ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

The Association of Divalent Cations with Acylated Histidine Derivatives[1]

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The association of the divalent cations copper, nickel, zinc, cobalt and cadmium with acetyl-L-histidine, β-alanyl-L-histidine (carnosine), glycyl-L-histidine and histidylhistidine has been studied by potentiometric methods. Copper(II) and nickel(II) ions promote the ionization of the peptide hydrogen in carnosine and histidylhistidine and both of these metals as well as zinc(II) induce this ionization in glycylhistidine. Copper(II) and nickel(II) ions also cause the ionization of the hydrogen bound at the pyrrole nitrogen of the imidazole ring in glycyl- and histidyl-histidines. In the case of nickel(II) complexes of the two latter peptides, the color of the solution changes from blue to yellow in the later stages of the titration with alkali. In the yellow products the maximum in the absorption band is found at 450 mμ, and the molar extinction coefficient is 110.

The acid dissociation of the amide hydrogen in certain glycine peptide complexes of copper(II) and nickel(II) ions has been demonstrated.[2] The binding of various divalent cations to imidazole has also received extensive study. This paper is concerned with a conjunction of these two lines of study, involving the effect on the amide linkage of the binding of divalent metal at the imidazole group in acylated histidine derivatives. The compounds investigated in this research are acetyl-L-histidine, β-alanyl-L-histidine (carnosine), glycyl-L-histidine and histidylhistidine. With regard to metal binding, the last compound is in a class with histidine and its derivatives which contain a free α-amino group but is included since the results obtained are not unrelated to the other compounds studied. Among the systems reported here, only the interaction of copper(II) ion with carnosine has been studied previously.[3]

Experimental

The techniques have been previously described.[2] The temperature was 25° and the ionic strength about 0.16 throughout the study. Acetyl-L-histidine monohydrate and carnosine were obtained from the California Corporation for Biochemical Research. Glycyl-L-histidine hydrochloride monohydrate was obtained from Mann Research Laboratories. The foregoing products were of good quality. Histidylhistidine was obtained from Mann Research Laboratories, lot No. B 1324, and Nutritional Biochemicals Corp. Titration of either of these products with one equivalent of base or two equivalents of acid indicated that approximately 80% of both preparations consisted of histidyl-histidine; they were presumably identical. Since the equivalent weight obtained by titrating with either acid or base was the same the indications were that the impurities are either water of hydration or inert salt. Drying to constant weight indicated that 20% of the original material was volatile at 110° *in vacuo*. Thus the sample contains 4.0 moles of water of hydration per mole of histidylhistidine. The optical configuration of the histidine residues in the peptide was not specified by the manufacturer and remains undetermined.

Results

The results are tabulated in Table I. The successive formation constants were determined at high ratios of amide to divalent metal ion in order to drive the equilibria toward the formation of the higher complexes and to minimize the effect of amide or other ionizations. No attempt was made to determine values of association constants for log k less than 1.5. The pK values of the additional acidic ionizations from the complex were determined in high concentrations of equimolar mixtures of amide and metal(II) ion and evaluated as previously described.[2] The method employed gives precise pK values only for the highest pK observed. The value quoted for pK_1 is a maximum value, the error being greatest for the copper complexes.

TABLE I

FORMATION AND IONIZATION CONSTANTS FOR ACYLATED HISTIDINES WITH DIVALENT METAL IONS AT 25° AND 0.16 IONIC STRENGTH

	Cu	Ni	Zn	Co	Cd
Acetyl-L-histidine pK_a = 7.08 (imidazole)					
$\log k_1$	4.35	2.85	2.50	2.35	2.70
$\log k_2$	3.40	2.20	2.30	1.80	1.95
$\log k_3$	2.55	1.7	2.15		
$\log k_4$	1.5		1.8		
L-Carnosine pK_a = 6.86, 9.40 (imidazole, α-amino)					
$\log k_1$	4.2	2.80	2.30	2.25	2.50
$\log k_2$		2.10	2.10	1.6	1.75
$\log k_3$		1.6	2.00		
$\log k_4$			1.7		
pK_1	5.00	7.35			8.50
pK_2	5.55	8.40			
Glycyl-L-histidine pK_a = 6.79, 8.20 (imidazole, α-amino)					
pK_1	4.00	6.10	6.50		7.30
pK_2	4.50	6.70	7.10		
pK_3	9.25	9.25			
Histidylhistidine pK_a = 5.40, 6.80, 7.95 (2 imidazole, 1 α-amino)					
pK_1	4.40	6.10			
pK_2	6.15	7.80			
pK_3	10.25	10.25			

