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Conformational Study of the Dipeptide Arginylglutamic Acid and of Its Complex with Nucleic Bases

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Abstract: Proton and ^{13}C magnetic resonance spectra are reported for Ac-Arg-Glu-NHEt, Boc-Arg-Glu-NHEt, and protected Arg and Glu peptides. An intramolecular complex with two hydrogen bonds is found between side chains of arginine and glutamic acid. In all protected peptides containing a carboxylate group, a hydrogen bond is found between COO^- and the peptidic NH (Glu). Replacing the carboxylate group (COO^-) by the acid function (COOH) leads to the vanishing of the intramolecular hydrogen bond. NMR investigation of the interaction of the dipeptide with nucleic acid bases has shown that only guanine forms a strong intermolecular complex. Guanine forms a complex with two hydrogen bonds with carboxylate groups and disrupts the intramolecular Arg-Glu complex. No strong interaction was seen between bases and arginine.

The specific recognition between nucleic acids and proteins is one of the fundamental molecular processes involved at every step of genetic expression. The most striking examples include the recognition of operators by repressors, of promoters by RNA polymerase, and of DNA base sequences by restriction endonucleases. It may be asked whether there are general rules which govern this recognition process.

Several authors have attempted to solve the problem of protein–nucleic acid recognition in terms of models based on structural complementarity between double-stranded DNA or RNA and the antiparallel β structure in proteins.[1-4]

The present work is based on the idea that besides an overall structural complementarity the interacting regions of the two macromolecules involved in a protein–nucleic acid complex must establish point interactions between the available chemical groups of both components. There are several ways by which peptides can interact specifically with other molecules. These include electrostatic interactions, which are very common but would not lead to highly specific binding, stacking interactions involving aromatic amino acids and bases,[5-9] and hydrogen-bonding interactions.[10-14]

It has been suggested by Seeman et al.[10] that a single hydrogen bond is unable to discriminate with any great precision a particular base pair in a nucleic acid double helix. However, two hydrogen bonds in the same functional group provide a mechanism for fixing the position of the two bonds relative to each other with a much higher degree of precision. An interesting analogy has been made by Davies,[15] who found many highly specific polynucleotide interactions, all of which utilize hydrogen bond pairs as the basis of specificity in the interaction. These analogies enabled Seeman et al.[10] and Hélène[11] to propose several types of complementary pairs involving nucleic acid bases or base pairs and some amino acid side chains. Among these side chains, two are expected to play a particularly important role. These are the carboxylate anions of glutamic and aspartic acids and the guanidinium cation of arginine. The former could form a highly specific hydrogen-bonded complex with guanine and experimental evidence is

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Table I. Summary of Physical Data of the Oligopeptides Synthesized

no.	compound	yield, %	mp, °C	$[\alpha]_{546}^{25}$, deg (c 1, MeOH)	R_f value (solvent system)
I	Nps-Glu(Bzl)-NHEt	77	89–91	−13.1	0.67 (A)
II	HCl,H-Glu(Bzl)-NHEt	85	hygr.		0.49 (B)
III	Ac-Glu(Bzl)-NHEt	74	158–159	−11.5	0.68 (A)
IV	Ac-Glu-NHEt	84	130–132	−23.3	0.32 (A)
V	Boc-Arg(NO_2)-NHEt	50	75–77	−0.4	0.46 (A)
VI	HCl,H-Arg(NO_2)-NHEt	98	hygr.		0.24 (B)
VII	Ac-Arg(NO_2)-NHEt	68	183–184	−9.2	0.56 (C)
VIII	Boc-Arg(NO_2)-Glu(Bzl)-NHEt	60	129–130	−16.7	0.54 (A)
IX	HCl,H-Arg(NO_2)-Glu(Bzl)-NHEt	86	195–196	18.8	0.50 (C)
X	Ac-Arg(NO_2)-Glu(Bzl)-NHEt	85	184–186	−17.0	0.25 (A)
XI	Ac-Arg-Glu-NHEt	88	185–187	−20.5	0.22 (B)
XII	Boc-Arg-Glu-NHEt	91	202–204 dec	−22.0	0.40 (B)
XIII	Nps-Nva-OH,DCHA	78	193–195	−48.9	0.62 (A)
XIV	Nps-Nva-OPcp	85	112–114	−95.8 (c 1, $CHCl_3$)	0.87 (A)
XV	Nps-Nva-NHEt	77	116–118	−46.4	0.69 (A)
XVI	HCl,H-Nva-NHEt	98	142–143	33.6	0.44 (B)
XVII	Boc-Arg(NO_2)-Nva-NHEt	41	118–120	−25.5	0.54 (A)
XVIII	HCl,H-Arg(NO_2)-Nva-NHEt	91	220–225 dec	14.7	0.35 (B)
XIX	Ac-Arg(NO_2)-Nva-NHEt	83	188–191	−30.2	0.21 (A)
XX	Ac-Arg-Nva-NHEt,$HClO_4$	91	94–98	−28.0	0.33 (B)

already available for this complex.[14] The latter has only hydrogen-bonding donor groups which could form a pair of hydrogen bonds with O(6) and N(7) of guanine involved in a G·C base pair and with cytosine O(2) and N(3) in a single strand. However, the arginine side chain bears a positive charge which makes it a good candidate for electrostatic interaction with a negatively charged phosphate group of the nucleic acid, and this residue may not be readily available to bind guanine. Hélène[11] proposed a model in which this difficulty could be overcome. In this model the positive charge of the arginine side chain could be neutralized by a carboxylate anion of glutamate or aspartate whose two oxygen atoms could form hydrogen bonds with two NH groups of arginine. Such a complex has been observed between arginine and the terminal carboxylic group of bradykinin.[16] From space-filling models it appears that the sequence Arg-Glu would be appropriate for such an interaction. The carboxylic group of Glu and the guanidinium group of Arg (which can potentially form two hydrogen bonds with $N_\epsilon H$ and one NH_2) could have a conformation where the planar guanidinium group[17] and the carboxylic group are coplanar. Such a structure has already been found in the crystalline complex of L-arginine-L-glutamate.[18] It should be noted that this Arg-Glu sequence is observed in several nucleic acid binding proteins. For example, in ribosomal S_4 protein the sequence Arg-Glu-Lys appears twice, Arg-Glu three times, and Glu-Arg twice. The sequence Arg-Glu-Lys appears also in position 35–37 of the N-terminal part of the *lac* repressor. However, the arginyl and glutamic acid residues need not necessarily be adjacent in the protein.

