Physicochemical Characterization of Native and Asialo Human Chorionic Gonadotropin

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SUMMARY

Human chorionic gonadotropin (HCG) was desialized by treatment with neuraminidase to yield asialo-HCG, and comparative physicochemical studies were carried out on HCG and asialo-HCG. The E276 nm value at 276 nm was 5.47 for HCG and 5.72 for asialo-HCG. Intrinsic viscosities of HCG and asialo-HCG at near isionic point were estimated to be 3.4 and 3.2 ml per g, respectively. HCG had an isionic point of 5.02 as compared with that of 6.13 for asialo-HCG. Acid-base titration curves of the two proteins were different, the difference corresponding to a loss of strongly acidic groups, the number of which equals approximately that of neuraminic acid residues removed from HCG. The electrostatic interaction factor obtained for side chain carboxyl groups of asialo-HCG showed good agreement with that calculated for the same group of a rigid sphere model of the same molecular weight. Only 4 of the 7 tyrosyl residues of HCG were reversibly titratable under normal conditions with an apparent pK of 10.3, and complete ionization of the tyrosyl groups was possible only in the presence of 5.4 M guanidine hydrochloride or after exposure of the protein to pH 12.8 at 5°C for 6 weeks. In asialo HCG the ionization was time-dependent and irreversible under normal conditions and all the seven tyrosyl groups were titratable when the sample was allowed to stand for 1 week at 5°C and pH 12.8. The intrinsic pK value and the interaction factor estimated for the four tyrosyl groups of HCG suggested that these groups are normally reactive. The circular dichroism spectra of HCG and asialo-HCG in the ultraviolet region were essentially the same at near-neutral or high pH and did not show the presence of any secondary structure in the molecule. A comparison of the near-ultraviolet spectra in neutral and alkaline media indicated that microconformational changes took place at high pH.

These results indicate that both HCG and asialo-HCG are compact, nearly spherical molecules and have a similar gross conformation. However, tyrosyl residues of asialo-HCG are more easily accessible to solvent molecules than those of HCG, suggesting that desialization of HCG results in minor conformational changes to reduce the stability of the molecule at alkaline pH.

It is established that AcNeu residues of the HCG molecule are essential for the biological but not for the immunological activity of the hormone (1–6). The loss of biological activity of HCG after removal of the AcNeu residues has recently been attributed to rapid removal of the desialized product (asialo-HCG) from the circulation by the liver (7). Also it has been shown that with in vitro systems asialo-HCG elicits biological activity to nearly the same extent as intact HCG (8, 9). However, little information is available as to the effect of desialization of HCG on its conformation.

This report describes the results of a series of experiments in which AcNeu was selectively removed from HCG by treatment with neuraminidase and comparative physicochemical studies were conducted on HCG and desialized HCG with such parameters as viscosity, acid-base titration, spectrophotometric titration, and CD.

EXPERIMENTAL PROCEDURE

A commercial preparation of HCG (APL, lot No. 91301, 3000 i.u. per mg) was processed in a manner similar to that described previously (5) to yield an apparently homogeneous HCG with a biological potency of 11,600 i.u. per mg as assayed by the seminal vesicle weight method in immature rats (10). This preparation served as native HCG in the present study.

Preparation of Asialo-HCG—Into a solution containing 80 mg of HCG in 3.0 ml of 0.01 M acetate buffer, pH 5.0, was added 0.5 ml of the same buffer containing 1.37 units of neuraminidase (1.4 units per mg; NEUP, Worthington), and the mixture was placed in a dialysis sac in 50 ml of the same buffer with continuous slow stirring for 30 hours at 25°C. During this period the dialyzate was replaced twice with a fresh 50-ml aliquot of the same acetate buffer. The reaction mixture was then dialyzed against distilled water overnight at 5°C and lyophilized to yield 59 mg of a final product. Dialyzates obtained at each step of enzymatic hydrolysis including the last desalting process were lyophilized and then were analyzed for AcNeu by the thiobarbituric acid method of Warren (11) with a synthetic AcNeu preparation (Sigma) as a standard. The presence of amino acids and neutral sugars in the lyophilizates was also examined, respectively by the ninhydrin

1 The following abbreviations are used: AcNeu, N-acetylneuraminic acid; sialic acid; HCG, human chorionic gonadotropin; and CD, circular dichroism.

2 Gift of Dr. A. D. Grieve, Ayerst Laboratories, Montreal.
reaction and by thin layer chromatography (5). The neuraminidase preparation was free of proteolytic activity as tested by incubating with Azocoll (Calbiochem) in pH 5.0 acetate buffer for 80 min at 37°.

