*Acta Chimica Academiae Scientiarum Hungaricae, Tomus 100 (1—4), pp. 275—288 (1979)*

# NEW METHOD FOR THE EVALUATION OF OVERLAPPING PROTONATION EQUILIBRIA OF POLYFUNCTIONAL LIGANDS

## EFFECT OF SOLVENT ON THE PROTONATION OF THE FUNCTIONAL GROUPS OF CORTICOTROPINE

B. NOSZÁL and K. BURGER

*(Institute of Inorganic and Analytical Chemistry, L. Eötvös University, Budapest)*

Received July 5, 1978

Accepted for publication August 7, 1978

A method has been developed for the simultaneous determination of the protonation constants characteristic of the single functional groups and of pairs of groups forming hydrogen bonds, and of the number of hydrogen bonds in systems of overlapping protonation equilibria. The group and structural constants of the polyfunctional ligands, $ACTH_{1-4}$ and $ACTH_{1-32}$ polypeptides in 50% propylene glycol solution have been determined by the method, and from the constants conclusions are drawn concerning the interaction of the functional groups with one another and with the solvent.

### Introduction

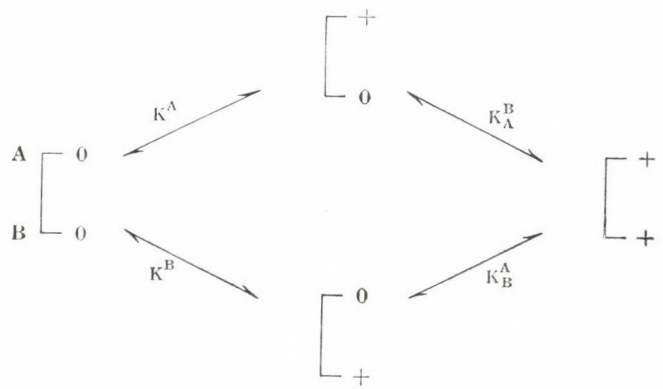
The interpretation of the protonation equilibria of ligands containing several donor groups of similar basicity is made rather difficult by overlapping processes. No evaluation method was elaborated so far suitable for characterizing the single functional groups in systems showing the overlap of more than two protonation steps. This is rather surprising as the basic relationships between macroconstants, characteristic of the stepwise protonation of the molecule, and microconstants, describing the protonation of a certain group of the molecule in a given state of protonation, were established by Niels BJERRUM in his classical work as early as 1923 for systems containing molecules of two and three groups without hydrogen bonding [1]. Since that time the literature on methods for the determination of macroconstants has vastly expanded [2—4] and the accumulated results fill several volumes [5]. This much less applies to microconstants, because in spite of the fact that several complex measuring methods including UV, Raman, NMR spectroscopy, *etc.* and methods of evaluation are known [6—10], the performance of these methods is limited to four microconstants characteristic of two groups, or to two times two groups contained in the molecule, thus to eight microconstants [11]. This is to be attributed primarily to the fact that the number of both the possible species of different protonation and the microconstants characteristic of

these particles increases rapidly with an increasing number of functional groups, causing difficulties in evaluation, insurmountable so far.

Polyfunctional bioligands, playing an important role in vital processes, can exert their action only at a suitable protonation of their groups. Therefore, the knowledge of the protonation constants of these groups is very important also from the aspect of the understanding and influencing biological systems. In the following the results of a work are summarized, the object of which was the elaboration of a method permitting in principle the simultaneous determination of the protonation constants of the functional groups and of the number of hydrogen bonds between these groups. The method is illustrated on the example of a protonation study of two fragments of the corticotropine (ACTH) molecule in propylene glycol—water mixture, where the effect of interaction of the functional groups with one another and with the solvent on the group protonation constants can be observed.

## Principles of the Method

A molecule containing two functional groups, in which the sites of coordination are not equivalent, is characterized by the protonation equilibria shown in Fig. 1.




(1)

*Fig. 1.* Protonation equilibria of molecules containing two groups, without formation of hydrogen bonds

The microconstants relevant to this system are:

$$K^A = \frac{[\square_0^+]}{[H^+][\square_0^0]} \qquad K_A^B = \frac{[\square_+^+]}{[H^+][\square_0^+]}$$

$$K^B = \frac{[\square_+^0]}{[H^+][\square_0^0]} \qquad K_B^A = \frac{[\square_+^+]}{[H^+][\square_+^0]}, \tag{2}$$

where the upper index denotes the group being protonated in the process, while the lower index the already protonated group.