The aim of this work was to test experimentally this model of specific interaction. The conformation of the dipeptide Arg-Glu blocked at its N- and C-terminal ends was investigated by proton and ^{13}C magnetic resonance spectroscopy and was shown to form an intramolecular ion pair. Also, hydrogen-bond formation between the dipeptide Arg-Glu and adenine, uracil, cytosine, and guanine derivatives was investigated by nuclear magnetic resonance spectroscopy.

Experimental Section

Materials. All amino acids (of the L configuration) were obtained from Merck and cytosine was from Sigma. 9-Ethyladenine (e^9A), 1-cyclohexyluracil ($(chx)^1U$), 9-ethylguanine (e^9G), and 2,2-*N*,*N*-dimethyl-9-methylguanine ($m_2^2m^9G$) were purchased from Cyclo Chemical. For thin-layer chromatography (TLC), precoated silica gel plates from Merck were used.

Peptide Synthesis. The starting materials, Boc-Arg(NO_2)-OH,[19] Nps-Glu(Bzl)-OH,DCHA,[20] and Nps-Nva-OH,DCHA,[20] were prepared by standard literature procedures. In order to suppress electrostatic contributions to the interactions with nucleic bases, the NH_2-terminal group of the peptide was blocked by acetylation[21] and the terminal carboxylic acid group of the peptide was blocked by ethylamide formation. The Nps and Boc protecting groups were removed by HCl/ether and HCl/acetic acid, respectively. The classical dicyclohexylcarbodiimide method at low temperature was used for the formation of the peptide bond between the Boc-Arg(NO_2) and the Glu(Bzl)-NHEt or Nva-NHEt derivatives. Traces of the two unreacted starting compounds were first eliminated by washing successively with a 5% $KHSO_4$ solution, distilled water, saturated $NaHCO_3$ solution, and distilled water, before recrystallization of the desired product. Compounds V and XVII (Table I) were further purified by preparative chromatography on silica gel with $CHCl_3$/MeOH (8:1) as eluting solvent.

For compounds VIII and X, the hydrogenolytic cleavage of the nitro group from N^η-nitroarginine and of the benzyl-protecting group of the side chain of glutamic acid was carried out simultaneously in water/methanol (1:6) solutions using palladium/activated charcoal (10% Pd) as the hydrogenation catalyst. For these hydrogenolyses, no acid was necessary because as soon as the amino groups of the arginine side chain were liberated they were protonated by the carboxylic groups of the glutamic acid side chains.

For compounds VII and XIX, the hydrogenolytic cleavage of the nitro group from N^η-nitroarginine in the absence of acid gave rise to several side reactions. As acetate or chloride counterions of the guanidinium group interact strongly with nucleic acid bases[22] (and guanine particularly), the hydrogenolytic cleavage was attempted in perchloric acid which does not interact with nucleic bases. However, under these conditions, one important side reaction proceeded. The best method to obtain these compounds in the perchlorate form was to make the hydrogenolytic cleavage in a water/methanol mixture (1:6) containing 10% acetic acid and then, after lyophilization, to displace the acetic acid by 1 equiv of perchloric acid and to lyophilize again.

After purification, all the peptides showed a single spot when subjected to thin-layer chromatography on silica gel using the following solvent systems: (A) chloroform/methanol/acetic acid (85:10:5), (B) 1-butanol/acetic acid/water (4:1:1), and (C) ethyl acetate/pyridine/water (20:10:11) (all ratios by volume). A summary of the reaction yields, melting points, optical rotations, and thin-layer chromatography R_f values of these new compounds is given in Table I. The greatest deviation in the elementary analysis of these com-

Table II. Proton Magnetic Resonance Data of Protected Peptides (5×10^{-2} M) in Me_2SO-d_6 at 295 K from Tetramethylsilane (Me_4Si = 0.00 ppm)

	NH (Glu)	$N_\epsilon H$	NH (Arg)	NH (Et)	NH_2 (Gdm)	$C_\alpha H$ (Arg)	$C_\alpha H$ (Glu)	$C_\delta H_2$ (Arg)	CH_2 (Et)	$C_\gamma H_2$ (Glu)	CH_3 (Ac or Boc)	$C_\beta H_2$ (Glu)	$C_\gamma H_2$ (Arg)	$C_\beta H_2$ (Arg)	CH_3 (Et)
Ac-Arg-Glu-NHEt[a]	9.19	9.08	8.05	7.73	7.33	4.22	4.02	3.05	3.05	2.10	1.85	1.70	1.50	1.50	0.98
Ac-Arg-NHEt perchlorate		7.59	7.97	7.94	7.07	4.19		3.08	3.08		1.85		1.50	1.48	1.00
Ac-Arg-NHEt acetate		9.29	8.14	8.14	7.81	4.19		3.07	3.07		1.86		1.62	1.49	1.00
Ac-Glu-NHEt[b]	7.90			7.90			4.20		3.07	2.22	1.85	1.70			1.00
Ac-Glu-NHEt, sodium	8.83			8.14			4.04		3.03	1.91	1.81	1.70			0.99
Ac-Arg-Nva-NHEt[c] perchlorate		7.60	7.87	7.88	7.04	4.21		3.08	3.08		1.87			1.50	1.00
Ac-Arg(NO_2)-Glu-(Bzl)NHEt	8.05	8.40	7.23	7.81	7.23	4.20	4.20	3.08	3.08	2.33	1.86	1.70	1.55	1.55	1.00
Boc-Arg(NO_2)-MHEt			6.76	7.78		3.84		3.11	3.11		1.38		1.50	1.50	1.01
Boc-Arg-Glu-NHEt	9.24	9.46	7.03	7.75	7.64	3.86	4.03	3.38	3.06	2.01	1.37	1.66	1.57	1.57	0.99

[a] 1.5×10^{-2} M. [b] COOH at 12.09 ppm. [c] NH(Nva) at 7.60 ppm and $C_\alpha H$(Nva) at 4.12 ppm.

