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

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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.

tions of these two components to the over-all free energy change during hydrolysis at a fixed *p*H value between a wide variety of compounds.

Most reactions occurring in the cell take place at fixed *p*H values, generally near seven. In considering these reactions, it is convenient to have a secondary convention, designated here as the *pH 7 convention*, which may be used for a tabulation of the over-all free energy change during hydrolysis at a *p*H value near that found under physiological conditions. Actually, the concept of "high energy bonds" or transfer potential was derived from considering the over-all free energy change at fixed *p*H values, generally near seven.[4,8] Comparison of the two conventions shows that they are related in much the same sense as standard electrode potentials (ϵ_0) and electrode potentials at a fixed *p*H value (ϵ_0')[9] in which the non-ionized compound convention is the primary standard from which values for the free energy change at any *p*H value may be calculated.

TABLE I

NOTATION[a]

$XH_n(a = 1)$	An aqueous soln. containing XH_n at unit activity
$X^{n-}(\Sigma a_{p\mathrm{H}} = 1)$	Sum of the activities of all the ionic and non-ionic forms of X, determined by a specified *p*H value, totals to unity in water
$H^+(a = f(p\mathrm{H}))$	The hydrogen ion activity in water as determined by a specified *p*H value
ΔFh^0	Increment of free energy in the hydrolysis at a specified absolute temperature of non-ionized reactant at unit activity in water by water in the liquid state to non-ionized products, each at unit activity in water
$\Delta Fh_{p\mathrm{H}}{}^{0'}$	Increment in free energy in the hydrolysis by water in liquid state of reactant ions ($\Sigma a_{p\mathrm{H}} = 1$) to product ions ($\Sigma a_{p\mathrm{H}} = 1$) at a specified absolute temp. and *p*H value
$\Delta Fi_{p\mathrm{H}}{}^{0'}$	Increment in free energy in the conversion of non-ionized compounds ($a = 1$) to the mixture of ions ($\Sigma a_{p\mathrm{H}} = 1$) determined by a specified *p*H value and absolute temp.
$\Delta Fhi_{p\mathrm{H}}{}^{0'}$	$\Delta Fh_{p\mathrm{H}}{}^{0'} - \Delta Fh^0$
K^*	Acidic ionization constant divided by a specified hydrogen ion activity [$K_a/(H^+)$]
$K_n{}^*$	$K_1{}^*K_2{}^*K_3{}^*\ldots K_n{}^*$

[a] Where applicable the notation of Lewis and Randall[10] was used. The table contains only the new notation.

The Free Energy Change during Hydrolysis at a Specified *p*H Value[10]; $\Delta Fh_{p\mathrm{H}}{}^{0'}$.—For most of the reactions of interest to the biochemist there is generally an enzyme available which will catalyze a rapid attainment of the equilibrium at temperatures near 25°. However, use of the enzyme to establish the equilibrium introduces certain complications: the reaction must be studied at some specified *p*H value, near the optimum for the action of the enzyme, which may result in both the reactants and products being present in several ionic forms; frequently either the buffer salts, necessary for the maintenance of *p*H, or metal ions, which may be necessary for the action of the enzyme, combine with substrate or products in such a manner as to give rise to still other ionic forms.[11] As a result the actual equilibrium state established in the presence of an enzyme may be between a very complex mixture of reactant and product ions. Despite this fact, it has been customary to calculate such equilibrium constants on the basis of the *chemically determined concentrations (without reference to ionic forms)* of the reactants and products and to calculate the free energy change in such a reaction (assuming unit activity coefficients) by multiplying the natural logarithm of such an equilibrium constant by $-RT$.[4,8,12] In this paper which is concerned primarily with *p*H and not metal ion effects the free energy change calculated in this manner in a hydrolysis reaction will be designated as $\Delta Fh_{p\mathrm{H}}{}^{0'}$ where the subscript will indicate the *p*H. It should be pointed out that several authors[4,6,8,12] have used the symbol ΔF^0 to indicate the free energy change in hydrolytic reactions under conditions described above. In the present paper the symbol ΔF^0 will be reserved for the free energy change in equations which involve only one molecular species of each reactant and product. To illustrate the difference between the meanings of the two notations consider the hydrolysis of a relatively simple compound such as ethyl acetate.

$$\mathrm{EtOAc}(a = 1) + \mathrm{H_2O}(l) \longrightarrow \mathrm{EtOH}(a = 1) + \mathrm{HOAc}(a = 1) \quad (\mathrm{I})$$

$$\mathrm{HOAc}(a = 1) \longrightarrow \mathrm{H^+}(a = f(p\mathrm{H})) + \mathrm{AcO^-}(\Sigma a_{p\mathrm{H}} = 1) \quad (\mathrm{II})$$

$$\mathrm{EtOAc}(a = 1) + \mathrm{H_2O}(l) \longrightarrow \mathrm{EtOH}(a = 1) + \mathrm{H^+}(a = f(p\mathrm{H})) + \mathrm{AcO^-}(\Sigma a_{p\mathrm{H}} = 1) \quad (\mathrm{III})$$

Reaction I fulfills the requirements for a standard state reaction as defined by Lewis and Randall[10] for reactions which normally occur in dilute aqueous solutions where the standard state of water is taken as the liquid form and the standard state of all others as the "hypothetical 1 molal" solution. As such the free energy change involved in reaction I could properly be designated as the ΔF^0 of hydrolysis of ethyl acetate. In reaction II the notation H^+ ($a = f(p\mathrm{H})$) is used to indicate that the hydrogen ion activity is determined by the *p*H specified and the notation, $AcO^-(\Sigma a_{p\mathrm{H}} = 1)$ is used to indicate that the sum of activities of the undissociated acetic acid and acetate ion, of which

(8) (a) O. Meyerhoff and H. Green, *J. Biol. Chem.*, **178**, 655 (1949); (b) P. Oesper in McElroy and Glass, "Phosphorus Metabolism," Vol. I, Johns Hopkins Press, Baltimore, Md., 1951, p. 523.

(9) M. J. Johnson in Lardy, "Respiratory Enzymes," 2nd Ed., Burgess Publishing Co., Minneapolis, Minn., 1949, p. 59.

(10) The notation of Lewis and Randall ("Thermodynamics and the Free Energy of Chemical Substances," McGraw-Hill Book Co., Inc., New York, N. Y., 1923) is used wherever applicable. New notation is shown in Table I.

(11) (a) L. Noda, S. A. Kuby and H. A. Lardy, *J. Biol. Chem.*, **210**, 83 (1954); (b) R. M. Smith and R. A. Alberty, THIS JOURNAL, **78**, 2376 (1956); (c) E. A. Robbins and P. D. Boyer, *J. Biol. Chem.*, **224**, 121 (1957); (d) K. Burton, *Biochem. J.*, **71**, 388 (1959).

(12) G. E. Vladimirov, V. G. Vlassova, A. Y. Kolotilova, S. N. Lyzlova and N. S. Panteleyeva, *Nature*, **179**, 1350 (1957).

the relative proportions are determined by the specified pH, is equal to unity. The free energy change involved in reaction II is that due to the ionization and dilution of the hydrogen ion to a specified pH value. The amount of this free energy change is a function of the ionization constant of acetic acid and the specified pH. The free energy change in reaction III is the value one would obtain in the hydrolysis of ethyl acetate at some specified pH value. It is relatively easy to show[13] that

$$\Delta F_{III} = \Delta Fh_{pH}{}^{0\prime} = -RT \ln \frac{(EtOH)[(HOAc) + (OAc^-)]}{(EtOAc)} \quad (3)$$

where parentheses are used to indicate the activities of the various species at the equilibrium point of the reaction at a specific pH and temperature.

The hydrolysis of ethyl acetate serves to illustrate the differences and relationships between the free energy change in hydrolysis under standard state conditions and that measured by calculations from the chemically determined equilibrium constant at some specified pH value. However, it should be noted that in most instances several standard state reactions can be written for the hydrolysis reaction. As a consequence the numerical value of the difference between the standard state hydrolysis reaction and the reaction at any specified pH will depend upon the convention selected for writing the hydrolysis reaction. Regardless of what standard state convention is used, the over-all free energy change at pH 7 ($\Delta Fh_7{}^{0\prime}$) can be of considerable use in biochemistry in establishing a scale of transfer potentials which approximates the conditions that exist in the cell when allowance is made for concentration effects. Burton and Krebs[14] have tabulated the free energy change in a number of reactions assuming the following conditions: 0.2 atm. O_2, 0.05 atm. CO_2, pH 7, and 0.01 molal concentration of all other reactants and products.[15] The values calculated under these conditions for hydrolytic reactions differ by a constant factor (−2,740 cal./mole) from those calculated by the pH 7 convention and as a result the relative order of "transfer potentials" is not changed.

