# Physical Characterization of *S*-Methylglucagon and Quantitation of Carbamino Adduct Formation†

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ABSTRACT: Methylation of the single methionine residue at position 27 of glucagon yields a highly soluble sulfonium derivative, *S*-methylglucagon. The increased solubility of *S*-methylglucagon makes natural abundance $^{13}C$ nuclear magnetic resonance studies accessible. These studies have resulted in the determination of $pK_z$ values for the proton dissociations of the histidine-1 $\alpha$-amino and imidazole functional groups of 7.23 and 5.32, respectively. Equilibration of *S*-methylglucagon with $^{13}CO_2$ yields a carbamino adduct which is identified and quantitated. The $pK_c$ value corresponding to the formation constant of the carbamate with the peptide $\alpha$-amino group is 4.66. These values of $pK_z$ and $pK_c$, which define the extent of carbamino adduct formation for all values of pH and $pCO_2$, imply that under normal physiological conditions the mole fraction of carbamate is near 0.35 and that this mole fraction varies significantly over the physiologically important range of extracellular pH and $pCO_2$ values. Since the $\alpha$-amino group of the hormone is necessary for apparent biological activity, the reversible carbamate formation which will occur to a variable extent in vivo may alter the biological effectiveness of the peptide hormone. Justification for the use of *S*-methylglucagon as a model for native glucagon comes from nuclear magnetic resonance and circular dichroism evidence, which indicates that the two peptides have similar secondary structures at dilute peptide concentrations and that the structure of *S*-methylglucagon appears to be independent of peptide concentration in contrast to the native hormone. This is consistent with *S*-methylglucagon resisting intermolecular association, thereby making this derivative an excellent model for the native hormone in its predominant physiological form.

Methylation of the single methionine residue of the 29-membered peptide hormone glucagon yields a highly soluble, functionally active sulfonium derivative, *S*-methylglucagon (Rothgeb et al., 1977). The increased solubility of *S*-methylglucagon over the native hormone makes possible a number of studies designed to assess the role of structural alterations of the molecule. Among these are physical studies to determine the extent of carbamino adduct formation arising from the reaction of carbon dioxide with the free $\alpha$-amino group of the sulfonium derivative.

Any peptide, unless its $\alpha$-amino group is chemically blocked or conformationally hidden, can be expected to form carbamino adducts with $CO_2$ under physiological conditions (Morrow et al., 1974a,b). Such adducts have been quantitated for several peptides of biological interest (Wittebort et al., 1978; Morrow et al., 1974a) and the mole fraction of carbamino adduct formed has been found to vary significantly over physiologically important ranges of pH and $pCO_2$ (Morrow et al., 1974a,b; Morrow et al., 1976; Matthew et al., 1977; Wittebort et al., 1978). However, whether or not such adducts are physiologically significant is determined to a large extent by the importance of the $\alpha$-amino group for the biological activity of the molecule.

Glucagon is an excellent candidate for a study of $CO_2$ interactions not only because of the apparent functional importance of its $\alpha$-amino group for biological activity (Desbuquois, 1975; Epand et al., 1973, 1976; Epand and Wheeler, 1975; Grand et al., 1972; Lande et al., 1972) but also because of its possible involvement in diabetes mellitus and other associated acid-base disturbances (Unger and Orci, 1977; Unger, 1973). The $^{13}C$ NMR[1] technique is well suited for the direct analysis of $CO_2$ interactions (Morrow et al., 1974a,b), and the functionally active *S*-methylglucagon derivative is an accessible and appropriate model to study.

The present report includes the determination by $^{13}C$ NMR techniques of the equilibrium constants for *S*-methylglucagon which define the extent of carbamino formation for all values of pH and $pCO_2$. These equilibrium constants are $K_z$,[1] the proton dissociation constant for the $\alpha$-amino group, and $K_c$,[1] the formation constant of the carbamino adduct. The proton dissociation for the histidine-1 imidazole group is also determined. Circular dichroism evidence is presented which indicates that at dilute peptide concentrations native and *S*-methylglucagon have similar secondary structures at pH values of 2 and 10.2 and that *S*-methylglucagon, in contrast to native glucagon, remains largely unstructured at higher peptide concentrations. The potential physiological role of carbamino adduct formation is also discussed.

## Experimental Section

### *Materials*

Crystalline porcine glucagon was provided through the courtesy of Eli Lilly and Co. *S*-Methylglucagon was prepared and purified as previously described (Rothgeb et al., 1977). Histidinamide, purchased from Cyclo Chemical Corp., was recrystallized as the dihydrochloride from ethanol–water before use. $^{13}C$-enriched $NaHCO_3$ (90 atom %) was obtained from Bio-Rad Laboratories and $^{13}C$-enriched $CO_2$ (90 atom %) from Mound Laboratories, Monsanto Chemical Corp. Sucrose, at natural isotope abundance, was obtained from Mallinckrodt. All other chemicals used were reagent grade.

† From the Department of Chemistry and the Medical Sciences Program, Indiana University, Bloomington, Indiana 47401. *Received May 10, 1978.* This is the 98th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper see Lehman et al. (1978). This work was supported by Public Health Service Research Grants HL-05556 and AM21121.

‡ Supported by the Insurance Medical Scientist Scholarship Fund, sponsored by American United Life.

[1] Abbreviations used are: $^{13}C$ NMR, carbon-13 nuclear magnetic resonance; $pK_z$ and $pK_c$, are respectively $-\log$ of the dissociation constant, $K_z$, and the formation constant, $K_c$; $Me_4Si$, tetramethylsilane.

0006-2960/78/0417-4564$01.00/0 

*Methods*

*Potentiometric Titration of Histidinamide.* Titrations were performed using the apparatus and procedure of Shire et al. (1974), at temperatures ranging from 10 to 40 °C and at ionic strengths, adjusted with KCl, from 0.005 to 0.205. Concentrations of histidinamide solution were determined by amino acid analysis following hydrolysis in 5.7 N HCl.

