in n-DNA structure is occurring.

Melting Temperature Studies. The effect of the various peptide systems on the T_m of the helix–coil transition of salmon sperm DNA has been reported (Gabbay *et al.*, 1972a). The earlier results may be briefly summarized. (1) A primary sequence effect on the stabilization of the DNA helix is observed. (2) With the exception of L-phenylalanyl-L-alaninamide (**9**) and L-alanyl-L-phenylalaninamide (**8**), the dipeptide amides with the aromatic residue on the C-terminal end stabilize the DNA helix to a greater extent than do the corresponding N-terminal isomers. (3) Dipeptide amides containing L-tyrosine stabilize the helix to a greater extent than do the corresponding L-phenylalanine-containing systems. (4) Cadaverine (1,5-diaminopentane) shows a greater stabilization of the helix than do the α-substituted cadaverines, *i.e.*, the lysyl dipeptide amides. (5) The dipeptide stabilizes the helix to a lesser extent than do the dipeptide amides, which is reasonable on the basis of electrostatic considerations.

Additional T_m data are presented in Table II for the lysyl and dilysyl tripeptide amides, **26–31**. Two observations may be made. (1) There is a sequence dependence in the ability of the peptides to stabilize the helix relative to the random coil and (2) the dilysyl containing tripeptide amides, **26–28**, increase the T_m to a greater extent than do the monolysyl systems, **29–31**. The increased stabilization of n-DNA helix by

TABLE I: Chemical Shifts (Parts per Million) from DSS[a] at 100 MHz of Free and DNA-Bound Peptide Systems at Variable Temperature.

Peptide System	Temp (°C)	$\Delta\delta_1$[b]	$\Delta\delta_2$[c]
L-Phe-L-Arg (**1**)	37	731	312
+DNA	37	730	311
+DNA	55	731	310
+DNA	90		
+d-DNA	37	729	311
L-Arg-L-Phe (**2**)	37	733	314
+DNA	37	729	313
+DNA	55	727	313
+DNA	90		
+d-DNA	37	722	311
L-Leu-L-Phe (**3**)	37	733	316
+DNA	37	729	312
+DNA	55	731	315
+DNA	90		
+d-DNA	37	728	314
L-Leu-L-PheNH$_2$ (**4**)	37	736	312
+DNA	37	725	B[d]
+DNA	55	724	B
+DNA	90	726	B
+d-DNA	37	718	B
L-Phe-L-LeuNH$_2$ (**5**)	37	739	322
+DNA	37	734	319
+DNA	55	733	317
+DNA	90	730	314
+d-DNA	37	725	314
L-Phe-GlyNH$_2$ (**6**)	37	737	323
+DNA	37	734	319
+DNA	55	733	317
+DNA	90	730	314
+d-DNA	37	725	314
Gly-L-PheNH$_2$ (**7**)	37	736	312
+DNA	37	728	308
+d-DNA	37	720	303
L-Ala-L-PheNH$_2$ (**8**)	37	734	312
+DNA	37	726	306
+DNA	55	726	B
+DNA	90	727	B
+d-DNA	37	720	B
L-Phe-L-AlaNH$_2$ (**9**)	37	739	323
+DNA	37	734	320
+d-DNA	37	729	317
L-His-L-PheNH$_2$ (**10**)	37	732	308
+DNA	37	726	308
+d-DNA	37	721	308
L-Phe-L-SerNH$_2$ (**11**)	37	737	324
+DNA	37	732	319
+DNA	55	733	319
+d-DNA	37	726	316
L-PheNH$_2$ (**12**)	37	740	324
+DNA	37	737	321
+DNA	55	737	320
+DNA	90	731	316
+d-DNA	37	728	316
β-L-Ala-L-PheNH$_2$ (**13**)	37	735	314
+DNA	37	731	313
+DNA	55	731	313
+DNA	90	729	314
+d-DNA	37	725	313
L-Phe-L-TrpNH$_2$ (**14**)	37	735	322
+DNA	37	730	310
+DNA	55	725	309
+DNA	90	720	305
+d-DNA	37	717	297
*D-Lys-D-Phe[g] (**15**)	37	727	298
+DNA	37	725	298
+DNA	55	725	297
+DNA	90	728	298
+d-DNA	37	732	299
L-Lys-L-Phe (**16**)	37	736	296
+DNA	37	726	296
+DNA	55	725	296
+DNA	90	727	297
+d-DNA	37	726	296
*L-Lys-L-PheNH$_2$ (**17**)	37	734	304
+DNA	37	722	304
+DNA	55	721	304
+DNA	90	723	304
+d-DNA	37	713	304
L-Phe-L-PheNH$_2$ (**18**)	37	735 ± 5	318	306
+DNA	37	736 and 724	B	B
+DNA	55	734 and 724	B	B
+DNA	90	730 ± 5	B	B
+d-DNA	37	734 and 716	B	B