The Contributions of Ionization–Neutralization Reactions, $\Delta Fhi_{pH}{}^{0\prime}$, and the Non-ionized Compound Convention, ΔFh^0.—Depending on the nature of the reaction, there may be several choices from which to select a standard state convention to describe the hydrolysis reaction. The actual selection of any one convention will be determined by the type of information which one desires to obtain. For example Hill and Morales discussed the hydrolysis of a number of phosphate compounds[6b] with reference to the standard state reaction IV

$$XPO_3^-(a = 1) + H_2O(l) \longrightarrow HX(a = 1) + HPO_4^{=}(a = 1) \quad (IV)$$

where X refers to a radical such as acetyl, adenosyl, etc. Their study was made to compare the contribution of electrostatic effects to the free energy change on the hydrolysis of a number of phosphate compounds at pH 7. For their study the standard state reaction IV served very well. However, in the present instance the selection of a standard state convention was delimited by the fact that it (1) must be applicable to all types of hydrolytic reactions and (2) must serve as a sound basis for the calculation of the relative contribution of ionization–neutralization reactions to the over-all free energy change in the hydrolysis of a variety of compounds at fixed pH values. The standard state reaction IV which was used by Hill and Morales in their study of electrostatic effects would not meet either one of these criteria.

Of the great number of possible conventions for expressing the standard state hydrolysis reaction, only one appears to be universally applicable. This is designated the *non-ionized compound* convention and is expressed as follows: *The standard free energy change on hydrolysis at a specified temperature* (ΔFh^0) *refers to the free energy change involved in the hydrolysis of non-ionized reactant at unit activity in water by water in the liquid state to yield non-ionized products at unit activity in water.* This convention can be applied without ambiguity to all compounds except those having a positive charge but lacking a dissociable hydrogen, such as quaternary ammonium and sulfonium compounds. For these, the reference state is the onium hydroxide in water.

The portion of the over-all free energy change involved in the hydrolysis of a compound at a specified pH value which can be attributed to ionization–neutralization reactions will be designated, $\Delta Fhi_{pH}{}^{0\prime}$, and is determined by formula 4.

$$\Delta Fhi_{pH}{}^{0\prime} = \Delta Fh_{pH}{}^{0\prime} - \Delta Fh^0 \quad (4)$$

Equations

In order to apply the non-ionized compound convention and also to calculate the contribution of ionization–neutralization reactions to the over-all free energy change in hydrolysis at a fixed pH value, it is necessary to derive formulas for calculating the free energy change involved in the ionization of an uncharged compound to the ion forms determined by the specified pH ($\Delta Fi_{pH}{}^{0\prime}$) or *vice versa*. For compounds that contain only uncharged acidic groups (dissociate to give negatively charged basic groups and protons) or for compounds containing only cationic acidic groups (positively charged groups which dissociate to give neutral basic groups and protons), the free energy change on ionization to a certain pH can be calculated from the formulas[16]

$$H_nR(a = 1) \longrightarrow nH^+(a = f(pH)) + R^{n-}(\Sigma a_{pH} = 1) \quad (V)$$

(13) See R. A. Alberty, R. M. Smith and R. M. Bock [*J. Biol. Chem.*, **193**, 425 (1951)] for a similar derivation.

(14) K. Burton and H. A. Krebs, *Biochem. J.*, **54**, 94 (1953); H. A. Krebs in Greenberg, "Chemical Pathways of Metabolism," Vol. I, Academic Press, Inc., New York, N. Y., 1954, p. 109.

(15) H. G. Bray and K. White, ["Kinetics and Thermodynamics in Biochemistry," J. and A. Churchill, Ltd., London, England, 1957, p. 256] have used the same conditions with the exception of the substitution of pH 7.5 for pH 7 and have used the notation ΔG^8 to designate the free energy change.

(16) These are given without proof as they can be derived by considerations similar to those used in the derivation for the mixed function compound.

$$\Delta Fi_{p\mathrm{H}}{}^{0\prime} = -RT \ln (1 + K_1{}^* + K_{21}{}^* + K_{31}{}^* + \cdots K_{n1}{}^*) \quad (5)$$

$$R(a = 1) + n\mathrm{H}^+(a = f(p\mathrm{H})) \longrightarrow \mathrm{H}_n\mathrm{R}^{n+}(\Sigma a_{p\mathrm{H}} = 1) \quad (\mathrm{VI})$$

$$\Delta Fi_{p\mathrm{H}}{}^{0\prime} = -RT \ln (1 + K_1{}^* + K_{21}{}^* + K_{31}{}^* + \cdots K_{n1}{}^*)/K_{n1}{}^* \quad (6)$$

where

K^* = acidic dissociation constant/hydrogen ion activity (7)

$$K_{21}{}^* = K_1{}^*K_2{}^*; \; K_{n1}{}^* = K_1{}^*K_2{}^*K_3{}^* \cdots K_n{}^* \quad (8)$$

In a compound which contains both uncharged and cationic acidic groups, such as the amino acids, the situation is somewhat more complex. According to Adams[17] one can visualize the ionization of glutamic acid as shown in Fig. 1.

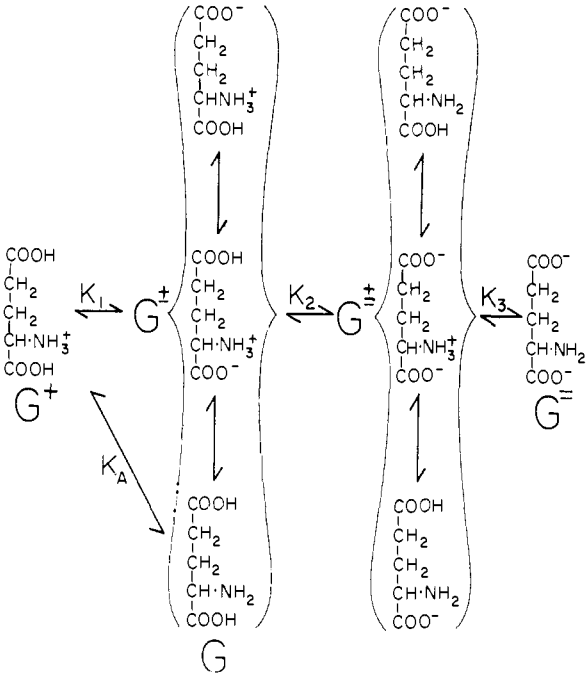


Fig. 1.—Schematic diagram of the ionization pathways of glutamic acid.

The acidic dissociation constants (K_1, K_2, K_3), as usually determined, reflect very complex equilibria between the various ionic forms enclosed in the brackets. One desires to determine the free energy change involved in converting the non-ionized form (G in Fig. 1) to a mixture of ions that is determined by the *p*H or *vice versa*. In order to make this calculation the free energy changes involved in reaction VII through X are summed.

$$\mathrm{G}(a = 1) + \mathrm{H}^+(a = 1) \longrightarrow \mathrm{G}^+(a = 1) \quad (\mathrm{VII})$$

$$\Delta F \approx RT \ln K_A \quad (9)$$

$$\mathrm{G}^+(a = 1) \longrightarrow 3\mathrm{H}^+(a = 1) + \mathrm{G}^{\equiv}(a = 1) \quad (\mathrm{VIII})$$

$$\Delta F = -RT \ln K_1K_2K_3 \quad (10)$$

$$2\mathrm{H}^+(a = 1) \longrightarrow 2\mathrm{H}^+(a = f(p\mathrm{H})) \quad (\mathrm{IX})$$

$$\Delta F = RT \ln (\mathrm{H}^+)^2 \quad (11)$$

$$\mathrm{G}^{\equiv}(a = 1) \longrightarrow \mathrm{G}^{\equiv}(\Sigma a_{p\mathrm{H}} = 1) \quad (\mathrm{X})$$

$$\Delta F = RT \ln \frac{K_1{}^*K_2{}^*K_3{}^*}{(1 + K_1{}^* + K_1{}^*K_2{}^* + K_1{}^*K_2{}^*K_3{}^*)} \quad (12)$$

$$\mathrm{G}(a = 1) \longrightarrow 2\mathrm{H}^+(a = f(p\mathrm{H})) + \mathrm{G}^{\equiv}(\Sigma a_{p\mathrm{H}} = 1) \quad (\mathrm{XI})$$

(17) E. Q. Adams, THIS JOURNAL, **38**, 1503 (1916).

$$\Delta Fi_{p\mathrm{H}}{}^{0\prime} = -RT \ln (1 + K_1{}^* + K_1{}^*K_2{}^* + K_1{}^*K_2{}^*K_3{}^*)/K_A{}^* \quad (13)$$

The free energy change involved in reaction X is calculated from the mass balance as follows where parentheses are used to indicate the activities of the various species.

$$(\mathrm{G}^+) + (\mathrm{G}^{\pm}) + (\mathrm{G}^{\pm}) + (\mathrm{G}^-) = 1 \quad (14)$$

Upon substitution of these equivalent expressions into (14)

$$(\mathrm{G}^+) = (\mathrm{G}^-)(\mathrm{H}^+)^3/K_1K_2K_3 = (\mathrm{G}^-)/K_1{}^*K_2{}^*K_3{}^* \quad (15)$$

$$(\mathrm{G}^{\pm}) = (\mathrm{G}^-)(\mathrm{H}^+)^2/K_2K_3 = (\mathrm{G}^{\equiv})/K_2{}^*K_3{}^* \quad (16)$$

$$(\mathrm{G}^{\pm}) = (\mathrm{G}^{\equiv})(\mathrm{H}^+)/K_3 = (\mathrm{G}^{\equiv})/K_3{}^* \quad (17)$$

and solving for $(\mathrm{G}^{\equiv})$ as a function of (H^+), one obtains

$$(\mathrm{G}^-) = K_1{}^*K_2{}^*K_3{}^*/(1 + K_1{}^* + K_1{}^*K_2{}^* + K_1{}^*K_2{}^*K_3{}^*) \quad (18)$$

from which the expression for the free energy change in reaction X is obtained.