*Circular Dichroism Measurements.* A Jasco ORD/UV-5 spectropolarimeter fitted with the Sproul Scientific SS-10 CD modification was employed to measure fresh solutions of native and *S*-methylglucagon at concentrations of ~0.5, 1.5, and 3.5 mg/mL in 0.01 N HCl (pH 2) and 0.2 M $K_2HPO_4$ (pH 10.2). Peptide concentrations were determined on a Cary Model 14 instrument using the extinction coefficient 8260 $M^{-1}$ $cm^{-1}$ at 278 nm (Gratzer and Beaven, 1969). Estimations of the $\alpha$-helix content were made using the parameters of Chen et al. (1974).

*Sample Preparation for $pK_z$ Determinations.* *S*-Methylglucagon samples were prepared by dissolving the lyophilized peptide in the appropriate volume of a solution containing 0.02 M KCl, 0.001 M $NaN_3$, and a trace of dioxane (10 $\mu$L/mL of solution). Peptide concentrations, typically ~5 mM, were determined as described above. Measurements of pH were made at the same temperature as that found during the respective NMR run. A Radiometer PHM-72 digital acid–base analyzer fitted with a Radiometer GK 2322C combination electrode was used, and immediately prior to each pH measurement the pH scale was linearized using primary standard phosphate, phthalate, and borate buffers (National Bureau of Standards). Adjustments in sample pH were made with either 2 N NaOH or 2 N HCl.

*Sample Preparation for $pK_c$ Determinations.* Solutions of histidinamide and *S*-methylglucagon were prepared by dissolving the amino acid or peptide to the desired concentration in a solution containing known concentrations of sucrose (~0.10 M) and $NaH^{13}CO_3$ (~0.05 M). The sample was then transferred to an NMR tube, placed under a known pressure of $^{13}CO_2$ (Morrow et al., 1973, 1974b), sealed, and allowed to equilibrate at least 1 h before NMR measurements were begun. Immediately after each NMR run, values of pH, total carbonates, total amine, and mole fraction of $^{13}CO_2$ were separately determined either as described above or as previously described (Matthew et al., 1977).

*NMR Measurements.* Carbon-13 NMR spectra were obtained at 25.2 and 67.9 MHz by means of the Fourier transform method (Glushko et al., 1972; Jones et al., 1976). Chemical shifts are reported relative to the $^{13}C$ resonance of $Me_4Si$[1] and were measured digitally relative to internal dioxane (67.86 ppm) at 67.9 MHz and to the single nonprotonated C-2 carbon resonance (104.86 ppm) at 25.2 MHz of the fructose moiety in sucrose (Oldfield et al., 1975a; Wittebort et al., 1978). Integration of resonances was performed digitally. Resonance assignments[2] for *S*-methylglucagon made use of histidinamide and other model compounds[3] (Gurd et al., 1972).

*Measurements for $pK_z$ Determinations.* NMR measurements for the $pK_z$ values of the $\alpha$-amino and imidazole functional groups of *S*-methylglucagon were made at 67.9 MHz. The spectrometer, described previously by Jones et al. (1976), has since been modified to accommodate 15-mm sample tubes and to operate in the quadrature phase-detection mode. The 90° $^{13}C$ radiofrequency pulse (18.5 $\mu$s) was set at 78.15 ppm relative to $Me_4Si$, and two 4096 data point quadrature time domain spectra were collected. All spectra were obtained at 28 ± 1 °C using a 16.129-kHz spectral width with a 1-s recycle time.

Spectra referred to as "proton decoupled" were obtained by applying proton irradiation, at 270 MHz, with a peak field strength of ~0.35 G and phase modulation of the decoupler carrier frequency with a 50% duty cycle 100-Hz square wave (Grutzner and Santini, 1975). Noise-modulated off-resonance proton-decoupled spectra were obtained using the same proton irradiation described above with a random noise modulation bandwidth of 300 Hz centered at 1.0-ppm upfield from the proton resonance of $Me_4Si$ (Oldfield et al., 1975b, Dill and Allerhand, 1977; Norton et al., 1977).

On all spectra obtained at 67.9 MHz the convolution difference method (Campbell et al., 1973) was used to enhance resolution as follows: two Fourier transformed spectra were obtained from each accumulated time domain spectrum, one (spectrum A) with a digital broadening of 4.0 Hz and the other (spectrum B) with a digital broadening of 20 Hz. The difference spectrum was obtained by the digital subtraction of 0.7 of spectrum B from A.

*Measurements for $pK_c$ Determinations.* NMR measurements for the $pK_c$ determinations of histidinamide and *S*-methylglucagon were made at 25.2 MHz using a Varian XL-100-15 spectrophotometer (Visscher and Gurd, 1975; Jones et al., 1976). The temperature at which these measurements were made was 25 ± 1 °C. Since in these experiments it was necessary to relate the area of the carbamate resonance to the areas of the reference sucrose resonances (see below), a gated proton-decoupling method was chosen which allows decoupled $^{13}C$ spectra without appreciable nuclear Overhauser effects to be obtained (Opella et al., 1976). Such a method exploits the separate time scales for scaler decoupling and the development of magnetization (Freeman et al., 1972). The sequence of events used in these experiments was as follows: (1) the proton decoupler was gated on (at 100 MHz) simultaneously with a 70° (16 $\mu$s) radiofrequency pulse at the $^{13}C$ frequency; (2) the free-induction decay was collected with proton irradiation during the acquisition time, $t$; and (3) the proton decoupler was gated off after the acquisition time for a relatively long period of time, $T$, before the sequence was repeated. For histidinamide, values of $t$ and $T$ were, respectively, 0.324 and 5.670 s, while the analogous values for *S*-methylglucagon were 0.537 and 6.463 s. Under these conditions, the errors introduced in the area calculations of the pertinent resonances due to nuclear Overhauser effects were not experimentally significant for our purposes.