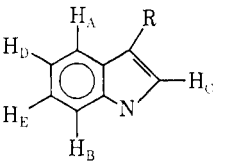
Peptide System	Temp (°C)	$\Delta\delta_1$[b]	Tyr (d_1)	Tyr (d_2)
L-Phe-L-TyrNH$_2$ (**19**)	37	737	717	689
+DNA	37	735	711	682
+DNA	55	736	713	683
+DNA	90	737	715	606
+d-DNA	37	734	711	683
L-Tyr-L-PheNH$_2$ (**20**)	37	731	710	683
+DNA	37	718	708	682
+d-DNA	37	711	697	670
L-Phe-Gly-GlyNH$_2$ (**21**)	37	734	300	
+DNA	37	732	300	
+d-DNA	37	728	297	

Table I (*Continued*)

Peptide System	Temp (°C)	$\Delta\delta_1$[b]	Tyr (d_1)	Tyr (d_2)
Gly-Gly-L-PheNH$_2$ (**22**)	37	734	305	
+DNA	37	730	303	
+DNA	55	730	304	
+DNA	90	729	303	
+d-DNA	37	725	301	
*L-Lys-D-PheNH$_2$ (**23**)	37	736	291	
+DNA	37	734	291	
+DNA	55	734	288	
+DNA	90	730	285	
+d-DNA	37	726	291	
*L-Phe-L-LysNH$_2$ (**24**)	37	734	305	
+DNA	37	734	300	
+DNA	55	732	300	
+DNA	90	728	300	
+d-DNA	37	727	300	
*L-Arg-L-PheNH$_2$ (**25**)	37	734	312	
+DNA	37	732	312	
+DNA	55	732	311	
+DNA	90	730	309	
+d-DNA	37	725	312	
*L-Lys-L-Lys-PheNH$_2$ (**26**)	40	736		
+DNA	40	732		
+DNA	50	731		
+DNA	90	722		
+d-DNA	40	724		
*L-Lys-L-Phe-L-LysNH$_2$ (**27**)	40	735	303	
+DNA	40	731	295	
+DNA	50	731		
+DNA	90	722		
+d-DNA	40	722	305	
*L-Phe-L-Lys-L-LysNH$_2$ (**28**)	40	733		
+DNA	40	733		
+DNA	50	733		
+DNA	90	722		
+d-DNA	40	718		
*L-Phe-L-Lys-L-PheNH$_2$ (**29**)	40	735		
+DNA	40	722		
+DNA	50	722		
+DNA	90	723		
+d-DNA	40	720		
*L-Lys-L-Phe-L-PheNH$_2$ (**30**)	40	731		
+DNA	40	727		
+DNA	50	726		
+DNA	90	719		
+d-DNA	40	716		
*L-Phe-L-Phe-L-LysNH$_2$ (**31**)	40	737		
+DNA	40	731		
+DNA	50	730		
+DNA	90	722		
+d-DNA	40	718		

Peptide System	Temp (°C)	$\Delta\delta$ (d_1)[e]	$\Delta\delta$ (d_2)[f]	$\Delta\delta_2$
Gly-L-TyrNH$_2$ (**32**)	37	717	685	377
+DNA	37	715	683	375
+d-DNA	37	705	673	375
L-Tyr-Gly-Gly (**33**)	37	716	687	392
+DNA	37	716	687	392
+d-DNA	37	711	682	391
Gly-Gly-L-TyrNH$_2$ (**34**)	37	715	685	394
+DNA	37	715	685	394
+d-DNA	37	707	677	392
L-His-L-TyrNH$_2$ (**35**)	37	713	683	299
+DNA	37	710	680	B
+d-DNA	37	704	675	B
L-Lys-L-Tyr (**36**)	37	718	685	301
+DNA	37	717	685	298
+d-DNA	37	708	675	295
L-Lys-L-TyrNH$_2$ (**37**)	37	727	690	298
+DNA	37	706	673	297
+DNA	55	709	678	298
+DNA	90	709	678	298
+d-DNA	37	704	676	296