Substitution of the proper dissociation constants into equation 13 should yield the free energy change involved in the conversion of a "hypothetical 1 molal" solution of non-ionized glutamic acid to the ionized forms determined by a specified *p*H value. However, with the data presently available, the equation cannot be solved without making a simplifying assumption with regard to the dissociation constant, K_A (Fig. 1). To date no direct method for the determination of K_A has been published. For compounds containing only one cationic acidic group and one or more uncharged acidic groups, K_A generally is assumed to be equal to the acidic ionization constant of the completely esterified compound.[18] If one makes this simplifying assumption, the general equation for the free energy change involved in the ionization of compounds containing one cationic acidic group and one or more neutral acidic groups becomes

$$\Delta Fi_{p\mathrm{H}}{}^{0\prime} = -RT \ln (1 + K_1{}^* + K_{21}{}^* + \cdots K_{n1}{}^*)/K_{\mathrm{ester}}{}^* \quad (19)$$

where $K_{\mathrm{ester}}{}^*$ is the acidic ionization constant of the completely esterified compound divided by the specified hydrogen ion activity.

For compounds containing several cationic and neutral acidic groups other simplifying assumptions would have to be made. Fortunately this latter situation rarely arises in the application of these formulas in the calculation of the contribution of ionization-neutralization reactions to the overall free energy change in hydrolytic reactions. The reason for this is that one can eliminate from consideration any groups whose *pK* values do not change appreciably during the hydrolytic reaction—generally those groups that are far removed from the site of the hydrolyzed bond.

Results and Discussion

Acetyl Compounds.—Unfortunately the thermodynamic data presently available do not allow one to calculate the free energy change involved in the hydrolysis in aqueous solution of some rather

(18) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 96.

simple compounds, such as acetic anhydride and acetyl chloride, which are considered to contain groups with "high transfer potential." A variety of values for ΔFh^0 can be estimated for ethyl acetate. These values range from -1.6 to -5.8 kcal./mole. If one accepts an approximate value of -4.2 kcal./mole for ΔFh^0 of ethyl acetate at 25° (*vide infra*) the calculated value of $\Delta Fh_7^{0\prime}$ amounts to -7.2 kcal./mole. According to the figures of Burton[19] the $\Delta Fh_7^{0\prime}$ for the hydrolysis of acetyl coenzyme A amounts to -8.3 kcal./mole at 25°. Assuming no change in ionization of the coenzyme A on hydrolysis, one calculates that $\Delta Fh^0 = -5.2$ kcal./mole for the hydrolysis of acetyl coenzyme A, which value is approximately 1.0 kcal./mole more negative than the approximate value for ethyl acetate. A crude idea of the $\Delta Fh_{p\mathrm{H}}^{0\prime}$ values of the oxygen and thio ester compounds can be obtained by use of the ΔFh^0 values of ethyl acetate or acetyl coenzyme A, respectively. It should be noted that in both of these cases, the value for $\Delta Fh_{p\mathrm{H}}^{0\prime}$ increases with *p*H at values above *p*H 4 (Fig. 2). The rate of increase at high *p*H values is greater for acetyl coenzyme A than for ethyl acetate because of ionization of the mercaptan.

Amide Bond.—The results obtained from available thermodynamic data on the hydrolysis of a number of different type amide bonds are shown in Table II.[20] The origin of the data, which were derived in some cases from combustion and entropy measurements and in other cases from equilibrium constant measurements, is shown in a later section. Study of Table II reveals that whereas the $\Delta Fh_7^{0\prime}$ for the hydrolysis of peptide bonds varies from -0.5 to -4.2 kcal./mole, the values for ΔFh^0 at 25° are confined to a much narrower range ($+4.7 \pm 0.7$ kcal./mole). In view of the limitations in accuracy of the combustion and entropy data, as well as some of the assumptions made in the use of activity coefficients and ionization constants, the observed spread of values for ΔFh^0 of peptide bonds is probably well within the range of experimental error. Consequently, it would appear that the free energy of hydrolysis of all typical peptide bonds has approximately the same value when compared by the non-ionized compound convention and that the differences observed in the free energy of hydrolysis at a fixed *p*H value ($\Delta Fh_{p\mathrm{H}}^{0\prime}$) are largely traceable to differences in the contribution of ionization–neutralization reactions ($\Delta Fhi_{p\mathrm{H}}^{0\prime}$).

Even the ΔFh^0 values for the hydrolysis of typical amide bonds (glutamine and asparagine) as well as for acyl-amino acids (benzoyl-glycine and benzoyl-/-glycyl-glycine) fall for the main part in the same range as that found for the peptide bond. In these cases because of the lack of substituents on the α-carbon of the acyl moiety and also on the nitrogen in the case of glutamine and asparagine, one might well expect a divergence from the ΔFh^0 values of a typical peptide bond. The close correspondence of the ΔFh^0 values of these amides with those of the peptide bonds would indicate that here also the variable contribution of the ionization–neutralization terms ($\Delta Fhi_{p\mathrm{H}}^{0\prime}$) plays the predominant role in variation in the free energy of hydrolysis at a fixed *p*H value. It is

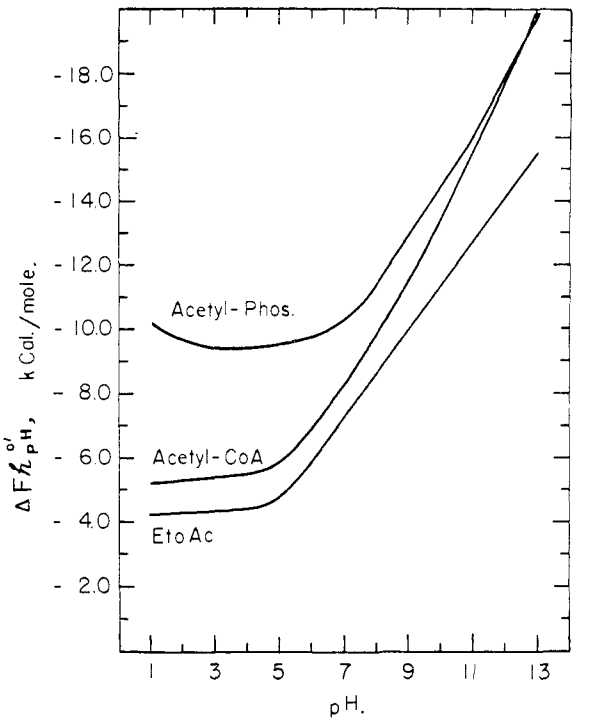


Fig. 2.—The variation of the free energy change on hydrolysis of several acetyl compounds as a function of *p*H.

TABLE II

SUMMARY OF FREE ENERGY CHANGE ON HYDROLYSIS OF VARIOUS COMPOUNDS[a]

Bond type	Compound	ΔFh^0, kcal./mole	$\Delta Fhi_7^{0\prime}$, kcal./mole	$\Delta Fh_7^{0\prime}$, kcal./mole
Ester	Ethyl acetate	−4.2	−3.0	−7.2
	Glucose 6-phosphate[b]	(−4.4)[c]	(+2.2)[c]	(−2.2)[c]
	Acetyl-coenzyme A	−5.2	−3.1	−8.3
Amide	Glycyl-glycine	+3.9	−8.2	−4.3
	DL-Alanyl-glycine	+3.9	−8.1	−4.2
	DL-Leucyl-glycine	+4.9	−8.3	−3.4
	L-Glutamine	+3.3	−6.7	−3.4
	L-Asparagine	+4.3	−7.3	−3.0
	Benzoyl-glycyl-/-glycine	+4.2	−7.3	−3.1
	Benzoyl-glycine	+4.9	−6.8	−1.9
	Benzoyl-/-glycyl-glycine	+5.2	−6.0	−0.8
	Benzoyl-L-tyrosyl-/-glycinamide	+5.4	−5.9	−0.5
	Creatine phosphate[d]	+2.8 (−13.1)[e]	−13.4 (+2.5)[e]	−10.6 (−10.6)[e]
Anhydride	Adenosine triphosphate[f]	−6.8	−0.8	−7.6
	Acetyl phosphate	−10.4	+0.2	−10.2

[a] Unless otherwise specified, values are for 25°. [b] Values at 38° from O. Meyerhof and H. Green, *J. Biol. Chem.*, **178**, 655 (1949). [c] Values recalculated to 25° from data of L. M. Ginodman, *Biokhimia*, **19**, 666 (1954). [d] Values at 30°. The assignment of ionization constants to creatine phosphate is that of Dekker and Chambers (see Table III). [e] The assignment of ionization constants is that of Kumler and Eiler (THIS JOURNAL, **65**, 2355 (1943)). [f] Values are for 30° (see text).

(19) K. Burton, *Biochem. J.*, **59**, 44 (1955).

(20) K. U. Linderström-Lang [*Stanford Univ. Pub. Med. Sci.*, **6**, 97 (1952)] has made similar calculations on some of these compounds for a different purpose. The present values differ somewhat from the values of Linderström-Lang owing in part to recalculations made by the present author of some of the older thermochemical data.

