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THE EQUILIBRIUM CONSTANTS AND ENTHALPY CHANGES FOR THE ESTERIFICATION OF GLYCINE BY ETHANOL IN WATER AND ETHANOL–WATER MIXTURES

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Equilibrium constants, defined on the basis of the moles of all reactants and products including water, were found to be 4.6, 1.0 and 0.8 for the esterification of the glycine cation at 20° in a dilute aqueous sytem, and systems having 0.5 : 1 and 1 : 1 molar ratios of ethanol to water, respectively. When corrections were made for deviations from ideality a value of 5 ± 1 was obtained for all three systems. Enthalpy changes were determined calorimetrically for the dilute aqueous and equimolar ethanol-water systems, and in each case was close to 1 kcal mole^{-1}. The entropy change was calculated as 6 cal mole^{-1} deg^{-1}. $\Delta G^{0'}$ for the *hydrolysis* of the glycine ethyl ester cation at 20° and pH 7 was calculated as −7.5 kcal mole^{-1}.

1. Introduction

It has long been known that ribosomal formation of proteins involves amino acid esters in the form of aminoacyl-tRNA's. In spite of this, little has been done to determine the thermodynamic parameters for reactions involving compounds of this type. Early investigations suggested that the activated forms of amino acids involved in protein biosynthesis were "high energy compounds" since the ATP-requiring reactions involved in their formation had equilibrium constants close to unity [1,2,3]. Systematic investigations to determine the thermodynamic parameters for reactions involving amino acid esters *per se* were not undertaken, however, until Jencks and his coworkers made their studies involving a variety of biologically important esters. As a result of these studies, they reported in 1960 an equilibrium constant of 2.3 for the esterification of glycine by ethanol in a dilute aqueous sytem at 39° [4]. Shortly thereafter Bender's group reported equilibrium constants for the hydrolysis of N-acetyl-L-phenylalanine methyl ester and N-acetyl-L-tryptophan ethyl ester [5]. To these equilibrium data involving amino acid esters Lumry's group subsequently added a calorimetrically-determined enthalpy change for the hydrolysis of N-acetyl-L-tryptophan ethyl ester [6].

More than a decade later the equilibrium constant was determined in this laboratory for the esterification of glycine in an equimolar mixture of ethanol and water at 20° [7]. Using the same convention that Jencks and his coworkers used to define this constant, a value of 0.82 was obtained in an equimolar mixture of ethanol and water at 20° in contrast to the value of 2.3 they observed in a dilute aqueous system at 39°. Knowing that the enthalpy changes for esterification reactions are commonly small, it would seem that the principal cause for the observed difference involving glycine esterification was the difference in reaction media rather than temperature. The suggestion that the reaction medium may play a significant role in reactions involving amino acid esters has been made by Wolfenden who explained the exergonic character of the aminolysis of amino acid esters of transfer RNA in terms of the stronger solvation of products as compared with reactant [8]. However, it has long been known that the equilibrium constant for the esterification of acetic acid by ethanol is practically independent of the relative amounts of reactants and thus differences in the relative amounts of ethanol and water may not have a measurable effect on amino acid esterification. Because of the above-mentioned difference in equilibrium constants in different reaction media and the question of whether reactions involving amino acid

Table 1
Equilibrium constants for the esterification of the glycine cation with ethanol in water and two ethanol–water mixtures at 20° [a]

Molar ratio of ethanol to water	K_{eq} obs	$\frac{\gamma \text{ ester}^+}{\gamma \text{ glycine}^+}$	$\frac{\gamma \text{ water}}{\gamma \text{ ethanol}}$	Corrected K_{eq}
0 : 1	4.6 ± 0.4	1.02	0.99	4.6 ± 0.4
0.5 : 1	1.0 ± 0.1	1.03	4.45	4.6 ± 0.5
1 : 1	0.8 ± 0.1	1.04	8.42	7 ± 0.9
			weighted mean	5 ± 1

[a] K_{eq} obs was calculated from the moles of all reactants and products including water. The deviations shown in column 2 are the standard deviations of the intercepts on the ordinate of the least squares lines relating K_{eq} obs to mole fraction of HCl (fig. 1).

esters are dependent on the compositions of the surrounding media, experimentation involving esterification of glycine was extended.

This paper reports observed equilibrium constants for the esterification of the glycine cation by ethanol in water and two ethanol-water mixtures at 20° and accounts for the differences between these on the basis of deviations from ideality. In addition, the enthalpy changes are reported for this reaction in a dilute aqueous system and an equimolar ethanol-water system at 20°.

