High-Resolution Proton Nuclear Magnetic Resonance Studies of Human Gastrin

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ABSTRACT: High-resolution ^1H nuclear magnetic resonance (NMR) spectroscopy at 300 MHz has been used to study the behavior of human gastrin in aqueous solution. A large number of resonances have been assigned by analysis of one- and two-dimensional NMR spectra and the effects of pH and by comparison with the spectrum of des-<Glu^1-gastrin. In gastrin, the ratio of cis to trans conformations around the Gly-2 to Pro-3 peptide bond is 3:7. This is reflected in splitting of the resonances of several neighboring residues and of a residue distant in the sequence, Tyr-12. The pK_a of Tyr-12 is 10.7. Sulfation of this residue perturbs the resonances of Tyr-12 and Gly-13 but has very little effect on the rest of the spectrum. A study of the temperature dependence shows that several perturbed resonances move toward their expected positions as the temperature is raised but with a linear dependence on temperature, consistent with a redistribution of populations among accessible local conformations rather than a cooperative conformational change. Addition of Na^+ or Ca^{2+} causes only minor changes in the spectrum. The paramagnetic metal ion Co^{2+} produces a number of spectral changes, reflecting strong binding to at least one site involving the Glu residues and weaker binding to Asp-16.

Human gastrin is a peptide hormone secreted by G cells located mainly in the antral mucosa of the stomach (Konturek, 1980). Although originally known for its ability to promote the stomach's secretion of gastric acid (Tracy & Gregory, 1964), gastrin was later found to act as a trophic hormone, promoting proliferation of cells lining the digestive tract (Johnson, 1980). The hormone exists in 14-, 17-, and 34-residue forms, which share a common C-terminal (Konturek, 1980) and are all processed from a 101-residue prohormone (Boel et al., 1983; Kato et al., 1983). The C-terminal tetrapeptide of gastrin, Trp-Met-Asp-Phe-NH_2, elicits all the effects of larger homologues, but biological potency increases by almost 2 orders of magnitude with increasing chain length, reaching a maximum for the nonapeptide (Tracy & Gregory, 1964).

In 1982 it was noted that there was greater than 50% sequence homology (Figure 1) between gastrin and a region of middle T antigen from Polyoma virus, an oncogenic virus of rodents (Baldwin, 1982). Middle T antigen is thought to be partly responsible for the transforming activity of Polyoma, since mutations affecting middle T antigen influence transforming ability (Eckhart, 1981) and since plasmids encoding middle T antigen alone can transform established cell lines but not primary rat embryo fibroblasts (Rassoulzadegan et al., 1982). Thus, in addition to their sequence homology, gastrin and middle T antigen share the ability to influence cellular proliferation.

This common trophic action may well be related to the presence of modified tyrosine residues in both molecules. In naturally occurring gastrin, one-third to one-half of Tyr-12 is sulfated (Gregory et al., 1964). Although this modification does not alter the hormone's ability to stimulate acid secretion (Konturek, 1980), Jensen et al. (1980) have reported that sulfation increases gastrin-stimulated exocrine secretion by the pancreas. Tyr-12 of gastrin can also be phosphorylated by human epidermal growth factor stimulated tyrosine kinase (Baldwin et al., 1983) although this modification is yet to be demonstrated in vivo. The corresponding Tyr (315) of middle T antigen can be phosphorylated in vivo. Although the significance of this modification has not been directly tested, deletion mutants in which Tyr-315 is absent are unable to transform (Ding et al., 1982; Nilsson et al., 1983). Substitution of Tyr-315 by phenylalanine has been variously reported to have no effect on (Oostra et al., 1983), or to drastically reduce (Carmichael et al., 1984), transforming ability. Because of the obvious importance of Tyr-315 in transformation by Polyoma virus and the presumptive importance of the homologous tyrosine of gastrin in the hormone's trophic actions, we have investigated the solution structure of synthetic human gastrin, and its sulfated derivative, by high-resolution ^1H nuclear magnetic resonance (NMR)[1] spectroscopy at 300 MHz.

NMR spectroscopy has been used previously to study the C-terminal tetrapeptide of gastrin and synthetic analogues thereof. Feeney et al. (1972) concluded that the tetrapeptide was an extended coil with the Trp and Phe aromatic rings separated by more than 5 Å and with no intramolecular hydrogen bonds. From measurements of ^1H and ^{13}C spin–lattice relaxation behavior of the tetrapeptide, Bleich et al. (1976) found that the backbone α-carbons had similar correlation times, whereas the side-chains had different degrees of motional freedom, Trp being the most rigid. Circular dichroism has also been used to study peptides related to gastrin. Pham Van Chuong et al. (1979) proposed that the tetrapeptide in organic solvents adopted a defined, compact conformation in

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[1] Abbreviations: NMR, nuclear magnetic resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate sodium salt; COSY, two-dimensional homonuclear correlated spectroscopy; SECSY, two-dimensional homonuclear spin-echo correlated spectroscopy; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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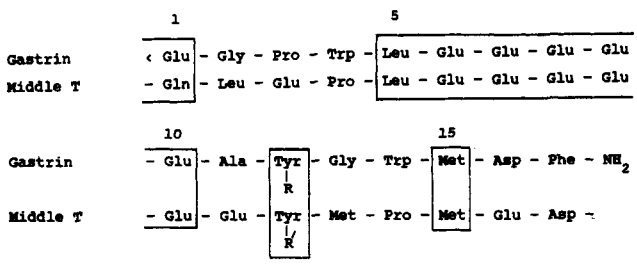

FIGURE 1: Comparison of amino acid sequences of human gastrin and a region of Polyoma middle T antigen. Boxed residues are common to both sequences. <Glu is the symbol for pyroglutamate. R can be either a hydroxy or sulfate group; R′ is a phosphate group [from Baldwin (1982)].

which the peptide backbone was folded and the aromatic side chains were <8 Å apart. It was further suggested that the structure of the tetrapeptide region was maintained in a series of larger analogues. By contrast, Peggion et al. (1981) found no evidence of conformational rigidity in the tetrapeptide in trifluoroethanol. They also showed that it did not maintain the same conformation when elongated in the N-terminal direction. Moreover, an analogue of human gastrin containing Nle at position 15 in place of Met was found to adopt a random-coil structure in aqueous solution. Thus, there is some disagreement in the literature concerning the structure of the C-terminal tetrapeptide of gastrin and the effect on its structure of the N-terminal region.