Material balances can be written as

$$C_L = [l_0^0] + [l_0^+] + [l_+^0] + [l_+^+] \tag{3}$$
$$C_H = [H^+] + [l_0^+] + [l_+^0] + 2[l_+^+],$$

where $C_L$ is the total ligand concentration and $C_H$ the total hydrogen ion concentration.

Rearrangement of Eqs (2) and their substitution into Eq. (3) gives;

$$C_L = [l_0^0] + (K^A + K^B)[l_0^0][H^+] + K^A K_A^B [l_0^0][H^+]^2 =$$
$$= [l_0^0] + (K^A + K^B)[l_0^0][H^+] + K^B K_B^A [l_0^0][H^+]^2$$
$$C_H = [H^+] + (K^A + K^B)[l_0^0][H^+] + 2K^A K_A^B [H^+]^2 [l_0^0] =$$
$$= [H^+] + (K^A + K^B)[l_0^0][H^+] + 2K^B K_B^A [H^+]^2 [l_0^0]. \tag{4}$$

It can be seen from Eqs (4) that on the basis of the material balance written for any of the total concentrations, the constant to be obtained from potentiometric data by the various known evaluation methods as the coefficient of hydrogen ion activity, is the sum of microconstants characterizing the different functional groups, while the constant obtained as the coefficient of the square of hydrogen ion activity is the product of corresponding microconstants (one of them is, however, different from the former microconstants):

$$\beta_1 = K^A + K^B$$
$$\beta_2 = K^A K_A^B = K^B K_B^A, \tag{5}$$

where $\beta_1$ and $\beta_2$ are complex products obtainable by the usual evaluation methods.

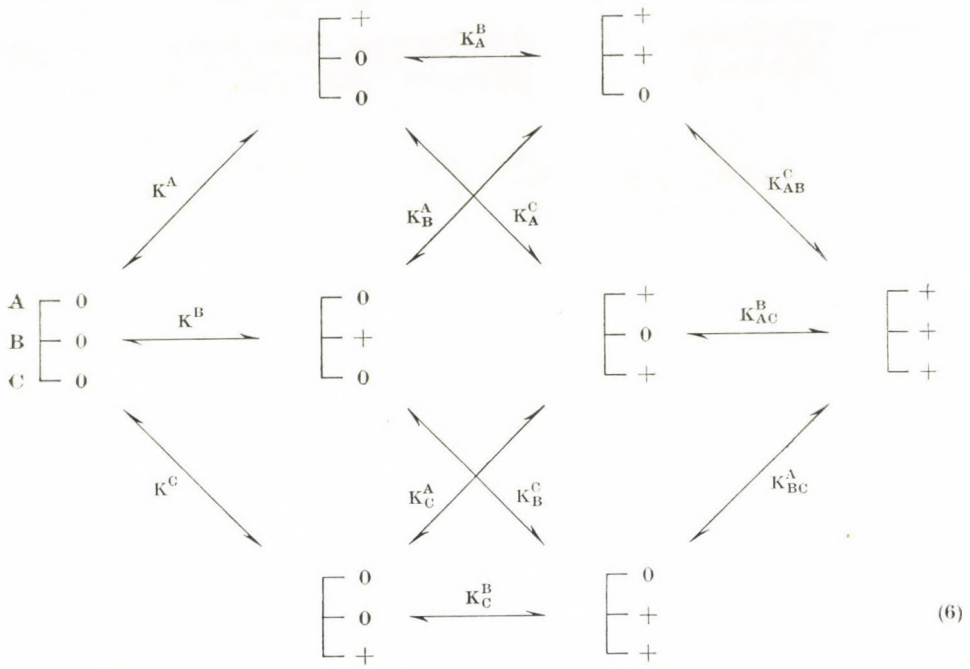
The same type of relationship for a system containing molecules with three groups is:

$$\beta_1 = K^A + K^B + K^C$$
$$\beta_2 = K^A K_A^B + K^A K_A^C + K^B K_B^C = K^B K_B^A + K^C K_C^A + K^C K_C^B$$
$$\beta_3 = K^A K_A^B K_{AB}^C = K^B K_B^C K_{BC}^A = K^C K_C^B K_{BC}^A. \tag{7}$$

The corresponding equilibria are shown in Fig. 2.

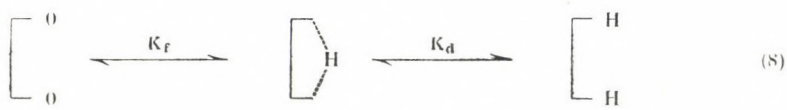
Models shown in Figs 1 and 2, and relationships (5) and (7) which can be derived from them, are characteristic of the protonation processes of molecules, in which the functional groups do not form intramolecular H-bonds with one another, and influence one another's protonation processes only by inductive and mesomeric effects, or possibly by hydrophobic interaction.

