with water, dried, and evaporated to a small volume, whereupon 2.75 g. of a crystalline product, m.p. 196–202°, separated. The analytical sample recrystallized from ether as prisms, m.p. 203–204°, $[\alpha]^{25}D$ −18.4°, transparent to ultraviolet.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.61; H, 8.62.

16α,17α-Epoxy-11α-hydroxy-4-pregnene-3,20-dione (16α,-17α-Epoxy-11α-hydroxy-progesterone) (XIX).—The preceding preparation was submitted to Oppenauer oxidation under the same conditions used to prepare XVII. The product was XIX identical in all respects with the product obtained by microbiological fermentation.[12b]

PHILADELPHIA 18, PENNA.

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Corticotropins (ACTH). XII. Acid–Base Equilibria of α-Corticotropin and Bovine Corticotropin[1]

BY JOSÉ LÉONIS[2] AND CHOH HAO LI

RECEIVED JULY 16, 1958

Titration curves for α-corticotropin and bovine corticotropin have been obtained in 0.1 *M* KCl solutions at 24.5°. The degree of ionization of phenolic groups has also been determined spectrophotometrically. From these data, intrinsic ionization constants (pK_i) of all acidic and basic groups in the corticotropins, as well as values for the electrostatic parameter (w), have been estimated. The relationship of the amino acid sequence in the corticotropin molecule to the estimated values for pK_i and w is discussed.

α-Corticotropin from sheep glands,[3] which has been characterized as a polypeptide of relatively low molecular weight[4] and whose structure has been established,[5] has recently been compared with a seemingly identical compound isolated in pure form from bovine glands.[6] Titration curves for both the ovine and bovine hormones, together with details of an investigation of the ionization of phenolic hydroxyl groups, will be presented here.

Experimental

Titration Assembly.—The cell used for the acid–base titration is shown in Fig. 1; its design permitted the investigation of the rather limited amount of material available. The compact combination glass and reference electrode is supported by the cork stopper of the cell. Standard acid or base is delivered from an Agla micrometer syringe, the tip of the needle (glass, or stainless steel) being immersed in the sample only during the time required for additions. Stirring of the sample is performed by a small magnetic stirrer, rotating simultaneously with the main magnetic stirrer of the thermostatic bath. Proper precautions are applied to exclude carbon dioxide while the sample is being dissolved or transferred to the cell, a slow flow of purified nitrogen being maintained above the solution while the titration is being performed; a saturator, inserted in front of the cell, prevents changes in the volume of the sample resulting from evaporation. In the course of these experiments, each sample was titrated throughout the entire *p*H range under investigation, in a semi-continuous fashion.

Measurement of the *p*H.—The electrode train used was a combination glass electrode/Ag–AgCl reference electrode, of the type described by Cannon[7]; its small size and excellent concentric shielding make it especially suitable for semi-continuous titration of small volumes.[8] The *p*H measurements were made with a Beckman model G *p*H meter.

In order to standardize the *p*H scale, five reference buffers were prepared according to directions given by Bates[9] (0.05 *M* K tetroxalate; saturated KH tartrate; 0.05 *M* KH biphthalate; 0.025 *M* KH_2PO_4, 0.025 *M* Na_2HPO_4; 0.01 *M* borax). During calibration of the Beckman instrument, all these buffers afforded very consistent readings (among all 5 buffers, discrepancies higher than 0.01 of a *p*H unit were never observed). Since the *p*H readings of the buffers showed no drift over a period of several hours, it would appear that no significant leakage of saturated KCl from the liquid junction of the electrode occurred.

After each addition of acid or base during a titration experiment, equilibration of the sample to constant *p*H was usually reached within 2–3 minutes; only when some insoluble material separated out was it necessary to allow longer equilibration, although the time interval never had to be extended beyond 10 minutes.