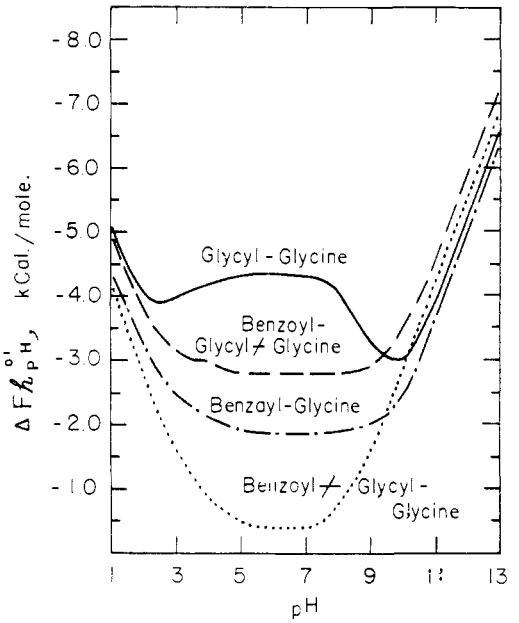


Fig. 3.—The variation of the free energy change on hydrolysis of several peptides as a function of *p*H.

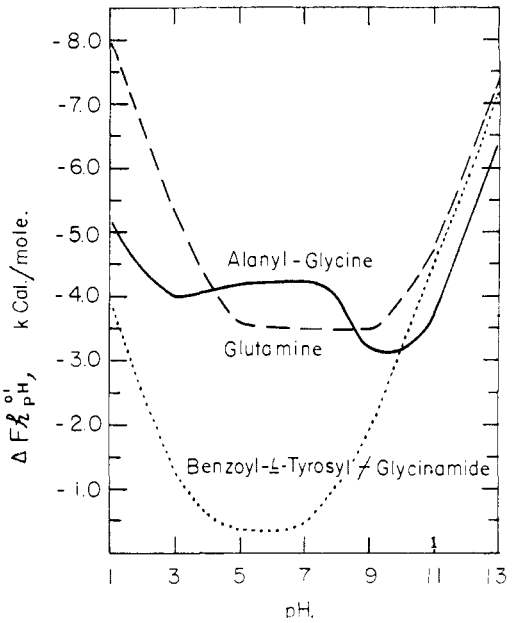


Fig. 4.—The free energy change on the hydrolysis of DL-alanyl-glycine (———), L-glutamine (·····), and benzoyl-L-tyrosyl-/-glycinamide (. . . . .) as a function of *p*H.

apparent from these results that the preferable method for extension of thermodynamic data from known compounds to unknown compounds in the amide field is on the basis of ΔFh^0 values along with measured ionization constants.

It may seem somewhat incongruous to obtain a value of about + 4.7 kcal./mole for the hydrolysis of a peptide bond by use of the non-ionized compound convention. Such a value would indicate net synthesis rather than hydrolysis of the bond under these conditions. However, it serves to point out the essential fact that the significant reason for the observed hydrolysis of peptide bonds at *p*H 7 is attributable, in a thermodynamic sense, to the release of an acidic and basic group during the hydrolysis. From the point of view of synthesis of peptide bonds at physiological *p*H values, considerable thermodynamic work is demanded for both the removal of a proton from a positively charged ammonium group and the addition of a proton to a negatively charged carboxyl group.

The effect of *p*H on the value of $\Delta Fh_{p\mathrm{H}}{}^{0'}$ for a number of amide bonds is shown in Figs. 3–4. In all cases the values are rather constant in the *p*H range 5 to 8, which range, except for the dipeptides, also yields their minimum value. The dipeptide yields two minima, a small one in the *p*H range 2-3 and a more pronounced one in the *p*H range 9–10. The $\Delta Fh_{p\mathrm{H}}{}^{0'}$ values for all amide bonds become progressively more negative at *p*H values below 2 and above 10. Figures 3–4 illustrate the marked difference that exists between $\Delta Fh_{p\mathrm{H}}{}^{0'}$ values for different peptide bonds in the physiological *p*H region. From the point of view of synthesis, the greatest amount of work is needed to bring two amino acids together to form a dipeptide (*cf.* glycyl-glycine), a somewhat smaller requirement is involved in adding an amino acid to an existing peptide chain (*cf.* benzoyl-glycyl + glycine), while the minimum need is exhibited in the addition of one peptide chain to another peptide (*cf.* benzoyl-L-tyrosine + glycinamide, Fig. 4).

Hydrolysis of Phosphate Compounds.—The calculated values of ΔFh^0, $\Delta Fhi_7{}^{0'}$ and $\Delta Fh_7{}^{0'}$ for several kinds of phosphate compounds are shown in Table II. The values derived in the table have been obtained from published equilibrium constant measurements and are subject to several uncertainties involving difficulties in the measurement of primary phosphate ionization constants, questionable assignment of *pK* values to the various ionizing groups and unknown contributions from ion complexes with metal activators or buffer salts. In view of these uncertainties only a few representative samples of phosphate compounds have been included and one can hardly attach more than qualitative significance to the values recorded in Table II.

For ordinary ester phosphates at *p*H 7, the contribution of ionization–neutralization reactions is a positive value ($\Delta Fhi_7{}^{0'} \cong +2.0$ kcal./mole) owing to the fact that both ionizations of the esterified phosphate are stronger than the first two of phosphoric acid. As a result the free energy of hydrolysis by the non-ionized compound convention is a larger negative number than that observed at *p*H 7. This is in contrast to what was noted for the acyl oxygen and thio esters. The ΔFh^0 value for the phosphate esters appears to lie in about the same range as the values for acyl oxygen. As the *p*H is decreased below 7, the value of $\Delta Fh_{p\mathrm{H}}{}^{0'}$ for phosphate esters becomes increasingly negative, approaching ΔFh^0 as a limit. As the *p*H is increased above 7, there is little change in $\Delta Fh_{p\mathrm{H}}{}^{0'}$ for phosphate esters that yield ordinary alcohols on hydrolysis until high *p*H values (>11) are attained (see Fig. 5). However, where a phenol (such as *p*-nitrophenol) is liberated on hydrolysis,

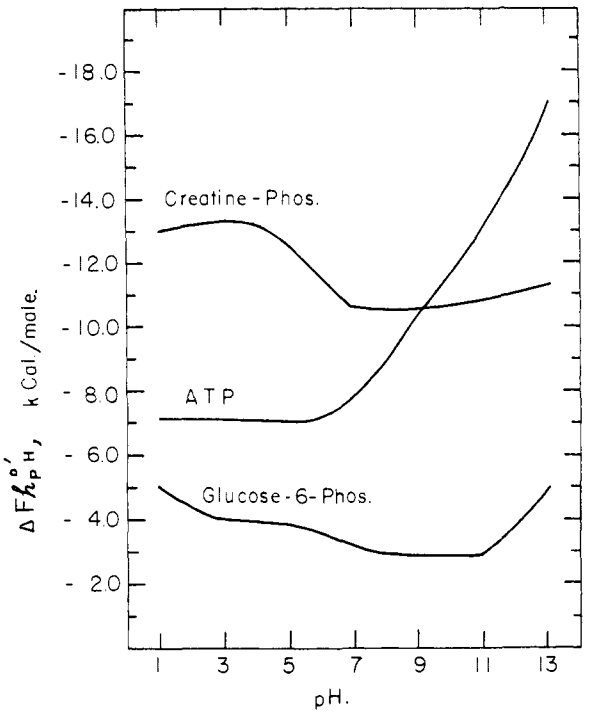


Fig. 5.—The free energy change on hydrolysis of several phosphate compounds as a function of pH.

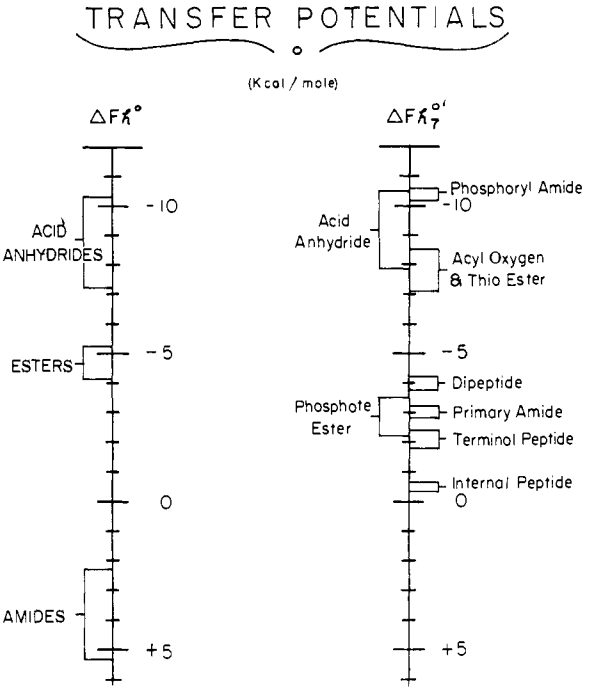


Fig. 6.—A scale of transfer potentials according to the non-ionized compound convention ($\Delta Fh°$) and the pH 7 convention ($\Delta Fh_7°'$).

increasing pH values would result in increasing negative values of $\Delta Fh_{p\mathrm{H}}{}^{0'}$ owing to the ionization of the phenol.