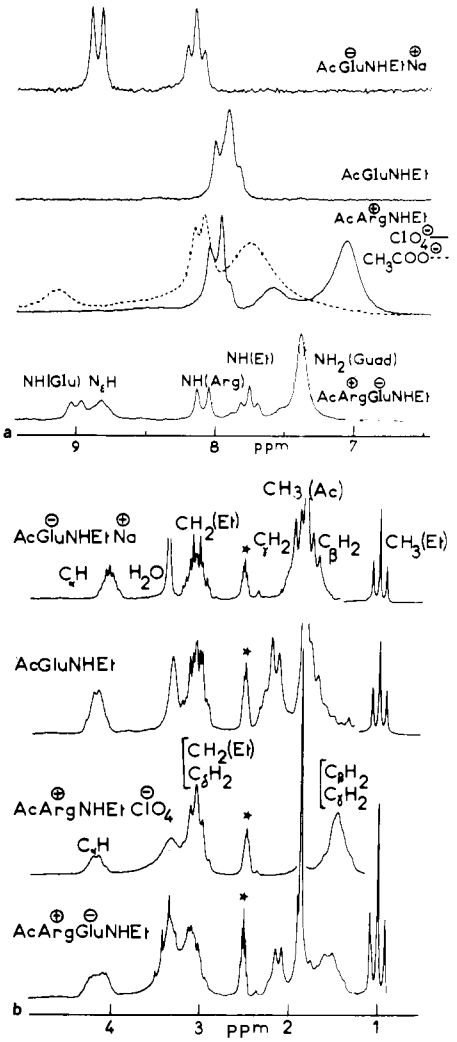

Figure 1. (a) ^1H NMR spectra of exchangeable protons of Ac-Arg-Glu-NHEt, Ac-Arg-NHEt perchlorate, Ac-Arg-NHEt acetate, Ac-Glu-NHEt, and Ac-Glu-NHEt sodium in Me_2SO-d_6 at 295 K. Parts per million are expressed from tetramethylsilane (Me_4Si = 0.00). The COOH resonance line at 12.09 ppm is not shown in Ac-Glu-NHEt spectra. (b) ^1H NMR spectra of nonexchangeable protons of Ac-Arg-Glu-NHEt, Ac-Arg-NHEt perchlorate, Ac-Glu-NHEt, and Ac-Glu-NHEt sodium in Me_2SO-d_6 at 295 K. Parts per million are expressed from tetramethylsilane (Me_4Si = 0.00). The CH_3 resonance line of ethyl is not shown in Ac-Arg-NHEt perchlorate. The asterisk denotes Me_2SO.

pounds between the experimental and the calculated values did not exceed 0.2% for C, 0.2% for H, 0.1% for N, 0.3% for O, and 0.2% for S.

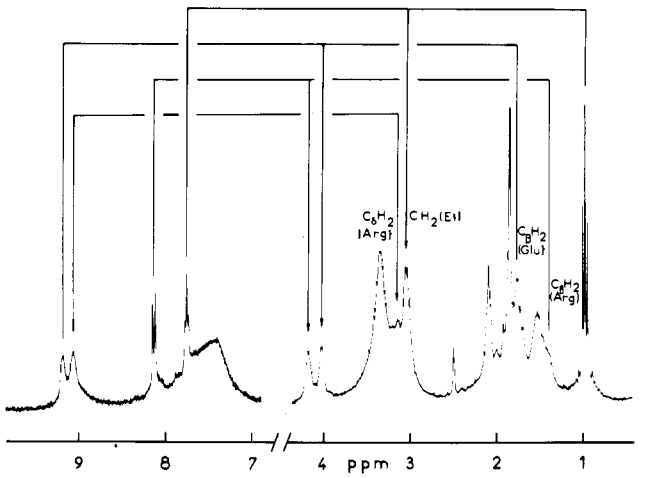

Figure 2. The 250-MHz spectrum of Ac-Arg-Glu-NHEt in Me_2SO-d_6 at 295 K. The arrows show the irradiated resonance lines and the full lines the corresponding observed collapsed lines. Parts per million are expressed from tetramethylsilane (Me_4Si = 0.00).

Methods. The ^1H (90 MHz) and ^{13}C (22.63 MHz) NMR spectra were recorded with a WH 90 Bruker spectrometer operating in the pulse Fourier transform mode with complete proton noise decoupling for ^{13}C recording. The temperature was measured to ±1 °C. Samples were dissolved in 99.95% Me_2SO-d_6 (C.E.A., France); the concentrations were in the range 10^{-3}–2×10^{-1} M for ^1H resonance and 0.1 M for ^{13}C resonance. The ^1H (250 MHz) NMR spectra were recorded with a Cameca spectrophotometer operating in the pulse Fourier transform mode.

Results and Discussion

1. Ac-Arg-Glu-NHEt. Assignments. The assignments of the different resonance lines could be partly solved by comparison with the previously reported spectra of peptides in Me_2SO[23] and the protected amino acid spectra of Ac-Arg-NHEt and Ac-Glu-NHEt (Figures 1a and 1b and Table II). All proton resonances were resolved in the 250-MHz spectrum (Figure 2). The assignment of the typical triplet structure of NH(Et) at 7.73 ppm (J = 5.4 Hz) is confirmed by the collapse of this triplet structure by irradiation of the ethyl CH_2 group. The NH_2 protons of guanidinium show a broad resonance with a maximum absorption at 7.33 ppm (Figures 1a and 2) and an integral curve corresponding to four protons. Homonuclear decoupling on one of the C_α resonances (4.02 ppm) led to a simultaneous collapse of the resonance lines of $C_\beta H_2$ (Glu) (1.70 ppm) and NH at 9.19 ppm. Therefore, the resonance lines at 9.19 and 8.05 ppm can be ascribed to NH(Glu) and NH(Arg), respectively. Confirmation of this assignment was obtained by substitution of the acetyl group by a *tert*-butyl-

Table III. ^{13}C Magnetic Resonance Data of Protected Peptides (0.1 M) in Me_2SO-d_6 at 303 K from Tetramethylsilane (Me_4Si = 0.00 ppm)