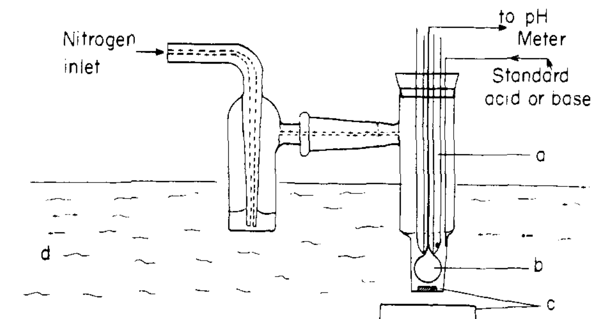

Fig. 1.—Titration cell: a, reference electrode; b, glass electrode; c, magnetic stirrer; d, thermostatic bath.

Acid–Base Titration Curves.—All titrations were carried out at 24.5°, with samples of 8–10 mg. (about 2 micromoles of α-corticotropin in 1 ml. of CO_2-free 0.1 *N* KCl. Standard 0.2 *M* HCl was added first, and the *p*H was read after each addition. When a *p*H around 2 had been reached, the sample was titrated in the other direction with standard CO_2-free 0.2 *M* KOH; finally, it was titrated again from a *p*H around 12 back to the initial *p*H value. By this time, the total volume in the cell has increased by approximately one half; however, the mutual neutralization of acid and base has restored the concentration of KCl to what it was at the start.

The protein concentration was averaged from the results of two types of analysis: namely, determination of total nitrogen and measurement of the ultraviolet absorption spectrum. The correction for free acid or base at extreme *p*H

(1) Presented in part at the 131st meeting of the American Chemical Society, Miami, Florida, April 1957. This work is supported in part by a grant from the National Institutes of Health of the United States Public Health Service (No. RG-2907).

(2) Fulbright Grantee 1955–1957, on leave of absence from the University of Brussels, Belgium.

(3) C. H. Li, I. I. Geschwind, J. S. Dixon, A. L. Levy and J. I. Harris, *J. Biol. Chem.*, **213**, 171 (1955).

(4) A. L. Levy, I. I. Geschwind and C. H. Li, *ibid.*, **213**, 187 (1955).

(5) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(6) C. H. Li and J. S. Dixon, *Science*, **124**, 934 (1956).

(7) M. D. Cannon, *ibid.*, **106**, 597 (1947).

(8) Electrodes of this type, free from any significant sodium ion error, were purchased from Radiometer, Denmark (catalogue number OK 2021-B).

(9) R. G. Bates, "Electrometric *p*H Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954.