2. Materials and methods

The samples of amino acid and ester used in this research were obtained from the following suppliers: glycine (Mallinckrodt Chemical Works), glycine ethyl ester hydrochloride (Sigma Chemical Co.), 1-^{14}C glycine and 1-^{14}C glycine ethyl ester hydrochloride (Research Products International Corp., 54 and 18.5 mCi/mmole, respectively). All other materials used were reagent grade from common suppliers.

Determination of equilibrium data involved three reaction media with differing mole fractions of ethanol and water: a dilute aqueous system and systems having 0.5 : 1 and 1 : 1 molar ratios of ethanol to water. For brevity the dilute aqueous systems have been designated as having a 0 : 1 molar ratio of ethanol to water in tables 1 and 2 even though small amounts of ethanol had been added initially to these systems that started with glycine. Three pairs of reactions were performed in each of these media. One member of each pair started with glycine; the other with ester. The initial mole fraction of glycine or ester was always ca 4×10^{-3}, but the mole fractions of HCl present as a catalyst varied, being ca 9 or 18 or 27×10^{-3}. All systems were prepared by weight using an analytical balance. After thorough mixing ca 1 ml of each reaction mixture was placed in a small reaction vessel and spiked with ^{14}C glycine or ^{14}C ester to give ca 10^6 CPM. These additions changed the concentrations of glycine or ester by no more than 0.1%. The resulting radioactive systems were then gently agitated at 20° until equilibrium was established.

Thin layer chromatography, autoradiography and scintillation counting were used to obtain equilibrium data. The glycine and glycine ethyl ester in each equilibrium mixture were separated on microcrystalline cellulose thin layer plates by use of butanol-acetic acid-water 60 : 15 : 25 v/v/v. Autoradiography allowed location of these on the plates and after the appropriate areas were removed from the plates the ratio of glycine to ester could be obtained by liquid scintillation counting. Knowing this ratio and the initial moles of reactants and products for each mixture, it was possible to determine observed equilibrium constants. The liquid scintillation counting involved use of Cocktail No. 3a708 from Research Products International Corp. and a Beckman Model LS-200B liquid scintillation counter.

The observed equilibrium constants (abbreviated K_{eq} obs) determined in this study were defined in terms of the moles of all reactants and products including water. The moles of ester and glycine were defined as the analytically determined amount of each (total of all species). No corrections were made for different ionic species that might be present since the pK_a's for the carboxyl group of glycine in water and ethanol-water mixtures indicated that these groups were essentially completely protonated under the conditions we used for experimentation [9]. Thus mathe-

Table 2
Enthalpy changes for the esterification of the glycine cation with ethanol in an ethanol–water mixture and water at 20° [a]

Molar ratio of ethanol to water	Reaction number as given in text	Mean ΔH (kcal mole^{-1})
1 : 1	1	−11 ± 1
	2	−1.1 ± 0.1
	3	−0.8 ± 0.1
	4	−9.7 ± 0.4
$\Delta H_{esterification} = \Delta H_2 + \Delta H_4 - \Delta H_1 - \Delta H_3 = 1 \pm 1$ kcal mole^{-1}		
0 : 1	5	−17 ± 1
	6	ca −10.5
	7	ca −1
	8	ca −13.7
$\Delta H_{esterification} = 2\Delta H_8 - \Delta H_5 - \Delta H_6 - \Delta H_7 =$ ca 1 kcal mole^{-1}		

[a] The deviations listed are standard deviations involving five or six determinations each. The enthalpy changes for reactions (6), (7) and (8) were obtained by averaging certain calorimetrically determined values from the literature and when necessary correcting them to 20°.

matically K_{eq} obs was defined as K_{eq} obs = (total moles of all species of glycine ethyl ester)(moles of water)/(total moles of all species of glycine)(moles of ethanol) and the reaction under study was considered to be

$$^{+}NH_3CH_2COOH + CH_3CH_2OH \rightarrow {}^{+}NH_3CH_2COOCH_2CH_3 + H_2O.$$

After calculation of the observed equilibrium constant for each member of the three pairs of reactions in each of the three reaction media, a plot was made of K_{eq} obs. versus mole fraction HCl. The method of least squares was then used to determine the observed equilibrium constants corresponding to zero mole fraction HCl. The standard deviations reported for these extrapolated values are the standard deviations of the intercepts of the least squares lines on the ordinates.