**Bioassays**—Biological activity of HCG and its desialized product was estimated by the seminal vesicle weight assay method (10).

**Chemical Analyses**—Individual amino acid and carbohydrate components of HCG and desialized HCG were determined by procedures previously described (5).

**Physicochemical Characterization**—Ultraviolet absorption spectra of HCG and desialized HCG were essentially similar and the $E_{1%}^{1cm}$ values for the two proteins at 276 nm were 5.47 ± 0.14 and 5.72 ± 0.12 (S.E.), respectively, when corrected for the moisture content of the preparations. In all of the four methods which are described below, sample concentrations were determined by the use of the $E$ value for HCG.

Viscosity measurements of HCG and its desialized product were carried out in concentrations of 0.4 to 1.2% in 0.1 M KCl buffered at pH 5.0 with 0.01 M sodium acetate at 25 ± 0.1° in an Ostwald-type viscometer with flow time of about 100 s for 0.1 M KCl. The intrinsic viscosities $[\eta]$ of the two proteins were estimated by extrapolation to zero concentration.

Acid-base titrations were conducted on isionic solutions of the two proteins in 0.1 M CO$_2$-free KCl in a titration vessel maintained at 25.0 ± 0.1°. The quantity of 0.2 M HCl or KOH added and the resultant pH were measured with a Radiometer Titror, model TTT1b, connected to a Radiometer SBRK2 1nigraph. Sample solutions in a volume of 2.0 ml, containing 12 to 21 mg of HCG or desialized HCG, were titrated from the respective isionic points with acid or alkali. Activity coefficients for H$^+$ and OH$^-$ of the blank at that pH and on the assumption that the molecular weights of HCG and desialized HCG are 37,700 (5) and 34,800 (see "Results"), respectively, the electrostatic interaction factor $\alpha$ for side chain carboxyl groups of the desialized product was computed from the slope of the plot of

$$\text{pH} - \log \left(\frac{1}{1 - \alpha} \right) = pK_{int} - 0.868\omega Z$$  \hspace{1cm} (1)

**RESULTS**

**Chemical Characterization of Asialo-HCG**—The desialized product gave a negative reaction (up to 2 mg) in the thiobarbituric acid assay for AcNeu. During enzymatic desialization 78% of the AcNeu in the original HCG was released during the first 3-hour period, 14% in the next 3-hour period, and the remaining 9% in the last 24-hour period (Table I). The dialyzates were also negative in the ninhydrin reaction and were found no neutral sugars or hexosamines.

The amino acid compositions of the untreated and desialized HCG were in complete agreement within the limits of experimental error (Table II). However, the number of neutral sugars (galactose and mannose) was slightly less in the desialized product than in the untreated HCG. The desialized HCG contained only a single component in polyacrylamide gel electrophoresis and its mobility toward the anode was from one-third to one-half that of the untreated HCG.

The biological potency of the untreated HCG was 11,600 (9,650 to 13,500; 95% confidence limits) i.u. per mg, whereas that of the desialized HCG was 74 (53 to 96) i.u. per mg.

**Viscosity**—When the reduced viscosities $\eta_{cv}/c$ of the untreated and desialized HCG were plotted against the concentrations, parallel straight lines were obtained (Fig. 1). Extrapolation of these
TABLE II
Composition of untreated and desialized HCG

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Untreated HCG</th>
<th>Desialized HCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>9.0</td>
<td>8.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>4.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>13.9</td>
<td>14.2</td>
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<tr>
<td>Aspartic acid</td>
<td>17.0</td>
<td>16.6</td>
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<tr>
<td>Threonine</td>
<td>14.6</td>
<td>14.4</td>
</tr>
<tr>
<td>Serine</td>
<td>20.1</td>
<td>19.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Proline</td>
<td>26.3</td>
<td>26.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.4</td>
<td>11.7</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>19.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Valine</td>
<td>15.6</td>
<td>16.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.3</td>
<td>5.5</td>
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<td>Leucine</td>
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<td>Tyrosine</td>
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<td>Phenylalanine</td>
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<td>b</td>
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<td>N-Acetylgalactosamine</td>
<td>16.4</td>
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<tr>
<td>N-Acetylgalactosamine</td>
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<td>4.4</td>
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<td>N-Acetylneuraminic acid</td>
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</tr>
<tr>
<td>Fucose</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

* Calculated for molecular weight of 37,700 for untreated HCG and 34,800 for desialized HCG.