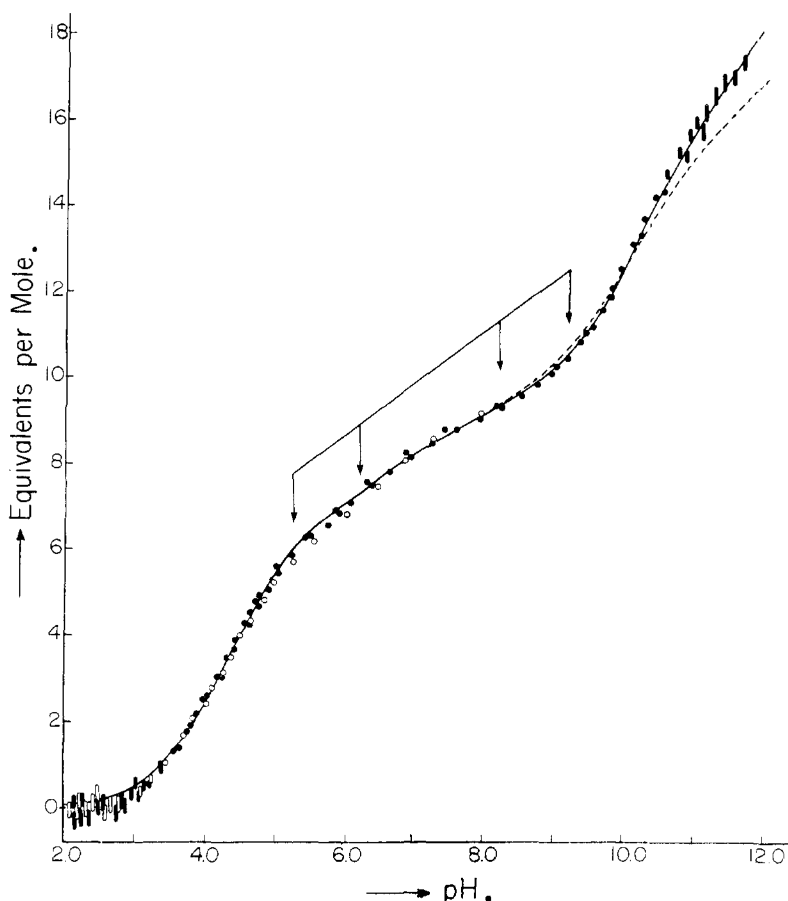

Fig. 2.—Electrometric titration curve of α-corticotropin trichloroacetate (24.5°, 0.1 M KCl). The inner arrows bracket the pH range of insolubility, the outer arrows indicate the limits of noticeable turbidity. Porcine corticotropin-A_1 is also shown for comparison (open circles; data from ref. 19). Solid and dotted curves are calculated as discussed in the text.

values was calculated according to a graphical procedure described previously.[10]

Studies on the Ionization of Phenolic Groups.—Measurements of optical density were made with a Beckman model DU spectrophotometer, provided with thermostatic equipment for maintaining the cell compartment at 24.5°. The samples, 1.0 to 1.5 mg. of corticotropin in 3 ml. of a solution containing 0.02 M in glycine and 0.1 M in KCl, were measured in quartz cells equipped with standard-tapered stoppers. The pH was progressively increased by the addition of KOH, optical density readings being taken after each addition. A mixture of appropriate pH indicators, previously standardized with buffers of known pH, was also included in the solvent. With this technique, the pH was obtained simply from a reading of optical density in the visible range; moreover, it was unnecessary to take extensive precautions against CO_2 since both the pH and the degree of ionization of phenolic groups could be determined simultaneously.

Optical density readings were recorded at wave lengths of 288, 291, 294, 297 and 300 mμ for measurement of the ionization of tyrosine, at 330 and 360 mμ for correction of the background absorbancy as discussed by Beaven and Holiday,[11] and at 458 and 556 mμ for measurement of the pH by means of alizarin yellow and phenolphthalein indicators. Suitable blanks including all components of the system except the corticotropin were adjusted to the same pH as the actual sample.

Results and Discussion

Acid–base titrations of sheep α-corticotropin were initially performed with several samples of a trichloroacetate salt, the usual end product of the isolation procedure[3]; these data are presented in Fig. 2. Reproducibility of the measurements was very satisfactory, even in the region of insolubility where longer equilibration was necessary; decreasing accuracy at either end of the pH scale is indicated in the plot of the data by the use of larger circles, as in Figs. 2 and 3.

Trichloroacetate ions can, however, be removed from corticotropins by using the anion-exchange resin IR-4B in the basic form; desalted lyophilized preparations as a rule show an increased content of nitrogen ($\sim$12% → 15.5%) and an insignificant amount of chloride (0.1–0.2%). Electrometric titration curves for desalted preparations of sheep α-corticotropin and beef corticotropin are presented in Fig. 3. It can be observed that removal of trichloroacetate ions does not appreciably modify the solubility limits of the corticotropin; if anything, with desalted samples a slightly greater discrepancy is observed between forward and reverse titration through the range of insolubility.