	COO^-	COOH	C=O (Arg)	C=O (Ac or Boc)	C_ζ (Arg)	C=O (Glu)	>CO (Boc)	$C_\alpha H$ (Arg or Glu)	CH_2 (Et)	$C_\beta H_2$ (Arg)	$(CH_3)_3$ (Boc)	CH_3 (Ac)	$C_\gamma H_2$ (Arg)	CH_3 (Et)
Boc-Arg-Glu-NHEt	178.07		171.38	170.79	157.34	155.65	78.12	53.68 53.16	33.27	28.66	28.08		24.31	14.56
Ac-Glu-NHEt		174.0		169.17		170.79		51.88	33.27	27.69		22.42		14.56
Ac-Glu-NHEt + TFA (1:1)		173.78		169.23		170.89		51.87	33.34	27.49		22.42		14.56
Ac-Glu-NHEt sodium	176.97			168.91		171.70		53.55	33.14	29.05		22.55		14.69
Ac-Arg-NHEt perchlorate[a]			169.17		156.56			51.93	33.27	29.25				
Ac-Arg-NHEt acetate[a]			169.10		157.08			51.93	33.27	29.05				
butyric acid ($C_\alpha H_3C_\beta H_2$-$C_\gamma H_2COOH$)[b]		174.50												
$CH_3CH_2CH_2$-COOH[c] + TFA (1:1)		174.69												

[a] 0.05 M. [b] C_α (13.52 ppm), C_β (18.07 ppm), C_γ (35.81 ppm). [c] C_α (13.65 ppm), C_β (18.26 ppm), C_γ (35.94 ppm).

oxycarbonyl group (Boc).[24,25] In Ac-Arg-NHEt this substitution led to an upfield shift (1.21 ppm) of the NH(Arg) resonance line, since this NH is covalently bound to the Boc group. The same effect was observed for the N_α protected Arg-Glu-NHEt peptide where the NH(Arg) resonances were 8.05 and 7.03 ppm in the Ac and Boc derivatives, respectively, while the other peptidic NH resonance shifted only slightly (Table II). The broad resonance line at 9.08 ppm could then be ascribed to one of the NH protons of the guanidinium group or to the proton of the carboxylic group, since Ac-Arg-Glu-NHEt had a net total charge equal to zero and could exist in two forms, $-NHC(NH_2)_2^+\cdots COO^-$ or $-N{=}C(NH_2)_2\cdots$ COOH. Since the irradiation of $C_\delta H_2$ of arginyl led to the collapse of this blurred triplet structure (Figures 1a and 2), the resonance line observed at 9.08 ppm could be attributed to the $N_\epsilon H$ proton. Moreover, the position of this resonance line is close to the resonance of $N_\epsilon H$(Gdm) in Ac-Arg-NHEt acetate (9.29 ppm) with the same line width (20 Hz). The COOH resonance is known to give rise to a resonance line around 12 ppm at this concentration (see Ac-Glu-NHEt, Table II) with a greater line width (about 100 Hz). Another direct and unambiguous assignment was provided by determining the ionization state of the two side chains.

2. Ionization State of the Side Chains. It is now well known that the ^{13}C resonance of a carboxylate group (COO^-) is shifted downfield with respect to a carboxylic group (COOH) by about 3-4 ppm in water, as shown by the titration curves of glutamic and aspartic acids.[26] This property could be used to determine the ionization state of the glutamyl residue in the dipeptide Arg-Glu. The ^{13}C NMR experiments were performed in Me_2SO-d_6 in the concentration range 5×10^{-2}-0.1 M, with the peptides protected by a *tert*-butyloxycarbonyl group since they were more soluble than the corresponding acetyl peptides. Assignments (Table III) were made by comparison with the known spectra in Me_2SO.[27] The ^{13}C NMR spectrum of Ac-Glu-NHEt showed a broad resonance line at about 174 ppm. In the presence of 1 equiv of trifluoroacetic acid (TFA), this resonance line became narrower and was located at 173.78 ppm. The ^{13}C resonance line of COOH in butyric acid showed no shift in the presence of TFA (174.50 ppm compared with 174.69 ppm). The ^{13}C resonance line found at 178.07 ppm in Boc-Arg-GluNHEt is close to the COO^- resonance (176.97 ppm) in Ac-Glu-NHEt sodium. This clearly shows that the glutamyl residue exists in the carboxylate form in the dipeptide.

3. Intermolecular Interactions. Since Ac-Arg-Glu-NHEt is only slightly soluble in Me_2SO, we have used Boc-Arg-Glu-NHEt to study the intermolecular interactions as a function of concentration. At 5×10^{-2} M, the resonance lines of the peptidic NH(Glu) and of the guanidium $N_\epsilon H$ (designated as NH(Gdm)) were well separated at 9.24 and 9.46 ppm, respectively, whereas the NH_2(Gdm) resonance lines overlapped with that of the NH(Et) and appeared with a maximum at about 7.64 ppm (Figure 3). At a lower concentration (10^{-3} M), the two NH(Glu) and NH(Gdm) resonances were shifted downfield and they overlapped at 10.04 ppm, whereas the NH_2(Gdm) resonance lines were shifted upfield to 7.14 ppm and broadened (Figure 3). The position of the NH(Et) resonance was nearly invariant; the NH(Arg) resonance was slightly shifted upfield (from 7.04 to 6.80 ppm). This concentration dependence showed that at 5×10^{-2} M part of the amino groups of the guanidinium were bound by intermolecular interactions with carboxylate or peptidic C=O groups. The downfield shifts of NH(Glu) and NH(Gdm) were more important at lower concentration, as a result of competition between intramolecular and intermolecular interactions. Figure 4 shows the variation of the chemical shifts of NH protons vs. the peptide concentration. It should be noted that intermolecular interactions involving NH_2(Gdm) disappeared almost completely at 10^{-3} M since the chemical shift of NH_2(Gdm) (7.14 ppm) is very close to the value extrapolated at zero peptide concentration (7.06 ppm). The concentration dependence was analyzed according to a model in which the dipeptide self-associates to form only dimers. The fit thus obtained was very good (see Figure 4). A dimerization constant of 12 ± 1 M^{-1} was calculated from a least-squares program analysis.[12] At low concentration the same behavior was observed for Ac-Arg-Glu-NHEt.