* Not determined.

lines to zero concentration gave values of 3.4 and 3.2 ml per g for [γ] at pH 5.0 of the untreated and desialized HCG, respectively.

**Acid-Base Titration**—The titration curves of the untreated and desialized HCG are shown in Fig. 2. These curves represent two independent titrations performed for each preparation, and the reproducibility of the two titrations was excellent below pH 11. However, the reproducibility above pH 11, particularly in the desialized product, was rather poor, the values of the deviation ranging from 6 to 17% of the mean. The two curves are essentially similar in shape above pH 3 but do show that the isionic points of the two preparations are different, i.e. 5.02 for the untreated HCG compared with 6.13 for the desialized HCG. The curve for the desialized HCG levels off below pH 2.4, whereas that for the untreated HCG is still in the upward trend. The intrinsic pK and ω for the aspartyl and glutamyl carboxyl groups of the desialized product were estimated to be 4.72 and 0.050, respectively, as shown in Fig. 3.

**Spectrophotometric Titration of Phenolic Hydroxyl Groups**—Ionization characteristics of tyrosyl residues of the two HCG preparations are shown in Figs. 4 and 5. In the untreated HCG the titration was reversible between pH 6.5 and 13 and the curve showed a constant Δp value of 0.70 above pH 12.5 with an apparent pK of 10.3. Since the Δp value for phenolic hydroxyl group in 0.1 M KCl was found to be 2.250 for the reference compound, N-acetyl-L-tyrosyl ethyl ester, the value of 9.740 corresponds to approximately four tyrosyl groups. However, the amino acid analysis of the same HCG preparation has shown a total of 7 tyrosyl residues per molecule (Table II). When a reaction mixture after forward retitration was left at pH 12.8, 5° for more than 6 weeks,
a maximum $\Delta \varepsilon$ of 16,700 was obtained, which corresponds to 7 tyrosyl residues. This reaction mixture was titrated in the reverse direction and the curve obtained gave a $pK$ value of 9.90 (▲ in Fig. 4). In the presence of 5.4 M guanidine hydrochloride, the titration of the untreated HCG proceeded in an almost identical manner with that seen in the reversed titration of the alkali-treated HCG and a $pK$ value of the titration curve was 9.75. When the spectrophotometric titration data for the four tyrosyl groups of the untreated II CG and the acid-base titration data (Fig. 2) of the same preparation were combined, a straight-line plot was obtained (Fig. 6). Extrapolation of this line to zero net charge ($Z$) yielded a $pK_{\text{int}}$ value of 9.77. The value of the interaction parameter $\omega$ for these tyrosyl groups were calculated as 0.039.

On the other hand, the phenolic hydroxyl titration of the desialized product was found to be time-dependent and irreversible as shown in Fig. 5. In each titration about 30 min were required to run between pH 6 and 13. When a retitrated sample of pH 12.8 was kept at 5°C for 1 week, the $\Delta \varepsilon$ value of the sample at 295 nm was increased to a maximum of 16,640, corresponding to 7 tyrosyl residues. Backtitration of this sample yielded a curve of $pK$ 9.88. The data obtained in the first forward and reversed, and the second forward titrations were combined with the data for the desialized product from Fig. 2 and the combined data were substituted in Equation 1 to produce Fig. 6 (bottom). A linear plot was obtained only in the first forward titration to give values of 9.52 and 0.054 for $pK_{\text{int}}$ and $\omega$, respectively, whereas the plots for the first reversed and the second forward titrations gave an anomalous nonlinear relation.

Circular Dichroism—The CD spectra between 195 to 300 nm of the untreated and desialized HCG are shown in Fig. 7. The $[\theta]_d$ values of the two in the far-ultraviolet region (195 to 240 nm) are expressed on the left-hand ordinate, whereas those at the near-ultraviolet region (240 to 300 nm) are shown on the right-hand ordinate. In the far-ultraviolet region the CD spectrum of each preparation at near-neutral pH was essentially similar to that at pH 12.0 and there was no marked difference between the two preparations at either pH. In this wavelength range both preparations showed smooth, featureless CD curves with a pattern of increasing negative ellipticities with decreasing wave length. In the near ultraviolet region the CD spectra of the two

Fig. 4. Spectrophotometric titration of phenolic OH- groups of HCG in 0.1 M KCl at 25°C. Samples were first titrated with alkali (○), back-titrated with acid to the original pH (●), and then retitrated forward (▲). When the reaction mixture after retitration was left at pH 12.8, 5°C, for 6 weeks and then was reversely titrated, a curve marked with ▲ was obtained. The curve (×) shows the results of the titration in 5.4 M guanidine hydrochloride in 0.1 M KCl at 25°C.