For the pyrophosphates and acyl phosphates, it would appear that ionization–neutralization reactions make relatively little contribution to the over-all free energy change in hydrolysis at pH 7; ΔFh^0 and $\Delta Fh_7{}^{0'}$ have about the same value. However, with increasing pH values above 7, there is an increasing contribution of the ionization–neutralization term resulting in a rapid increase in the negative value of $\Delta Fh_{p\mathrm{H}}{}^{0'}$[13,21] (Figs. 2 and 5).

The results with creatine phosphate (and also the other phosphagens) are subject to an uncertainty in the assignment of pK values to the various dissociating groups. The recent assignment of Dekker and Chambers[22] (Table III) refutes the generally accepted proposals of Kumler and Eiler[23] and is similar to the original assignments of Meyerhof and Lohmann (Table III). According to the interpretation of Dekker and Chambers the very weakly acidic guanidinium group becomes a relatively strong cationic acid upon substitution with phosphate. At the same time the relatively strong primary dissociation of phosphate becomes a somewhat weaker dissociation upon substitution with the guanidine group, as does also the secondary phosphate group. As a consequence of this interpretation the hydrolysis of the phosphoryl amides resembles the acyl amides in that hydrolysis leads to the formation of an acidic and basic group both of which undergo ionization–neutralization reactions. This results in a large contribution of the ionization–neutralization terms to the over-all free energy change at pH 7. The value of ΔFh^0 is a positive number (+ 2.8 kcal./mole) surprisingly close to the value estimated for ΔFh^0 of the carbonyl amide bond. $\Delta Fh_{p\mathrm{H}}{}^{0'}$ remains practically constant at pH values above 7 but becomes increasingly negative at values below 7, reaching a maximum at about pH 4 and then becomes somewhat more positive at values below pH 4 (Fig. 5).

Making use of the same equilibrium constant data but substituting the assignments of ionizing groups according to the interpretation of Kumler and Eiler, results in quite a different picture as shown by the values in parentheses for creatine phosphate in Table II. ΔFh^0 becomes a large negative value (−13.1 kcal./mole) while $\Delta Fhi_7{}^{0'}$ is a positive value (+ 2.5 kcal./mole). The values calculated for $\Delta Fh_7{}^{0'}$ from the two different assignments of ionizing groups are the same and do not depend on the assignment of ionizing groups.

Only a few representative samples of phosphate compounds have been included in Table II but they should be useful as a guide in considering other compounds. Thus, phosphoarginine and phosphoramidic acid may be expected to behave somewhat similarly to phosphocreatine. Mention should be made of phospho-enol-pyruvate which represents a case where undoubtedly a large contribution to the over-all free energy change during hydrolysis is traceable to the tautomeric conversion of enol–pyruvate to pyruvate. Although such tautomeric conversions are not generally considered as acid–base reactions, it is perfectly reasonable to do so, as is illustrated in the reactions. Ionization of the enol-pyruvic acid gives rise

(21) M. Dixon, "Multi-enzyme Systems," Cambridge University Press, Cambridge, Eng., 1949.
(22) C. A. Dekker and R. A. Chambers (unpublished data).
(23) W. D. Kumler and J. J. Eiler, THIS JOURNAL, **65**, 2355 (1943).

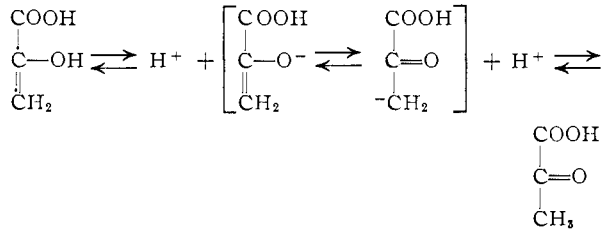
TABLE III

IONIZATION CONSTANTS (AS pK_a VALUES) USED IN CALCULATION OF $\Delta Fhi_{pH^0}'$

Compound	pK_a or pK_a' values[a] at 25° 1st	2nd	3rd	Ester[b] (pK_E')
Acetic acid[c]	4.76*			
Acetyl phosphate[d]	1.20	4.90		
Adenosine diphosphate[e]	1.00 (pK_{a4}' = 6.68)	1.00	3.95	3.63[f]
Adenosine triphosphate[e]	1.00 (pK_{a4}' = 4.00;	1.00 pK_{a5}' = 6.95)	2.00	3.63[f]
DL-Alanine[g]	2.35*	9.87*		7.80[h]
DL-Alanylglycine[i]	3.11	8.18		7.75[j]
Ammonia	9.26			
L-Asparagine[k]	2.02	8.80		6.50[l]
L-Aspartic acid[m]	1.88	3.65	9.60	6.50[h]
Benzoic acid[n]	4.20*			
N-Benzoylglycine[o]	3.81*			
N-Benzoylglycyl-glycine[p]	3.81			
N-Benzoyl-L-tyrosine[p]	3.81			
Creatine[q]	2.66	14.28		13.40[r]
Creatine phosphate[s]	2.00 (pK_{a4}' = 12.00)[u]	2.70	4.50	2.00[t] (13.40)[v]
Glucose-6-phosphate[w]	0.94	6.11		
L-Glutamine[x]	2.17	9.13		7.04[y]
L-Glutamic acid[z]	2.16*	4.32*	9.96*	7.04[z]
Glycinamide[aa]	7.93			
Glycine[ab]	2.35*	9.78*		7.78[ac]
Glycyl-glycine[ad]	3.15*	8.25*		7.75[h]
DL-Leucine[ae]	2.33	9.75		7.63[h]
DL-Leucylglycine[af]	3.18	8.29		7.75[j]
Phosphoric acid[ag]	2.00	6.70	12.0	

[a] An asterisk is used to indicate thermodynamic pK_a values. [b] The values of K_E*, used in equation 19, were calculated from these pK_a values. [c] H. S. Harned and R. W. Ehlers, THIS JOURNAL, **54**, 1350 (1932). [d] F. Lipmann and L. C. Tuttle, *Arch. Biochem.*, **13**, 373 (1947). [e] R. A. Alberty, R. M. Smith and R. M. Bock, *J. Biol. Chem.*, **193**, 425 (1951); R. M. Smith and R. A. Alberty, THIS JOURNAL, **78**, 2376 (1956). Owing to lack of data on the primary phosphate ionizations in ADP and ATP, it has been assumed that $K_{a1}' = K_{a2}' = 10^{-1}$ in ADP and ATP and that $K_{a3}' = 10^{-2}$ in ATP. The use of these values along with those noted for phosphoric acid results in virtual elimination from consideration any changes that may occur in primary phosphate ionizations during the hydrolysis of ATP. [f] Assumed to be the same as the value for adenosine (see [e]). [g] P. K. Smith, A. C. Taylor and E. R. B. Smith, *J. Biol. Chem.*, **122**, 109 (1937). [h] J. T. Edsall and M. H. Blanchard, THIS JOURNAL, **55**, 2337 (1933). [i] L. J. Harris, *Proc. Roy. Soc.* (London) B**95**, 440 (1923–1924). [j] Assumed to be identical with the value for glycyl-glycine ethyl ester (see [h]). [k] A. C. Chibnall and R. K. Cannan, *Biochem. J.*, **24**, 945 (1930). [l] Assumed to be identical with the value for aspartic acid diethyl ester. [m] S. Miyamoto and C. L. A. Schmidt, *J. Biol. Chem.*, **90**, 165 (1931). [n] A. V. Jones and H. N. Parton, *Trans. Faraday Soc.*, **48**, 8 (1952). [o] B. A. Josephson, *Biochem. Z.*, **267**, 74 (1933). [p] Assumed to have the same value as N-benzoyl-glycine. [q] R. K. Cannan and A. Shore, *Biochem. J.*, **22**, 920 (1928); A. Hahn and H. Fasold, *Z. Biol.*, **82**, 473 (1925). [r] Assumed to have the same value as N,N-dimethylguanidine (S. J. Angyal and W. K. Warburton, *J. Chem. Soc.*, 2492 (1951)). [s] O. Meyerhof and K. Lohmann, *Biochem. Z.*, **196**, 49 (1928). [t] Based on the interpretation of Dekker and Chambers in which the first dissociation of creatine phosphate is assigned to the cationic acidic group, and the assumption that the dissociation of this group will change very slightly upon esterification of the neutral acidic groups. [u] Assumed. [v] Based on the interpretation of Kumler and Eiler[23] with the assumption that the ester would have the same value as N,N-dimethylguanidine (see [r]). [w] O. Meyerhof and K. Lohmann, *Biochem. Z.*, **185**, 113 (1927). [x] J. Melville and G. M. Richardson, *Biochem. J.*, **29**, 187 (1935). [y] Assumed to have the same value as L-glutamic acid diethyl ester. [z] A. Neuberger, *Biochem. J.*, **30**, 2085 (1936). [aa] M. Zief and J. T. Edsall, THIS JOURNAL, **59**, 2245 (1937). [ab] B. B. Owen, *ibid.*, **56**, 24 (1934). [ac] O. H. Emerson and P. L. Kirk, *J. Biol. Chem.*, **87**, 597 (1930). [ad] E. R. B. Smith and P. K. Smith, *ibid.*, **146**, 187 (1942). [ae] P. K. Smith, A. C. Taylor and E. R. B. Smith, *ibid.*, **122**, 109 (1937). [af] Assumed to have the same value as glycyl-leucine (H. S. Simms, *J. Gen. Physiol.*, **11**, 629 (1928)). [ag] Approximate values for solutions with ionic strengths of 0.2: J. Sendroy, Jr., and A. B. Hastings, *J. Biol. Chem.*, **71**, 785 (1927); L. F. Nims, THIS JOURNAL, **55**, 1946 (1933); **56**, 1110 (1934); R. G. Bates and S. F. Acree, *J. Research Natl. Bur. Standards*, **34**, 373 (1945); R. G. Bates, *ibid.*, **39**, 411 (1947); also see [e].

through its mesomeric forms to a strong base which accepts the proton. Lacking knowledge of the equilibrium constant for the over-all conversion of enol-pyruvic acid to pyruvic acid, it is not possible at this time to calculate the effect of this contribution to the over-all free energy change, but it does appear reasonable to assign such effects to the ionization–neutralization terms of the reaction



Scale of Transfer Potentials.—Figure 6 summarizes the present information on transfer potentials in a form of a scale. At the left are shown the values for ΔFh^0 of several classes of hydrolytic reactions while the scale on the right gives the relative values for $\Delta Fh_7^{0'}$ of a number of compounds at *p*H 7. It will be noted that on the scale at the left, the amides fall into one rather narrow range, esters in another range and anhydrides in a somewhat broader range. In the scale on the right, these compounds are relatively widely distributed and intermixed without any general correlation between their structure and the value of the free energy change on hydrolysis.