A role for the N-terminal peptide in metal binding has also been suggested. Palumbo et al. (1980) utilized circular dichroism to show that in trifluoroethanol the C-terminal tridecapeptide of gastrin containing Nle in place of Met bound two Ca^{2+} ions, one with an association constant of $>5 \times 10^8$ M^{-1} and the other of 4.3×10^5 M^{-1}. Subsequently, they showed that there were in fact three Ca^{2+} binding sites and that elongation of the peptide chain from 13 to 17 residues (to give Nle^{15}-gastrin) did not provide any additional sites (Peggion et al., 1983). Possible interactions with Ca^{2+} in aqueous medium were not investigated.[2] In this paper we have examined the effects of Na^+, Ca^{2+}, Co^{2+}, and Eu^{3+} on the ^{1}H NMR spectrum of gastrin in aqueous solution.

Materials and Methods

Materials. Synthetic human gastrin and des-<Glu^1-gastrin were obtained from Research Plus Laboratories Inc., Bayonne, NJ, and Tyr(SO$_3$H)^{12}-gastrin was from Cambridge Research Biochemicals Ltd., Cambridge, U.K. All batches were used without further purification, although the sample of sulfated gastrin contained approximately 17% normal gastrin. All other reagents were analytical reagent grade.

Samples for NMR Spectroscopy. For spectra in ^2H_2O the sample was deuterated by at least one lyophilization from ^2H_2O and dissolved in 0.45 mL of ^2H_2O (≥99.75% ^{2}H). pH was measured at 22 °C with an Activon Model 101 pH meter fitted with an Ingold 6030-02 combination microelectrode. pH was adjusted by addition of 0.05 or 0.5 M ^{2}HCl or NaO^{2}H and was measured before and after each experiment. Reported values are the average of these two measurements uncorrected for deuterium isotope effects. pK_a values were obtained by nonlinear least-squares fits of the chemical shifts to the Henderson–Hasselbalch equation for a single ionization, assuming fast exchange between the conjugate acid and base.

Metal ion titrations were carried out by addition of stock solutions of the chloride salts in ^2H_2O. Gastrin concentrations were measured spectrophotometrically with a value for ϵ_{280} of 12 320 (Peggion et al., 1981). Dissociation constants were obtained from plots of $1/\Delta\delta$ vs. $1/[Co^{2+}]$, where $\Delta\delta$ is the change in chemical shift caused by addition of Co^{2+}.

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 300.07 MHz on a Bruker CXP-300 spectrometer operating in the pulsed Fourier-transform mode with quadrature detection. Spinning sample tubes of 5-mm o.d. (Wilmad Glass Co., 527-PP grade) were used, and the probe temperature was maintained with a Bruker B-VT 1000 variable-temperature unit. Typical spectral acquisition parameters were as follows: sweep width 2994 Hz, 8192 addresses, 90° radio-frequency pulses, 2.0-s recycle time, and probe temperature 25 °C. Chemical shifts in one-dimensional spectra were measured digitally, with 1,4-dioxane, at 3.751 ppm downfield from DSS, as internal standard. Taking into account measurements from two-dimensional spectra, the accuracy of chemical shifts is ±0.01 ppm.

Two-dimensional homonuclear correlated spectra (COSY) were recorded with the pulse sequence (Aue et al., 1976; Wagner et al., 1981)

$$(t_0\text{–}90°\text{–}t_1\text{–}90°\text{–}t_2)_n$$

where t_1 and t_2 are the evolution and observation periods, respectively. Quadrature detection was employed in both dimensions, with appropriate phase cycling to select N-type peaks. Data block sizes were 2048 addresses in t_2, zero filled to 4096 addresses, and 256 or 512 equidistant t_1 values, zero filled to 2048 addresses. Before Fourier transformation, both time domains were multiplied by a sine-bell function. All two-dimensional spectra are presented in the absolute value mode without symmetrization. The water signal was suppressed by selective irradiation during the period t_0. The COSY in ^2H_2O was acquired with the following: sweep width 2703 Hz, t_0 1.421 s, 512 t_1 values from 0–190 ms, t_2 0.379 s, and 144 transients at each t_1 value. Only the first 256 t_1 values were used in processing the COSY spectrum shown under Results. The COSY in 90% H_2O/10% ^2H_2O was acquired with the following: sweep width 3496 Hz, t_0 1.707 s, 256 t_1 values from 0.01–73 ms, t_2 0.293 s, and 256 transients at each t_1 value.

The two-dimensional homonuclear spin-echo correlated spectrum (SECSY) in ^2H_2O was acquired with the pulse sequence (Nagayama & Wüthrich, 1981)

$$(t_0\text{–}90°\text{–}t_1/2\text{–}90°\text{–}t_1/2\text{–}t_2)_n$$

where t_1 and t_2 are the evolution and observation periods, respectively. Spectral parameters were as follows: t_0 1.638 s, t_1 0.01–213 ms, t_2 0.362 s, sweep width in f_1 1202 Hz, sweep width in f_2 2825 Hz, 256 t_1 values, 240 transients at each t_1 value, and 2048 addresses in t_2. The data in each time domain were multiplied by a sine-bell function and zero filled to 1024 addresses in t_1 and 4096 addresses in t_2 before Fourier transformation.

The two-dimensional homonuclear J-resolved spectrum in ^2H_2O was acquired with the pulse sequence (Nagayama & Wüthrich, 1981)

$$(t_0\text{–}90°\text{–}t_1/2\text{–}180°\text{–}t_1/2\text{–}t_2)_n$$

where t_1 and t_2 are the evolution and observation periods, respectively. Spectral parameters were as follows: t_0 1.693 s, t_1 0.01–307 ms, t_2 0.307 s, sweep width in f_1 52 Hz, sweep width in f_2 3333 Hz, 16 t_1 values, 1616 transients at each t_1 value, and 2048 addresses in t_2. The data in each time domain

[2] Peggion et al. (1983) state that no interaction of the hormone with calcium has been observed in aqueous solution, citing the previous paper by Palumbo et al. (1980) as a reference. We can find no data on aqueous solutions in the latter.