Two functional groups (A and B) form an H-bond only if this is energetically favourable for the system. Since the H-bond between A and B represents




(6)

*Fig. 2.* Protonation equilibria of molecules containing three groups, without formation of hydrogen bonds




(8)

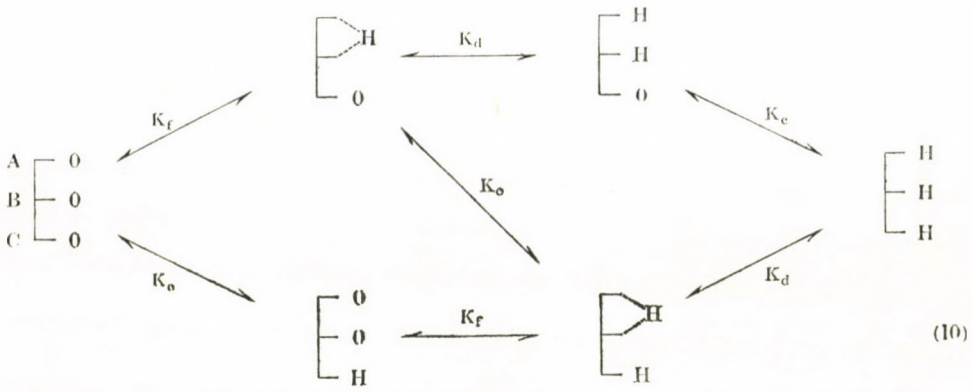
*Fig. 3.* Protonation equilibria of molecules containing two groups in the case of hydrogen bond formation

the singly protonated state of the lowest energy level, the H-bond is formed regardless of whether the proton approaches the molecule from group A or B. This reduces the possible number of particles occurring in the system (Fig. 3). In this case

$$\beta_1 = K_f; \quad \beta_2 = K_f \cdot K_d, \tag{9}$$

where $K_f$ is the micro- and at the same time group constant (to be introduced in the following), characteristic of hydrogen bond formation, while $K_d$ that characteristic of its rupture and the uptake of the second proton.

For molecules containing three functional groups (A, B and C), the scheme of protonation in the case of H-bond formation between groups A and B is




(10)

*Fig. 4.* Protonation equilibria of molecules containing three groups in the case of hydrogen bond formation

shown in Fig. 4 [where the symbols according to Eqs (16) are indicated in a simplified form]. From this the following relationships are obtained:

$$\beta_1 = K^f + K^C$$

$$\beta_2 = K^f K_f^C + K^f K_f^d = K^C K_C^f + K^f K_f^d$$

$$\beta_3 = K^f K_f^C K_{fC}^d = K^C K_C^f K_{fC}^d = K^f K_f^d K_{fd}^C . \qquad (11)$$

Tables I and II show the relationships between the number of groups ($n$) to be found in the molecule, the number of hydrogen bonds ($k$) linking the groups, and the number of species and microconstants, respectively.

It becomes evident from a comparison of Eqs (5) and (9) as well as (7) and (11) the protonation schemes (6) and (10) and data in Table I, that in the case of hydrogen bond formation the number of species formed is less than without H-bond formation, and thus, the number of microconstants needed for the characterization of the whole equilibrium system is also inversly proportional to the number of hydrogen bonds (Table II).

**Table I**

*Number of species in the system as a function of the number of groups ($n$) contained in the molecule, and of the number of hydrogen bonds ($k$) linking the groups*

| $k$ \ $n$ | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 0 | 2 | 4 | 8 | 16 | 32 | 64 |
| 1 | — | 3 | 6 | 12 | 24 | 46 |
| 2 | — | — | — | 9 | 18 | 35 |
| 3 | — | — | — | — | — | 27 |

**Table II**

*Number of microconstants serving for the complete equilibrium characterization of the system as a function of the number of groups (n) contained in the molecule and of the number of hydrogen bonds (k) linking the groups*

| k \ n | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 0 | 1 | 4 | 12 | 32 | 80 | 192 |
| 1 | — | 2 | 7 | 20 | 52 | 128 |
| 2 | — | — | — | 12 | 33 | 84 |
| 3 | — | — | — | — | — | 54 |

On comparison of the system of Eqs (5) and (9), as well as (7) and (11), it is remarkable and of basic importance for the determination of the number of hydrogen bonds to be described later, that for any of the systems the number of terms in the $i$-th $\beta$ constant is equal to the number of $i$-times protonated species present in the solution.