Tryptophan Systems



Peptide System	Temp (°C)	$\Delta\delta$ (H_C)	$\Delta\delta$ (H_B)	$\Delta\delta_2$
L-TrpNH$_2$ (**38**)	37	729	756	344
+DNA	37	714	743	335
+DNA	55	712	741	335
+DNA	90	717	741	333
+d-DNA	37	700	724	325
D-TrpNH$_2$ (**39**)	37	729	756	343
+DNA	37	713	743	335
+DNA	55	712	743	335
+DNA	90	719	743	337
+d-DNA	37	703	729	328
*Gly-L-TrpNH$_2$ (**40**)	37	725	754	330
+DNA	37	713	739	323
+DNA	55	713	740	323
+DNA	90	716	743	324
+d-DNA	37	702	727	317
L-Trp-GlyNH$_2$ (**41**)	37	730	757	344
+DNA	37	715	744	338
+DNA	55	715	744	337
+DNA	90	718	744	337
+d-DNA	37	704	730	331
*L-Ala-L-TrpNH$_2$ (**42**)	37	726	754	330
+DNA	37	712	739	318
+DNA	55	712	740	324
+DNA	90	716	743	325
+d-DNA	37	700	724	310
L-Trp-L-LeuNH$_2$ (**43**)	37	726	754	332
+DNA	37	710	738	327
+DNA	55	710	737	325
+DNA	90	714	742	325
+d-DNA	37	699	724	317
*L-Lys-L-TrpNH$_2$ (**44**)	37	730	750	332
+DNA	37	719	B	328
+d-DNA	37	709	B	320

Table footnotes are on the following page.

FOOTNOTES TO TABLE I

[a] 2,2-Dimethyl-2-silapentanesulfonic acid. [b] Chemical shift of phenyl aromatic protons. [c] Chemical shift of benzyl protons of the phenylalanine residue. [d] The proton signal is not distinguishable from base-line noise. [e,f] Chemical shift of the aromatic ring protons of the tyrosine residue which appear as doublets (*i.e.*, d_1 and d_2). All spectra taken on a Varian XL-100 spectrometer equipped with a variable-temperature probe. Sonicated salmon sperm DNA was used at 0.16 mol of DNA-P/l. in D_2O at pD 7.2 ± 2. d-DNA was obtained by heating native DNA at 100° for 10 min followed by quenching in ice-water at 0°. The concentration of the peptide systems was 0.02 M. [g] The asterisk means synthesized in our laboratory.

26–28 as compared to **29–31** is readily explained on the basis of electrostatic arguments. The sequence dependence, however, is not clear at present.

As has been pointed out earlier (Gabbay *et al.*, 1972a), the interpretation of the T_m data is complicated by at least two competing processes, *i.e.*, relative interaction of the peptide (P) with the helix (H) and with the random coils (C). For example, a knowledge of the value of K_H and K_C *at* or *near* the *melting temperature* is necessary before any valuable conclusions can be drawn.

$$H + P \overset{K_H}{\rightleftharpoons} H \cdot P \rightleftharpoons C \cdot P \overset{K_C}{\rightleftharpoons} C + P$$

Viscosity Studies. It has been shown previously that peptides containing aromatic amino acids cause dramatic decreases in the specific viscosity of n-DNA solutions which cannot be accounted for on the basis of simple electrostatic constriction of the polymer at high ionic strength (Gabbay *et al.*, 1972a). A nonclassical intercalation model was proposed whereby the aromatic residue of the peptide amide is partially inserted between base pairs resulting in slight bending of the helix at the point of complexation. Such a model is totally consistent with that suggested by the pmr studies. It should be noted that Müller and Crothers (1968) also observed a decrease in viscosity of high molecular weight DNA solution upon intercalation of actinomycin. They propose, however, a model whereby an enhanced cross-linking of the DNA chains occurs in the presence of actinomycin. Such a mechanism is highly unlikely for the DNA–peptide complexes since all the peptides examined show no tendency to self-aggregate as evidenced by pmr spectroscopy.