The degree of ionization of the phenolic groups in both ovine and bovine corticotropin was determined spectrophotometrically with material isolated both before and after the purification step involving countercurrent distribution.[3,6] The results obtained in every instance were identical within the limits of the method; a typical curve is shown in Fig. 4. The degree of ionization (α) was computed separately from measurements of optical density at five wave lengths between 288 and 300 mμ, after correction for background absorbancy; in all instances the values for α at a given pH but at different wave lengths, were found to fall within a range of $\pm$0.01. The variation in molar extinction coefficient per tyrosine residue at 295 mμ, between $\alpha = 0$ and $\alpha = 1$, was 2,150–2,250 for ovine and 2,000–2,200 for bovine corticotropin. These values compare very favorably with Tanford's determinations for free tyrosine (2,300), ribonuclease (2,630) and serum albumin (2,430)[12]; they also agree with values obtained from growth hormones of different species (2,250 to 2,400).[13]

Since electrometric and spectrophotometric titration curves appear to be fully reversible, their interpretation can be based upon equilibria involving hydrogen ions. Although the relative simplicity of the corticotropin molecule would seem to permit the assigning of particular pK values to individual ionic groups, assignment of these values among some 20 groups would still be largely arbi-

(10) J. Léonis and A. G. Léonis, *Ind. Chim. Belge*, **18**, 244 (1953).

(11) G. H. Beaven and E. R. Holiday, *Adv. Prot. Chem.*, **7**, 319 (1952).

(12) C. Tanford, J. D. Haucnstein and D. G. Rands, THIS JOURNAL, **77**, 6409 (1955).

(13) J. Léonis and C. H. Li, *Biochim. Biophys. Acta*, in press.

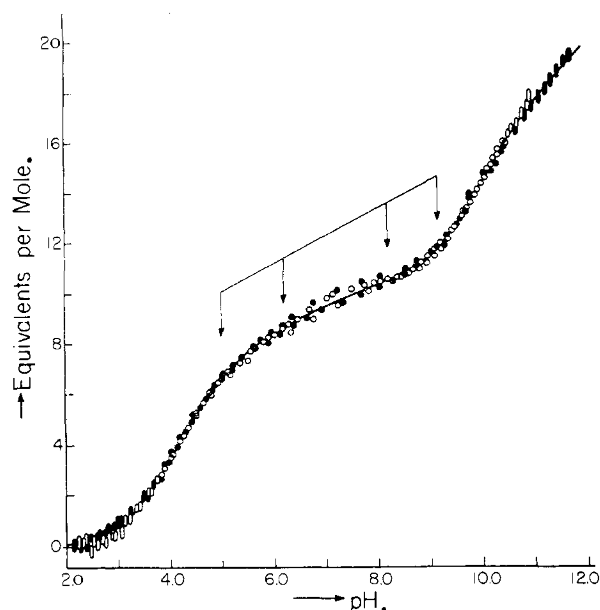

Fig. 3.—Electrometric titration curve of desalted corticotropins (24.5°, 0.1 M KCl): solid circles represent ovine α-corticotropin; open circles, bovine corticotropin; arrows indicate limits of solubility, as in Fig. 2. The solid curve is calculated from values in Tables II and III.

trary. It was thus thought preferable to follow the procedure currently used for larger proteins,[14] where the ionic groups are distributed among limited numbers of classes such as carboxyls, ϵ-amino groups, etc. Each class is analyzed by means of the relationship

$$pH - \log \frac{r}{n - r} = pK_i + 0.868w(-Z) \qquad (1)$$

where n is the total number of groups in the class, r of which are dissociated. The tendency to dissociation is ultimately expressed both by an intrinsic dissociation constant (K_i) and by a factor w which measures the sensitivity to changes in the net charge Z of the protein.

Stoichiometry.—The number of titratable groups found in various corticotropin preparations is summarized in Table I. Their nature was inferred from the amino acid composition of the molecule[4,6] and from a consideration of pK_i values (see below). Values for imidazole, α-amino, ϵ-amino, guanidino and phenolic groups are entirely congruent with the known structure of the hormone.