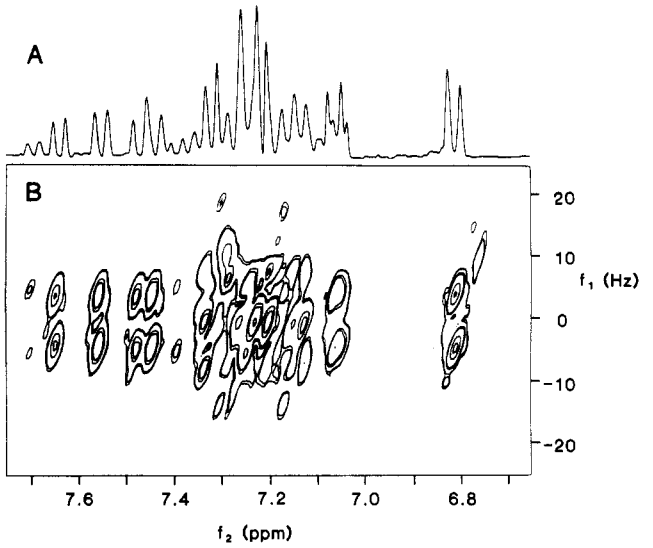

FIGURE 2: Aromatic region of 300-MHz ^1H NMR spectra of gastrin in ^2H_2O at 25 °C. (A) One-dimensional spectrum of 0.6 mM gastrin, pH 6.4. Time-domain data, consisting of 1500 transients acquired in 8192 addresses, were multiplied by a Lorentz–Gauss window function and zero filled to 16 384 addresses prior to Fourier transformation. (B) Contour plot of two-dimensional homonuclear *J*-resolved spectrum of 3.0 mM gastrin, pH 7.4. Spectral conditions are given under Materials and Methods.

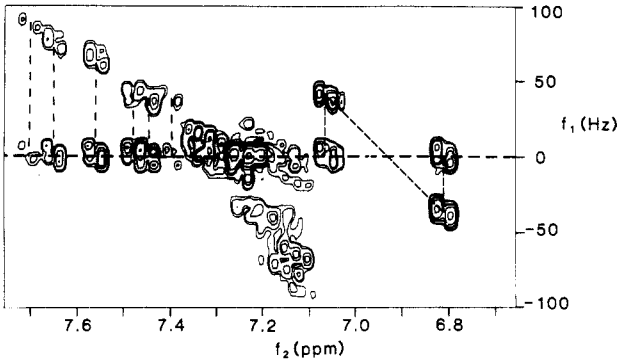

FIGURE 3: Aromatic region of a 300-MHz ^1H two-dimensional spin-echo correlated (SECSY) spectrum of 3.0 mM gastrin in ^2H_2O, pH 7.4, at 25 °C. Spectral conditions are given under Materials and Methods. Spin–spin coupling connectivities between ortho protons are indicated for the resonances of Tyr-12; partial connectivities are shown for Trp C(4) H and C(7) H resonances. Splitting of the Tyr-12 C(2,6) H resonances is evident in the additional contour on the cross-peak.

were multiplied by a sine-bell function and then zero filled to 128 addresses in t_1 and 16 384 addresses in t_2 before Fourier transformation. Spectra were displayed after application of a 45° tilt to the transformed data matrix.

RESULTS

Assignments. A large number of resonances in the ^1H NMR spectrum of human gastrin in ^2H_2O have been specifically assigned. The assignment procedures for the aromatic region of the spectrum are outlined here.

Figure 2 shows the aromatic region of a two-dimensional homonuclear *J*-resolved spectrum, together with the corresponding region of a one-dimensional spectrum. The *J*-resolved spectrum provides information on chemical shifts and peak multiplicities. For example, the apparent triplet at 7.46 ppm is revealed as a pair of doublets with chemical shifts of 7.44 and 7.47 ppm. However, for the assignment of complete spin systems, the two-dimensional NMR techniques of homonuclear correlated spectroscopy (COSY) and spin-echo correlated spectroscopy (SECSY) have been used. Figure 3 shows the aromatic region of a SECSY spectrum of gastrin with connections indicated between the C(2,6) H and C(3,5) H doublets of Tyr-12. Connections are also shown between Trp C(4) H and C(7) H doublets on the $f_1 = 0$ axis and cross-peaks indicating spin–spin coupling between these doublets and their corresponding C(5) H and C(6) H triplets.

One of the Trp spin systems is split into two components. This is most readily seen for the C(4) H doublets (Figure 2). The C(4) H doublet of one Trp, at 7.55 ppm, has an intensity of one proton, whereas C(4) H of the other gives rise to two doublets at 7.64 and 7.70 ppm, with intensities of 0.7 and 0.3 proton, respectively. Similar splitting is observed for the C(7) H doublet (Figure 2) and for the C(5) H and C(6) H triplets (Figure 3). A COSY spectrum recorded in H_2O (Figure 4) shows that the indole N(1) H and C(2) H resonances are also split.

These observations indicate that gastrin in aqueous solution exists in two forms, which interchange slowly on the NMR time scale (Dwek, 1973) and have relative populations of 7:3. As outlined under Discussion, this conformational heterogeneity is ascribed to slow exchange between the cis and trans forms of the peptide bond preceding Pro-3. Of the two Trp residues in gastrin, Trp-4 is closer to Pro-3 than Trp-14, so the split spin system is assigned to Trp-4.

This conformational heterogeneity also affects the C(2,6) H doublet of Tyr-12 (Figures 2 and 3) but not its C(3,5) H doublet. The peaks from the remaining aromatic residue, Phe-17, display second-order splittings and partially overlap peaks from the Trp residues, so possible effects of heterogeneity on Phe-17 cannot be observed.

The chemical shifts and assignments of the aromatic resonances are summarized in Table I. In general, chemical shifts in gastrin match those found in small peptides (Bundi &

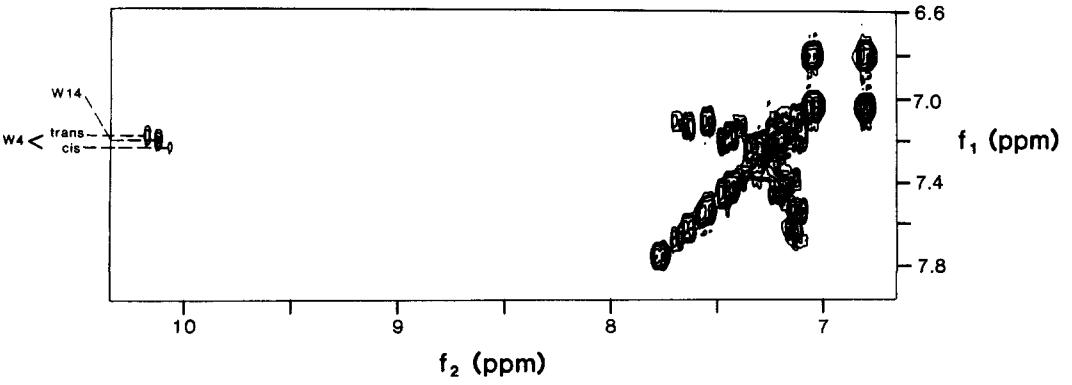

FIGURE 4: Region of a 300-MHz ^1H two-dimensional correlated (COSY) spectrum of 2.4 mM gastrin in 90% H_2O/10% ^2H_2O (v/v), pH 6.1, at 25 °C. Spectral conditions are given under Materials and Methods.