In this paper the effect of additional peptide amide systems on the specific viscosity, η_{sp}, of n-DNA is reported (Table III). (It should be emphasized that intrinsic viscosity measurements were carried out for many of the peptides listed in Table III. The results are found to be uninformative since the value of the intrinsic viscosity at infinite DNA dilution in the presence of other molecules *will* and *does* approach the value of the intrinsic viscosity of free DNA at infinite dilution, *i.e.*, since the binding constant of the small molecule to DNA is finite, the complex will be disassociated at the lower concentrations. Instead, the effect of increasing concentrations of various molecules on the η_{sp} of DNA solution at *near infinite* dilution of the latter, *i.e.*, at 2.6×10^{-4} mol of P/l., is presented.)

In order to interpret the viscometric data given in Table III, it is important to note that the η_{sp} of a DNA solution is inversely dependent on ionic strength (Cohen and Eisenberg, 1969, and references therein). Presumably, the effect is due to shielding of neighboring negatively charged phosphate groups by the positively charged counterions which would lead to electrostatic constriction of the DNA polymer. The magnitude of this effect is dependent on the nature of the cation, *i.e.*, the greater the number of positive charges on the molecule the greater the lowering of η_{sp}. For example, the diammonium salt, $N^+H_3(CH_2)_5N^+H_3$, which has been shown to specifically bind to two adjacent phosphate groups of DNA (Mahler and Mehrota, 1963; Gabbay, 1967; Gabbay and Shimshak, 1968) decreases the η_{sp} to a greater extent than Na^+ (Table III). Therefore, in order to compare the effect of various dipeptide amides (which contain two positive charges) on the η_{sp} of DNA solution, the diammonium salt, $N^+H_3(CH_2)_5N^+H_3$, is used as a reference since (i) it contains two positive charges, and (ii) the N-terminal lysyl dipeptides are chemically related, *i.e.*, α-carboxyamido-substituted 1,5-diamines. The viscometric data (Table III) show that the bis positively charged dipeptide amides may be divided into two groups. Group I peptides show a decrease in η_{sp} of the same magnitude as the diammonium salt, $N^+H_3(CH_2)_5N^+H_3$. Group II peptides, on the other hand, show a more dramatic decrease in the η_{sp} of DNA which cannot be explained on the basis of an ionic effect alone. The two types of peptides are listed as

group I peptides: L-Lys-D-PheNH$_2$ (**23**), L-Phe-L-LysNH$_2$ (**24**), L-Lys-L-LeuNH$_2$ (**46**), L-Leu-L-LysNH$_2$ (**47**), and L-Arg-L-LeuNH$_2$ (**48**)

group II peptides: L-Lys-L-PheNH$_2$ (**17**), L-Lys-L-TyrNH$_2$ (**37**), and L-Lys-L-TrpNH$_2$ (**44**)

It is noted that all of group II dipeptide amides contain an aromatic ring at the C-terminal position and not only show a higher decrease in η_{sp} of DNA but also exhibit a greater upfield chemical shift and line broadening of the pmr signals of the aromatic protons in the n-DNA complex (Table I) than the dipeptide amides of group I, *i.e.*, L-lysyl-D-phenylalaninamide and L-phenylalanyl-L-lysinamide.

Equilibrium Dialysis Studies. Direct binding studies of a number of dipeptide amides with several types of DNA were carried out using equilibrium dialysis. The quantitative analysis of the peptide concentrations was accomplished *via* the fluorescent reagent fluorescamine (see Experimental Section for details). The results of these studies are shown in Table IV. (It should be noted that the binding studies were carried out in duplicates at a single peptide and DNA concentration, *i.e.*, at 7×10^{-4} mol of P/l. of DNA and 5×10^{-5} M peptide.