From earlier determinations of amide-nitrogen, it was concluded that two of the eight carboxylic groups in α-corticotropin trichloroacetate are present as amides.[4] Values of 1.9 ± 0.2 amides/mole were obtained with the present preparations, by means of a micromodification of the method of Rees.[15,16] The number of acidic groups obtained by titration would thus appear to be high (Table

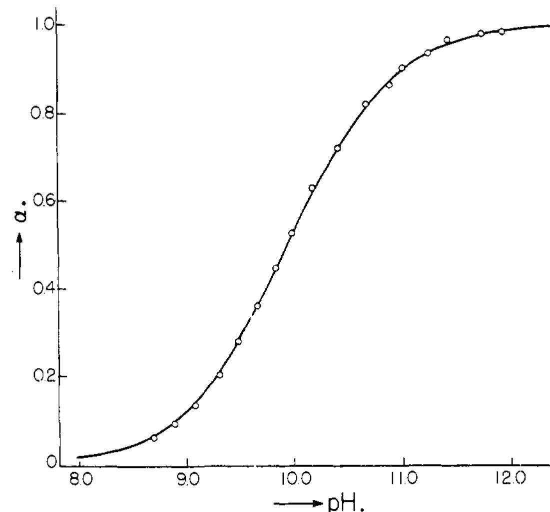

Fig. 4.—Phenolic hydroxyl ionization of α-corticotropin trichloroacetate (24.5°, 0.1 M KCl + 0.02 M glycine). The data at each pH represent the averages of measurements at five wave lengths. The solid curve is calculated from values in Tables II and III.

I). Since the trichloroacetate ion cannot contribute significantly to the titration curve above pH 2, the discrepancy might possibly arise from the presence in the preparations of foreign materials (see below). However, it may also be that the procedure for amide determination yields erroneously high quantities of ammonia, owing, among other possibilities, to instability of constituents of the corticotropin molecule under the conditions required for the estimation (including prolonged hydrolysis with strong acid, and microdiffusion from alkaline solutions). One amide per mole is certainly a minimal value, as was indicated by recent structural investigations.[17] Desalted samples contain two more ionic groups than do trichloroacetate preparations. These groups, which apparently have arisen from the desalting procedure,[18] behave like one carboxylic and one amino group.

TABLE I

IONIC GROUPS IN CORTICOTROPIN PREPARATIONS

Nature of the group	Equiv. of titratable groups per mole corticotropin: Sheep α-corticotropin, Trichloroacetate	Sheep α-corticotropin, Desalted	Beef corticotropin (desalted samples)
Carboxylic	7	8	8
Imidazole	1	1	1
α-Amino	1	1	1
Phenolic	2	2	2
ϵ-Amino	4	5	5
Guanidino	3	3	3
(Extraneous material)	~1	~1	~1

Approximately one equivalent per mole of an unexpected component, with a pK around 6, was observed in all preparations investigated here; it

(14) C. Tanford, in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955, p. 248.

(15) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(16) After conventional hydrolysis with concentrated HCl for 10 days at 37°, ammonia from the material (0.1–0.3 μM amide-N per aliquot) was isolated by microdiffusion in a Conway cell, nesslerized and estimated photometrically.

(17) J. Léonis, C. H. Li and D. Chung, THIS JOURNAL, **81**, 419 (1959).

(18) De-amidation reactions were also encountered during the desalting of porcine corticotropin-A₁ with IRC-50 resin.[19]

(19) H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).

was also found in intermedin (MSH) peptides.[20] Although this component is not removed by desalting with IR-4B resin, its foreign origin is emphasized by its nonstoichiometrical occurrence in the intermedin preparations (1.6 equiv./mole). Such an impurity might easily result from the handling and evaporation of comparatively large volumes of acidic solvents during countercurrent distribution. The apparent amount is greater when the titration is carried out at a higher temperature, and then the amount also becomes time-dependent; clearly, under these conditions this material undergoes some base-consuming reaction, possibly involving corticotropin. Consequently, any further investigation of the effect of temperature upon titration curves was discontinued. The ionization curve of this extraneous material was always deducted from the total titration curves in the following calculations; it also was taken into account for the comparison with porcine corticotropin-A_1,[19] shown in Fig. 2.