In order to determine the enthalpy changes for the esterification of glycine, a microcalorimeter similar to that described by Kitzinger and Benzinger was used [10]. To find the enthalpy change associated with glycine esterification in an equimolar mixture of ethanol and water the following reactions were studied in this reaction medium:

$$^{+}NH_3CH_2COOCH_2CH_3 + 2OH^- \rightarrow NH_2CH_2COO^- + CH_3CH_2OH + H_2O \quad (1)$$

$$^{+}NH_3CH_2COO^- + OH^- \rightarrow NH_2CH_2COO^- + H_2O \quad (2)$$

$$^{+}NH_3CH_2COO^- + H^+ \rightarrow {}^{+}NH_3CH_2COOH \quad (3)$$

$$H^+ + OH^- \rightarrow H_2O \quad (4)$$

The enthalpy change for the esterification reaction is then equal to

$$\Delta H_2 + \Delta H_4 - \Delta H_1 - \Delta H_3.$$

The enthalpy change for glycine esterification in a dilute aqueous system was determined in an analogous manner but made use of the following reactions in this reaction medium:

$$^{+}NH_3CH_2COOCH_2CH_3 + 2OH^- \rightarrow NH_2CH_2COO^- + CH_3CH_2OH + H_2O \quad (5)$$

$$NH_2CH_2COO^- + H^+ \rightarrow {}^{+}NH_3CH_2COO^- \quad (6)$$

$$^{+}NH_3CH_2COO^- + H^+ \rightarrow {}^{+}NH_3CH_2COOH \quad (7)$$

$$H^+ + OH^- \rightarrow H_2O \quad (8)$$

The enthalpy change for the esterification reaction in this case is equal to $2\Delta H_8 - \Delta H_5 - \Delta H_6 - \Delta H_7$.

The enthalpy changes shown in table 2 for reactions (1), (2), (3), (4) and (5) were determined experimentally. The enthalpy changes shown for reactions (6), (7) and (8), however, were obtained by averaging the calorimetrically determined values for either 20° or 25° and for ionic strengths comparable to those used in our studies as listed in the compilation of Christensen, Hansen and Izatt [11]. The values used for water were for 20°; those for glycine were for 25°. The latter values were corrected to 20° by use of the ΔC_p's for the glycine ionizations as listed in the compilation of Edsall and Wyman [12]. We also determined experimentally the enthalpy change for reaction (8). The reason for this was to assess the accuracy of our procedures after having obtained the low value shown for reaction (4) which involved the reaction of the hydrogen and hydroxyl ions in an equimolar ethanol–water reaction medium. Our experimental value

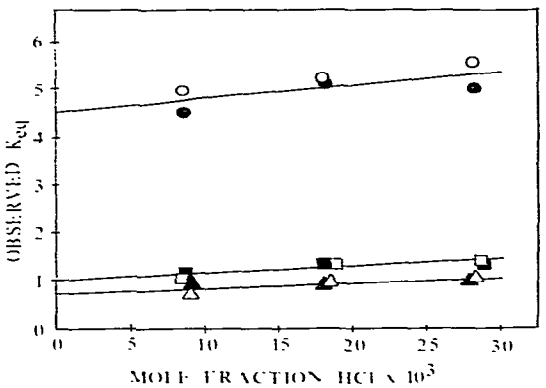

Fig. 1. Observed equilibrium constants for the esterification of the glycine cation by ethanol in various reaction media at 20° as a function of mole fraction HCl (catalyst). The constants were calculated from the moles of all reactants and products including water. The circles, squares and triangles represent a dilute aqueous system and systems having 0.5 : 1 and 1 : 1 molar ratios of ethanol to water, respectively. The open figures represent experiments that started with glycine; the filled figures represent experiments that started with glycine ethyl ester hydrochloride. The lines shown were obtained by the method of least squares.

for reaction (8), which used essentially pure water as the reaction medium, agreed well with the literature values. Thus we feel the low value for reaction (4) reflects the change in reaction medium rather than experimental error.

A typical calorimetric experiment was done as follows. Five ml aliquots of reactant solutions were placed in a bicompartmented microcalorimeter cell. This and the appropriate blank cell were then put into the microcalorimeter. After initial thermal equilibrium had been established, the reactants were mixed and the resulting potential recorded as a function of time. This was done with a Keithley 150B microvolt ammeter and power supply and a Leeds and Northrup Speedomax G recorder. The heat changes associated with dilution of the reactants were then determined. By subtracting these from the observed heat changes and knowing the moles of limiting reactant, we could determine the enthalpy change for the reaction under examination. Six calorimetric experiments were performed for each reaction. No data were discarded unless Chauvenet's criterion indicated that such should be done. Accordingly, one experimental point was discarded from the data for each of the following reactions: (2), (3) and (5).