TABLE II: Effect of Lysyl and Dilysyl Tripeptide Amides on the ΔT_m of the Helix–Coil Transition of Salmon Sperm DNA ($\Delta T_m = T_m - T_{m0}$, where T_m and T_{m0} is the Melting Temperature in the Presence and Absence of Peptide).[a]

System	ΔT_m
L-Lys-L-Lys-L-PheNH$_2$ (**26**)	15.5
L-Lys-L-Phe-L-LysNH$_2$ (**27**)	14.7
L-Phe-L-Lys-L-LysNH$_2$ (**28**)	15.5
L-Phe-L-Lys-L-PheNH$_2$ (**29**)	3.4
L-Lys-L-Phe-L-PheNH$_2$ (**30**)	2.9
L-Phe-L-Phe-L-LysNH$_2$ (**31**)	2.6

[a] T_m studies were carried out in 0.01 M 2-(*N*-morpholino)-ethanesulfonate (Mes) buffer (pH 6.2) using 8.4×10^{-5} mol of DNA-P/l. of DNA and peptide concentrations of 1.5×10^{-4} M.

TABLE III: Effect of Various Peptide Systems on the Viscosity of Salmon Sperm DNA.[a]

No.	Peptide	Peptide Concn × 10^4					
		0.00	0.93	1.86	2.79	3.72	5.58
	NaCl	1.00	0.96	0.91	0.91		0.87
	$N^+H_3(CH_2)_5N^+H_3$	1.00	0.87	0.79	0.77		0.72
17	L-Lys-L-$PheNH_2$	1.00	0.79	0.69	0.66	0.62	0.60
23	L-Lys-D-$PheNH_2$	1.00	0.87	0.81	0.78	0.74	0.73
24	L-Phe-L-$LysNH_2$	1.00	0.88	0.82	0.80	0.78	0.73
37	L-Lys-L-$TyrNH_2$	1.00	0.77	0.71	0.67	0.64	0.61
44	L-Lys-L-$TrpNH_2$	1.00	0.83	0.76	0.70	0.66	0.62
46	L-Lys-L-$LeuNH_2$	1.00	0.89	0.83	0.79	0.76	0.73
47	L-Leu-L-$LysNH_2$	1.00	0.93	0.87	0.83	0.79	0.76
48	L-Arg-L-$LeuNH_2$	1.00	0.88	0.84	0.81	0.78	0.75

[a] The results are shown as the ratio of the specific viscosity of the DNA–peptide complex to the specific viscosity of DNA ($\eta_{sp}{}^C/\eta_{spDNA}$) and its dependency on peptide concentration. Studies conducted with a low-shear Zimm viscometer at 37.5°. The DNA concentration was 2.6×10^{-4} mol of DNA-P/l. in 0.01 M Mes buffer (pH 6.2) (0.005 M Na^+).

TABLE IV: Apparent Binding Constant, K_a, of Various Dipeptide Amides with Several DNA.[a]

Peptide	× 10^{-3}				
	$K_{salmon\ sperm}$ (58% A-T)	$K_{poly[d(A-T)]}$ (100% A-T)	$K_{M.\ luteus}$ (28% A-T)	K^{AT}/K^{SS}	K^{SS}/K^{ML}
*Lys-$TrpNH_2$[b] (**44**)	1.8 ± 0.25	6.4	1.2 ± 0.10	3.50	1.52
Lys-$TrpNH_2$ (**37**)	1.4 ± 0.21	3.9	1.2 ± 0.15	2.79	1.17
*Lys-$PheNH_2$ (**17**)	1.2 ± 0.25	4.0	0.9 ± 0.10	3.24	1.30
*Phe-$LysNH_2$ (**24**)	1.5 ± 0.10				
*Lys-D-$PheNH_2$ (**23**)	1.4 ± 0.08	0.6	2.3 ± 0.19	0.43	0.61
*Arg-$PheNH_2$ (**25**)	1.4 ± 0.12	3.3	0.8 ± 0.28	2.39	1.70
*Lys-$LeuNH_2$ (**46**)	1.2 ± 0.08	2.0	0.8 ± 0.17	1.69	1.52
*Leu-$LysNH_2$ (**47**)	1.3 ± 0.01	1.2	0.3 ± 0.05	0.93	4.80
*Arg-$LeuNH_2$ (**48**)	1.4 ± 0.09	2.4	0.6 ± 0.15	1.70	2.40