For the copper complexes of acetylhistidine additional ionizations are observed, which may be, at least in part, amide hydrogen ionizations. However, since they occur at pH values greater than 8 and precipitates ultimately form, it is likely that they are due to hydroxo complex formation and

(1) This work was supported by grants from the National Science Foundation (G-3230) and the United States Public Health Service (H-3169).

(2) R. B. Martin, M. Chamberlin and J. T. Edsall, THIS JOURNAL, **81**, 495 (1960). References to earlier studies are given in this paper.

(3) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59**, 246 (1955).

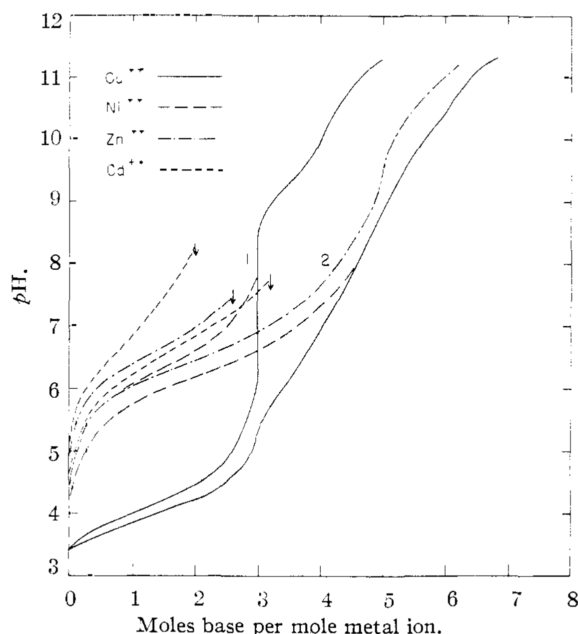


Fig. 1.—Titration curves of solutions of glycyl-L-histidine hydrochloride and 10^{-2} *M* metal ion. The numeral near the curves indicates the molar ratio of peptide to divalent metal ion. Arrows indicate onset of precipitation. Above *p*H 8 the curves for copper and nickel are similar, so that the solid lines represent both ions in this region.

olation.[4] This effect should be investigated further.

In contrast with a previous formulation,[3] the formation constants of carnosine are calculated on the assumption that the initial association of the metal ion occurs at an imidazole nitrogen rather than at the amino nitrogen. This is a good approximation in the case of carnosine because though the formation constants of imidazole and ammonia with metal ions are comparable,[5] imidazole is a much weaker base and hence will be the more likely to react in the lower *p*H range. In the *p*H region where both the imidazole and amino groups are predominantly in the basic form, the metal will be more evenly distributed between the two sites. Little chelation is likely initially at either site as it would involve the formation of seven-membered, or even larger, rings. No attempt was made to evaluate the higher formation constants for copper because of the low *p*H at which the first ionization from the complex occurs.

The pK_1 value of 8.50 for the equimolar cadmium complex of carnosine as compared with 9.40 in the free peptide is about what would be expected on electrostatic considerations for the value of the amino ionization with an additional positive charge at the imidazole ring. This supports the formulation in terms of initial binding at the imidazole ring. Precipitation of the zinc complex prevented the determination of pK_1, and oxidation of the cobalt complex occurred before this *p*H region could be attained.

(4) A. E. Martell, S. Chaberek, Jr., R. C. Courtney, S. Westerback and H. Hyytiainen, THIS JOURNAL, **79**, 3036 (1957); R. C. Courtney, R. L. Gustafson, S. Chaberek, Jr., and A. E. Martell, *ibid.*, **81**, 519 (1959); R. L. Gustafson and A. E. Martell, *ibid.*, **81**, 525 (1959).