Table I: Assignment of Aromatic Proton Resonances in the ^{1}H NMR Spectrum of Gastrin[a]

residue	N(1) H	C(2) H	C(4) H	C(5) H	C(6) H	C(7) H	C(2,6) H	C(3,5) H	C(2) H–C(6) H
Trp-4									
cis[b]	10.07	7.26	7.70	7.12	7.14	7.39			
trans	10.18	7.21	7.64	7.15	7.23	7.47			
Trp-14	10.13 (10.21)[c]	7.23 (7.23)	7.55 (7.63)	7.13 (7.15)	7.21 (7.23)	7.44 (7.49)			
Tyr-12									
cis							7.05	6.81	
trans							7.06 (7.13)	6.81 (6.84)	
Phe-17									7.25, 7.29, 7.31, 7.34, 7.36 (7.32)

[a] At pH 6.4 and 25 °C. Chemical shifts are in ppm downfield from DSS. [b] Cis and trans refer to the conformation of the Gly-2 to Pro-3 peptide bond. [c] Values in parentheses are chemical shifts of corresponding protons in small peptides obtained by subtracting 0.015 ppm from the data of Bundi & Wüthrich (1979) to correct for the difference between the chemical shifts of DSS and sodium (trimethylsilyl)propionate at pH 7.

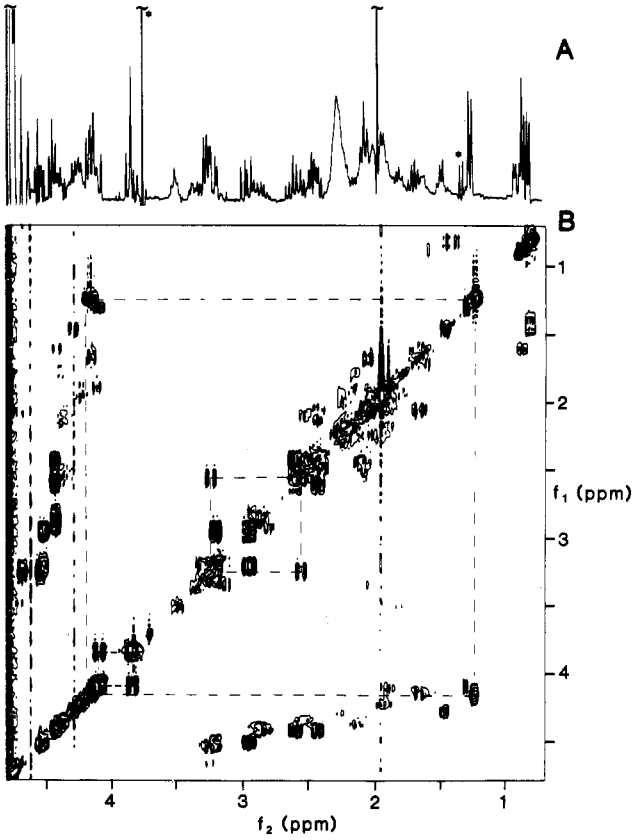

FIGURE 5: Aliphatic region of 300-MHz ^{1}H NMR spectra of gastrin in ^2H_2O at 25 °C. (A) One-dimensional spectrum of 0.6 mM gastrin, pH 6.4. Time-domain data, consisting of 1500 transients acquired in 8192 addresses, were multiplied by a Lorentz–Gauss window function and zero filled to 16 384 addresses prior to Fourier transformation. Resonances from impurities are marked with asterisks. (B) Contour plot of two-dimensional homonuclear correlated (COSY) spectrum of 3.0 mM gastrin at pH 6.5. Spectral conditions are given under Materials and Methods. Spin–spin coupling connectivities are shown for Gly-2 α-CH_2 in the cis and trans forms and for Ala-11 α-CH–β-CH_3.

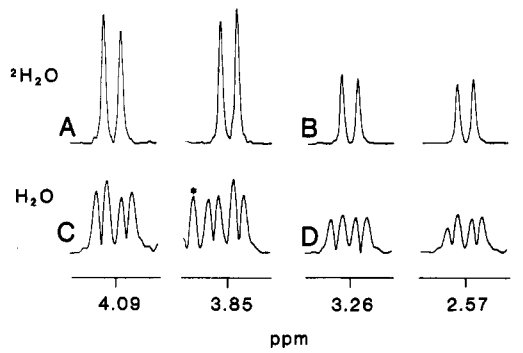

FIGURE 6: Cross sections of 300-MHz ^{1}H COSY spectra showing off-diagonal cross-peaks from Gly-2 in trans (A and C) and cis (B and D) conformers. (A and B) Same spectral data as in Figure 5 but zero filled to 1024 addresses in t_1 and 8192 in t_2 before Fourier transformation. (C and D) Same spectral data as in Figure 4 but zero filled to 1024 addresses in t_1 and 8192 in t_2 before Fourier transformation. A resonance from another spin system is labeled with an asterisk in (C). Each cross-section is 0.2 ppm wide.

Wüthrich, 1979) quite closely, indicating that none of the aromatic residues is strongly perturbed by other residues. The greatest difference is observed for N(1) H of Trp-4 (0.14 ppm upfield), all other chemical shifts differing by ≤0.1 ppm from corresponding values in peptides.

A number of resonances in the aliphatic region of the spectrum of gastrin in ^2H_2O have also been assigned. A COSY and a one-dimensional spectrum of this region are shown in Figure 5, and chemical shifts are summarized in Table II.