[a] All solutions were 0.01 M Mes buffer (0.01 M Na^+), pH 6.3 at 7.0°. DNA concentrations were salmon sperm DNA (7.0×10^{-4} mol of DNA-P/l.), poly[d(A-T)] (4.90×10^{-4} mol of DNA-P/l.), and *M. luteus* DNA (6.80×10^{-4} mol of DNA-P/l.). The peptide concentration was 5×10^{-5} M. The stereochemistry of all amino acid residues is L unless otherwise specified. [b] The asterisk means synthesized in our laboratory.

under identical buffer and ionic strength conditions. We have attempted unsuccessfully to carry out Scatchard-type binding studies in order to ascertain the maximum number of binding sites as well as the average binding constant. The most probable reason for the failure is due to the fact that the binding constants of the peptides to DNA are very low compared to systems such as Acridine Orange, proflavin, ethidium bromide, etc., where Scatchard plots have been successfully employed. For example, (1) the Donnan effect (Tanford, 1963) limits the maximum DNA concentration which can be used at the low ionic strength which is necessary to obtain significant binding of the peptide, and (2) lowering the peptide concentration significantly affects the accuracy of the quantitative assay determination employed in this work. It should be noted that the duplicate determinations of the apparent binding constant, K_a, reported in Tables IV and V varied by not more than ±15% in most cases. The apparent binding constant, K_a, was evaluated according to eq 1, where R_f equals the free peptide concentration, R_b equals the concentration of bound peptide, and P_t is the total DNA phosphate concentration. This model assumes that each DNA phosphate group binds independently to a peptide molecule and that the maximum number of binding sites on DNA is 1, *i.e.*, 1 peptide molecule/phosphate group. Obviously, this may not be true; however, since the binding data from the interactions of peptides to various nucleic acids are always evaluated in the same manner (*i.e.*, according to eq 1), the differences in values of K_a shown in Tables IV and V do reflect the differences in strength of binding. This situation would be especially true if the total DNA phosphate, P_t, is present in a large excess as compared to R_b. It should be noted that in all cases examined (Tables IV and V), P_t/R_b is found to be greater than 30.

$$K_a = \frac{R_b}{(P_t - R_b)R_f} \quad (1)$$

A number of observations may be made from the data given in Table IV. (1) L-Lysyl-L-tyrosinamide (**44**) binds more strongly to salmon sperm DNA than L-lysyl-L-tyrosinamide (**37**) which in turn binds more strongly than L-lysyl-L-phenylalaninamide (**17**). (2) The dipeptide amides containing nonaromatic residues (*e.g.*, **46**–**48**) bind as strongly to salmon sperm DNA as do some of the aromatic-containing dipeptide

TABLE V: Apparent Binding Constant, K_a, of Various Peptide Amides to Salmon Sperm DNA at Low (K_1) DNA Concentration and at High (K_2) DNA Concentration.

Peptide	K_1[a]	K_2[b]
L-Lys-L-Lys-L-PheNH$_2$ (**26**)	3.53×10^3	4.50×10^3
L-Lys-L-Phe-L-LysNH$_2$ (**27**)	2.99×10^3	2.58×10^3
L-Phe-L-Lys-L-LysNH$_2$ (**28**)	3.35×10^3	3.23×10^3
L-Phe-L-Lys-L-PheNH$_2$ (**29**)	5.10×10^2	8.40×10^2
L-Lys-L-Phe-L-PheNH$_2$ (**30**)	1.23×10^3	1.55×10^3
L-Phe-L-Phe-L-LysNH$_2$ (**31**)	5.80×10^2	1.03×10^3

[a] Salmon sperm DNA concentration was 7.00×10^{-4} mol of DNA-P/l. [b] Salmon sperm DNA concentration was 1.30×10^{-3} mol of DNA-P/l. All solutions were 0.01 M MES buffer (0.01 M Na^+), pH 6.3, at 7.0°. The peptide concentration was 5×10^{-5} M.