The difference between the microconstants relevant to the same group in different surroundings can be traced back to two basic factors. One of these is the electron-attracting, (*i.e.* basicity-reducing) effect, occuring on protonation of the adjacent group, and perceptible also on the selected group. This effect is operative when the adjacent group is separated by an adequately low number of carbon (or other) atoms from the group in question, *i.e.* the static inductive effect actually reaches the given functional group.

The other essential effect to be taken into account is the steric proximity established by the protonation more often of the adjacent and more seldom of the more distant functional group, causing changes in rotational energy levels, which results in the formation of a hydrogen bond, ensuring a lower energy level for the system.

In the case of molecules having unknown structure in the solution, the first effect cannot be investigated separately from the second effect by the potentiometric method, because the change of the protonation constant observed cannot be separated into static inductive effect and H-bond formation. Therefore, independent experimental data are needed to establish electron attraction and electron release through the chain. Data of this kind are furnished by the proton resonance measurements of SUDMEIER and REILLEY [12]. These authors have found that the change in chemical shift occurring on protonation of the primary amino group can still be perceived at the third ($\gamma$) carbon atom from the amino group, but not at more distant atoms. In the case of carboxylate the effect cannot be measured any more at the third ($\gamma$) carbon atom. This means that, with the exception of conjugated systems, electron attraction occurring on protonation of group B (or electron donation on loss of a proton) will cause a perceptible change in the electron density of

group A only if not more than 2 atoms are located between the two functional groups. In peptides this occurs only in the single case when the *C*-terminal amino acid is aspartic acid. In the case of *C*-terminal glutamic acid the number of intermediate carbon atoms is already three, while in the next interchain case, when the — Asp — Asp sequence is present, the number of intermediate atoms is already six.

Thus, disregarding those peptides the *C*-terminal amino group of which is aspartic acid, or to be on the safe side, glutamic acid, it can be established that the basicity of the functional groups in the side chain of peptides occurring in practice, and thus the value of the protonation microconstants of the said functional groups cannot be affected by electron shifts through the chain even due to the nearest functional group. This means that microconstants relevant to identical groups are to be considered as identical, independently of the protonation of the molecule in the environment of the group. Accordingly, in the case of a molecule with two groups it follows from Eqs (2) that

$$K^{\mathrm{A}} = K_{\mathrm{B}}^{\mathrm{A}} = K_{\mathrm{A}}$$
$$K^{\mathrm{B}} = K_{\mathrm{A}}^{\mathrm{B}} = K_{\mathrm{B}} \tag{12}$$

and substituting Eqs (12) into Eq. (5):

$$\beta_1 = K_{\mathrm{A}} + K_{\mathrm{B}}$$
$$\beta_2 = K_{\mathrm{A}} K_{\mathrm{B}} \,. \tag{13}$$

In an analogous way, in a molecule containing three groups, in the absence of H-bonds:

$$K^{\mathrm{A}} = K_{\mathrm{B}}^{\mathrm{A}} = K_{\mathrm{C}}^{\mathrm{A}} = K_{\mathrm{BC}}^{\mathrm{A}} = K_{\mathrm{A}}$$
$$K^{\mathrm{B}} = K_{\mathrm{A}}^{\mathrm{B}} = K_{\mathrm{C}}^{\mathrm{B}} = K_{\mathrm{AC}}^{\mathrm{B}} = K_{\mathrm{B}}$$
$$K^{\mathrm{C}} = K_{\mathrm{A}}^{\mathrm{C}} = K_{\mathrm{B}}^{\mathrm{C}} = K_{\mathrm{AB}}^{\mathrm{C}} = K_{\mathrm{C}} \tag{14}$$

and upon substitution of Eqs (14) into Eqs (7):

$$\beta_1 = K_{\mathrm{A}} + K_{\mathrm{B}} + K_{\mathrm{C}}$$
$$\beta_2 = K_{\mathrm{A}} K_{\mathrm{B}} + K_{\mathrm{A}} K_{\mathrm{C}} + K_{\mathrm{B}} K_{\mathrm{C}}$$
$$\beta_3 = K_{\mathrm{A}} K_{\mathrm{B}} K_{\mathrm{C}} \,. \tag{15}$$

In the case of H-bond formation in a molecule containing two groups the reductions are not feasible.