(5) R. B. Martin and J. T. Edsall, *ibid.*, **80**, 5033 (1958).

In the presence of copper(II) or nickel(II) ions L-carnosine nitrate on titration with alkali yielded one equivalent per mole metal ion, in addition to the imidazole and amino hydrogens, for any ligand to metal ratio greater than unity. Since this additional hydrogen is titrated in the *p*H region in which peptide hydrogens of other amide complexes of each metal ion are released,[2] it is assumed that it is the peptide hydrogen which gives rise to the additional equivalent.

The titration curves for glycyl-L-histidine hydrochloride in the presence of divalent metal ions are shown in Fig. 1. No values for association constants are presented in Table I because the amino pK_a is much closer to the imidazole pK_a than in carnosine; and, moreover, a strengthening of the metal ion combination at the former group is now probable due to the possibility of chelation. Thus though greater metal association at the imidazole site is expected, it will not be nearly as predominant as in the case of carnosine. However, the lowering of the curves on the *p*H scale corresponds to what would be expected from the known combining properties of these metals.[5]

Two equivalents of acid, in addition to the imidazole and amino hydrogens, are titrated in equimolar mixtures of glycyl-L-histidine hydrochloride and either copper(II) or nickel(II) ions. The values of pK_1, pK_2 and pK_3 of Table I represent the pK values of the ionizations of the second, third and fourth equivalents, respectively, for the equimolar mixtures of Fig. 1.

In the equimolar mixture of glycyl-L-histidine hydrochloride and nickel(II) ion, the color of the solution changes from blue to yellow on addition of the fourth equivalent of base. The same color is obtained on addition of the sixth equivalent in a 2:1 mixture. The maximum molar extinction coefficient of 110 is obtained at 450 mμ.

The problem for a solution of histidylhistidine dinitrate and metal ion is more complex. The principal initial reaction involves, almost certainly, a chelation between the imidazole nitrogen and the amino nitrogen of the NH_2-terminal histidyl residue. The resulting structure is similar to the chelate complexes formed by histidine and metal ions[6]; this is confirmed by the depression of the curve on the *p*H scale at the start of the titration by about one unit more than in the case of glycylhistidine hydrochloride and the same metal ion. This is shown for copper(II) and nickel(II) in Fig. 2. However, as for the case of histidine and copper(II) ion[7] other binding sites are probably significant. For histidylhistidine the problem is compounded by the overlapping of the three ionizations from the nitrogen atoms; to interpret these adequately would necessitate a knowledge of the microconstants.[8] Thus an analysis similar to that reported for glutathione[9] is necessary.

(6) F. R. N. Gurd and P. E. Wilcox, *Advances in Protein Chem.*, **11**, 311 (1956).

(7) R. Leberman and B. R. Rabin, *Nature*, **183**, 746 (1959); *Trans. Faraday Soc.*, **55**, 1660 (1959).

(8) J. T. Edsall, R. B. Martin and B. R. Hollingworth, *Proc. Natl. Acad. Sci., U. S.*, **44**, 516 (1958).

(9) R. B. Martin and J. T. Edsall, THIS JOURNAL, **81**, 4044 (1959).

The pK_a values reported in Table I were evaluated by the technique already suggested[8] but are subject to some uncertainty, due to the fact that the histidylhistidine was not pure (see Experimental). However, the results compare favorably with those previously reported by Greenstein[10] and vary from his in the direction to be expected because of the different ionic strength.

The pK values reported in Table I for histidylhistidine represent the ionization constants of the third, fourth and fifth equivalents titrated for the equimolar mixtures of histidylhistidine dinitrate and metal ion in Fig. 2. Two equivalents in addition to the two imidazole and one amino hydrogens are titrated.

In the equimolar mixture of histidylhistidine dinitrate and nickel(II) ion the color changes from blue to yellow on the addition of the fourth equivalent of base. In a 2:1 mixture the color change does not occur until the addition of the eighth equivalent of base. The molar extinction coefficient and the maximum wave length for both of these solutions is the same as that mentioned above for glycyl-L-histidine and nickel(II) ion.