amides. (3) The binding constants for the aromatic peptide amides are greater than the nonaromatic systems with poly[d(A-T)]-poly[d(A-T)] and with *M. luteus* DNA. (4) The ratios of the apparent binding constant of the peptides to poly[d(A-T)]-poly[d(A-T)] and salmon sperm DNA, K^{AT}/K^{SS}, and salmon sperm DNA and *M. luteus* DNA, K^{SS}/K^{ML}, given in Table IV are expected to reflect the base pair binding selectivity of the peptides since the base compositions of the nucleic acids are different (100, 58, and 28% A-T for poly[d(A-T)]-poly[d(A-T)], salmon sperm, and *M. luteus* DNA, respectively). It is noted that the selectivity ratios, K^{AT}/K^{SS} and K^{SS}/K^{ML}, are consistent with the concept that the aromatic containing peptide amides are more selective for A-T sites than nonaromatic containing peptide amides. (5) The arginine dipeptide amides, L-arginyl-L-phenylalaninamide (**25**) and L-arginyl-L-leucinamide (**48**), bind more strongly to salmon sperm DNA than the lysine analogs. This relationship does not hold true for poly[d(A-T)]-poly[d(A-T)] and *M. luteus* DNA. (6) In two instances, L-lysyl-L-phenylalaninamide (**17**) *vs.* L-phenylalanyl-L-lysinamide (**24**) and L-lysyl-L-leucinamide (**46**) *vs.* L-leucyl-L-lysinamide (**47**), the peptide which has the nonbasic residue on the N-terminal end of the peptide binds more strongly to DNA. (7) The selectivity ratios of the lysyl dipeptide amides (*e.g.*, **17**, **37**, **44**, and **46**) suggest the following order for increasing A-T selectivity: tryptophan > phenylalanine > tyrosine > leucine.

The importance of hydrophobic type forces in controlling the binding of dipeptide amides to DNA is suggested by the data in Table IV, *i.e.*, selective binding of peptide amides which contain hydrophobic amino acid residues to A-T sites is observed. These results are completely in line with the observations of Shapiro *et al.* (1969) that hydrophobic cations, *e.g.*, N^+H_4 and $(CH_3)_4N^+$, have a higher affinity to A-T than to G-C sites. There are at least two types of hydrophobic forces that have been experimentally verified for small molecule–DNA interactions. They are (i) intercalation of planar aromatic residues between base pairs of DNA and (ii) external hydrophobic type binding of large "hydrophobic" cations, *e.g.*, steroidal amines (Gabbay *et al.*, 1970). The second type of hydrophobic binding is possible for the nonaromatic containing dipeptide amides, *e.g.*, L-lysyl-L-leucinamide, L-leucyl-L-lysinamide, and L-arginyl-L-leucinamide. However for the aromatic containing systems, intercalation may be specific for sites containing only A-T base pairs and/or A-T–G-C combinations. Since there are ten different intercalation sites possible in DNA (Gabbay *et al.*, 1972b), specificity for those sites that contain only A-T base pairs would be reflected in a greater binding constant for the peptides with poly[d(A-T)]-poly[d(A-T)] than for salmon sperm DNA. This effect is observed. The A-T selectivity of aromatic containing peptides with DNA may be the result of electronic and/or steric factors. The binding data indicate that the three aromatic amino acid residues have different affinities to A-T sites. Although the indole nucleus of the tryptophan residue is larger than the phenylalanine and tyrosine residues, the selectivity difference between these latter two residues is hard to explain without invoking specific electronic interactions between the aromatic residues and the DNA bases. However, definitive evidence for the selectivity mechanism(s) of the aromatic containing peptide amides with DNA is not yet available.

Binding studies of the lysyl and the dilysyl tripeptide amides were also carried out with salmon sperm DNA at two different base pair to peptide ratios (*i.e.*, 14/1 and 26/1). The results (Table V) show that the binding constant of the bis(lysyl) tripeptide amides is much greater than the monolysyl tripeptide amides. Clearly, the addition of an extra positive charge to the peptide system enhances its binding affinity to DNA. Also, the sequence of the amino acid residues is important with respect to the magnitude of the binding constant. For the monolysyl systems a greater binding constant is obtained when two aromatic phenylalanine residues are adjacent to one another. For the bis(lysyl) systems a greater binding is observed when the two lysine residues are adjacent to one another. The explanation for these effects is not clear.