In the case of H-bond formation in a molecule containing three groups:

$$K^{f} = K_{\mathrm{C}}^{f} = K_f$$
$$K^{\mathrm{C}} = K_f^{\mathrm{C}} = K_{fd}^{\mathrm{C}} = K_{\mathrm{C}}$$
$$K_f^d = K_{f\mathrm{C}}^d = K_d \,, \tag{16}$$

where $K^f$ is a constant characteristic of hydrogen bond formation, $K^d$ a constant characteristic of the rupture of the same by protonation; upon substituting Eqs (16) into (11) one obtains

$$\beta_1 = K_f + K_C$$

$$\beta_2 = K_f K_C + K_f K_d$$

$$\beta_3 = K_f K_C K_d. \tag{17}$$

In general formulation for the case of $n$ groups and $k$ hydrogen bonds:

$$\beta_1 = \sum_{i=0}^{n-k} K_i$$

$$\beta_2 = \sum_{\substack{a=0 \\ b=a+1}}^{\substack{n-k \\ n-k-1}} \prod_{i=a}^{b} K_i + \sum_{s=0}^{k} K_s K_{s+n-k}$$

$$\beta_3 = \sum_{\substack{a=0 \\ b=a+1 \\ c=b+1}}^{\substack{n-k \\ n-k-1 \\ n-k-2}} \prod_{i=a}^{c} K_i + \sum_{s=0}^{k} K_s K_{s+n-k} \sum_{\substack{t=0 \\ t=s+1}}^{\substack{n-k \\ s-1}} K_t$$

$$\vdots$$

$$\beta_n = \prod_{i=1}^{n} K_i, \tag{18}$$

where $K_i$ and $K_s$ are group constants carrying the symbols of current indexes $i$ and $s$. Constants denoted from $K_1$ till $K_k$ are the structural constants characterizing the formation of H-bonds, those from $K_{k+1}$ till $K_{n-k}$ are the protonation constants of groups without H-bond formation, while constants from $K_{n-k+1}$ till $K_n$ refer to rupture of H-bonds by protonation.

It can be seen from equations (13), (15) and (16) that the number of unknown group constants ($K_A$, $K_B$, $K_C$) and that of the group + structural constants ($K_f$, $K_d$), respectively, is equal to that of the known macroconstants ($\beta_1$, $\beta_2$, $\beta_3$) to be determined from potentiometric data. Thus, knowing the H-bond forming properties of the molecule, protonation constants characteristic of the groups and of the structural elements can be determined.*

* It should be noted that the simplifying reductions in systems of equation (12), (14) and (16) can be performed only in the case of adequately distant groups, because otherwise incorrect results (like those described in Ref. [14]) will be obtained.

## Simultaneous determination of group constants and the number of hydrogen bonds from protonation macroconstants

If the number of hydrogen bonds in the molecule investigated is known, from relationships (13), (15) or (16), though they cannot be made explicite both for group and structural constants, the values of $K_A$, $K_B$, $K_C$, $K_f$, $K_d$ can be obtained.

However, in the majority of cases, particularly for complicated molecules, the number of H-bonds in unknown.

As becomes evident from the comparison of equations (9) and (12) as well as (15) and (17) and from the interpretation of (18), the corresponding complex products ($\beta$) are determined by the group constants and by the number of species (which depends, besides the known number of functional groups, on the usually unknown number of hydrogen bonds ; see also Table I). This offers at the same time a possibility for the simultaneous determination of the group constants and the number of hydrogen bonds, when the $\beta$ values are known.

It can be seen that from the $n$ complex products of a molecule containing $n$ groups, the $n$ group or structural constants can be obtained in any case as the roots of the system of equations by a suitable mathematical (*e.g.* Newton—Raphson iteration) method, whichever relationship is used. Naturally, reality is correctly reflected only by those group and structural constants, in the calculation of which the number of the assumed H-bonds has been the same as in reality.

If the chemical model is not correct (incorrect number of H-bridges) some of the roots of the equation system may be complex or negative. In view of the fact that a protonation constant is always a real positive number, this in itself would be sufficient proof of the incorrectness of the model, if the macroconstants obtained from measured data were not subject to random errors, which are similarly sources of distortion in the type and particularly in the value of the roots obtained. Thus, the most suitable method of determination of the group (or structural) constants of a molecule containing an unknown number of hydrogen bonds, and of the number of hydrogen bonds in real systems is the following.

From the difference of the complex products, obtained from data measured, and from the group constant combinations yielding the same, a quadratic error function is generated in each member of the equation system. Constants obtained at zero value (which is the minimum of the error function) of the partial derivatives with respect to all the group constants of all the members of the equation system will give the group or structural constants with the best fit to the data.