Fig. 5. Spectrophotometric titration of phenolic OH- groups of desialized HCG in 0.1 M KCl at 25°C. The symbols for each curve are the same as used in Fig. 4, except for a curve (▲) that was obtained by reversed titration of a retitrated sample solution that was kept at pH 12.8, 5°C, for 1 week.

Fig. 6. Intrinsic pH and electrostatic interaction factor for phenolic OH- groups of native (top) and asialo (bottom) HCG. The data were taken from Figs. 2, 4, and 5. In asialo-HCG the results of the first forward titration are shown in ○, those of the first reversed titration in ●, and those of the second forward titration in ▲.
preparations at near-neutral pH were again nearly identical and were characterized by a broad, weak, negative dichroic band with a maximum at 275 to 280 nm. However, at pH 12.0 in both preparations a positive dichroic band appeared with a maximum at 250 nm for the untreated HCG but at 252 nm for the desialized HCG. The broad negative dichroic bands of the two preparations shown at pH 7.6 were somewhat sharpened at pH 12.0 and 250 nm for the untreated HCG but at 252 nm for the desialized HCG. The broad negative dichroic bands of the two preparations shown at pH 7.6 were somewhat sharpened at pH 12.0 and their negative maxima underwent a red shift from 280 to 285 nm. However, at pH 12.0 in both preparations a positive dichroic band appeared with a maximum at 250 nm for the untreated HCG but at 252 nm for the desialized HCG. The broad negative dichroic bands of the two preparations shown at pH 7.6 were somewhat sharpened at pH 12.0 and their negative maxima underwent a red shift from 280 to 285 nm for untreated HCG and from 275 to 280 nm for asialo-HCG.

**DISCUSSION**

The facts that the recovery of AcNeu in the dialyzates was complete and that the AcNeu content of the enzyme-treated HCG was nil, clearly indicate that all of the AcNeu residues were removed from HCG by the enzymatic desialization procedure. The chemical composition of the desialized product had only a few less neutral sugar residues than the untreated HCG minus AcNeu, indicating that the procedure was selective for desialization of HCG.

As expected, the removal of AcNeu residues from HCG had no apparent effect on the pattern of the ultraviolet absorption spectrum. Based on the assumptions that the molecular weight of native HCG is 37,700 (5) and that of asialo-HCG is 34,800 (obtained by subtracting the molecular weight of 9.4 AcNeu residues), the observed molar extinction coefficients (εobs) for the two preparations are calculated as 20,620 and 19,910, respectively, and are essentially the same. When the extinction coefficient values of native and asialo-HCG are calculated from the molar absorptivities of the constituent optically active amino acids (13)

\[ \varepsilon_{obs} = \frac{[\theta]_{\lambda}}{\varepsilon + \omega/\rho} \]

of the two proteins (εcalc), the ratio of εobs:εcalc for the two are 1.12 and 1.07, respectively. The E\textsubscript{220} value of 5.47 found for native HCG in this study is considerably higher than the value of 3.88 reported by Bahl (14). Since a comparison of the composition of those optically active amino acids in terms of molar ratios shows good agreement between our and his HCG preparations, this discrepancy is difficult to explain.

To obtain an estimate of the molecular shape and appearance of the two HCG preparations, the viscosity increments of the hydrated proteins v\textsubscript{H} are calculated from the respective intrinsic viscosities [\eta] according to the following equation as described by Yang (15)

\[ \varepsilon_{obs} = \frac{[\theta]_{\lambda}}{\varepsilon + \omega/\rho} \]