In summary, the peptide–nucleic acid studies reported in this paper are consistent with a model whereby the aromatic residues are partially intercalated between base pairs of DNA. The pmr data indicate a sequence-dependent selective broadening and upfield chemical shift of the peptide aromatic protons on binding to DNA. For instance, the extent of the upfield shift and degree of broadening of the phenylalanine residues of dipeptide amides, $N^+H_3CHRCONHCH(CH_2C_6H_6)CONH_2$, are increased as the size of the apolar side chain (R group) of the adjacent amino acid is increased. In addition, the extent of the upfield chemical shift and the degree of broadening are not indications of the binding affinity of the peptide to DNA but rather are probes of the geometry of the complex. This point is made clear if the pmr data of L-lysyl-L-phenylalaninamide (**17**) and L-lysyl-L-lysyl-L-phenylalaninamide are compared with the binding data. The selective lowering of the specific viscosity of DNA by the aromatic amino acid containing peptides as opposed to the nonaromatic amino acid containing peptides is also in agreement with the partially intercalated model. It is postulated that the aromatic residue of the peptide causes tilting of adjacent base pairs and hence a decrease in the effective length of the helix. Denaturation of the helix at the point of complex formation is ruled out by the results of the T_m, uv, and CD studies. The peptides are found to stabilize the helix to heat denaturation and show little effect on the uv and CD spectra of DNA. The results of Helene *et al.* (1971a,b) utilizing fluorescence and pmr techniques on the studies of tyramine and tryptamine binding to DNA and its monomer components are in agreement with our data and indicate that the planar aromatic indole ring is intercalated between base pairs of DNA.

Direct binding studies of peptides with DNA suggest that the peptides may bind selectively to A-T sites. It is shown that the affinity to A-T binding sites for the aromatic containing lysyl dipeptide amides is tryptophan > phenylalanine > tyrosine.

The biological significance of the intercalation specificity found for the aromatic-containing peptides is difficult to evaluate at present. It has been proposed that the ten distinctly different intercalation sites possible in DNA may serve as a specific means for recognition of DNA sequence by an intercalating molecule (Gabbay *et al.*, 1972b). In a sense, the different intercalation sites can be considered as pages in a book with the intercalating molecule acting as a selective bookmark. Thus, the "bookmark" hypothesis proposed earlier by Brown (1970) takes on added significance, now that it has been shown that the aromatic amino acid residues not only can intercalate, but do so with some degree of selectivity with respect to primary sequence of the peptide as well as base-pair specificity.

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Nature and Possible Functions of Association between Glutamate Dehydrogenase and Cardiolipin†

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ABSTRACT: Addition of cardiolipin to glutamate dehydrogenase inhibited the enzyme and induced aggregation of lipid and protein. The inhibition was dependent on enzyme concentration. Both apolar and polar parts of cardiolipin were implicated in the binding. Indeed, hydrolysis of cardiolipin fatty acid chains or addition of a detergent such as Lubrol WX which destroyed hydrophobic bonds prevented the inhibition. The fact that acetylation of glutamate dehydrogenase amino groups hindered the aggregation suggested that they were involved in binding the cardiolipin polar head. The amount of lipid and enzyme found in the aggregates depended upon their relative concentrations and on the presence of cofactors. Glutamate increased and NADH diminished the aggregation. Additional cardiolipin, ADP, GTP, or glutamate could release this inhibition. The presence of glutamate could also selectively prevent the release of glutamate dehydrogenase from inner membrane–matrix mitochondrial fractions, suggesting that glutamate increased the binding of the enzyme to the membrane. The possible role that this association can play in the assembly of mitochondrial membranes and in the regulation of glutamate dehydrogenase activity *in situ* is discussed.

The key to an understanding of the assembly and functions of mitochondrial membranes is the nature of the interactions between lipids and proteins and the new properties that these associations may generate. The active role played by lipids in the activity of membrane-bound enzymes of the mitochondria has been demonstrated in numerous studies (*cf.* Rothfield and Romeo, 1971). However, interferences of lipids with more soluble mitochondrial enzymes have received less attention.

Glutamate dehydrogenase (EC 1.4.1.3) (Norum *et al.*, 1966; Schnaitman and Greenawalt, 1968) and cardiolipin (Getz *et al.*, 1962) are both selectively located in the mitochondria. Earlier investigations on model systems have shown that cardiolipin is a potent inhibitor of glutamate dehydro-

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