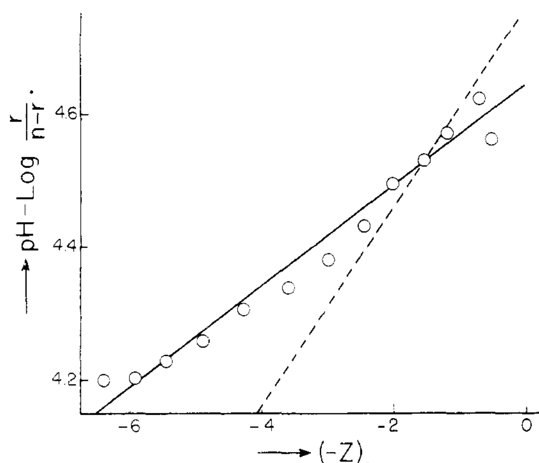

Fig. 5.—Data for the carboxylic groups in α-corticotropin trichloroacetate plotted according to equation 1. Readings were taken at 0.2 pH intervals from a smooth curve drawn through the data from Fig. 2. The dotted line is calculated for a compact sphere the size of α-corticotropin.

The value of pH 8.4–8.5 obtained for the isoionic point of ovine and bovine corticotropins from desalted samples (Fig. 3) is in reasonable agreement with the value of 8.7–8.8 calculated for trichloroacetate preparations according to maximal acid binding and the total number of cationic sites. Accordingly, the isoelectric point (pH 6.6) previously reported[21] for α-corticotropin trichloroacetate would indicate the binding of approximately 1.5 anions per mole of corticotropin; trichloroacetate samples, obtained directly from countercurrent distribution, usually contain 2 to 3 trichloroacetate ions in excess of this amount.[22]

Intrinsic Ionization Constants.—According to equation 1, the intrinsic pK is obtained from a plot of the experimental quantity (pH − log $r/(n - r)$) against $(-Z)$; typical examples are shown in Figs. 5 and 6. For these calculations the net charge at any pH was read from the titration

(20) J. Léonis, I. I. Geschwind and C. H. Li, unpublished results.

(21) I. D. Raacke and C. H. Li, *J. Biol. Chem.*, **215**, 277 (1955).

(22) C. H. Li, *Adv. Prot. Chem.*, **11**, 101 (1956).

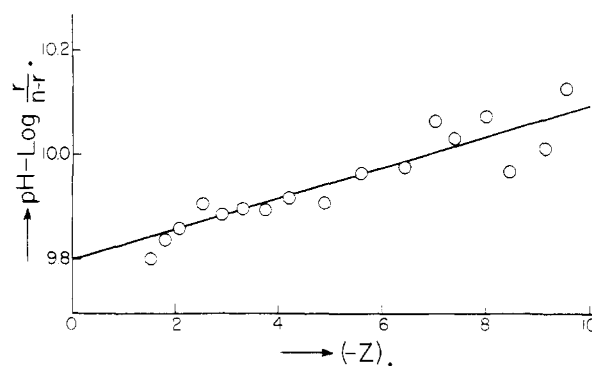

Fig. 6.—Data for the phenolic groups in α-corticotropin trichloroacetate, plotted according to equation 1. Data are the same as in Fig. 4.

curve, with the zero net charge assumed to be at pH_I 6.6. Some degree of overlapping usually had to be corrected for, as for instance that between carboxylic and imidazole groups, or between phenolic, ϵ-amino and guanidino groups. The linear regression line for these plots was determined according to the principle of least squares; in this manner pK_i values were obtained which compare favorably with those obtained by Tanford from the study of larger proteins[23] (Table II).