Use of the non-ionized compound convention as a primary standard in writing hydrolysis reactions removes a number of ambiguities from the concept of the "transfer potential" and makes possible the extension of this concept to a wide variety of compounds. Although any number of secondary conventions could be defined, the *pH 7 convention* has the advantage of giving a reasonable picture of the relative transfer potentials of various compounds at *p*H values approaching those in the cell. When used in connection with the non-ionized compound convention, it sheds light on the contribution of ionization–neutralization reactions to the over-all free energy change in hydrolytic reactions at *p*H 7. Neither the non-ionized compound convention nor the *p*H 7 convention takes into account two factors that actually play a role in the over-all free energy change during hydrolysis in the cell. One of these, the contribution attrib-

utable to the formation of metal or other ion complexes within the cell, is difficult to assess from the present data. For example the report of Smith and Alberty[11b] indicated that the differential complexing of magnesium ions between adenosine triphosphate and adenosine diphosphate made a relatively small contribution (*ca.* 0.7 kcal./mole at 25°) to the over-all free energy change in the hydrolysis of adenosine triphosphate in the presence of magnesium ions. However, the more recent work of Burton[11d] indicates that the differential binding of metal ions may be of considerable importance in determining the magnitude of the free energy change in the hydrolysis of adenosine triphosphate in the presence of magnesium ions. Another factor that must be considered in regard to the free energy changes during reaction within the cell is the steady-state concentration of the various compounds in the cell. The contribution of concentration effects can be calculated from ΔFh^0 or $\Delta Fh_{p\mathrm{H}}{}^{0\prime}$ by substitution of the concentration terms into the classical formulas.[7]

Thermochemical Data and Calculations

Thermochemical Constants.—The following values for the defined and derived constants were used[24]: one calorie = 4.1840 absolute joules = 4.1833 international joules; 25°C. = 298.16°K.; R = 1.9872 cal. deg.$^{-1}$ mole^{-1}; ΔHf^0 of CO_2(g) from carbon (graph) and O_2(g) = −94,052 cal. mole^{-1}, of H_2O (l) from H_2(g) and O_2(g) = −68,317 cal. mole^{-1} at 25°; entropy of the elements at 25°, O_2(g) = 49.003, carbon (graph) = 1.3609, H_2(g) = 31.211, N_2(g) = 45.767 cal. deg.$^{-1}$ mole^{-1}.

Ionization Constants.—In calculating the contribution of ionization–neutralization reactions to the over-all free energy change by use of the proper combination of equations 5, 6 and 19, Brönsted (acidic) ionization constants, determined for the main part at 25°, were used. Except in the case of phosphate compounds, the thermodynamic ionization constants (denoted by an asterisk in Table III) were used when available, otherwise the apparent ionization constants (based on concentrations, K'_a) were used. Although the thermodynamic constant for phosphoric acid is known, this is not the case for most of the organic phosphates where the apparent constants have been measured in media of various ionic strengths. Acting on the assumption that use of apparent ionization constants would lead to a partial cancellation of concentration effects in the phosphate compounds because both reactants and products are involved in the ionization terms, the apparent ionization constants of phosphoric acid measured in approximately 0.2 ionic strength were used. The actual values introduced into the calculations are shown in Table III in the form of pK_a or pK'_a values. The footnotes to Table III refer to the source and/or assumptions made in arriving at the values.

ΔFh^0 of Ethyl Acetate.—Various values for the free energy change in the hydrolysis of ethyl acetate to give non-ionized products were obtained in the following manner.

A.—By making the questionable assumptions of a perfect solution and a temperature independent equilibrium constant, one obtains a value of −1.6 kcal./mole from the liquid phase equilibrium constant of Berthelot and Saint-Gilles.[25]

B.—By summation of the following reactions a value of −5.8 kcal./mole at 25° is obtained.

	$\Delta F_{298.16}$, kcal./mole	
$2C(graph) + 3H_2(g) + {}^1/_2O_2(g) \longrightarrow C_2H_5OH(g)$	−40.40[24]	(XII)
$C_2H_5OH(g) \longrightarrow C_2H_5OH(a = 1)$	− 3.04[26]	(XIII)
$2C(graph) + 2H_2(g) + O_2(g) \longrightarrow CH_3COOH(l)$	−93.80[25]	(XIV)
$CH_3COOH(l) \longrightarrow CH_3COOH(a = 1)$	− 1.79[27]	(XV)
$H_2O(l) \longrightarrow H_2(g) + {}^1/_2O_2(g)$	+56.69[24]	(XVI)
$CH_3COOC_2H_5(g) \longrightarrow 4C(graph) + 4H_2(g) + O_2(g)$	+75.27[28]	(XVII)
$CH_3COOC_2H_5(a = 1) \longrightarrow CH_3COOC_2H_5(g)$	+ 1.20[29]	(XVIII)
$CH_3COOC_2H_5(a = 1) + H_2O(l) \longrightarrow C_2H_5OH(a = 1) + CH_3COOH(a = 1)$	− 5.78	(XIX)

In this tabulation, the values taken from Rossini, *et al.*,[24] should be given considerable weight, the values for gas to aqueous transformations, because of their small magnitudes, can hardly be in error by more than a few hundred calories, which leaves the value used for the free energy of formation of ethyl acetate(g) from the elements as the major suspect in contributing to the value for the ΔFh^0 of ethyl acetate. This unexpected result indicates the sore need for a new determination of the free energy of formation of ethyl acetate from combustion and entropy measurements.

C.—What appears to be the best value at present is obtained by substituting into the above series of equations the recent value of −76.86 kcal./mole for ΔFf^0 of ethyl acetate(g) obtained from equilibrium measurements[30a] on the system, 2 ethanol $\rightleftarrows$ ethyl acetate + $2H_2$. The result gives a value of −4.17 kcal./mole for ΔFh^0 of ethyl acetate at 25°. This value for the ΔFh^0 of ethyl acetate is close to that proposed for the hydrolysis of a thio ester (−5.2 kcal./mole for ΔFh^0 of acetyl-Co A). In this connection it is interesting to note that the measurements of Wadsö[30b] have indicated that there is no large difference between the enthalpies of

(24) F. D. Rossini, *et al.*, "Selected Values of Chemical Thermodynamic Properties," Circular of the National Bureau of Standards, 500, U. S. Govt. Printing Office, Wash., D. C., 1952.

(25) See G. S. Parks and H. M. Huffman, "The Free Energies of Some Organic Compounds," The Chemical Catalog Company, Inc., New York, N. Y., 1932, p. 173.

(26) J. A. V. Butler, C. N. Ramchandani and D. W. Thomson, *J. Chem. Soc.*, 280 (1935).

(27) W. A. Kaye and G. S. Parks, *J. Chem. Phys.*, **2**, 141 (1934). A correction was made for ionization (see ref. 25, p. 146).

(28) Calculated from the heat of combustion at constant pressure and 25° [W. A. Roth and F. Müller in Landolt-Börnstein-Roth Scheel, "Tabellen," Erster Ergänzungsband, 1927, p. 876] along with the more recent data on heats of combustion of carbon and hydrogen and the entropy of the elements (see ref. 24) and the values of Parks and Huffman (ref. 25) for the entropy of ethyl acetate (l) and the vapor pressure data of S. Young and G. L. Thomas [*J. Chem. Soc.*, **63**, 1191 (1893)] for ethyl acetate.

(29) J. A. V. Butler and C. N. Ramchandani, *ibid.*, 952 (1935).

(30) (a) A. A. Vvedenskii, P. Ya. Ivannikov and V. A. Nekrasova, *Zhur. obshchei Khim.*, **19**, 1094 (1949); *J. Gen. Chem., USSR.*, **19**, 1087 (1949); (b) I. Wadsö, *Acta Chem. Scand.*, **11**, 1745 (1957).