For example, for a molecule with three groups and no H-bond the error functions are:

$$Z_1 = [\beta_1 - (K_A + K_B + K_C)]^2$$
$$Z_2 = [\beta_2 - (K_A K_B + K_A K_C + K_B K_C)]^2$$
$$Z_3 = [\beta_3 - (K_A K_B K_C)]^2 \qquad (18)$$

The quadratic matrix obtainable from the partial derivatives is:

$$\left.\begin{array}{lll} \frac{\partial Z_1}{\partial K_A} = 0; & \frac{\partial Z_1}{\partial K_B} = 0; & \frac{\partial Z_1}{\partial K_C} = 0 \\ \frac{\partial Z_2}{\partial K_A} = 0; & \frac{\partial Z_2}{\partial K_B} = 0; & \frac{\partial Z_2}{\partial K_C} = 0 \\ \frac{\partial Z_3}{\partial K_A} = 0; & \frac{\partial Z_3}{\partial K_B} = 0; & \frac{\partial Z_3}{\partial K_C} = 0 \end{array}\right\} \begin{array}{l} K_A \\ K_B \\ K_C . \end{array} \qquad (19)$$

A proof of the validity of the model reflecting the equilibrium constants and the number of H-bonds thus calculated is a good agreement between the experimental $\beta$ values and those calculated from the group constants; the measure of this agreement can be characterized by the mean quadratic error. Another possibility for the selection of the models and thus of the number of hydrogen bonds is to follow the protonation of one of the functional groups by selective spectroscopy. If the constant calculated in this way is in exclusive agreement (whithin the limits of error) with one of the group constants of one of the models, this is also proof of the correctness of the given H-bond number.

The applicability in practice of the evaluation method described above is shown on the example of the protonation equilibria of corticotropin fragments consisting of 32 amino acids (henceforth $ACTH_{1-32}$) and its *N*-terminal tetrapeptide fragment ($ACTH_{1-4}$), measured in 50 vol. % propylene glycol.

## Experimental

The *N*-terminal fragment of the $\alpha_H$ adrenocorticotropic hormone consisting of 32 amino acids ($ACTH_{1-32}$), and $ACTH_{1-4}$ consisting of the *N*-terminal four amino acids of this hormone were twice lyophilized, perprotonated preparations of G. Richter Chemical Works (Budapest). The solvent mixture was a homogenous 50 vol. % mixture of distilled propylene glycol and water, in which the ionic strength has been adjusted with $NaClO_4$ to 0.3 *M*. Devices used for the measurements: digital pH-meter Model Radiometer PHM 64 (precision of display + 0.1 mV), automatic burette Model Radiometer ABU 12 (precision of reading 0.001 ml); measuring electrode: glass electrode Radiometer G202B, reference electrode: Ag electrode Model Radiometer P 401, which was immersed into 0.01 *M* $AgNO_3$ solution (ionic strength adjusted with $NaClO_4$ to 0.3 *M*) and was connected through 0.3 *M* $NaClO_4$ dissolved in 50% propylene glycol with the measuring cell.

The macroconstants ($\beta$ complex products) were calculated by the well known computerized evaluation. For the checking of the accuracy of the constants, the experimental mV *vs.* $cm^3$ 0.01 *M* NaOH curves were recalculated with their aid. The good fit of the experimental

points to the calculated curves was considered as proof of the reliability of the data. From the macroconstants obtained in this way the group constants and structural constants characteristic of the single functional groups have been calculated with the evaluation method described above.

## Results and discussion

Constants obtained in the protonation investigation of $ACTH_{1-4}$, a Ser-Tyr-Ser-Met tetrapeptide, are summarized in Table III. The protonation

**Table III**

*Protonation constants of the tetrapeptide $ACTH_{1-4}$ in a* **50**% *mixture of* **1,2**-*propylene glycol and water*

| Complex product | Macroconstant | Group constant |
|---|---|---|
| $\lg\beta_1 = 10.04$ | $\lg K_1 = 10.04$ | $\lg K_A = 10.04$ |
| $\lg\beta_2 = 16.91$ | $\lg K_2 = 6.87$ | $\lg K_B = 6.87$ |
| $\lg\beta_3 = 20.75$ | $\lg K_3 = 3.84$ | $\lg K_C = 3.84$ |

macroconstants ($K_1$, $K_2$, $K_3$) belonging to the tetrapeptide and the values of the group constants obtainable from them are the same. The reason for this is that between the macroconstants there is a difference of at least 3 orders of magnitude, which at an accuracy of $\pm 0.06$ lg $K$ in the alkaline range and of $\pm 0.04$ lg $K$ in the acid range does not permit a distinction between the numerical values of the macroconstants and of the group constants. Moreover, the overlap of such distant equilibria is also too small for significantly changing the constants.

When the values of the protonation constants of the functional groups (phenolic hydroxy, terminal amino, terminal carboxy) obtained in the given solvent are compared with values obtained in aqueous solution [13], conclusions can be drawn on solvent — solute interactions.

Evidently, the value of lg $K_C = 3.84$ can be assigned to the terminal carboxylate group. As compared to the value of lg $K = 3.20$ measured in water, here primarily a decrease in permittivity ($\varepsilon = 59$), favourable for the formation of neutral particles, exerts its action.