TABLE II

INTRINSIC IONIZATION CONSTANTS OF ACIDIC AND BASIC GROUPS IN CORTICOTROPINS

Nature of the group	Sheep α-corticotropin trichloroacetate	Sheep and beef corticotropins, desalted samples	Expected values[20]
Carboxylic	4.64(4.60–4.67)[a]	4.53(4.49–4.57)	4.6
Imidazole	*ca.* 7.0	*ca.* 6.8	6.5–7.0
α-Amino	*ca.* 8.3	*ca.* 8.3	7.8
Phenolic (from u.v. absorption data)	9.80(9.78–9.81)	9.79(9.76–9.82)	9.5–9.9
ϵ-Amino	10.07(10.01–10.13)	10.02(9.95–10.08)	10.1–10.6
Guanidino	≥11.6	≥11.8	>12

[a] The 95% confidence interval is given in parentheses.

Compared with the range of expected values, the intrinsic pK values found for the ϵ-amino and guanidino groups appear to be rather low. This is presumably related to the occurrence of most of these groups in the very basic sequence –Lys.Lys.Arg.Arg.Pro.Val.Lys– of corticotropin.[5] The pK of the α-amino group, on the other hand, although determined with only limited accuracy (owing to poor solubility of the material), appears higher than is the case with more conventional proteins. The same behavior was observed in earlier studies of the reaction of this group with fluorodinitrobenzene and other reagents[24]; again, this effect might be explained in part by the vicinity of the relatively negative sequence NH_2-Ser.Tyr.Ser.Met.Glu–. Finally, during a preliminary study of the phenol/phenoxide equilibrium in α-corticotropin, the results had been expressed by means of a "titration constant," $pK' = 10.01 \pm 0.08$, because of lack of information concerning charge effects.[25] This value is en-

(23) C. Tanford and J. D. Hauenstein, THIS JOURNAL, **78**, 5287 (1956).

(24) A. L. Levy and C. H. Li, *J. Biol. Chem.*, **213**, 487 (1955).

(25) I. I. Geschwind and C. H. Li, *Arch. Biochem. et Biophys.*, **63**, 316 (1956).

tirely consistent with the results found in the present investigation, which was carried out at slightly higher temperature, where the mid-point of the curve is found in the range 9.92–9.98.

Electrostatic Interactions.—Values for the electrostatic parameter w (see equation 1) are presented in Table III; limits of reliability, accounting for randomness of the data, are also included. In the carboxylic region, however, a definite curvature appears in the plot of (pH *vs.* log $r/(n - r)$) *versus* ($-Z$) (*cf.* Fig. 5). Deviations from the linearity implicit in equation 1 could arise from any one of the following reasons, or from a combination.

TABLE III

VALUES OF THE ELECTROSTATIC PARAMETER (w) IN CORTICOTROPINS

Nature of the group	Sheep α-corticotropin trichloroacetate	Sheep and beef corticotropins, desalted samples
Carboxylic	0.086(0.076–0.096)[a]	0.090(0.080–0.099)
Phenolic	0.034(0.026–0.042)	0.025(0.010–0.040)
ε-Amino	0.027(0.013–0.041)	0.039(0.025–0.053)

[a] The 95% confidence interval is given in parentheses.

First, it might be an oversimplification to assume a single pK_1 value for all carboxylic groups. Indeed, four of the carboxyl groups appear in the very unusual sequence –Glu.Asp.Asp.Glu– in corticotropin.[5] Alternately, the value for Z obtained from the titration curve might be inadequate, if pH-dependent ion binding occurs. In view of the significant difference between isoionic and isoelectric pH, this alternative possibility must be considered. Finally, it might be that the value of w is not constant throughout the pH range of from 3 to 6. Since the w factor is directly related to the size and shape of the molecule,[26] this would mean that α-corticotropin is endowed with appreciable deformability or flexibility.