4. Hydrogen Bonding between Guanidinium and Carboxylate Groups. Perchlorate ions do not interact with nucleic acid bases in solution.[22] Adding sodium perchlorate to a solution of *N*-methylguanidinium chloride in Me_2SO left the ^1H NMR spectrum invariant. So, the ^1H NMR spectrum of Ac-Arg-NHEt perchlorate, which is very similar to the spectrum of *N*-methylguanidinium chloride or nitrate, represents the resonance lines of the guanidinium group interacting with the

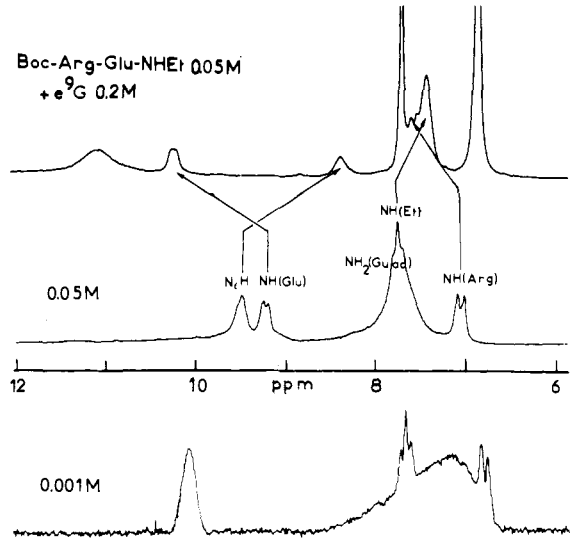

Figure 3. ^1H NMR spectra of exchangeable protons of Boc-Arg-Glu-NHEt, 10^{-3}, 5×10^{-2}, and 5×10^{-2} M in the presence of 0.2 M e⁹G.

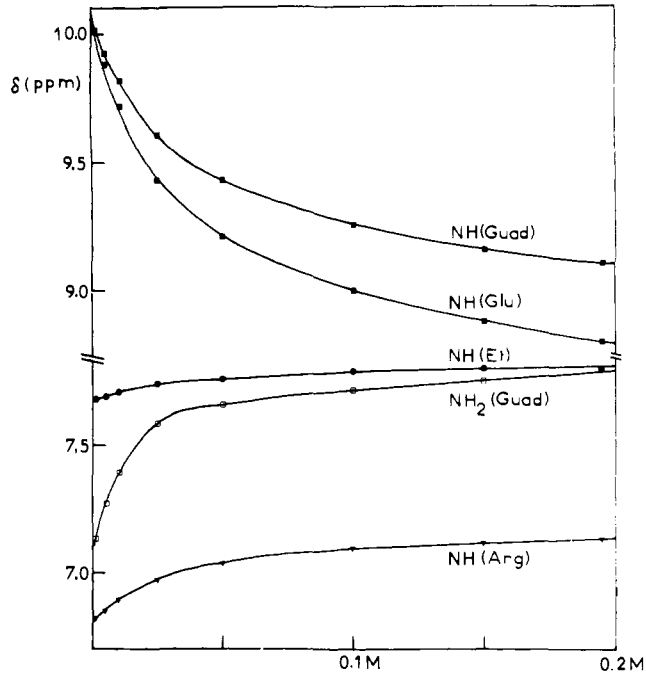

Figure 4. Chemical shifts of NH protons vs. concentration of Boc-Arg-Glu-NHEt in Me_2SO-d_6 at 295 K. Full lines represent the best fit obtained with a dimerization constant of 12 M^{-1}.

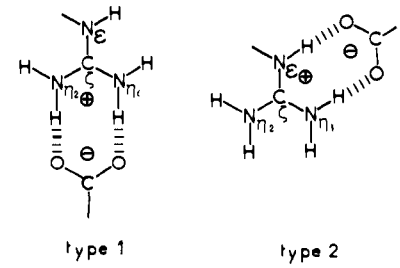

Figure 5. Two possible complexes with two hydrogen bonds between side chains of arginine and glutamate.

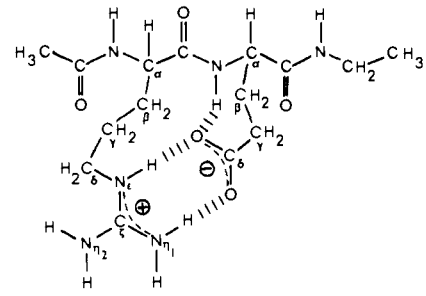

Figure 6. Proposed conformation of Ac-Arg-Glu-NHEt.

solvent (Figure 1a). Substitution of the perchlorate ion by an acetate ion led to a downfield shift and a broadening of the guanidinium amino resonances which extended from 7.5 to 9.4 ppm (Figure 1a), with two maxima at 7.81 and 9.29 ppm. This spectrum was composed of the five NH resonance lines which could be solvated by Me_2SO or bound to acetate ion in two different 1:1 complexes with two hydrogen bonds (Figure 5).

Possible intramolecular interactions between the arginyl and glutamyl residues in the dipeptide Arg-Glu are slightly different. A Corey-Pauling-Koltun (CPK) model showed that type 1 complex (Figure 5) could not exist because of steric hindrance. The type 2 complex was sterically allowed and gave the structure represented in Figure 6. Thus, the spectra of Ac-Arg-NHEt acetate could not represent the limiting spectrum obtained when two NHs are bound to the carboxylate group in the protected Arg-Glu peptide.

5. Strength of Hydrogen Bonds. Since intramolecular hydrogen bond effects are generally independent of concentration, their formation is usually tested by comparing spectra obtained in the absence or in the presence of hydrogen bond disrupting agents. Depending on the strength of the hydrogen bonds, their disruption can occur by using either mild physical techniques of chemical disrupting agents (e.g., temperature increase, addition of methanol or water) or strong disrupting agents such as trifluoroacetic acid.

The temperature dependence of the chemical shifts for the different NH protons of the dipeptide Boc-Arg-Glu-NHEt (0.05 M) is shown in Figure 7. A linear dependence was observed with slopes of 4×10^{-4} ppm/deg for NH(Gdm), 5.7×10^{-3} ppm/deg for NH(Et) and NH_2(Gdm), and 9.4×10^{-3} ppm/deg for NH(Arg). The NH(Glu) resonance was nearly independent of temperature between 296 and 346 K. These results provide strong evidence for the involvement of NH(Gdm) and NH(Glu) in strong intramolecular hydrogen bonds.