Gastrin contains two Gly residues. Gly-2 is affected by cis–trans isomerism around its peptide bond with Pro-3. Its resonances in the predominant trans form occur at 3.85 and 4.09 ppm (Figure 5) and have a total intensity of approximately 1.4 protons. The resonances of Gly-2 in the cis form are highly shifted, occurring at 2.57 and 3.26 ppm [1.39 and 0.70 ppm, respectively, upfield of the position found in small peptides (Bundi & Wüthrich, 1979)]. The assignment of these peaks to Gly α-CH protons is indicated by the clear doublet pattern of the cross-peaks in the COSY spectrum in ^2H_2O (Figure 6A,B) and confirmed by the additional splitting into doublets of doublets in H_2O, due to coupling to the α-NH (Figure 6C,D). The intensities of the upfield doublets could not be measured at ambient temperature because of peak overlap, but integrations at temperatures of 45 and 50 °C, where they are resolved (see below), confirm that each doublet corresponds to 0.3 proton. The other Gly residue, at position 13, gives a two-proton resonance at 3.84 ppm, close to the position found in small peptides (Table II).

The full spin system of Leu-5 was readily assigned from COSY and SECSY spectra. Its δ-CH_3 and γ-CH resonances are split (Figure 5), the δ-CH_3 peaks showing an intensity ratio of 7:3. The spin systems of Ala-11 (Figure 5) and Met-15 were also readily assigned. Certain resonances of Leu-5 and Met-15 display significant upfield shifts relative to their corresponding values in small peptides.

Gastrin contains five Glu residues at positions 6–10. Peaks from individual Glu residues could not be clearly resolved, so only the average chemical shifts are given in Table II. The spin systems of <Glu-1 and the trans form of Pro-3 have also been assigned, although chemical shifts for the β- and γ-CH_2 peaks of these residues are less precise because of peak overlap. The remaining resonances in the aliphatic region come from the AMX spin systems of Trp-4, Tyr-12, Trp-14, Asp-16, and Phe-17. These occur at the following chemical shifts (ppm) (α-CH, β-CH, β-CH): 4.43, 2.44, 2.61; 4.43, 2.8, 2.9; 4.54,

Table II: Assignment of Resonances from Aliphatic Residues in the ^1H NMR Spectrum of Gastrin[a]

residue	α-NH	α-CH	β-CH	others
<Glu-1		4.36	2.14, 2.5	γ-CH_2 2.4, 2.5
Gly-2				
cis[b]	8.13 (8.38)[c]	2.57, 3.26 (3.96)		
trans	8.25 (8.38)	3.85, 4.09 (3.96)		
Pro-3 trans		4.23 (4.46)	1.9–2.0 (1.97, 2.28)	γ-CH_2 1.8 (2.02); δ-CH_2 3.51 (3.64)
Leu-5				
cis				γ-CH 1.61 (1.63); δ-CH_3 0.87, 0.91 (0.88, 0.93)
trans	8.48 (8.41)	3.92 (4.37)	1.48 (1.63)	γ-CH 1.42 (1.63); δ-CH_3 0.81, 0.85 (0.88, 0.93)
Glu-6–10[d]	8.3–8.4 (8.35)	4.1 (4.28)	2.07–2.09 (1.95, 2.08)	γ-CH_2 2.19–2.30 (2.27, 2.30)
Ala-11	8.35 (8.23)	4.19 (4.33)	1.25 (1.38)	
Gly-13	8.25 (8.38)	3.84 (3.96)		
Met-15	8.13 (8.40)	4.17 (4.50)	1.64, 1.71 (1.99, 2.15)	γ-CH_2 2.07 (2.62); ε-CH_3 1.96 (2.11)

[a] At pH 6.4 and 25 °C. Chemical shifts are in ppm downfield from DSS. [b] cis and trans refer to the conformation of the Gly-2 to Pro-3 peptide bond. [c] Values in parentheses are chemical shifts of corresponding protons in small peptides, obtained by subtracting 0.015 ppm from the data of Bundi & Wüthrich (1979) to correct for the difference between the chemical shifts of DSS and sodium (trimethylsilyl)propionate at pH 7. [d] Range of chemical shifts is given because of overlap of peaks from the cluster of Glu residues.

2.96, 3.22; 4.55, 3.25; (not measured) 3.2–3.4. Some of these may be tentatively assigned on the basis of the observation of weak cross-peaks in the COSY spectrum in ^2H_2O, which represent long-range couplings between β-CH and aromatic protons. The Tyr-12 C(2,6) H peak at 7.06 ppm shows a cross-peak to 2.8–2.9 ppm, suggesting that the AMX system having β-CH peaks at 2.8 and 2.9 ppm corresponds to Tyr-12. The C(2) H resonances from Trp-4 and -14 show cross-peaks to the region 3.2–3.3 ppm, indicating that at least two of the AMX systems having β-CH resonances in this region correspond to these Trp residues.

Des-<Glu[1]-gastrin. The spectrum of gastrin lacking its N-terminal pyroglutamate residue differs from that of intact gastrin not only in the lack of resonances from <Glu-1 but also in the ratio of the cis and trans conformers around the Gly-2 to Pro-3 peptide bond.[3] Whereas in gastrin the cis–trans ratio was 30:70, in des-<Glu[1]-gastrin it is 15:85.

The chemical shifts of Gly-2 and Pro-3 are affected by removal of <Glu-1 and, together with Trp-4, sense the deprotonation of α-NH_3^+ of Gly-1 at high pH (see below). All other resolved resonances have chemical shifts within 0.1 ppm of their counterparts in intact gastrin.

Tyr(SO_3H)[12]-gastrin. Spectra of gastrin sulfated on the phenolic hydroxyl of Tyr-12 were also compared with those of intact gastrin. The only significant change is in the aromatic proton resonances of Tyr-12, which now give large peaks at 7.22 and 7.24 ppm. A similar pattern of peaks from sulfated Tyr has been observed recently in the spectrum of the sulfated derivative of cholecystokinin fragment CCK_{27-33} (Durieux et al., 1983). The α-CH_2 resonance of Gly-13 shows slight splitting in sulfated gastrin although its chemical shift is unchanged. All other well-resolved peaks in sulfated gastrin have chemical shifts essentially identical with those of gastrin, and the ratio of cis to trans conformers is unchanged. Because of peak overlap, it cannot be ascertained whether the resonances of sulfated Tyr-12 are still affected by cis–trans isomerism.