*Determination of $pK_z$ Values.* Estimations of the $pK_z$ values for the $\alpha$-amino and imidazole functional groups were made by fitting either the potentiometric or NMR titration data to the best double p$K$ Henderson–Hasselbalch function by a nonlinear least-squares method. Temperature and ionic-strength corrections determined from potentiometric titrations of histidinamide were applied to $pK_z$ values of *S*-methylglucagon to adjust for small differences in the experimental conditions (Butler, 1964). Such treatment entails a number of assumptions (Tanford, 1957); however, the corrections were deemed sufficiently accurate over the small range of temperature (3 °C) and at the low ionic strengths (less than 0.075 M) used.

*Determination of $pK_c$.* The pH dependence of carbamino formation is dictated by the following equilibria:

[2] The convention suggested by the IUPAC–IUB Commission on Biochemical Nomenclature [*Biochemistry 14*, 449 (1975)] is used for histidine carbon nomenclature. See inset of Figure 1.

[3] Glushko, V., Keim, P., Lawson, P. J., and Gurd, F. R. N., unpublished observations.

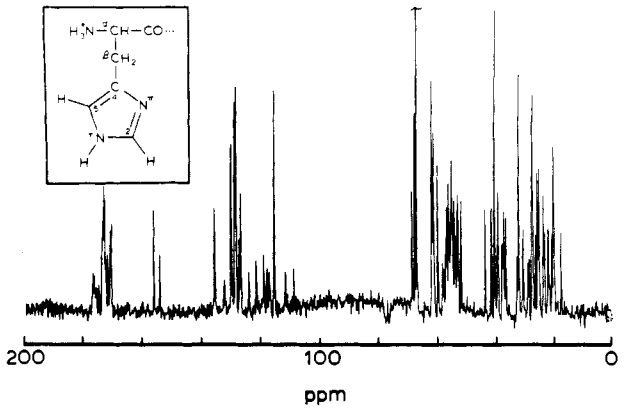


FIGURE 1: The fully proton-decoupled convolution-difference natural-abundance $^{13}C$ NMR spectrum of *S*-methylglucagon at 67.9 MHz, peptide concentration of 5 mM (pH 8.2), and 16 192 accumulations at a 1-s recycle time. The spectrum is referenced to external $Me_4Si$ using internal dioxane at 67.86 ppm. *Inset:* An N-terminal histidine residue to illustrate the IUPAC-IUB histidine carbon nomenclature. The tautomeric form shown for the imidazole side chain is the $N^{\tau}$–H form.

$$RNH_3^+ \overset{K_z}{\rightleftharpoons} RNH_2 + H^+ \quad (1)$$

$$CO_2 + RNH_2 \overset{K_c}{\rightleftharpoons} RNHCOO^- + H^+ \quad (2)$$

$$CO_2 + H_2O \overset{K_1}{\rightleftharpoons} HCO_3^- + H^+ \quad (3)$$

$$HCO_3^- \overset{K_2}{\rightleftharpoons} CO_3^{2-} + H^+ \quad (4)$$

Here $K_z$ is the proton-dissociation constant of the amino group in question, $K_c$ is the formation constant of the carbamino adduct, expressed to include the step of dissociation of the relatively strong carbamic acid (Roughton and Rossi-Bernardi, 1969), and $K_1$ and $K_2$ are, respectively, the dissociation constants for carbonic acid and bicarbonate.

The mole fraction of carbamino adduct, $Z$, is related to free $CO_2$ and protons by (Kilmartin and Rossi-Bernardi, 1973; Morrow et al., 1974a):

$$Z = \frac{K_cK_z[CO_2]}{K_cK_z[CO_2] + K_z[H^+] + [H^+]^2} \quad (5)$$

With eq 3, 4, the equation for total carbonates ($[TC] = [CO_2] + [HCO_3^-] + [CO_3^{2-}] + [RNHCO_2^-]$), and that for total amines ($[TA] = [RNH_2] + [RNH_3^+] + [RNHCO_2^-]$), it is possible to recast eq 5 in terms of the more easily measured total carbonates. This result is expressed below, and for the purpose of our work is solved for $K_c$.

$$K_c = \frac{Z(K_1'K_2'K_z + [H^+](K_zK_1'K_2') + [H^+]^2(K_1' + K_z) + [H^+]^3)}{K_z[H^+](Z^2[TA] - Z([TC] + [TA]) + [TC])} \quad (6)$$

The ionization constants, $K_1'$ and $K_2'$ (eq 3 and 4), for carbonic acid and bicarbonate (Harned and Davis, 1943) were evaluated at each experimental ionic strength by the use of the Davies equation (Butler, 1964). Similarly, the dissociation constant, $K_z$, was evaluated as above at each experimental ionic strength.

Experimental values of $Z$ were obtained from the gated decoupled NMR spectra by the following relation (Matthew et al., 1977; Wittebort et al., 1978):