Whereas glycyl-L-histidine gave no biuret color with copper(II) ion at any point in the titrations, the formation of this color occurred during the addition of the fifth equivalent of base for an equimolar mixture of histidylhistidine dinitrate and during the eighth equivalent for the 2:1 mixture.

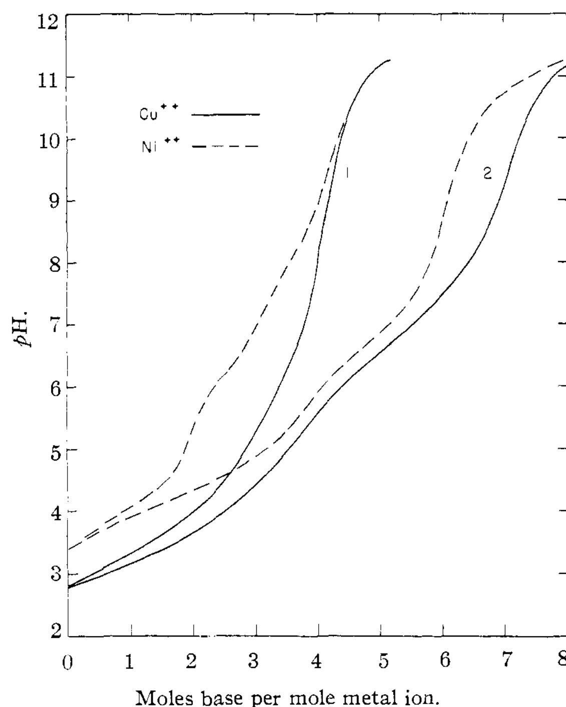


Fig. 2.—Titration curves of solutions of histidylhistidine dinitrate and 10^{-2} M metal ion. The numeral near the curves indicates the molar ratio of peptide to divalent metal ion.

Discussion

In the series carnosine, acetylhistidine and imidazole[5] the respective pK_a values of the imidazolium group, at 25° and ionic strength 0.16, are 6.86, 7.08 and 7.11. Considering zinc(II) as a representative of metal ion binding the respective values of the logarithm of the first association constants (log k_1) are 2.30, 2.50 and 2.57. Thus the values of the logarithm of the first association constants parallel the pK_a values. However, 4-methylimidazole[11] does not fit the series, since the pK_a is 7.69 and the logarithm of the first association constant for zinc binding only 2.44. A similar disparity is obtained if the comparison is made with copper(II) ion. The binding of 4-methylimidazole is thus anomalous.

An explanation for the unexpectedly weak association of 4-methylimidazole is possible if the nature of the binding is considered further. The comparable strength of the metal binding of imidazole and ammonia, in spite of the greater basicity of ammonia,[6] is due to the donation of electrons in the d orbital of the metal ion to the π orbitals of the imidazole. The hyperconjugative effect of the methyl group in 4-methylimidazole results in a less strengthening interaction between the electrons in the metal d orbital and the ligand π orbital due to greater electron repulsion.[12]

The zinc complexes of both acetylhistidine and carnosine, like other zinc complexes, are characterized by successive association constants which are greater than expected on statistical grounds. On the ionization of the peptide hydrogen in the copper(II) and nickel(II) complexes of carnosine the peptide nitrogen is available as a chelating site and two six-membered rings my be formed. For both metals only an equimolar chelated carnosine complex is present after the peptide ionization even at ratios of carnosine to metal ion greater than unity. Structure 10 B of Dobbie and Kermack[3] may be taken as the structure of the complex, except that the double bond and the hydrogen should be shifted so that the pyridine and not the pyrrole nitrogen of the imidazole ring is involved in the chelate ring.[13] Carnosine is a more effective buffer in the presence of metal ions than in their absence.