Figure 8 shows the dependence of the chemical shifts on the concentration of trifluoroacetic acid (TFA). In the presence of 5.6×10^{-2} M TFA, the spectrum of Boc-Arg-Glu-NHEt (5×10^{-2} M) showed resonance lines at 7.80 ppm for NH(Glu), 7.59 ppm for NH(Gdm), 7.10 ppm for NH_2(Gdm), 7.94 ppm for NH(Et), and 7.06 ppm for NH(Arg). This corresponds to shifts of −1.54, −1.97, −0.65, +0.20, and +0.05 ppm, respectively, when compared with the spectrum obtained without TFA. The comparison with the chemical shifts obtained in the absence of hydrogen-bonding interactions (Table II) shows that TFA destroys all hydrogen bonds.

6. Conformation of the Dipeptide. Proton and ^{13}C magnetic resonance data have shown that the arginyl and glutamyl residues are in their ionized form in both Ac-Arg-Glu-NHEt and Boc-Arg-Glu-NHEt dipeptides. Moreover, the guanidinium group as well as the NH(Glu) are hydrogen bonded, but the nature of these hydrogen bonds remains to be determined. In Ac-Arg(NO_2)-Glu(Bzl)-NHEt, the resonance lines are found at 8.05 ppm for NH(Glu), 7.81 ppm for NH(Et),

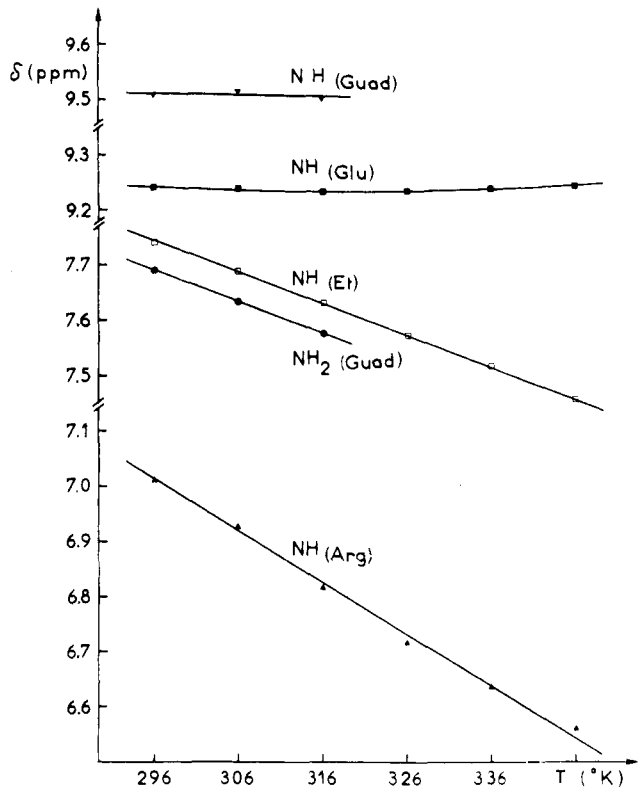

Figure 7. Temperature dependence of NH chemical shifts of Boc-Arg-Glu-NHEt (5×10^{-2} M) in Me_2SO-d_6.

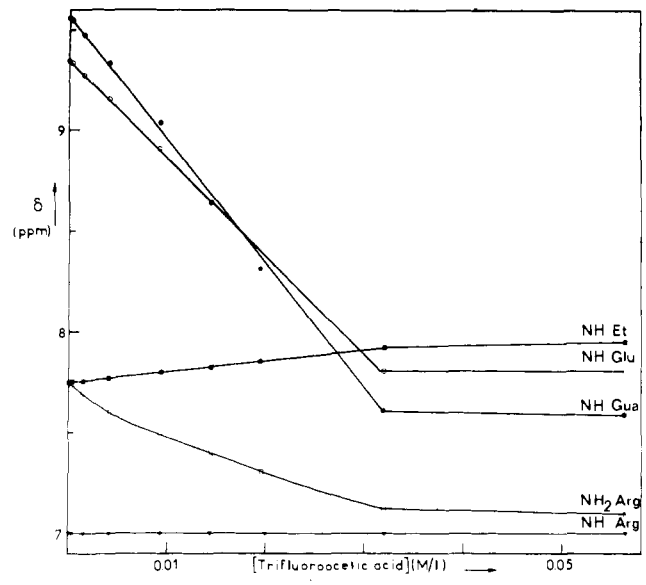

Figure 8. Chemical shifts observed of NH protons of Boc-Arg-Glu-NHEt vs. concentration of trifluoroacetic acid.

and 7.23 ppm for NH(Arg) (Table II). These data show that the three peptidic NHs are not hydrogen bonded when the guanidinium and the carboxylate groups are protected. Moreover, the ^{1}H NMR spectrum of Ac-Arg-Nva-NHEt shows (Table II) that the three peptidic NHs as well as the guanidinium group are not hydrogen bonded in the absence of a carboxylate group. We conclude that the carboxylate group is hydrogen bonded to the NH(Glu) and to the guanidinium group of the arginyl residue in both Arg-Glu dipeptides. Other evidence for the existence of the NH(Glu)···COO^- hydrogen bond was provided by the ^{1}H NMR spectrum of the sodium salt of Ac-Glu-NHEt, where the resonance line of NH(Glu) is observed at 8.83 ppm showing the formation of a hydrogen bond which does not exist in Ac-Glu-NHEt where the glutamyl residue is in its acid form (NH(Glu) at 7.90 ppm). A CPK model shows that for the structure of the dipeptide shown in Figure 5 the dihedral angle between NH(Glu) and C_αH(Glu) is in the range of 240° in agreement with the coupling constant found, $J = 4.6$ Hz, which in turn yields $\theta = 230°$ and $\phi = -70°$,[28] corresponding to a stable structure. Moreover, the breaking of hydrogen bonds by trifluoroacetic acid modifies the conformation and increases this coupling constant to 7.6 Hz (corresponding to $\phi = -90°$), whereas the other coupling constants remain unchanged. Increasing the temperature by 60 deg has no effect on the coupling constant $J = 4.6$ Hz. Since a small variation of the dihedral angle must lead to a measurable variation of the coupling constant (for $J = 4.6$ Hz, $\partial J/\partial\phi = 0.13$ Hz/deg of arc), we can conclude that the conformation shown in Figure 5 is particularly stable.