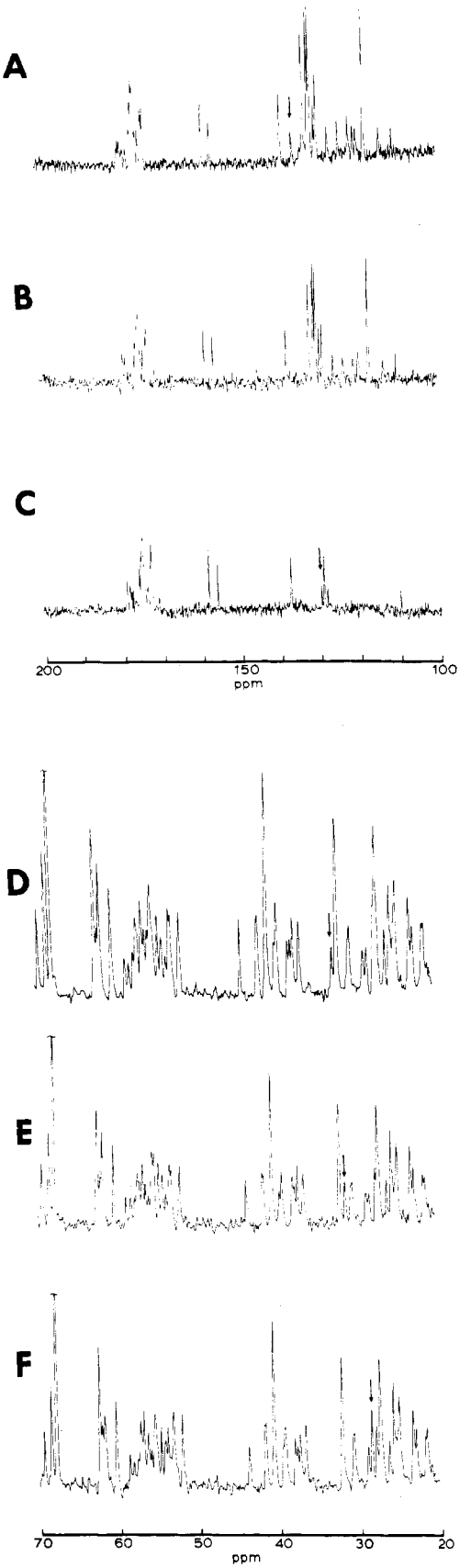


FIGURE 2: The expanded carbonyl aromatic regions (A–C) and expanded $\alpha$-carbon aliphatic regions (D–F) of Figure 1 at various pH values. The respective pH values of A–F were 8.2, 4.6, 4.6, 8.2, 7.2, and 4.6. All spectra were recorded using the conditions specified in the legend of Figure 1, except spectrum 2C which was obtained under conditions of noise-modulated off-resonance proton decoupling. A base-line adjustment routine was used to remove the broad methine carbon resonance features. The arrows in spectra A and C designate the histidine-1 $C^4$ imidazole resonance and in spectra D–F the histidine-1 $C^{\beta}$ resonance.

$$Z = \frac{I_{cam}}{I_{ref}} \times \frac{\text{concn of reference}}{\text{concn of total amine}} \times \frac{1.1 \times 10^{-2}}{\chi^{13}CO_2} \quad (7)$$

Here $I_{cam}$ and $I_{ref}$ are the digitally integrated intensities of the carbamino resonance and the reference sucrose resonances. $\chi^{13}CO_2$ is the measured mole fraction of $^{13}C$ in the enriched carbonates and the factor $1.1 \times 10^{-2}$ represents the natural abundance of $^{13}C$ in the reference. The values of $Z$ were applied to eq 6 to calculate values of $K_c$ which were then averaged for the $Z$ determinations at different pH values (Table I). For the $Z$ determinations, pH values ranged from ~7.0 to 9.0 and total carbonates from 40 to 70 mM.

## Results and Discussion

*NMR Studies of S-Methylglucagon Titration.* Presented in Figures 1 and 2 are the natural-abundance $^{13}C$ NMR spectra obtained on *S*-methylglucagon at 67.9 MHz. Figure 1 shows the complete proton-decoupled spectrum at pH 8.2, and Figure 2 shows expanded regions of Figure 1 at various pH values. It is apparent in Figure 1 that the spectra contain a number of well-resolved narrow resonances. Especially important in this regard is the $\alpha$-carbon region from 50 to 60 ppm, which contains at least 11 resolved resonances of the possible 29. Line widths are typically less than 20 Hz. Figure 2 reveals little or no change with pH in the nontitrating resonances, indicating no relevant structural changes in the molecule discernible from the NMR spectra. These observations are consistent with a nonrigid random conformation of the molecule in solution. This conclusion is supported by the circular dichroism studies below. At pH 4.0, *S*-methylglucagon at these high concentrations appeared to gel and the NMR lines broadened markedly. The tendency for *S*-methylglucagon to gel under these conditions may be similar to the formation of glucagon gels and fibrils observed under similar conditions (Gratzer et al., 1967, 1968). The effect of dioxane on the gel formation of *S*-methylglucagon was not investigated (Beaven et al., 1969).

The titrating histidine-1 $C^{\beta}$ and $C^4$ imidazole resonances are easily assigned[3] (Gurd et al., 1972) and can be followed from pH 4 to 10 which allows the determination of the $\alpha$-amino and imidazole functional-group $pK_z$ values. However, the nonprotonated $C^4$ imidazole carbon resonance titrates below pH 6.7 under an envelope of resonances arising from the phenylalanines and tyrosines near 131 ppm. Nevertheless, the resonance can be followed below pH 6.7 by the technique of noise-modulated off-resonance proton decoupling, as illustrated by comparison of the spectra of Figure 2B and C.

Figure 3 presents the titration behavior of (A) the histidine-1 $C^4$ imidazole and (B) $C^{\beta}$ resonances of *S*-methylglucagon. The points are experimental, and the curves are the best-fit Henderson–Hasselbalch functions applied to the two titration steps. The $pK_z$ values obtained from these curves, given in the figure legend, are the same within experimental error. These $pK_z$ values and those obtained for histidinamide are corrected to 25 °C and summarized in Table I, along with the appropriate chemical-shift data.

The $pK_z$ values obtained here for *S*-methylglucagon agree qualitatively with estimates obtained under somewhat different conditions for glucagon (Epand et al., 1973; Epand and Wheeler, 1975). The ionization properties of the N-terminal histidine residue of *S*-methylglucagon are very similar to those of histidinamide (Table I). This indicates that the terminal histidine residue of *S*-methylglucagon, like that of glucagon (Epand et al., 1973; Epand and Wheeler, 1975), is freely accessible to the solvent and is probably not involved in intramolecular bonding.