On the ionization of the peptide hydrogen in glycyl-L-histidine one six- and one five-membered ring may be formed. The greater stability of five-membered as compared to six-membered rings[14] as well as the lower value for pK_a of the ammonium group explain the lower pK values for glycylhistidine as compared with carnosine. Even the binding of zinc(II) presumably induces ionization of the peptide hydrogen in glycylhistidine; this is probably the first evidence for this type of ionization in zinc complexes. After ionization of the peptide hydrogen, only equimolar chelate complexes are formed

(10) J. P. Greenstein, *J. Biol. Chem.*, **93**, 479 (1931). The values reported by Greenstein are *titration* and not *ionization* constants. Allowance for this difference would make the agreement even closer.

(11) Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, THIS JOURNAL, **79**, 2123 (1957).

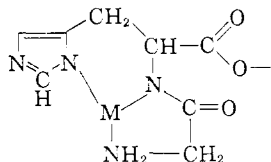
(12) J. G. Jones, J. B. Boole, J. C. Tomkinson and R. J. P. Williams, *J. Chem. Soc.*, 2001 (1958).

(13) N. C. Li, J. M. White and E. Doody, THIS JOURNAL, **76**, 6219 (1954); N. C. Li, B. E. Doody and J. M. White, *ibid.*, **79**, 5859 (1957). Following previous authors (see for instance K. Hofmann, "Imidazole and its Derivatives, Part I," Interscience Publishers, Inc., New York, N. Y., 1953, pp. 19–25) the –NH– nitrogen in imidazole is denoted as the pyrrole nitrogen, and –N= as the pyridine nitrogen. In free imidazoles the two are indistinguishable due to tautomeric exchange.

(14) H. Irving, R. J. P. Williams, D. J. Ferrett and A. E. Williams, *J. Chem. Soc.*, 3494 (1954).

with all three metals, even with ligand to metal ion ratios greater than unity.

The most unusual feature of the glycylhistidine hydrochloride titration curves is apparent in Fig. 1 where the titration of equimolar mixtures of peptide and either copper(II) or nickel(II) ion yields a fourth equivalent. The pK value of the group involved (pK_3) is 9.25 for both metal ions. The color of the nickel complex changes from blue to yellow on the addition of the fourth equivalent of base presumably indicating a structural change from octahedral to planar.[2] As the copper complexes are usually planar the identical geometry and pK_3 value for the complexes of the two metal ions indicates that the ionization is due to electrostatic effects. The pK_a for the ionization of imidazole as an acid has been estimated[15] to be about 14.2, so that assignment of the pK_3 value of 9.25 to the ionization at the pyrrole nitrogen of the imidazole ring is not unreasonable for the complex. After this ionization, the net charge on the complex is -1, the metallic ion contributing $+2$ and the peptide -3. The resonance form with the most even charge distribution, apart from the ionized carboxyl group, is that described by the formula



This form probably makes the major contribution to the actual structure of the complex. For the zinc(II) ion complex precipitation in the equimolar mixture prevents interpretation of the data, but it is evident from Fig. 1 that the ionization of the sixth equivalent in a 2:1 mixture occurs at a higher pH than for copper or nickel. This may be due to tetrahedral geometry in the case of zinc. In the imidazole complex of ferrihemoglobin an observed pK_a value of 9.5 has been ascribed to the imino nitrogen of the imidazole ring.[16]

The first two equivalents of the titration curve of the equimolar mixtures of histidylhistidine dinitrate and nickel(II) ion in Fig. 2 may be assigned mainly to the chelation of the nickel(II) ion to the amino and imidazole nitrogen atoms of the N-terminal histidine residue. The third equivalent ($pK_1 = 6.10$) is then the pK of the COOH-terminal imidazole (about 6.8) reduced by the electrostatic effect of the combined nickel ion. The fourth equivalent ($pK_2 = 7.80$) is due to peptide hydrogen ionization. On the addition of this equivalent of base the solution changes from blue to yellow. Since the position and intensity of the peak giving rise to the yellow color are the same as in glycylhistidine, yet quite different in both particulars from similar yellow colors previously reported for nickel(II) ion with triglycine, tetraglycine and glycinamide,[2] it is assumed that the structure of the yellow complexes of glycylhistidine and histidylhistidine with nickel(II) ion must be identical insofar as the atoms actually bonded to nickel are concerned. Therefore, during the addition of the fourth equivalent of base to a mixture of nickel(II) ion and histidylhistidine dinitrate the complex changes structure from that described for the initial association at the beginning of this paragraph to that described above for glycylhistidine after ionization of the peptide hydrogen. The addition of the fifth equivalent of base would then again be due to the ionization of the hydrogen on the pyrrole nitrogen and the simultaneous electronic rearrangement described for glycylhistidine. It is interesting to note, however, that for glycylhistidine the yellow color appears on ionization of the pyrrole hydrogen and for histidylhistidine on ionization of the peptide hydrogen. In other respects the corresponding ionizations take place at higher pH values in histidylhistidine as is expected.