The lg $K_B = 6.87$ value, belonging to the terminal amino group, is somewhat decreased, as compared to the respective constants measured in water (lg $K = 7.17$), which can be attributed to the specific solvation of the unprotonated amino group. In the value of lg $K_A = 10.4$ to be assigned to the phenolic hydroxy group (of lower value than that found in water), most different type of forces play a role, of which the interaction between propylene glycol and phenolate oxygen, leading probably to the formation of an H-bonded chelate is dominant.

**Table IV**

*Various protonation constants obtained in* **50%** **1,2-propylene glycol**

| Macroconstant | Group constants | | | | |
|---|---|---|---|---|---|
| | $k=$ | 0 | 1 | 2 | 3 |
| $\lg K_1 = 10.27$ | $\lg K_A$ | 9.70 | 9.75 | 9.85 | 9.84 |
| $\lg K_2 = 10.13$ | $\lg K_B$ | 9.70 | 9.75 | 9.84 | 9.84 |
| $\lg K_3 = 9.89$ | $\lg K_C$ | 9.70 | 9.75 | 9.83 | 9.84 |
| $\lg K_4 = 9.50$ | $\lg K_D$ | 9.70 | 9.74 | 9.75 | 9.84 |
| $\lg K_5 = 9.28$ | $\lg K_E$ | 9.70 | 9.73 | 9.71 | 9.73 |
| $\lg K_6 = 8.58$ | $\lg K_F$ | 9.20 | 8.88 | 8.80 | 8.56 |
| $\lg K_7 = 6.90$ | $\lg K_G$ | 6.81 | 6.81 | 6.80 | 6.82 |
| $\lg K_8 = 6.11$ | $\lg K_H$ | 6.09 | 6.06 | 6.17 | 6.06 |
| $\lg K_9 = 5.41$ | $\lg K_I$ | 5.15 | 5.29 | 5.16 | 5.29 |
| $\lg K_{10} = 4.98$ | $\lg K_J$ | 5.00 | 5.00 | 5.06 | 5.02 |
| $\lg K_{11} = 4.61$ | $\lg K_K$ | 4.68 | 4.46 | 4.45 | 4.46 |
| $\lg K_{12} = 4.07$ | $\lg K_L$ | 4.07 | 4.21 | 4.40 | 4.21 |
| $\lg K_{13} = 3.95$ | $\lg K_M$ | 4.00 | 4.07 | 4.00 | 4.00 |
| | $\hbar_{OH}$ | 0.107 | 0.091 | 0.042 | 0.043 |
| | $\hbar_H$ | 0.057 | 0.045 | 0.043 | 0.044 |

where $\hbar_{OH}$ and $\hbar_H$ denote the means of the absolute error in solutions of pH $>$ 7.5 and pH $<$ 8.0, respectively.

The results of the investigation of $ACTH_{1-32}$ protonation are summarized in Table IV. The first column of Table IV contains the macroconstants of stepwise protonation, while the other columns the group and structural constants, under the assumption of $k = 0, 1, 2, 3$ H-bonds both in the acid and alkaline regions, divided by a broken line. The means of the absolute error ($\hbar$) of recalculation are given below these columns. The division of the complete protonation pH-range into two parts was made because of computational considerations. The division into constant series below $K_7$ and above $K_6$ is made possible by the fact that the difference between the logarithms of these two constants is 1.68, owing to which there is only an overlap of about 2% between the two protonation processes. Differences between the logarithms of the macroconstants vary between 0.12 and 1.68, so that their value exceeds in all the cases the limits of error. It can be seen at the same time that group and structural constants, particularly in the alkaline region, are very similar whichever model is used for their calculation. The best agreement, both in the alkaline and acid regions can be observed when calculating with two and three hydrogen bonds. The difference between these latter two cannot be considered

significant any more. This is due partly to the fact that as yet the accuracy of measurements does not make possible the determination of equilibrium constants, correct to 3—4 decimal logarithmic units. On the other hand, in the acid region there are differences even of an order of magnitude between the various constants, so that their sum reflects mainly the value of the larger constant. Nevertheless, both in the acid and alkaline regions the model with two hydrogen bonds reveals the smallest (though not significantly smallest) error, and the appropriateness of this variant is supported also by chemical evidences.

*Chemical conclusions to be drawn on the basis of the $k = 2$ model*

The method of calculation presented here does not give direct information on the assignment of the individual constants to the functional groups, and even less so on the location of the hydrogen bonds in the peptide sequence. However, the method yields the number of hydrogen bonds between the functional groups, and the constants characterizing the structural elements forming hydrogen bonds with one another. The participation in other intramolecular hydrogen bonds (or possibly in hydrophobic interaction) of those groups forming no hydrogen bonds with one another but having constants different from that expected for the functional group, is highly likely.