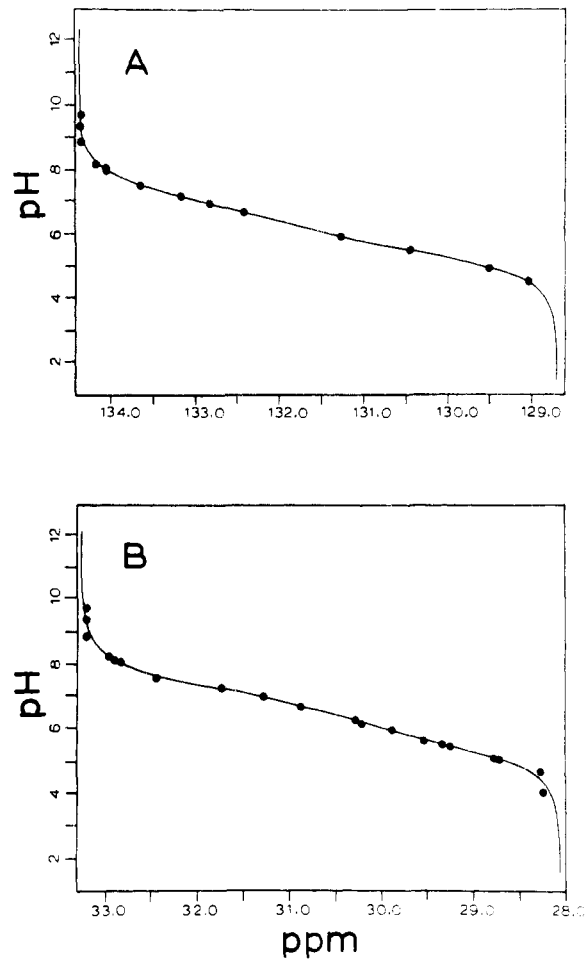


FIGURE 3: The respective titration behavior of the histidine-1 $C^4$ imidazole resonance (A) and the histidine-1 $C^{\beta}$ resonance (B) of *S*-methylglucagon. The points are experimental, and the curves are the best-fit Henderson–Hasselbalch functions for two $pK$ values derived from the experimental data. The $pK_z$ values obtained for curve A are $pK_{NH_2} = 7.17$ (0.08), $pK_{Im} = 5.47$ (0.09), and for curve B are $pK_{NH_2} = 7.23$ (0.05), $pK_{Im} = 5.45$ (0.08). The values in parentheses indicate the standard deviations.

On deprotonation, the histidine residue imidazole assumes one of two tautomeric forms which are distinguishable by NMR spectroscopy (Reynolds et al., 1973). If deprotonation yields the $N^{\tau}$–H imidazole tautomer the $C^4$ imidazole resonance exhibits a downfield shift of near 6 ppm, whereas if deprotonation yields the $N^{\pi}$–H imidazole tautomer the shift is approximately 2-ppm upfield. From Figures 2A–C and 3A it is seen that a downfield shift of the $C^4$ imidazole resonance has occurred and, hence, the $N^{\tau}$–H imidazole tautomer is the predominant species. This particular tautomer also predominates in histidinamide, several histidine-containing peptides[3] (Deslauriers et al., 1974; 1975; Gurd et al., 1972), and some of the histidine residues of several proteins (Ugurbil et al., 1977; Wilbur and Allerhand, 1977). At extracellular sites where glucagon is active, the deprotonated tautomeric forms are in large excess, and it has been suggested that the tautomeric form of the imidazole moiety in histidine-containing peptide hormones may be important in hormone–receptor interactions (Deslauriers et al., 1974). It would seem unlikely that the tautomeric equilibrium of glucagon would differ from that of *S*-methylglucagon.

Evidence of carbamino adduct formation of *S*-methylglucagon is presented in Figure 4. Shown are typical gated-decoupled $^{13}C$ NMR spectra of *S*-methylglucagon equilibrated with $^{13}CO_2$ at pH values of 8.9, 7.7, and 7.4. In Figure 4, peak

TABLE I: Summary of Chemical-Shift Data and $pK_z$ and $pK_c$ Values for Histidinamide and *S*-Methylglucagon.

| | chemical shifts (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $C^{\beta}$ resonance | | $C^4$-imidazole resonance | | $pK_z$ values[c] | | |
| | δA | δB | δA | δB | imidazole | α-amino | $pK_c$ values[d] |
| histidinamide | 27.47[a] | 33.29[a] | 127.18 | 134.29 | 5.02 | 7.37 | 4.76 (0.06) |
| *S*-methylglucagon | 28.07[b] | 33.22[b] | 128.70 | 134.33 | 5.32 | 7.23 | 4.66 (0.11) |

[a]The experimentally observed acid and base chemical-shift limits at respective pH values of 2.0 and 11.0. [b]The acid and base chemical-shift limits obtained from the best-fit double $pK$ Henderson-Hasselbalch functions. [c]All $pK_z$ values are corrected to 0 ionic strength at 25 °C as explained under Methods. Uncertainty in these values is estimated to be less than ±0.10. [d]Values in parentheses indicate the standard deviations obtained from at least six determinations.