7. Interaction with Nucleic Acid Bases. Each of the four bases of nucleic acids possesses several donor or acceptor sites for hydrogen bond formation. This is also true for several amino acid side chains. Examination of different possibilities of interactions between side chains of amino acids and bases has led Seeman et al.[10] and Hélène[11] to postulate that some specificity of interaction can be induced by formation of pairs of hydrogen bonds. The arginine side chain can be selectively associated with two bases by forming two hydrogen bonds, namely cytosine and guanine, both of which have two acceptor sites of hydrogen bonds in the appropriate positions.[10,11] Interaction of a carboxylate group with bases has already been studied and a highly selective interaction has been found with guanine.[14] With a dipeptide such as Boc-Arg-Glu-NHEt, several interactions are possible with nucleic acid bases: the two interactions already described (base-guanidinium and base-carboxylate), as well as the interactions between the peptidic C=O or NH groups and the complementary groups in the nucleic acid bases such as NH, NH_2, -N=, or C=O. The ribose or deoxyribose substituents also possess groups (-O- and OH) that are able to form hydrogen bonds with the dipeptide. In order to avoid this type of interaction, substituted bases have been used (e^9A, (chx)^{1}U, e^9G) where the sugar was substituted by a chemical group which was not able to form hydrogen bonds. The observed chemical shifts of the protons of Boc-Arg-Glu-NHEt (5×10^{-2} M) in the presence or in the absence of the four bases (0.1 M) are given in Table IV. Interaction of the dipeptide with cytosine, e^9A, or (chx)^{1}U led only to small chemical shifts (equal to or less than 0.23 ppm) of the NH(Glu) and NH(Gdm) resonances, while the other chemical shifts of both the dipeptide and the bases were invariant within experimental error. In the presence of e^9G (0.2 M), the NH(Glu) resonance line was shifted downfield by 1.16 ppm, while the NH(Gdm) and NH_2(Gdm) proton resonances were shifted upfield by 1.18 and 0.37 ppm, respectively (Figure 3, Table IV). These variations of chemical shifts increased with the concentration of e^9G, while the downfield shift observed for the NH and NH_2 protons of guanine decreased with concentration (Table IV). Interaction of Boc-Arg-Glu-NHEt with $m_2^2m^9$G (0.05 M) led to a variation of the chemical shifts of only 0.06 ppm for NH_2(Gdm) and approximately 0.01 ppm or less for the other protons (Table IV). Such a behavior of the resonance lines of amino protons of guanine is also observed when guanine interacts with carboxylate ions such as acetate or butyrate[14] or the sodium salt of Ac-Glu-NHEt (Table IV). Self-association of guanine is very weak[29] ($K = 0.18$ M^{-1}) as compared to the interaction of guanine with carboxylate ions[14] ($K = 110$ M^{-1}) and can be neglected. Therefore, we conclude that the major hydrogen-bonding interaction observed in the system Boc-Arg-Glu-NHEt···e^9G is the complexing of guanine by carboxylate ions as shown in Figure 9. This interaction breaks the hydrogen bonds between guanidinium and car-

Table IV. Proton Magnetic Resonance Data of Boc-Arg-Glu-NHEt (0.05 M) and Ac-Glu-NHEt (0.05 M) in Interactions with Nucleic Acid Bases in Me_2SO-d_6 at 295 K[a]

	NH(Glu)	NH(Gdm)	NH (Et)	NH_2 (Gdm)	NH (Arg)	NH_2 (e^9G)	N_1H (e^9G)	NH_2 (e^9A)	N_3H (chx)^1U	N_1H (C)	NH_2 (C)
Boc-Arg-Glu-NHEt	9.30	9.46	7.75[b]	7.75[b]	7.03						
+ e^9G, 0.02 M	9.59	9.16	7.66[b]	7.63[b]	7.20	7.11 (6.42)	11.84 (10.52)				
+ e^9G, 0.05 M	9.85	8.91	7.60[b]	7.53[b]	7.34[b]	7.03 (6.42)	11.59 (10.52)				
+ e^9G, 0.1 M	10.19	8.57	7.55[b]	7.45[b]	~7.47[b]	6.89 (6.44)	11.39 (10.55)				
+ e^9G, 0.2 M	10.44	8.27	7.54[b]	7.38[b]	7.49[b]	6.75 (6.45)	11.14 (10.59)				
+ e^9A, 0.1 M	9.53	9.27	7.69[b]	7.76[b]	7.03[b]			7.17 (7.17)			
+ (chx)^1U, 0.1 M	9.26	9.53	7.71[b]	7.71[b]	7.03				11.18 (11.19)		
+ cytosine, 0.1 M	9.40[b]	9.40[b]	7.74[b]	7.71[b]	7.13[b]					10.40[b] (10.38)	7.10[b] (7.08)
+ $m_2^2Gm^9G$, 0.05 M	9.31	9.46	7.76	7.81	7.03						
Ac-Glu-NHEt sodium	8.59		8.05			7.22	12.39				
+ e^9G, 0.05 M	(8.83)		(8.14)			(6.42)	(10.52)				

[a] Values in parentheses give chemical shifts observed in the absence of interaction. [b] Assignment obtained by homonuclear decoupling.

boxylate ions. This is in agreement with the observed upfield shift of NH(Gdm). Moreover, the intramolecular hydrogen bonds between guanidinium and carboxylate ions which exist at 5×10^{-2} M peptide concentration strongly decrease, since most of the carboxylate groups are complexed by e^9G in agreement with the observed upfield shift of NH_2(Gdm).

A quantitative analysis of the association between e^9G and Arg-Glu was carried out by varying e^9G concentration from 0.02 to 0.2 M while keeping a constant Arg-Glu concentration (0.05 M). Self-association of Arg-Glu and e^9G was taken into account. An association constant of 38 M^{-1} was calculated for the formation of the Arg-Glu/e^9G complex.

The formation of two intramolecular hydrogen bonds in the dipeptide Arg-Glu requires a well-defined conformation of both arginyl and glutamyl side chains. In this conformation, CPK models show a restricted rotation of CH_2 groups of both side chains. The interaction with e^9G leads to a new conformation of the two side chains. The appearance of well-resolved resonance lines at 1.70 and 1.81 ppm in the presence of e^9G as well as the downfield shift of NH(Glu) (increase of the strength of the NH(Glu)···COO^- hydrogen bond) are characteristic of the structure already found in Ac-Glu-NHEt sodium salt (Table I) and support the assumption that stabilization of the glutamyl side-chain conformation is due to the hydrogen bond COO^-···NH(Glu).