Effects of pH. The spectrum of gastrin was examined over the pH range 4–12.6. At pH ≤5, significant precipitation occurred, and complete spectra could not be observed. Thus, although the Glu γ-CH_2 resonances began to move downfield below neutral pH, accurate pK_a values could not be determined. Similarly, the titration of Asp-16 could not be observed. Precipitation of Nle[15]-gastrin at low pH has been reported previously (Peggion et al., 1981).

Over the pH range examined, the only complete ionization is that of Tyr-12, which has a pK_a of 10.74 ± 0.1. In its ionized form, the phenolic ring of Tyr-12 no longer senses the cis–trans isomerism at Pro-3, the splitting of the C(2,6) H peak disappearing at pH ≥ pK_a. Titration of Tyr-12 is reflected in the chemical shifts of protons of Ala-11 (β-CH_3, $\delta_A - \delta_B$ −0.03 ppm) and Gly-13 (α-CH_2, $\delta_A - \delta_B$ 0.03 ppm). At pH <5.5, several resonances, including those of both Trp residues, begin to shift. This may reflect protonation of the Glu residues or the aggregation resulting therefrom.

Des-<Glu[1]-gastrin was examined over the pH range 4.7–12.2. At the lowest pH, considerable precipitation occurred. Its Tyr residue has values of δ_A, δ_B, and pK_a (10.82 ± 0.2) essentially identical with those in gastrin itself. Titration of the free N-terminus of this analogue could also be observed via its effects on the resonances of δ-CH_2 of Pro-3 (pK_a = 7.94 ± 0.2, δ_A 3.45, δ_B 3.39) and C(4) H and C(7) H of Trp-4 (pK_a = 8.12 ± 0.3, δ_A 7.63, δ_B 7.67 and pK_a = 8.43 ± 0.2, δ_A 7.47, δ_B 7.53, respectively). At high pH, the upfield α-CH doublet of Gly-2 in the trans form resonates at 2.73 ppm; it moves downfield as the pH is lowered, but peak overlap prevents observation of its full titration. The pK_a of Gly-2 is nearly identical with that of Gly in pentapeptides, viz., 8.1–8.2 (Keim et al., 1973).

Temperature Dependence. Spectra of gastrin at pH 6.4 were examined over the temperature range 25–85 °C. The most significant changes are observed for resonances that are either shifted substantially from their random-coil positions or split as a result of cis–trans isomerism. Other well-resolved resonances shift by less than 0.05 ppm.

With increasing temperature the γ-CH_2 and ε-CH_3 resonances of Met-15 both move downfield toward their positions in small peptides. However, the dependencies of their chemical shifts on temperature are linear (correlation coefficients 1.00), with slopes of 3×10^{-3} and 0.7×10^{-3} ppm/°C for γ-CH_2 and ε-CH_3, respectively. The most high shifted resonances of all, those from α-CH_2 of Gly-2 in the cis form, also move downfield but are obscured at high temperature by overlap with other peaks. The upfield doublet moves from 2.64 ppm at 25 °C to 2.85 ppm at 65 °C, again in a linear fashion (6.9×10^{-3} ppm/°C, r = 1.00).

The splitting of the C(2,6) H resonance of Tyr-12 disappears at 55 °C. This is clearly due to a downfield movement of the minor doublet, rather than a merger of the major and minor doublets to yield a peak with a weight-average chemical shift, suggesting that the long-range interactions that cause Tyr-12 to be affected by cis–trans isomerism are disrupted around 50 °C.

The effects of temperature on the splitting of peaks from Trp-4 are shown in Figure 7. With increasing temperature the minor component of the Trp-4 C(4) H peak tends to merge

[3] The residue numbering system of gastrin is also used for des-<Glu[1]-gastrin.

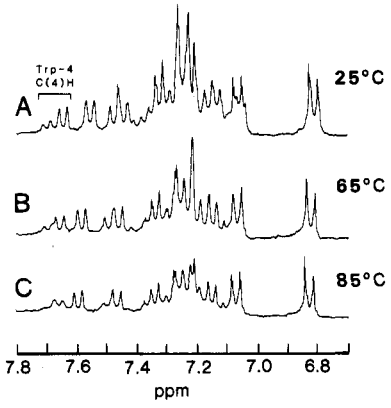

FIGURE 7: Region of aromatic protons in 300-MHz ^{1}H NMR spectra of 0.6 mM gastrin in ^2H_2O, pH 6.4, at temperatures of (A) 25, (B) 65, and (C) 85 °C. Time-domain data, consisting of 1500 transients acquired in 8192 addresses, were multiplied by an exponential function and zero filled to 16 384 addresses prior to Fourier transformation.

with the major component, but even at 85 °C this process is incomplete. Similar behavior is observed for the δ-CH_3 resonances of Leu-5. At 85 °C, broadening of the composite Trp-4 C(4) H doublet is evident, suggesting that the rate of exchange between the two conformers may now be in the intermediate range (Dwek, 1973). Because of peak overlap at 75 and 85 °C, it is not possible to determine the relative populations of the two forms, but no change is apparent up to 65 °C.

Metal Binding. Neither NaCl at concentrations up to 250 mM (pH 7.1) nor $CaCl_2$ at concentrations up to 86 mM (pH 7.4) causes any significant change in the spectrum of gastrin. In each case there are essentially linear upfield shifts of the Met-15 γ-CH_2 peak and the two doublets of the trans form of Gly-2, each by about 0.02 ppm. There are also slight changes in the Glu γ-CH_2 peak. In order to check that a possible strong metal binding site was not already occupied, gastrin was treated with Chelex-100 and dissolved in Chelex-100-treated ^2H_2O. Its spectrum was identical with that of untreated gastrin and, moreover, was not affected by the addition of 10 mM EGTA.

The interactions of gastrin with the paramagnetic cations Eu^{3+} and Co^{2+} were also examined. $EuCl_3$ at the lowest concentrations used, 1 and 0.5 mM, respectively, precipitated gastrin at pH 6.5 in ^2H_2O or at pH 7.2 in 10 mM Tris buffer in ^2H_2O. $CoCl_2$ over a concentration range of 0–82 mM caused a number of changes in the spectrum of gastrin at 0.17 mM, pH 7.3 in ^2H_2O. At the lowest $CoCl_2$ concentration examined, 1.4 mM, the peaks assigned to β- and γ-CH_2 of Glu residues 6–10 broaden so as to merge with the base line. With increasing $CoCl_2$ concentrations, a large number of resonances are shifted. One of the largest shifts is 0.35 ppm upfield for the β-CH_3 resonance of Ala-11. This presumably reflects binding of Co^{2+} to at least one site involving the Glu residues. Assuming that a single site is involved, a K_d of 2 mM is obtained. A slightly higher value of 5 mM is calculated from data for the Leu-5 δ-CH_3 peaks. The γ-CH_2 and ϵ-CH_3 resonances of Met-15 and the aromatic resonances of Phe-17 also shift upfield, presumably as a result of Co^{2+} binding to Asp-16. However, as the data give poor fits to a straight line, K_d values have not been determined.