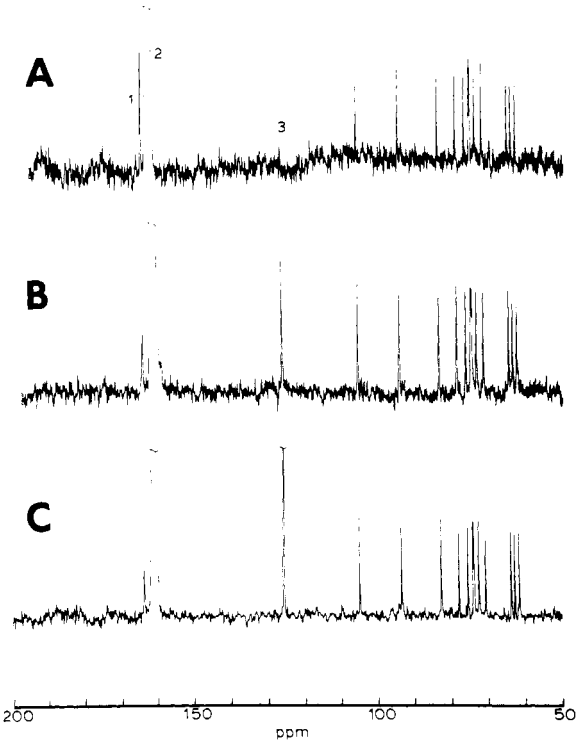


FIGURE 4: Typical gated-decoupled $^{13}C$ NMR spectra at 25.2 MHz of *S*-methylglucagon equilibrated with $^{13}CO_2$ at respective pH values for A, B, and C of 8.9, 7.7, and 7.4. Average peptide concentration was 2.9 mM at 66 mM total carbonates and 100 mM sucrose as the internal reference. Spectrum A represents 6000 accumulations, while spectra B and C each represent 10 000 accumulations collected under the conditions specified under Methods. Resonance 1 arises from the $^{13}C$-enriched carbamate, resonance 2 from the $^{13}C$-enriched bicarbonate, and resonance 3 from the $^{13}CO_2$, and the 12 resonances from 105 to 60 ppm arise from the natural-abundance $^{13}C$ in the internal reference sucrose. The spectra are referenced to external $Me_4Si$ using the most upfield resonance of internal sucrose at 104.86 ppm.

1 (164.5 ppm) arises from the $^{13}C$-enriched carbamino adduct, peak 2 (161.5 ppm) from the H $^{13}CO_3^-$ species, and peak 3 (125.8 ppm, barely visible in Figure 4A) from the dissolved $^{13}CO_2$. The 12 resonances from 105 to 60 ppm arise from the natural abundance $^{13}C$ of the internal reference sucrose. Adduct formation with any functional group other than the α-amino group can be ruled out in the pH range of these studies (Morrow et al., 1974b; Caplow, 1968). The carbamate resonance (peak 1) and the $CO_2$ resonance (peak 3) are in slow exchange, and hence a lower limit of the lifetime of the carbamino adduct can be estimated to be in this case approximately 30 ms (Gutowsky et al., 1953). This result is in agreement with other studies on the release of $CO_2$ from model carbamate compounds (Caplow, 1968). It should be pointed out that the absence of resonances arising from *S*-methylglucagon in Figure 4 is due to the low peptide concentrations employed in these experiments. NMR spectra of histidinamide indicate adduct formation to a similar extent.

As indicated by eq 2, only the nonprotonated form of the amine reacts with $CO_2$, and hence small pH changes at fixed total carbonates in the region near the $pK_z$ of the amine should lead to rather large changes in the extent of adduct formation. This is clearly illustrated by peak 1 in Figure 4. As the pH increases, there is a concomitant increase in the concentration of nonprotonated amine, which results in a greater extent of adduct formation. At pH values higher than those shown in Figure 4, the concentration of free $CO_2$ becomes limiting such that a bell-shaped curve of adduct concentration vs. pH is generated (Morrow et al., 1974a,b).

From the integrated intensities of the resonances such as in Figure 4, the determined enrichment of $CO_2$, and known concentrations of hormone and reference, $Z$ was computed (eq 7), and from these $K_c$ values were calculated according to eq 6. The $K_c$ values were averaged, and the results of these determinations at 25 °C for histidinamide and *S*-methylglucagon are reported in Table I. The $K_c$ values were found to be nearly temperature independent in the range from 15 to 40 °C as expected (Caplow, 1968).

The $pK_c$ and $pK_z$ values reported in Table I define the extent of the carbamino formation of *S*-methylglucagon for all values of pH and $pCO_2$, as illustrated in Figure 5. The mole fraction of carbamino adduct, $Z$, is illustrated as a family of downward sloping lines from left to right, and is superimposed on the standard pH-bicarbonate-$pCO_2$ diagram (Davenport, 1974). As described in the legend of Figure 5, a series of points which might be encountered under various conditions of acid-base imbalance has also been included.

Figure 5 shows that the magnitude of $Z$ varies significantly over the physiologically important range of extracellular pH and bicarbonate values. Maximum variation occurs on going from metabolic acidosis (point A) to metabolic alkalosis (point C), and this variation is much greater than that which occurs on going from respiratory-induced acidosis (point X) to respiratory-induced alkosis (point Y). The in vivo relevance of these observations has not yet been determined.

*Circular Dichroism Studies.* Justification for the use of *S*-methylglucagon as a model for native glucagon in experiments of this type is apparent from circular dichroism studies. Figure 6 shows the circular dichroism spectra of native and *S*-methylglucagon at pH values of 2 and 10.2 at various peptide concentrations. At low peptide concentrations (Figure 6A,B) which favor the monomeric state of the native hormone (Gratzer and Beaven, 1969; Formisano et al., 1977), native and *S*-methylglucagon have nearly identical secondary structures. Helicity values were low, near 10%, as expected (Gratzer and Beaven, 1969).

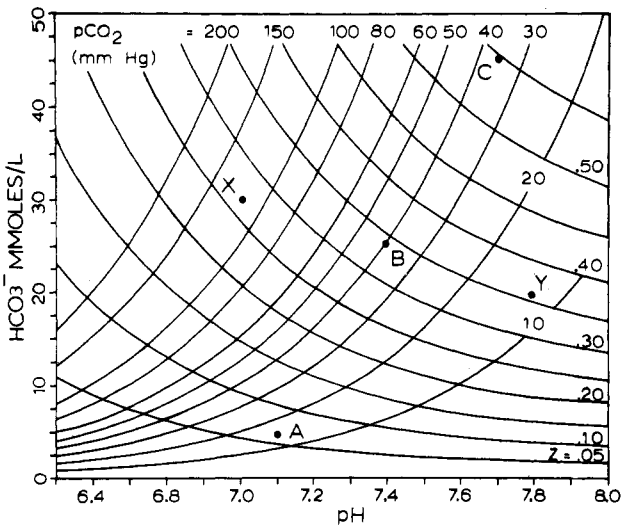


FIGURE 5: Web plot showing the relation between pH, $pCO_2$, bicarbonate concentration, and the mole fraction, $Z$, of carbamate for the $\alpha$-amino group of $S$-methylglucagon. The upward sloping lines from left to right are $pCO_2$ isobars, and the downward sloping lines indicate constant $Z$ values. The plot is based on an ionic strength of 0.075; and the parameters $pK_z$, $pK_1$, and $pK_2$ and Henry's law constant have been adjusted accordingly (Harned and Davis, 1943). Point B indicates normal conditions of acid–base balance, points A and C represent, respectively, conditions of uncompensated metabolic acidosis and alkalosis, and points X and Y represent, respectively, conditions of uncompensated respiratory acidosis and alkalosis.