These results demonstrate that, in Me_2SO, guanine is able to break the intramolecular complex formed between the arginyl and glutamyl side chains in the dipeptide Arg-Glu and strongly binds to the carboxylate ion via N_1H and NH_2 groups. Guanine is the only base able to form these two hydrogen bonds with carboxylate ions; none of the other bases have two donor groups in the correct location. This explains the highly selective interaction of carboxylate ion with guanine. The small downfield shift of NH_2(Gdm) observed in the presence of $m_2^2m^9G$ shows that interaction between guanidinium and O_6 and N_7 atoms of guanine is weak compared to the interaction with carboxylate. This confirms the small chemical shifts of NH(Gdm) and NH_2(Gdm) previously observed in Me_2SO in the presence of guanine.[30] Although the chemical shift observed was the average between the chemical shifts of these noninteracting protons and two interacting protons in this sort of complex, we can conclude that the preferential interaction of guanine with the dipeptide Arg-Glu is via two hydrogen bonds between NH_2 and N_1H of guanine and the carboxylate ion.

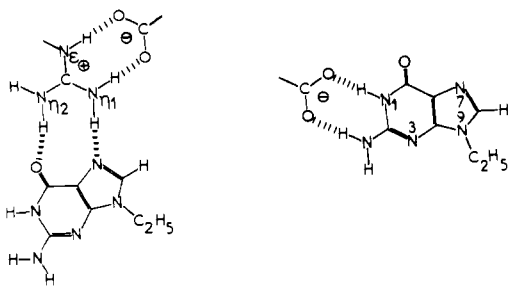

Figure 9. Possible complexes with two hydrogen bonds between guanine and side chains of arginine or glutamate.

From model building studies the sequence Glu-Arg should be favorable to the formation of the ion pair between the two side chains. The conformations of this dipeptide as well as other oligopeptides containing Arg-Glu or Glu-Arg sequences are under investigation.

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Comparison of Half-Met and Met Apo Hemocyanin. Ligand Bridging at the Binuclear Copper Active Site

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Abstract: Two series of *Busycon canaliculatum* hemocyanin derivatives have been prepared which have allowed a systematic study of ligand binding to the binuclear copper active site. Half-met-L hemocyanin, where L = CN^-, NO_2^-, N_3^-, SCN^-, OCN^-, F^-, Cl^-, Br^-, I^-, $CH_3CO_2^-$, and aquo contains a [Cu(II)···Cu(I)] active site; for the met apo-L form, however, one copper has been selectively removed and the remaining copper oxidized, producing a [Cu(II)···( )] active site. A comparison of the ligand substitution chemistry of these forms has led to two general observations. First, ligands bind far more tightly to the half-met active site. This, combined with spectroscopic data and the effects of CO coordination, requires the exogenous ligand to bridge the coppers. Second, an additional coordination position is shown to be available at the Cu(II) site for only certain half-met-L forms (L = CN^-, N_3^-, SCN^-), where the ligand is expected to keep the coppers >5 Å apart. No second coordination position is observed for any met apo derivative. These observations strongly support the presence of an endogenous protein bridge between the coppers. Furthermore, spectroscopic results have shown that certain half-met-L forms (L = Cl^-, Br^-, I^-, N_3^-) exhibit class II mixed valence properties (intervalence-transfer transitions and delocalized EPR) which directly correlate with the nature of the bridging ligand. Finally, half-met-L's, where L = N_3^-, Cl^-, $CH_3CO_2^-$, and aquo, are found to undergo reversible CO reactions which perturb the binding of L to the half-met active site.

Hemocyanin is a metalloprotein found in molluscs and arthropods which binds one oxygen molecule per binuclear copper active site.[1] The spectral features associated with the oxyhemocyanin active site are quite unusual as compared to known inorganic copper complexes.[2] In particular, while the copper is believed to be divalent, no EPR signal can be observed. This is generally ascribed to a reasonably strong magnetic coupling between the coppers, and susceptibility studies using the high sensitivity of a superconducting magnetometer have allowed a lower limit to be placed on antiferromagnetic exchange interaction of 550 cm^{-1} based on the lack of a detectable signal.[3] The optical spectral properties of oxyhemocyanin are also unusual, with an intense band at 350 nm ($\epsilon \sim 20\,000$ M^{-1} cm^{-1} per binuclear active site) and a reasonably intense transition at 570 nm ($\epsilon \sim 1000$ M^{-1} cm^{-1}) dominating the visible-UV spectral region.[4] Resonance Raman studies[5] into the visible absorption band allowed the O-O stretch to be observed at 749 cm^{-1}, a value in the lower range of peroxide complexes,[6] and suggested that the oxygen atoms were equivalent in oxyhemocyanin. However, the mode of coordination of the peroxide to the binuclear copper site and the possibility of a protein residue bridging the two coppers have not been spectroscopically determined. These unique spectral properties make this protein an extremely interesting but difficult system to study in detail.

Several valid regenerable derivatives of this metalloprotein have been prepared by different chemical routes. The met form of the protein (2Cu(II)) has been prepared by the action of peroxide on deoxyhemocyanin (2Cu(I))[7] or by artificial aging of oxy in excess ligand (N_3^-, F^-).[8] Like oxy-, methemocyanin is EPR-nondetectable,[9,10] the small EPR signal reported[11,12] for this form being associated with a damaged active site (~5%), resulting from the met preparation.[9] Alternatively, reacting deoxyhemocyanin with NO produces dimer hemocyanin which exhibits a large dipolar coupled EPR signal (the two Cu(II)'s in dimer are ~6 Å apart, based on computer simulation of the EPR signal[13,14]). Met and dimer hemocyanin can be interconverted and their optical spectral properties are quite similar, demonstrating that the interactions between the coppers leading to the large differences in ground-state EPR spectra are antiferromagnetic in nature.[9] The dimer preparation also shows an EPR signal associated with a single cupric site.[13] This could be obtained exclusively in large yield by the action of $NaNO_2$ at acid pHs on deoxyhemocyanin. In a preliminary communication,[15] we reported that this form undergoes complicated ligand substitution chemistry permitting a half-met-L [Cu(I)···Cu(II)L] series to be generated. Finally, we have shown[16] that for mollusc but not arthropod hemocyanin, one copper can be selectively removed from the active site and the remaining copper oxidized by a variety of small molecule oxidizing agents producing the met apo [Cu(II)···( )] derivative.

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