3. Results and discussion

The observed equilibrium constants for the esterification of the glycine cation with ethanol in systems having different molar ratios of ethanol to water and mole fractions of HCl are plotted in fig. 1. The values obtained by linear extrapolations to zero mole fraction of HCl are shown in column 2 of table 1. To one significant figure the value obtained from this study for the equimolar ethanol-water system is identical to the value obtained earlier in this laboratory by another analytical method [7]. However, the observed equilibrium constants for the other systems differ and change systematically as the reaction medium is changed, becoming smaller as one goes from a dilute aqueous system to an equimolar ethanol-water system.

From these results one can now qualitatively account for the difference between the equilibrium constant found in this laboratory involving an equimolar mixture of ethanol and water at 20° [7] and the value reported earlier by Jencks and his coworkers involving a dilute aqueous system at 39° [4]. However, the value we now have for a dilute aqueous system (at 20°) still differs, being larger, from their value (at 39°). Originally it was thought that the temperature difference between these two systems may have a role, although not major, in creating the difference for these dilute aqueous systems. However, in view of our finding that the esterification of the glycine cation by ethanol is endothermic, this notion must be discarded. Two factors that may have contributed to this difference, however, are differences in experimental approach and the fact that the observed equilibrium constants for this reaction are very dependent upon the mole fraction of ethanol in dilute aqueous systems. Relatively small decreases in the mole fraction of ethanol in dilute aqueous sytems translate into relatively large differences in observed equilibrium constants. It appears that our dilute aqueous sytems contained less ethanol than theirs and thus one would expect our value to be higher than theirs.

In the preceding paragraphs it has been pointed out that the observed equilibrium constants for the esterification of the glycine cation with ethanol change as

the ratio of ethanol to water changes in the reaction medium. Since all of these constants were calculated from the moles of reactants and products rather than their activities and since it was thought that these changes might simply reflect deviations from ideality, calculations were made to obtain equilibrium constants based on activities. This was done by multiplying the observed equilibrium constants by two ratios: the ratio of the activity coefficient for the glycine ethyl ester cation to that for the glycine cation, and the ratio of the activity coefficient for water to that for ethanol.

The activity coefficients (γ) for the glycine ethyl ester cation and the glycine cation and the corresponding ratios were estimated by use of the extended Debye-Hückel equation

$$\log \gamma = \frac{-Az_i^2\sqrt{\mu}}{1 + Ba_i\sqrt{\mu}}$$

where A and B are constants for any given solvent at a specified temperature, z_i is the charge on ion i, q_i is the "effective diameter" of this ion, and μ the ionic strength of the system. Values for A and B were estimated from published dielectric constants for various ethanol--water mixtures [13]. A value of a for the glycine cation has been reported [14]. A value for the glycine ethyl ester cation was estimated by assuming it could be related to the value for the glycine cation in the same manner that values for the methyl ammonium and n-propyl ammonium cations are related to each other [14]. Activity coefficients were then calculated at ionic strengths corresponding to those of our experimental systems and from these the corresponding ratios of activity coefficients. The results from these calculations showed that in all of our experimental systems the ratios of activity coefficients of the glycine ethyl ester cation to the glycine cation were essentially unity (column 3 of table 1).

The ratios of the activity coefficients of water to ethanol were calculated by use of the following equation:

$$\frac{\gamma_{water}}{\gamma_{EtOH}} = \frac{p_{water}km_{EtOH}}{p^0_{water}N_{water}p_{EtOH}}$$

where p_{water} and p_{EtOH} are the vapor pressures for water and ethanol, respectively, in a water-ethanol mixture at a specified temperature, p^0_{water} is the vapor pressure of pure water at that temperature, N_{water} the mole fraction of water in the ethanol--water mixture, m_{EtOH} the molal concentration of ethanol in that mixture and k the limit at infinite dilution of the proportionality constant relating p_{EtOH} to m_{EtOH} according to Henry's Law. This equation arises from the use of Raoult's Law to calculate the activity coefficients for water and Henry's Law to calculate those for ethanol. Since biochemists often use different standard states from those used here it should be emphasized that these calculations used pure water as the standard state for water and a hypothetical one-molal solution of ethanol which obeyed the limiting form of Henry's Law as the standard state for ethanol. To make the desired calculations vapor pressure data for ethanol-water mixtures at 20° were obtained from the International Critical Tables [13]. They were then used to provide data for a nonlinear least squares computer program which in turn provided values of p_{water} and p_{EtOH} corresponding to our experimental systems. The resulting ratios of activity coefficients are shown in column 4 of table 1.