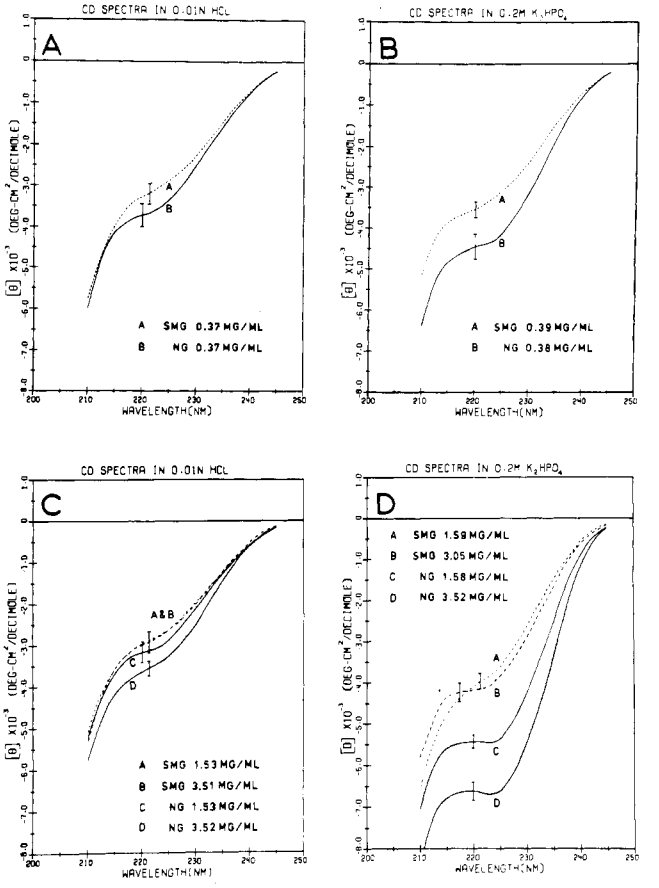


FIGURE 6: The circular dichroism spectra of native and $S$-methylglucagon in 0.01 M HCl (pH 2) and in 0.2 M $K_2HPO_4$ (pH 10.2). The dashed curves represent $S$-methylglucagon (SMG), and the solid curves represent native glucagon (NG). Each curve is an average of at least four scans at 2.2 nm/min with a time constant of 4 s, and the error bars ($I$) indicate the respective noise levels. The peptide concentration at which each curve was obtained is noted on the respective curve.

At higher peptide concentrations, both the native and $S$-methylglucagon appeared to have similar secondary structures at pH 2. These structures were only slightly concentration dependent, as shown in Figure 6C. However, at higher pH (Figure 6D) native glucagon secondary structure was concentration dependent as expected (Gratzer and Beaven, 1969), whereas $S$-methylglucagon tended to retain the same degree of secondary structure irrespective of concentration.

It is well established that in dilute solutions glucagon has a dynamic structure with few stable intramolecular bonds (Gratzer et al., 1968; Edelhoch and Lippolt, 1969; Gratzer and Beaven, 1969; Panijpan and Gratzer, 1974) and that the structure of the molecule under these conditions may be taken as the structure of the monomeric molecule. With increasing native hormone concentrations there appears to be a concentration-dependent equilibrium which involves the formation of associated forms with greater amounts of secondary structure (Gratzer et al., 1967; Gratzer and Beaven, 1969; Srere and Brooks, 1969). This effect, although present in acidic solution, is more pronounced in dilute alkaline solutions (Gratzer and Beaven, 1969; Formisano et al., 1977). These observations on native glucagon and the observation that $S$-methylglucagon does not exhibit concentration-dependent changes in ellipticity at alkaline pH indicate that $S$-methylglucagon undergoes little intermolecular association under these conditions. This conclusion is consistent with the previous NMR observations that the molecule has a high degree of conformational flexibility and does not appear to associate intermolecularly over a wide range of pH values.[4] These results indicate that $S$-methylglucagon is reasonably representative of the monomeric form of glucagon, the one likely to predominate under physiological conditions (Bromer, 1972). The tendency for $S$-methylglucagon to resist intermolecular association makes this derivative ideal for the determination of such thermodynamic constants as $pK_z$ and $pK_c$ (Gratzer and Beaven, 1969), and such values should be representative of those for the native molecule in its predominant physiological form.

*Implications of Carbamino Formation.* If the constants given in Table I for $S$-methylglucagon are, in fact, representative of those for the native hormone, then the parallel may be extended to changes in the mole fraction of carbaminoglucagon, as indicated by the changes in the mole fraction of carbamino-$S$-methylglucagon shown in Figure 5. This is the type of behavior to be anticipated if carbamino formation were to serve a regulatory role by changing the effective levels of the hormone in vivo.

The assignment of a regulatory role to carbamino formation requires the following assumptions: (1) carbaminoglucagon can bind to the glucagon receptor and (2) this complex once formed will elicit a different biological response from that of the native glucagon–receptor complex. Such assumptions appear reasonable in light of other work indicating that there are discrete regions of the primary structure of glucagon which are separately involved in receptor binding and biological activation (Epand and Wheeler, 1975; Epand et al., 1976; Rodbell et al., 1971; Lin et al., 1975). Des-histidine-glucagon (Hruby et al., 1976; Lin et al., 1975) and an acrylonitrile-modified imidazole derivative of glucagon (Epand et al., 1976) both are reported to behave as partial agonists of adenylate cyclase activity, while the acrylonitrile-modified derivative was

[4] These results do not preclude intermolecular interactions if they are fast on the NMR time scale. However, the likelihood of such interactions is small in light of the similarities of the $pK$ values for $S$-methylglucagon and histidinamide.

able to compete with glucagon to lower the activity of the native hormone (Epand et al., 1976). These results indicate that the carboxyl-terminal region of the glucagon molecule is most important in the process of recognition at the receptor, while the amino-terminal region, although contributing to the recognition process, plays a more important role in the expression of hormone action.