The very large difference in w observed between the acidic and the basic portions of the titration curve also favors this latter view. That this difference is real is indicated by a significant departure of the data from a titration curve calculated with a constant value of w throughout, the same value as that of the carboxylic groups (dotted curve in Fig. 2); in order to obtain a reasonably close fit between the data and the calculated curve around pH 12, the very unlikely value of $pK_1 \leqslant 11$ would have to be used for the guanidino groups. However, agreement between calculated and observed values is, as expected, extremely close if the set of values for w given in Table III are used (solid curves in Figs. 2 and 3). The change in w might in part reflect the variation between values for the sedimentation constant measured in acid and in alkaline solutions.[27] By means of equations discussed by Tanford,[26] it is calculated that a change of w from 0.09 to 0.035 (or 0.025) would correspond to an increase in molecular weight by a factor of at least 5 (or 10) if the molecule is assumed to retain spherical symmetry and to undergo no change in hydration. Actually, a spherical, unhydrated molecule of the size of corticotropin would have a value for w quite different (namely, 0.17) from those recorded in Table III. Figure 5 clearly indicates that the model of a rigid sphere becomes a reasonable approximation only in the neighborhood of the isoelectric pH. Accordingly, it seems justified to assume that the molecule has a compact configuration in the pH range of limited solubility but undergoes some expansion outside of this range.

(26) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(27) C. H. Li and H. Papkoff, unpublished experiments.

BERKELEY, CALIF.

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Corticotropins (ACTH). XV. The Action of Chymotrypsin on α-Corticotropin[1]

BY JOSÉ LÉONIS,[2] CHOH HAO LI AND DAVID CHUNG

RECEIVED JULY 16, 1958

The following peptide fragments have been isolated from chymotryptic digests of α-corticotropin and identified: Ser.Tyr; Ser(Tyr,Ser,Met,Glu)His.Phe; Arg.Try; Arg(Try,Gly,Lys,Pro,Val); Gly(Lys,Pro)Val.Gly.Lys; Lys(Arg$_2$,Pro)Val; Lys-(Glu$_2$,Asp$_2$,Ala$_2$,Val,Tyr,Pro,Gly,Ser)GluNH$_2$.Ala.Phe; Ala(Phe,Pro)Leu; and Glu.Phe. These sequences are consistent with the proposed structure for α-corticotropin. One of the two amide groups in the peptide hormone has been located as glutamine at amino acid position 33. The specificity of chymotryptic hydrolysis of peptide bonds has been discussed.

The structure of α-corticotropin has been deduced from peptide fragments isolated after cleavage of the hormone by means of peptic, tryptic and acid hydrolysis.[3] The peptide pattern obtained when α-corticotropin was digested with chymotrypsin, however, was so complex that only relatively limited information could be derived from it during the early stages of the structural investigation; and, indeed, the data presented here indicate that the number of peptide bonds susceptible to the enzyme is unexpectedly high. Furthermore, since the rate of splitting of susceptible bonds seems to differ and also to vary with the average extent of digestion, it was found that isolation of the resultant peptides in nearly stoichiometrical amount could not be expected. It is the purpose of this paper to demon-

(1) This work was aided in part by grants from the National Institutes of Health of the United States Public Health Service (G 2907) and the Albert and Mary Lasker Foundation. For Paper XIII of this series see C. H. Li, R. D. Cole, D. Chung and J. Leonis, *J. Biol. Chem.*, **227**, 207 (1957); and for Paper XIV see C. H. Li, J. S. Dixon and D. Chung, THIS JOURNAL, **80**, 2587 (1958).

(2) Fulbright Grantee 1955–1957, on leave of absence from the University of Brussels, Belgium.

(3) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).