The v values, the degree of hydration of a protein, were assumed in this study to be in the order of 0.2 g of water absorbed per g for both native and asialo-HCG. The v\textsubscript{H} values, the partial specific volume, for the two proteins were calculated from their amino acid and carbohydrate composition (16, 17). Then from the calculated v\textsubscript{H} values the axial ratios of the ellipsoid of revolution for the two proteins are estimated with the tables given by Yang (15) (Table III). These data as well as the remarkable similarity in the ultraviolet CD spectrum of the two preparations suggest that the gross conformation of native HCG did not appear to undergo any appreciable change after desialization and that the two protein molecules are compact and slightly asymmetrical, resembling a prolate ellipsoid of axial ratio 2.8 to 3.2 or an oblate ellipsoid with axial ratios of 3.2 to 3.7. The small difference in the \([\eta]\) value between native and asialo-HCG may reflect a slight difference in the molecular weight of the two proteins, i.e. asialo-HCG is about 8% less than native HCG. The rather compact over-all size of native HCG had been expected, since this protein is extremely rich in cystine (5, 14, 18), inferring that the molecule is tightly held by many disulfide bonds. An additional high content of proline of HCG also permits a high degree of irregular structure; thus it is likely that little or no a helical structure exists in the molecular conformation of this hormone. Indeed, the far-ultraviolet CD spectra of these two proteins at near-neutral or strongly alkaline pH did not indicate the presence of helical or \(\beta\) structure. No apparent contribution of the AcNeu residue to the gross conformation of glycoproteins has also been reported in α\(_1\)glycoprotein (19) and fetuin (20).

The slightly asymmetric shape of the native HCG molecule might result in part from a certain contribution by the AcNeu residues to the net charge of the molecule, as postulated by Gottschalk and McKenzie (21). The results of the acid-base
titerations indicate that the AcNeu carboxyl group of native HCG imposes a considerable electrostatic force on the over-all molecule. For example, the isionic point of native HCG rose from 5.02 to 6.13 upon complete removal of the AcNeu residues. At the isionic point of asialo-HCG the difference in the charge between the untreated and AcNeu-free HCG was 8, the number of which is close to the expected number (9.4) of AcNeu residues for the untreated HCG. When the difference between the two titration curves below pH 5 were plotted against pH, a curve with a pK value of 2.28 was obtained. This pK value is in fairly good agreement with the pK of 2.6 reported for free AcNeu (22). Hence the acidic nature of native HCG is attributable to the relatively high AcNeu content. This is by no means unique for HCG, since similar observations have also been made on other AcNeu-rich glycoproteins (23, 24). These findings and the relative ease with which virtually all of the AcNeu was released from the untreated HCG. When the difference between the two titration curves below pH 5 were plotted against pH, a curve with a pK value of 2.28 was obtained. This pK value is in fairly good agreement with the pK of 2.6 reported for free AcNeu (22). Hence the acidic nature of native HCG is attributable to the relatively high AcNeu content. This is by no means unique for HCG, since similar observations have also been made on other AcNeu-rich glycoproteins (23, 24). These findings and the relative ease with which virtually all of the AcNeu was released from the untreated HCG.

The acidic-base titration curves for native and asialo-HCG can each be divided into three regions; an acidic region from pH 2 to 6.4, a neutral region of pH 6.4 to 9, and an alkaline region of pH 9 to 12. Three of these regions correspond to the titration zones of carboxyl groups, imidazole plus α-amino groups and phenolic plus ε-amino groups, respectively. When the number of titrated groups measured in each region is compared with that calculated from the results of amino acid analysis of the two HCG preparations, the measured values in the acidic and neutral regions are in excellent agreement with the calculated ones, as shown in Table IV. However, in the alkaline region the observed number slightly exceed the expected number in the two proteins. The total number of cations at the isoelectric point expected from the known amino acid content of the desialized HCG is 29 (the sum of 9 lysines, 14 arginines, 4 histidines, and 2 ε-amino groups). In the acid-base titration curve the total number of acidic groups counted from the isoionic point was 28 for asialo-HCG but 26 for native HCG. The low value for the latter is apparently due to the low pK value of the AcNeu groups.

The pKint of 4.72 found for the β- and γ-carboxyl groups of asialo-HCG is close to the expected value to 4.6 for the intrinsic ionization constant of these groups. Also, the value of the electrostatic interaction parameter ω obtained for the side chain carboxyl groups agrees with the theoretical value of 0.048 calculated on the assumption that the charge on the protein molecule is evenly distributed over a compact impenetrable sphere (12). This approximation is compatible with the findings of the viscosity experiments. No estimation of ω or pKint for the side chain carboxyl groups was made on native HCG, since the titration curve of the carbohydrate groups has been reported to overlap partially that of AcNeu (27).

In the neutral region the number of titratable groups found in native and asialo-HCG agreed with the expected number of histidine residues and α-amino groups. Since the amino groups of the hexosamine residues, if free, are known to be titrated within this region, a majority of the hexosamine residues in native or asialo-HCG is presumably not in the free but in the N-acetyl form.