In a 2:1 mixture of histidylhistidine dinitrate to nickel(II) ion a 2:1 complex is initially formed. The seventh equivalent of base in Fig. 2 represents ionization of a peptide hydrogen and formation of what is probably an asymmetric 2:1 complex, since the yellow color now does not appear until the ionization of the pyrrole hydrogen on the addition of the eighth equivalent of base.

Inspection of Fig. 2 indicates that the association of copper(II) ion with histidylhistidine is both similar to, and different from, that of nickel(II) ion. In the 2:1 mixture the pH value of about 8.0 at 6.5 equivalents of base indicates that one amino group is being titrated in an un-complexed ligand and therefore at this pH only an equimolar complex exists. This is in contrast to the case of nickel (II) ion as described above. However as for glycylhistidine the pK_3 values for the copper and nickel complexes are identical.

One conclusion from this work is that interaction of a metal ion with the peptide bond after association at the imidazole site in a protein is unlikely (acetylhistidine) unless histidine is the second residue from the N-terminus where the complexing tendency of the terminal amino nitrogen may help to promote peptide ionization by chelation (glycylhistidine) even at physiological pH values. This latter ionization may be prevented by lengthening the chain so that histidine is at least three residues from the N terminus. However, even in the latter case interaction is possible if a many-membered chelate ring were to form, anchored at the ends by an imidazole and an amino group. In this instance two peptide hydrogens would be expected to ionize.

Histidine appears as the second amino acid from the N terminus in the β-chain of normal adult human hemoglobin where the sequence valine-histidine-leucine- is found. The propinquity of two such bulky residues to the histidine will probably result in a slightly weaker tendency for a cation to chelate than in the case of glycylhistidine. However, this effect should not be greater than an order of magnitude and therefore coördination with a heavy metal ion may result in the ionization of the first peptide hydrogen at physiological pH values.

A recent study of the binding of copper(II) and zinc(II) ions with carbobenzoxy-L-prolyl-L-histidylglycinamide indicates that there is no interaction with the peptide bond on the C-terminal side of the

(15) H. Walba and R. W. Isensee, This Journal, **77**, 5488 (1955).

(16) C. D. Russell and L. Pauling, *Proc. Nat. Acad. Sci.*, **25**, 517 (1939); C. D. Coryell and L. Pauling, *J. Biol. Chem.*, **132**, 769 (1940).

histidyl residue.[17] This is the expected result for association of the metal ions at the imidazole group as the formation of a seven-membered ring is not favored. Therefore, exclusive of the exceptions discussed above, simple association of metal ions with the imidazole moiety of the histidyl residue of proteins is to be expected in most cases.

(17) W. L. Koltun, R. E. Clark, R. N. Dexter, P. G. Katsoyannis and F. R. N. Gurd, THIS JOURNAL, **81**, 295 (1959).

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[CONTRIBUTION FROM THE DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA AT BERKELEY]

The Free Energy Change in Hydrolytic Reactions: The Non-ionized Compound Convention[1]

BY FREDERICK H. CARPENTER

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A convention, called the "non-ionized compound convention," for use in determining the free energy change involved in hydrolytic reactions is proposed. This convention can be applied without ambiguity to the hydrolysis of all types of compounds. It serves as a reference state for the comparison of the free energy change involved in the hydrolysis of similar type bonds in related compounds and, as such, should be useful in the estimation of unknown values. A portion of the over-all free energy change associated with the hydrolysis of a number of compounds at a fixed *p*H value can be attributed to ionization–neutralization reactions. An accurate measure of the contribution of such reactions can be ascertained by reference to the "non-ionized compound convention." This in turn allows the calculation of the variation of free energy change in hydrolytic reactions as a function of *p*H. Illustrative examples are given for the free energy change involved in the hydrolysis of acyl-oxygen esters, acyl-thio esters, amides, peptides and a number of phosphate compounds.