DISCUSSION

In this paper we have assigned a large number of resonances in the ^{1}H NMR spectrum of gastrin and examined the effects of pH, temperature, and metal ions on the spectrum. The spectra of Tyr(SO_3H)^{12}-gastrin and des-<Glu^1-gastrin have also been examined.

In the spectrum of gastrin a number of resonances have chemical shifts close to those found in small, unstructured peptides (Tables I and II). However, there are also several perturbed resonances, notably those from α-CH_2 of Gly-2 in the cis form, α-CH and γ-CH of Leu-5, and α-CH and β- and γ-CH_2 of Met-15. These perturbations probably arise from interactions with neighboring residues. Their temperature dependencies, where they can be followed, are linear, suggesting that the changes in chemical shifts reflect the disruption of local interactions rather than a cooperative conformational transition. The upfield shifts of peaks from Leu-5 and Met-15 may arise from interactions with the aromatic rings of nearby residues, Trp-4 in the case of Leu-5 and Trp-14 or Phe-17 in the case of Met-15, or with nearby negative charges, from Glu residues and Asp-16, respectively. The upfield shift of the α-CH_2 resonances of Gly-2 in the cis form but not in the trans form may be due to proximity to a peptide carbonyl group or aromatic ring.

Clear evidence for a long-range interaction is contained in the splitting of the Tyr-12 C(2,6) H resonance due to cis–trans isomerism around the Gly-2 to Pro-3 peptide bond. In the presumed family of conformations containing the cis form of this peptide bond, the time-average interactions of the Tyr-12 ring with other residues must differ from those of its counterpart in conformations containing the trans form. The effect on Tyr-12 may be direct, reflecting contributions to the average conformation from structures in which Tyr-12 is in close proximity to Pro-3, or indirect, reflecting interactions of Tyr-12 with intermediate residues that are in turn affected by the Gly-2 to Pro-3 peptide bond. The effects of cis–trans isomerism are propagated at least as far as Leu-5. Because of overlap of resonances from Glu residues 6–10, it cannot be ascertained how many of these residues are also affected, but Ala-11 shows no effects. Removal of the <Glu-1 residue changes the cis to trans ratio but does not eliminate the effect of isomerism on Tyr-12.

The rate of cis–trans isomerism around the Gly-2 to Pro-3 peptide bond is slow. From the observation that the doublets of Tyr-12 C(2,6) H in the two forms are quite sharp even though they are only 3 Hz apart, it may be calculated that the lifetime of each form at 25 °C is $\gg$50 ms. For each isomer there is probably a family of rapidly interconverting conformers, but transitions between individual conformers containing the cis isomer and those containing the trans are slow. At 75 and 85 °C, exchange broadening is observed for resonances of Trp-4 C(4) H in the cis and trans forms. A similar phenomenon is observed for the δ-CH_3 resonances of Leu-5. These observations do not necessarily indicate that the rate of exchange between the cis and trans forms of the Gly-2 to Pro-3 peptide bond is in the intermediate range.

Tyr-12 of human gastrin is a substrate for epidermal growth factor stimulated tyrosine kinase (Baldwin et al., 1983). As the environment of Tyr-12 seems to be affected by cis–trans isomerism, it is possible that the two populations of gastrin are not equally good substrates for the enzyme. In this context, it would be significant that the rate of interconversion of the two populations is much slower than the likely rate of association between enzyme and substrate. The local environment of Tyr-12 appears also to be affected by the pentaglutamate sequence. This is inferred from the phenolic pK_a of Tyr-12, which is about 0.5–0.6 unit higher than the value of 10.1–10.3 found in small peptides (Bundi & Wüthrich, 1979; Endo et al., 1981). Such a change is consistent with proximity of the

ring to one or more negative charges. Indeed, an elevated phenolic pK_a may be characteristic of many Tyr residues that are substrates for tyrosine kinases, as it has been noted that such Tyr residues are often preceded in the N-terminal direction by clusters of negatively charged residues (Hunter, 1982).

The effect of sulfation of Tyr-12 on the conformation of gastrin, as reflected in its ^1H NMR spectrum, is minimal. The only resonances affected, apart from those of Tyr-12 itself, are those of Gly-13. The ratio of cis to trans isomers is unchanged. These observations are consistent with the known lack of effect of sulfation on the biological activity of gastrin (Konturek, 1980).

It is of interest to consider the conformation of the C-terminal tetrapeptide, which is the smallest fragment exhibiting the range of gastrin-like activities, and possible effects of the N-terminal 13 residues of gastrin thereon. From a 100-MHz ^1H NMR study of the tetrapeptide and several analogues in $(C^2H_3)_2SO$ and ^2H_2O, Feeney et al. (1972) concluded that the tetrapeptide was an extended coil, with the Trp and Phe aromatic rings separated by more than 5 Å and with no intramolecular hydrogen bonds. ^1H and ^{13}C NMR spin–lattice relaxation measurements on the tetrapeptide in $(C^2H_3)_2SO$ indicated that the backbone α-carbons had similar correlation times, while amongst the side chains Trp had the greatest rigidity relative to the backbone and Met, Asp, and Phe showed internal motions (Bleich et al., 1976). Circular dichroism studies of the tetrapeptide and several analogues in trifluoroethanol suggested that the tetrapeptide had a defined conformation that was not altered significantly when the chain was elongated in the N-terminal direction (Pham Van Chuong et al., 1979). By contrast, in another circular dichroism study Peggion et al. (1981) found no evidence for conformational rigidity in the tetrapeptide in trifluoroethanol and showed that it did not maintain the same conformation upon elongation from 4 to 13 residues. Conformational calculations also gave conflicting results, with Kier & George (1972) predicting an extended planar conformation with the two aromatic rings 11 Å apart, Yamada et al. (1976) assuming an α-helical backbone and concluding that several side-chain conformations were possible, and Abillon et al. (1981) favoring conformations having β-structure at the level of the Trp residue, C_7 structure at the Asp residue, and the centers of the two aromatic rings about 5 Å apart.