The importance of the $\alpha$-amino group for the apparent activity of glucagon is demonstrated by a variety of chemical modifications (Epand et al., 1973; Desbuquois, 1975; Lande et al., 1973; Epand and Wheeler, 1975; Grande et al., 1972). The possible mechanisms by which these modifications affect biological activity remain unclear. A decrease in activity may result from a decreased affinity of the derivative for the receptor, as was observed for $N^{\alpha}$-acetylglucagon (Desbuquois, 1975), a decreased intrinsic ability of the derivative to activate the adenylate cyclase once it is bound to the receptor, or a combination of both possibilities (Lin et al., 1975; Epand et al., 1976; Hruby et al., 1976).

The effect of chemical modification on the $\alpha$-amino group of glucagon is critically dependent on both the nature and the size of the substituent (Epand et al., 1973; Epand and Wheeler, 1975). Therefore, it is not possible to predict a priori the behavior of carbamino glucagon. In particular, carbamino adducts are expected to be formed to a variable extent depending upon the extracellular physiological state, they add bulk to a critical region of the molecule, and they confer a negative charge on that region (Roughton and Rossi-Bernardi, 1969). Furthermore, their existence may alter the affinity of the molecule for possible cofactors such as $Mg^{2+}$ or $Ca^{2+}$. Such in vivo posttranslational modification may be sufficient to cause altered biological effectiveness of this hormone.

## Acknowledgments

The authors are grateful to Dr. Frank R. N. Gurd for his many helpful discussions and provision of laboratory space and equipment. We are also grateful to James B. Matthew and Stephen H. Friend for the excellent advice throughout this work. The technical help of James Gutfleisch and Diana Embry is also gratefully acknowledged.

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# Allergic Encephalomyelitis: Evidence for Lack of Significant Encephalitogenic Activity of Purified Peptide L in the Monkey†

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ABSTRACT: A highly purified preparation of peptide L of basic protein from bovine spinal cord was obtained by Cellex-P ion-exchange chromatography and repeated Sephadex G-50 filtration; the peptide L preparation was shown to be free from peptide T contamination. When tested for activity, it induced experimental allergic encephalomyelitis (EAE) in the Rhesus monkey after 5–15 weeks at the concentration above 5 mg, suggesting the presence of a weak encephalitogenic site. When peptide L was further cleaved by cyanogen bromide, two peptides, peptide J (residues 1–20) and peptide D (residues 21–115), were obtained. Peptide J was inactive in inducing EAE, while peptide D was as weakly active as peptide L. Conformational studies and identical rate of tryptic hydrolysis of native and denatured peptide L show that it exists in a highly unfolded conformation. Two regions, CD1 (residues 1–43) and peptide R (residues 44–89) present in peptide L, have been previously reported to be encephalitogenic in the monkey; on a molar basis these peptides were much more active than our peptide L preparation. The high activity observed in these peptides is probably due to contamination with the basic protein. It was concluded from our previous studies that the major disease-inducing site in the monkey is a 14-residue peptide (peptide M) localized near the COOH-terminal end of the molecule (Karkhanis, Y. D., Carlo, D. J., Brostoff, S. W., & Eylar, E. H. (1975) *J. Biol. Chem. 250,* 1718).

Experimental allergic encephalomyelitis is an autoimmune disease induced by the basic protein of CNS[1] myelin (Laatsch et al., 1962; Einstein et al., 1962; Eylar et al., 1969) which comprises 30% of the total myelin protein. The protein is believed to have a highly ordered and folded structure and is best described as a prolate ellipsoid with an axial ratio 10:1 (Epand et al., 1974). The ease with which the basic protein can be isolated from myelin suggests that its location in the membrane is that of a so-called *peripheral* protein. The determination of the amino acid sequence of this protein has facilitated the definition of different encephalitogenic sites in this protein which are: a nonapeptide (residues 113–121) active in the guinea pig, peptide R (residues 44–89) active in the rabbit and two peptides, peptides CD1 and M (residues 1–43 and 154–167) active in the monkey (Eylar et al., 1970; Kibler et al., 1972; Jackson et al., 1972; Karkhanis et al., 1975). The rabbit peptide (residues 44–89) has also been shown to be active in the monkey (Kibler et al., 1972). Since the basic protein contains a single tryptophan residue, it is susceptible to treatment with BNPS-skatole (Omenn et al., 1970), the split product being peptides T and L. Peptide T is a COOH-terminal 54-residue peptide which contains one of the monkey sites, whereas peptide L contains two encephalitogenic regions, peptides CD1 and R. On a molar basis peptide L should be more active than peptides T, Y, and M alone. The data obtained so far on peptide L have not supported this interpretation (Eylar et al., 1972) and could be due to the difficulty in obtaining peptide L free from basic protein, since on ion-exchange and gel filtration columns it is eluted close to the basic protein. In the present communication we report the isolation and characterization of peptide L, containing 1% or less of basic protein, which shows weak encephalitogenic activity in the monkey. Since peptide L is comprised of peptides CD1 and R regions, the high activity on a molar basis observed by other workers (Brostoff et al., 1974; Kibler et al., 1972) is perhaps

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[1] Abbreviations used: CNS, central nervous system; peptide L, residues 1–115 of basic protein; peptide T, residues 116–170; peptide Y, residues 154–170; peptide M, residues 154–167; peptide CD1, residues 1–43; peptide R, residues 44–89; peptide J, residues 1–20; peptide D, residues 21–115; fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-diane; CD, circular dichroism; UV, ultraviolet; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; BNPS-skatole, a bromide adduct of 2-(2-nitrophenylsulfenyl)-3-methylindole; EAE, experimental allergic encephalomyelitis.

0006-2960/78/0417-4571$01.00/0 