The discrepancy between the measured and the expected number of titratable groups in the alkaline region may have resulted from the formation of new groups during titration or from experimental errors. Within the pH range of 9 to 12, tyrosyl and ε-amino groups account for the major titratable groups. However, the results of the spectrophotometric titration of phenolic OH− groups have shown that in native HCG only 4 of the 7 tyrosyl residues are normally titrated. When this is taken into consideration, the discrepancy becomes substantial. Since native as well as asialo-HCG are characterized by a high content of cystine (i.e. disulfide bonds), it is highly possible that the anomalous titration behavior of native and asialo-HCG on the alkaline side was at least in part due to the β-elimination reaction of the disulfide bonds as discussed by Nozaki and Tanford (28). In this context, the results of the CD measurements of the two HCG preparations in the near-ultraviolet region are of interest. At near-neutral pH the CD spectra of the two showed a pattern of very weak side chain effects. At high pH these side chain effects were intensified, suggesting that at strongly alkaline pH native and asialo-HCG undergo alterations in the tertiary structure in the immediate vicinity of the aromatic residues, e.g. ionization of tyrosyl groups (29-31), and of the disulfide residues (32, 33). The appearance of a positive CD peak at alkaline pH, similar to the ones observed in this study, has also been reported in ovine follicle-stimulating hormone (34).

The titration profile of the tyrosyl residues of native HCG has clearly shown that 4 residues are easily accessible, but the remaining 3 residues are almost inaccessible, to the solvent and that the inaccessible residues became titratable only after prolonged exposure of the protein to a high pH or denaturation with guanidine hydrochloride. Since the titration curve of phenolic OH− groups of asialo-HCG was irreversible and time dependent, a clear differentiation of the easily accessible from the inaccessible tyrosyl residues was not possible. However, some of the tyrosyl groups of asialo-HCG were apparently rather slowly becoming ionizable and these could be normalized after a short exposure of the protein to a high pH. Hence the tyrosyl residues of asialo-HCG are more amenable to alkali than those of native HCG. In both native and asialo-HCG certain tyrosyl residues are probably bound more tightly or buried more deeply in the molecule than others so that these become accessible to the solvent only after the original conformation of the molecule is destroyed. This is in accord with the observation that tyrosyl side chains are among the most hydrophobic of protein side chains (35). The intrinsic pK value estimated for the four normally titratable tyrosyl

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**Table IV**

<table>
<thead>
<tr>
<th>pH region</th>
<th>Untreated HCG</th>
<th>Desialized HCG</th>
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<tr>
<td></td>
<td>Calculated</td>
<td>Found</td>
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<tr>
<td>2 to 6.4*</td>
<td>37</td>
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<tr>
<td>6.4 to 9*</td>
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<td>9 to 12*</td>
<td>16</td>
<td>19</td>
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<tr>
<td>Total</td>
<td>59</td>
<td>58</td>
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</tbody>
</table>

* The number of amide groups in the desialized HCG was determined by the procedure described by Spiro (26) and was 10.9 amoles/100 mg, corresponding to 3.8 residues per 34,800 g.
* The NH₂ terminal amino acids of the untreated HCG were alanine, serine, and valine, 0.8, 0.6, and 0.3 mole/46,000 g, respectively. The analysis was generously conducted by Dr. F. Morgan of Columbia University.
groups of native HCG is within the expected range and their interaction parameter is in reasonably good agreement with that calculated for a rigid sphere model of the same molecular weight (12), suggesting that these 4 tyrosyl residues are normally reactive. A similar case was noted in the tyrosyl groups of asialo-HCG titrated in the first forward direction. However, the irreversible nature of the ionization behavior of tyrosyl groups in asialo-HCG may limit the usefulness of the quantitative analysis with regard to electrostatic interaction, and thus the normal values of pK\textsubscript{a} and \(\omega\) obtained in the first forward titration may have been an artifact. Rather, the emphasis should be placed on the anomalies observed in the first reversed and the second forward titrations (Fig. 6), and these anomalies may be related to certain irreversible conformational changes of the asialo-HCG molecule occurring within the pH range of titration.

The ease with which tyrosyl groups are accessible to solvents can be taken as one measure of the general stability of the protein conformation. It is thus reasonable to assume that the conformational stability at high pH is less in asialo-HCG than in native HCG. This may account in part for the poor reproducibility of the acid-base titration curves of asialo-HCG in an alkaline region. Of interest to note is that the stability of native HCG to alkali is reflected by the resistance of the biological and immunological activities of this hormone to exposure to pH 12.0 for 30 min (5).

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