Having attained estimates of the corrections that should be applied to our observed equilibrium constants we were able to obtain corrected equilibrium constants (column 5, table 1). Examination of these shows that the variation from one solvent system to another is no longer systematic and thus it was thought that is simply reflected experimental error. Accordingly, a weighted mean and standard deviation were calculated from these corrected constants (each weighted according to the reciprocal of its variance). The resulting value was 5 ± 1 for all of our solvent systems at 20°. Thus it appears that the differences among our observed equilibrium constants are due to departures from ideality.

Going next to the results from our calorimetric studies, we find in table 2 the enthalpy changes for the various reactions used to determine the enthalpy changes for the esterification of the glycine cation by ethanol in equimolar ethanol-water and dilute aqueous systems. Using these algebraically as outlined in Materials and methods gave the results shown in this table. In summary, the esterification of the glycine cation by ethanol appears to be slightly endothermic in both equimolar ethanol-water and dilute aqueous systems. Within experimental error the enthalpy changes for these two systems appear to be identical and close to 1 kcal mole^{-1}.

Having calculated the equilibrium constant for the esterification of the glycine cation (corrected for differences in activity coefficients) and the corresponding enthalpy change, one can now assess the contribution that entropy makes to this reaction. Using 5 for the equilibrium constant and 1 kcal mole $^{-1}$ for the enthalpy change the entropy change at 20° is 6 cal mole^{-1} deg^{-1}.

Finally, biochemists are most commonly interested in equilibrium constants and free energy changes associated with a different convention from that we have used in this paper. Usually they are interested in numerical values based on total molar concentrations and the convention that selects as the standard state the state in which all reactants and products (other than the hydrogen ion and water) are 1 M. The standard state for the hydrogen ion is usually set at 10^{-7} M, and even if water is involved in the reaction it does not appear in the mathematical definition for the equilibrium constant. The free energy change for this set of conditions is the biochemists' $\Delta G^{0\prime}$. According to this convention $\Delta G^{0\prime}$ for the *hydrolysis* of the glycine ethyl ester in a dilute aqueous system at 20° and pH 7 is found to be −7.5 kcal mole^{-1}. To obtain this value we used our equilibrium constant (5), the molarity of pure water (55.5) and estimated pK_a's for the glycine ethyl ester cation, the glycine cation and dipolar glycine at 20° (7.86, 2.37 and 9.92, respectively). These estimates were obtained from published values for the pK_a's of these substances and their temperature dependences [11,12].

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References

[1] F. Lipmann, W.C. Hulsmann, G. Hartman, H.G. Boman, and G. Acs. J. Cell and Comp. Physiol. 54 (1959) 75.
[2] P. Berg, F.H. Bergmann, E.J. Ofengand and M. Dieckmann, J. Biol. Chem. 236 (1960) 1726.
[3] J. Leahy, E. Glassman and R.S. Schweet J. Biol. Chem. 235 (1960) 3209.
[4] W.P. Jencks, S. Cordes and J. Carriuolo, J. Biol. Chem. 235 (1960) 3608.
[5] M. L. Bender and F.J. Kézdy, J. Amer. Chem. Soc. 86 (1964) 3604.
[6] S. Rajender, R. Lumry and M. Han, J. Phys. Chem. 75 (1971) 1375.
[7] R.K. Burkhard, Fed. Proc. 38 (1979) 350.
[8] R. Wolfenden, Biochemistry 17 (1978) 201.
[9] L. Michaelis and M. Mizutani, Z. Physik. Chem. 116 (1925) 135.
[10] C. Kitzinger and T.H. Benzinger, Methods Biochem. Anal. 8 (1963) 309.
[11] J.J. Christensen, L.D. Hansen and R.M. Izatt, Handbook of proton ionization heats (John Wiley, New York, 1976) pp. 93-4, 207-209.
[12] J.T. Edsall and J. Wyman, Biophysical chemistry Vol. 1 pp. 452-3 (Academic Press, New York, 1958).
[13] E.W. Washburn, Ed., International Critical Tables of Numerical Data, Physics, Chemistry and Technology, Vol. 6 (McGraw-Hill, New York, 1926) pp. 23, 290.
[14] J. Kielland, J. Amer. Chem. Soc. 59 (1937) 1675.