The constants referring to the formation and rupture of hydrogen bonds between the appropriate pairs of groups in the alkaline region for $k = 2$ are 9.85 and 9.71, and 9.84 and 9.75, respectively. In view of the fact that there is a group in the same region which does not participate in a hydrogen bond and has a constant of 9.83, it can be established that all the hydrogen bonds formed are very weak, and their formation means only a slight gain in energy for the system. Since the deprotonation of the phenolic hydroxy group and of the $\varepsilon$-ammonium group of lysine proceeds in a very close pH-range, the above constants cannot be exactly assigned to groups and pairs of groups.

The structural constant of 8.80, similarly in the alkaline region, has no hydrogen bonding pair in this region. However, its unusually low value, as compared to both the phenolic hydroxy and the $\varepsilon$-amino group, indicates that the deprotonated form of one of the groups (presumably of the $\varepsilon$-amino group of lysine) forms a strong hydrogen bond with a group (*e.g.* the alcoholic hydroxy group of serine or the guanidino group of arginine), the deprotonation of which proceeds only at very high pH-values.

The lg $K$ values in the acidic region in the absence of hydrogen bonding are : 6.80, 6.17 and 4.00. These can be safely assigned to terminal amino, histidine imidazole and terminal carboxy group, respectively. Their values reflect the same effects that have been observed (with the exception of imidazole) also in the case of the tetrapeptide.

The structural constants of formation and rupture of hydrogen bonds belonging together are: 5.16 and 4.40, and 5.06 and 4.45, respectively. Since the solvent mixture (similarly as in the case of the tetrapeptide) increases the protonation constants of the carboxylate group by about 0.7 lg units as compared to that in water [13], it can be established that each of these H-bonds has aspartic acid-glutamic acid carboxylate bridgehead groups. In 50% propylene glycol-water glutamic acid $\gamma$-carboxylate should have a protonation group constant of about $\lg K = 4.95$, while that of aspartic acid is approximately $\lg K = 4.60$. The differences of $\sim \pm 0.20$ and $\sim \pm 0.15$ lg $K$ units from these values are proportional to the gain in energy by H-bond formation and to the loss in energy by H-bond rupture, respectively.

*

The authors thank G. Richter Chemical Works (Budapest) for making available the ACTH substances and propylene glycol, and Dr. Sándor B. Nagy for the determination of the relative permittivity of the solvent.

## REFERENCES

[1] Bjerrum, N.: Z. phys. Chem., **106,** 219 (1923)
[2] Rosotti, F. J. C., Rosotti, H. S.: The Determination of Stability Constants. McGraw Hill, New York 1961
[3] Butler, J. N.: Ionic Equilibrium. Addison—Wesley, Reading 1964
[4] Beck, M. T.: Chemistry of Complex Equilibria. Van Nostrand, London 1970
[5] Sillén, L. G., Martell, A. E.: Stability Constants of Metal-Ion Complexes. Special Publication No. 17. The Chemical Society, London 1964; Supplement No. 1, Special Publication No. 25. The Chemical Society, London 1971
[6] Edsall, J. T., Wyman, J.: Biophysical Chemistry, p. 474. Academic Press, New York 1958
[7] Sarneski, J. E., Reilley, C. N.: The Determination of Proton-Binding Sites by "NMR Titrations", in Analytical Chemistry, Essays in Memoriam of Anders Ringbom. (Ed. E. Wänninen.) Pergamon Press, p. 35—49. 1977
[8] Shrager, R. J., Cohen, J. S., Heller, R. S., Sachs, D. H., Shechter, A. N.: Biochemistry, **1,** 541 (1972)
[9] Benesch, R. E., Benesch, R.: J. Am. Chem. Soc., **77,** 5877 (1955)
[10] Martin, R. B.: J. Phys. Chem., **75,** 2657 (1971)
[11] Rabenstein, D. L.: J. Am. Chem. Soc., **95,** 2797 (1973)
[12] Sudmeier, J. L., Reilley, C. N.: Anal. Chem., **36,** 1698 (1964)
[13] Burger, K., Gaizer, F., Noszál, B., Pékli, M., Takácsi-Nagy, G.: Bioinorg. Chem., **7,** 335 (1977)
[14] Jameson, R. F.: J. Chem. Soc. Dalton Trans., **1978,** 43

Béla Noszál
Kálmán Burger } H-1088 Budapest, Múzeum krt. 4/b.