TABLE IV

THERMOCHEMICAL DATA ON AMINO ACIDS AND PEPTIDES AT 25°[aa]

(1) Compound	(2) ΔH_R (solid),[b] kcal. mole^{-1}	(3) ΔHf^0 (solid), kcal. mole^{-1}	(4) S (solid), cal. mole^{-1} deg.$^{-1}$	(5) ΔSf^0 (solid), cal. mole^{-1} deg.$^{-1}$	(6) ΔFf^0 (solid), kcal. mole^{-1}	(7) Solubility, m.	(8) γ (satd. soln.)	(9) ΔF of soln.,[c] kcal. mole^{-1}	(10) ΔFf_{pI}^0 (aq.)[d] kcal. mole^{-1}	(11) ΔFf^0 (aq., non-ionized),[e] kcal. mole^{-1}
DL-Alanine	− 386.6[f]	−134.7	31.6[g]	−154	− 88.9	1.9[h]	1.046[h]	−0.4	− 89.3	− 81.9
DL-Alanyl-glycine	− 625.9[i]	−185.9	51.0[j]	−251	−117.0	3.161[k]	0.73[l]	−0.5	−117.5	−110.9
L-Asparagine	− 460.6[m]	−188.8	41.7[n]	−208	−126.9			+0.5[o]	−126.4	−120.2
L-Aspartic acid	− 382.6[m]	−232.7	41.5[n]	−194	−174.9	0.0377[p]	.78[p]	+2.1	−172.8	−166.5
Benzoic acid	− 771.6[q]	− 92.06	40.0[r]	−112	− 58.6	0.028[s]	.95[t]	+2.2	.....	− 56.4
Glycine	− 232.6[f]	−126.3	26.1[u]	−127	− 88.6	3.33[v]	.729[w]	−0.5	− 89.1	− 81.8
Glycyl-glycine	− 471.4[i]	−178.1	45.4[j]	−204	−117.2	1.7[x]	.685[x]	−0.1	−117.3	−110.8
Hippuric acid	−1007.8[y]	−146.1	57.2[j]	−192	− 88.8	0.0205[z]	.91[t]	+2.4	.....	− 86.4
Hippuryl-glycine	−1245.4[i]	−199.1	75.2[j]	−271	−118.4	.0142[k]	.89[t]	+2.6	.....	−115.8
DL-Leucine	− 855.2[f]	−153.1	49.5[g]	−233	− 83.5	.0756[z]	1.0[aa]	+1.5	− 82.0	− 74.8
DL-Leucyl-glycine	−1093.4[i]	−205.6	67.2[j]	−313	−112.3	.126[k]	1.0[aa]	+1.2	−111.1	−104.9

[a] Except for the entropy data, the values in the table have been rounded off to the nearest 0.1 kcal./mole. Although no rigorous analysis was made, it was concluded from a consideration of the errors involved in the various measurements (combustion, entropy, solubility, activity coefficients and dissociation constants) that the values quoted in the table for the free energy of formation of the non-ionized compounds in aqueous solution have an uncertainty of about ±0.5 kcal./mole. [b] The heat of combustion at constant pressure (1 atm.) and 25°. [c] Free energy change in going from the saturated solution to the "hypothetical 1 molal solution." [d] Free energy change in the formation from the elements of the zwitter ion form of the compound at unit activity in aqueous solution at the *p*H of the iso-electric point. [e] Free energy of formation from the elements of the non-ionized compound at unit activity in aqueous solution. See Table III for values of dissociation constants used in these calculations. [f] H. M. Huffman, S. W. Fox and E. L. Ellis, THIS JOURNAL, **59**, 2144 (1937). [g] H. M. Huffman and E. L. Ellis, *ibid.*, **59**, 2150 (1937). [h] P. K. Smith and E. R. B. Smith, *J. Biol. Chem.*, 121, 607 (1937). [i] H. M. Huffman, *J. Phys. Chem.*, **46**, 885 (1942). [j] H. M. Huffman, THIS JOURNAL, **63**, 688 (1941). [k] Unpublished data of H. Borsook. [l] Assumed to be the same as that of glycine in the saturated solution (see *w*). [m] H. M. Huffman, E. L. Ellis and S. W. Fox, THIS JOURNAL, **58**, 1728 (1936). The value is for anhydrous asparagine. [n] H. M. Huffman and H. Borsook, *ibid.*, **54**, 4297 (1932). [o] Calculated from the solubility of asparagine hydrate (0.189 molal) (T. L. McMeekin, E. J. Cohn and J. H. Weare, *ibid.*, **57**, 626 (1935)) and from the free energy of formation of asparagine hydrate (solid) (−184.0 kcal./mole; recalculated from data of Huffman, Ellis and Fox[m]) and water(*l*)(−56.7 kcal./mole). [p] Represents the proportion of the total solute in solution that is present in the iso-electric form (as calculated from the *pK* values in Table III) which is assumed to have unit activity coefficient. [q] R. S. Jessup, *J. Research Natl. Bur. Standards*, **36**, 421 (1946). [r] G. T. Furukawa, R. E. McCoskey and G. J. King, *ibid.*, **47**, 256 (1951). [s] H. Borsook and J. W. Dubnoff, *J. Biol. Chem.*, **132**, 307 (1940). [t] Represents that proportion of the total solute which is present in the non-ionized form in the saturated solution (as calculated from the ionization constant in Table III) which is assumed to have a unit activity coefficient. [u] G. S. Parks, H. M. Huffman and M. Barmore, THIS JOURNAL, **55**, 2733 (1933). [v] L. S. Mason, *ibid.*, **69**, 3000 (1947). [w] E. R. B. Smith and P. K. Smith, *J. Biol. Chem.*, **117**, 209 (1937). [x] E. R. B. Smith and P. K. Smith, *ibid.*, **135**, 273 (1940). [y] H. M. Huffman, THIS JOURNAL, **60**, 1171 (1938). [z] J. B. Dalton and C. L. A. Schmidt, *J. Biol. Chem.*, **103**, 549 (1933). [aa] Assumed.

hydrolysis (in the liquid state) of oxygen and thio esters. Also, as noted by Wadsö, it is unlikely that there are large differences in the entropy terms during the hydrolysis of the two types of esters. Consequently one would expect to find a rather close agreement between the ΔFh^0 values for hydrolysis of the two types of esters.

Acetyl Coenzyme A.—In the calculation of ΔFh^0 from the recent value of −8.3 kcal./mole[19] for $\Delta Fh_7^{0\prime}$ of acetyl coenzyme A, it was assumed that the thiol groups of the liberated coenzyme A did not ionize at *p*H 7 and that none of the other ionizable groups of the coenzyme A moiety changed their values during hydrolysis.

Peptide and Amide Bonds.—Except for L-glutamine and N-benzoyl-L-tyrosylglycinamide, the free energy change involved in the hydrolysis of the various peptide and amide bonds was derived from thermochemical data (Table IV) and the following values for the free energy change in the formation from the elements of water(*l*), −56.69 kcal./mole and ammonia(a = 1), −6.37 kcal./mole.[24] Most of the experimental values for the heats of combustion and entropy of the compounds were obtained by Huffman and co-workers during the 1930's. During and after this period, the accepted values for the heats of combustion of carbon and hydrogen changed significantly as well as minor changes in the entropy of the elements. In order to obtain the best internal consistency of the values, as well as the best comparison with data derived from equilibrium constant measurements, the values of ΔHf^0, ΔSf^0 and ΔFf^0 (columns 3, 5 and 6 of Table IV) of the various compounds (solid state) were recalculated from Huffman's combustion and entropy data making use of the new values for the heats of combustion of carbon and hydrogen and for the entropy of the elements.[24]

The free energy change in going from the solid state to the "hypothetical 1 molal" solution (column 9, Table IV) was calculated from solubility data (column 7, Table IV) and molal activity coefficients in the saturated solution (column 8, Table IV). Owing to lack of information it was sometimes necessary to assume values for activity coefficients (see footnotes to Table IV) leading to an uncertainty of several hundred calories in these and subsequent calculations. The values in column 10, Table IV, refer to the free energy of formation from the elements of the compounds in the zwitterion form at unit activity in aqueous solution at the *p*H *of the isoelectric point.* The values in column 11, Table IV, were derived from those in column 10 by subtraction of the free energy change involved in the ionization of the compounds from the non-ionized form to a *p*H determined by the isoelectric point ($\Delta Fi_{pI}^{0\prime}$). This latter value was obtained by substitution of

the proper ionization constants from Table III and hydrogen ion activity into equation 19.

The values for ΔFh^0 of the various compounds shown in Table II were calculated by the proper combination of the values in column 11, Table IV. In order to determine the values for $\Delta Fh_7^{0\prime}$ shown in Table II, the proper combination of reactions involving the ionization of the reactants and products (calculated by use of equation 19) were added to the ΔFh^0 reaction.