Perhaps unique to the field of biochemical energetics is the concept of the "group transfer potential."[2] In essence, this concept, which had its origins in the papers of Kalckar[3] and Lipmann,[4] is designed to give a measure of the relative tendency or potential of a group or radical to participate in a transfer reaction. In practice this transfer potential is measured by determining the free energy change involved in the transfer of the various groups in diverse compounds to the same recipient compound, water. In other words the free energy change involved in the hydrolytic removal of the group in question is used as a measure of the transfer potential of that particular group in the compound. When the free energy change involved in the hydrolytic splitting of a bond in a compound is a relatively large negative number, the bond has been designated a "high energy bond" by Lipmann.[4] However, since the reaction involved in hydrolysis is entirely different from the conditions normally used in determining bond energies,[5] the application of the term "high transfer potential" to such a group in a compound would obviate confusion.

Despite the rather wide use of the concept of "transfer potential" in biochemistry, the full utility of this notion awaits the development of a universal and unambiguous convention for writing the hydrolysis reaction. This paper contains a proposal for a convention to describe the hydrolysis reaction, called the *non-ionized compound* convention, which has the following attributes: (1) In theory it can be applied to all types of hydrolysis reactions—not just those involving the liberation of phosphate. (2) It yields a realistic comparison of the free energy change involved in the hydrolysis of similar type bonds in various compounds. This property is of considerable use in the prediction of unknown values by the extension of known data. (3) It serves as a true basis for the calculation of the contribution of ionization–neutralization reactions to the over-all free energy change involved in the hydrolysis of compounds at a fixed and specified *p*H value.

The fundamental reasons for differences observed in the free energy of hydrolysis of various compounds has been attributed in part to changes between the original compound and the hydrolytic products in resonance and/or electrostatic effects[3,6] as well as in acidic or basic groups.[4,7] Although differences in acidic and basic properties may in part be considered as secondary effects reflecting changes in resonance or electrostatic effects, the former can be evaluated directly by experimentation, while resonance and electrostatic effects, as a rule, can only be qualitatively estimated by indirect means. Part of the over-all free energy change involved in the hydrolysis at a *fixed pH value* of compounds in which new acidic and/or basic groups are liberated may be attributed to the ionization and neutralization of these groups. In a purely formal sense, the free energy change involved in hydrolysis at a fixed *p*H value can be divided into two components: one assignable to the hydrolysis reaction and another to ionization–neutralization reactions. Obviously the division of the over-all free energy change between these two components will depend upon the convention used for writing the hydrolysis reaction. The non-ionized compound convention makes possible an accurate comparison of the relative contribu-

(1) Part of this study was performed while on leave from the Department of Biochemistry and Virus Laboratory of the University of California at Berkeley where correspondence regarding this paper should be addressed.

(2) I. M. Klotz, "Energetics in Biochemical Reactions," Academic Press, Inc., New York, N. Y., 1957, p. 27.

(3) H. M. Kalckar, *Chem. Revs.*, **28**, 71 (1941).

(4) F. Lipmann, *Adv. in Enz.*, 1, 99 (1941).

(5) L. Pauling, "Nature of the Chemical Bond," 2nd Ed., Cornell University Press, Ithaca, N. Y., 1940.

(6) (a) P. Oesper, *Arch. Biochem.*, **27**, 255 (1950); (b) T. L. Hill and M. F. Morales, THIS JOURNAL, **73**, 1656 (1951).

(7) A. B. Pardee, in Greenberg, "Chemical Pathways of Metabolism," Vol. I, Academic Press, Inc., New York, N. Y., 1954, p. 21.