Most recently, Durieux et al. (1983) examined cholecystokinin fragment CCK_{27-33} in $(C^2H_3)_2SO$ and ^2H_2O. This peptide has the sequence Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 and thus contains the same C-terminal tetrapeptide as gastrin. They proposed that in both solvents the sulfated peptide contained a β-turn involving the sequence Gly-Trp-Met-Asp and a γ-turn for the sequence Met-Gly-Trp. In the non-sulfated analogue folding was less well-defined. Gastrin lacks a Met residue between Tyr and Gly, making the γ-turn less probable. Furthermore, as the proposed β-turn was stabilized by an ionic interaction between α-NH_3^+ of Tyr and β-COO^- of Asp, which would not occur in gastrin, and by sulfation of the Tyr residue, this structure probably does not occur in gastrin. It is interesting to note, therefore, that the chemical shifts of the nonlabile protons of Gly-13 and Met-15 of gastrin are nearly identical with those of the corresponding residues of sulfated and non-sulfated forms of CCK_{27-33} in ^2H_2O [taken from Figures 2 and 3 of Durieux et al. (1983)]. In the case of Gly-13, this is perhaps not surprising as its chemical shift is nearly identical with that of Gly residues in small, unstructured peptides (see Table II) and it shows no splitting due to spin–spin coupling. Both observations are consistent with a lack of structure involving Gly-13. Indeed, it is possible that this may act as a flexible hinge joining the C-terminal tetrapeptide of gastrin to its N-terminal region.

By contrast, the chemical shifts of Met-15 differ significantly from those in small peptides, as discussed above, and might be expected to be sensitive indicators of conformation. The similarities between values in gastrin and those in CCK_{27-33} indicate that the local interactions amongst the C-terminal residues that perturb the Met resonances are maintained in gastrin despite the likely absence of the β- and γ-turns proposed by Durieux et al. (1983) for CCK_{27-33}.

A further comparison is possible in the case of Asp-16. In our spectra of gastrin and des-<Glu^1-gastrin, the β-CH_2 protons of an AMX spin system occur at 2.44 and 2.61 ppm, well separated from those of the other AMX systems. An identical spin system in CCK_{27-33} in ^2H_2O was assigned to Asp-16 by Durieux et al. (1983). Assuming that this assignment is also valid for gastrin and des-<Glu^1-gastrin, the populations of the three rotamers around the α-C–β-C bond may be calculated for the latter, where these peaks are free of overlap. Using the vicinal coupling constants and nomenclature of Feeney et al. (1972), we obtain values for $P_{(I)}$, $P_{(II)}$, and $P_{(III)}$ of 0.37, 0.47, and 0.15, respectively.[4] Corresponding values for pentagastrin are 0.40, 0.44, and 0.16 (Feeney et al., 1972). It appears, therefore, that the average side-chain conformation of Asp-16 in des-<Glu^1-gastrin differs little from that in pentagastrin.

Finally, we consider the interaction of gastrin with metal ions. NaCl at concentrations up to 250 mM or $CaCl_2$ at concentrations up to 86 mM caused only minor spectral changes. The paramagnetic metal ion Co^{2+} caused significant changes. Binding of Co^{2+} to at least one site involving one or more of the five Glu residues is indicated by the fact that K_d's calculated from the effects on Leu-5 and Ala-11 are similar (5 and 2 mM, respectively). Weaker binding to Asp-16 is reflected by shifts in resonances from Met-15 and Phe-17. These dissociation constants may be compared with a value of 18 mM found by McDonald & Phillips (1969) for Co^{2+} binding at pD 5.5 and 55 °C to a lysozyme site presumed to involve the carboxylates of both Glu-35 and Asp-52. These results show that gastrin is able to bind divalent metal ions in aqueous solution and contains at least two binding sites. Because the spectral changes caused by Ca^{2+} are so small, however, the strength of its binding to gastrin in aqueous solution, and the possible physiological significance thereof, could not be determined.

Registry No. Na, 7440-23-5; Ca, 7440-70-2; Co, 7440-48-4; human gastrin I heptadecapeptide, 10047-33-3; (1-depyroglutamate) human gastrin I heptadecapeptide, 22655-78-3; human gastrin II heptadecapeptide, 19361-51-4.

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[4] This analysis assumes that the larger vicinal coupling constant corresponds to J_{AX}, as found for Asp in the model compounds examined by Feeney et al. (1972).

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Histones of the Unicellular Alga *Olisthodiscus luteus*†

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ABSTRACT: The four major histones of the marine unicellular alga *Olisthodiscus luteus* were prepared from isolated nuclei and fractionated by molecular exclusion chromatography and preparative gel electrophoresis. The isolated histones were characterized by amino acid analysis and peptide mapping and compared to calf thymus histones. Using the above and various other criteria, we have identified these four histones in terms of their vertebrate counterparts. We conclude that *O. luteus* chromatin contains an H1-like histone, in addition to the highly conserved histones H3 and H4. A new histone (HOl) is described that serves the role of H2A and H2B in the nucleosome core. It is possible that H2A and H2B of higher organisms arose from an archetypal protein similar to HOl, through the usual gene duplication process.

Although the histones of higher plants and animals are now well characterized, little is known about histones of the lower plants that are collectively known as the algae. Histones have been reported in *Chlorella* (Iwai, 1964), *Volvox* (Bradley et al., 1974), *Euglena* (Jardine & Leaver, 1978; Bre et al., 1980), *Olisthodiscus luteus* (Rizzo, 1980), the endosymbiont nucleus of *Peridinium balticum* (Rizzo, 1982), and the red alga *Porphyridium* (Barnes et al., 1982). Only for *Euglena* (Jardine & Leaver, 1978) have these histones been characterized in terms of their vertebrate counterparts.

O. luteus is a wall-less unicellular marine alga, belonging to the Chrysophyceae (Gibbs et al., 1980). It can be grown to high cell densities in a chemically defined medium (McIntosh & Cattolico, 1978) and has been used successfully for chloroplast studies (Cattolico, 1978; Ersland et al., 1981; Aldrich et al., 1982). Since this alga is naturally wall-less, nuclei can be isolated in high yields (Rizzo & Burghardt,

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