	ΔF, kcal./mole	
$H_2N—CH(CH_3)CO—NHCH_2COOH$ $(a = 1) + H_2O(l) \longrightarrow$ $H_2N—CH(CH_3)COOH(a = 1) +$ $H_2N—CH_2COOH(a = 1)$	+3.9	(XX)
$H_2N—CH(CH_3)CO—NHCH_2COO^-$ $(\Sigma a_7 = 1) + H^+(a = f(p\text{H } 7) \longrightarrow$ $H_2N—CH(CH_3)CO—NHCH_2-$ $COOH(a = 1)$	+6.6	(XXI)
$H_2N—CH(CH_3)COOH(a = 1) \longrightarrow$ $H_2NCH(CH_3)COO^-(\Sigma a_7 = 1) +$ $H^+(a = f(p\text{H } 7))$	−7.4	(XXII)
$H_2N—CH_2COOH(a = 1) \longrightarrow$ $H_2N—CH_2COO^-(\Sigma a_7 = 1) +$ $H^+(a = f(p\text{H } 7))$	−7.3	(XXIII)
$H_2N—CH(CH_3)CO—NHCH_2COO^-$ $(\Sigma a_7 = 1) + H_2O(l) \longrightarrow$ $H^+(a = f(p\text{H } 7)) +$ $H_2N—CH(CH_3)COO^-(\Sigma a_7 = 1) +$ $H_2N—CH_2COO^-(\Sigma a_7 = 1)$ $\Delta Fh_7^{0\prime} = -4.2$		(XXIV)[31]

The contribution of ionization–neutralization reactions to the over-all free energy change during hydrolysis at a fixed pH ($\Delta Fhi_{p\text{H}}^{0\prime}$) is evaluated from equation 4 or by simple addition of the ionization reactions (reactions XXI to XXIII in the case of DL-alanyl-glycine, $\Delta Fhi_7^{0\prime} = -8.1$ kcal./mole).

Dobry, Fruton and Sturtevant[32] obtained the equilibrium constant value of 3.91 at pH 7.9 and 25° for the hydrolysis of N-benzoyl-L-tyrosyl-glycinamide to give N-benzoyl-L-tyrosine and glycinamide. Assuming unit activity coefficients, this gives a value of $\Delta Fh_{7.9}^{0\prime} = -0.81$ kcal./mole. By making use of this value, the ionization constants given in Table III and the assumption that the ionization of the phenolic hydroxyl group of the tyrosine does not change during the hydrolysis at this pH, the values shown in Table II were calculated by procedures similar to those shown above.

Benzinger and Hems[33] reported a value of −3.5 kcal./mole for the free energy change on the hydrolysis of glutamine at 25°. Recently,[34] this value was revised slightly to give $\Delta Fh_{5.5}^{0\prime}$ of − 3.42 kcal./mole at pH 5.5 and 25° for the free energy change in the hydrolysis of glutamine. Calculations based on this value and the ionization constants of Table III yield the values reported in Table II for ΔFh^0 and $\Delta Fhi_{p\text{H}}^{0\prime}$.

It should be noted that the two values for ΔFh^0, obtained from equilibrium constant measurements, fall at the upper and lower extremes for the ΔFh^0 values of peptide and amide bond hydrolysis shown in Table II. Although it is possible that the differences noted here reflect real differences in the free energy of hydrolysis of the non-ionized compounds, elimination of some of the uncertainties arising from assumptions made with regard to ionization constants and activity coefficients might well result in a considerable decrease in the numerical value of these differences.

Phosphate Compounds.—The values recorded in Table II for the hydrolysis of various phosphate compounds were all obtained from equilibrium constant measurements of various hydrolysis or transfer reactions with the assumption of unit activity coefficients for the compounds under the conditions of the equilibrium measurements. Although some of the equilibrium constants were measured at temperatures of 30 or 38°, it was assumed in calculating the ionization terms ($\Delta Fhi_{p\text{H}}^{0\prime}$) that the ionization constants of the various groups did not differ appreciably at these higher temperatures from the values measured at 25° (Table III).

Glucose 6-Phosphate.—The first values quoted in Table II were calculated from the equilibrium constant measurements of Meyerhof and Green[8a] on the hydrolysis of glucose 6-phosphate at pH 8.5 and 38° by making the, in this case, questionable assumption of unit activity coefficients. The values so obtained are 0.5 kcal./mole more negative than those reported in the more recent work of Ginodman[35] at 25° (shown in parentheses in Table II). Both determinations contain an unknown, but probably minor, contribution due to the presence of Mg^{++} ion complexes.

Adenosine Triphosphate.—The values recorded in Table II were calculated from the work of Robbins and Boyer[11c] who combined their measurements of the gluco-kinase catalyzed equilibrium between glucose 6-phosphate, glucose, ATP and ADP with the values of Meyerhof and Green[8a] for glucose 6-phosphate hydrolysis to give $\Delta Fh_7^{0\prime} = -7.6$ and -7.8 kcal./mole for the hydrolysis of ATP at zero and excess Mg^{++} ion concentration.

Benziger and Hems[33] have derived almost the same value ($\Delta Fh_7^{0\prime} = -7.73$ kcal./mole) for ATP hydrolysis from their measurements of the glutamine hydrolysis equilibrium combined with the measurements of Levintow and Meister[36] on the glutamine synthetase reaction. However, both of these values are probably subject to some revision in view of the recent report of Burton[11d,34] on the

(31) In this reaction taking place at pH 7, the peptides and amino acids would be present for the main part in the zwitterion forms. The fact that the reaction is written as taking place between the anions is a result of the use of the generalized equation for ionization reactions which is valid at any pH value and the significance of the notation, ($\Sigma a_{p\text{H}} = 1$). The latter means that the activities of all possible ionized and non-ionized forms, determined by the pH, totals to unity. In order to make this fit at any pH the most anionic form is used in writing the reaction, even though at pH 7 only the zwitterion forms would be present to any large extent.

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magnitude of the formation constants of the magnesium ion complexes of the nucleotides. It should be noted that the value of Vladimirov, *et al.*,[12] ($\Delta Fh_{7.25}{}^{0\prime} = -5.6$ kcal./mole), measured from the same reactions used by Robbins and Boyer, differs considerably from the above values. Part but not all of the difference in this last instance can be traced to the use of the value of Ginodman (*vide supra*) for the free energy change in the hydrolysis of glucose 6-phosphate.

Acetyl Phosphate.—The values quoted in Table II for the hydrolysis of acetyl phosphate were derived from the data of Burton[19] on the hydrolysis of acetyl coenzyme A and the measurements made by Stadtman,[37] apparently performed at *p*H 6.8 and 28°, on the equilibrium constant (K_{Total} = 74) for the transfer of acetyl from phosphate to coenzyme A.

Creatine Phosphate.—The measurements of Noda, Kuby and Lardy[11a] on the equilibrium constant ($K_{\text{Total}} = 0.25$) in the transfer of phosphate from ATP to creatine at *p*H 8.9, 30° and 0.02 *M* $MgSO_4$ were combined with the values of Robbins and Boyer for ATP hydrolysis in the presence of excess Mg^{++} and the stability constants of Smith and Alberty[11b] for the magnesium complexes of the various components of the system.

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Immunochemical Studies on Blood Groups.[1] XXIV. Some Oligosaccharides Isolated from Dialysates After Mild Acid Hydrolysis of Human Blood Group B Substances from Ovarian Cyst Fluid

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Blood group B substance derived from human pseudomucinous ovarian cyst fluid was hydrolyzed at *p*H 1.6 for 2 hr. at 100° and dialyzed. The dialysate was fractionated by charcoal column chromatography. Five fucose-containing oligosaccharides were isolated. The structures of these compounds are inferred from the composition, behavior on oxidation with sodium periodate before and after sodium borohydride reduction, reaction with triphenyltetrazolium chloride and optical activity. They are: (1) fucosyl (1 → 6) galactose, (2) fucosylgalactose, (3) fucosyl (1 → 3) fucose, (4) (fucosyl, galactosyl) (1 → 3) N-acetylglucosamine, (5) fucosylfucosyl (1 → 3) N-acetylglucosamine. None of the compounds isolated was as active in inhibiting B-anti-B precipitation as galactinol or melibiose, two of the most potent inhibitors available. Only 2.6% of the total fucose in the dialysate was free.

Studies on blood group substances[2-5] have clearly shown that biological activity is associated with oligosaccharide side chains which are split off by mild acid hydrolysis[6] at *p*H 1.5 to 2.0 for 2 hr. at 100° leaving a high molecular weight non-dialysable fraction,[7] PI, possessing little or none of the original blood group activity. This procedure also splits from these substances mono and other oligosaccharides unrelated to the blood group activity. Coté and Morgan[4,8] have identified six disaccharides from a partial acid hydrolysate in 0.1 *N* HCl for 3 hr. or in 1.0 *N* HCl for 30 minutes both at 100°, of blood group A substances. One of these, O-α-N-acetylgalactosaminoyl 1 → 3-D-galactose, was more active in inhibiting the hemagglutination of A cells by anti-A than any other compound thus far reported, in agreement with earlier work showing the importance of a terminal non-reducing N-acetylgalactosamine residue in the specificity of blood group A substance in its reaction with a plant hemagglutinin[9] and with human anti-A.[10]

This report describes the chromatographic separation and isolation of five hitherto undescribed oligosaccharides from the dialysate of a human ovarian cyst B substance which had been hydrolyzed in mild acid. The composition, partial identification and biological activity of these compounds is given.

Experimental

Materials and Methods.—Blood group B substance, Beach phenol insol 12.1 g., isolated from human ovarian cyst fluid, was hydrolyzed at *p*H 1.6 for 2 hr. at 100° at a concentration of 35 mg. per ml., dialyzed and the dialysate, 2.8 g., lyophilized. Two hundred seventeen mg. of the dialysate was chromatographed on a 100 g. Darco–100 g. Celite column[11] with an ethanol gradient as previously described.[12] The chromatogram thus obtained showed a

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