CCXCIII. DISSOCIATION CONSTANTS AND STRUCTURES OF GLUTAMIC ACID AND ITS ESTERS.

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It has been shown [Neuberger, 1936] that the dissociation constants of simple zwitterions which contain only one carboxyl and one amino-group are quantitatively related to their structures; similarly the change of their apparent dissociation constants with the ionic strength of the solution of the different amino-acids can be satisfactorily explained on such a basis. This paper represents an extension of the previous work to a more complex zwitterion, namely glutamic acid. It was deemed desirable to determine also the dissociation constants of the different esters of glutamic acid and in this connexion the preparation of α -ethyl hydrogen glutamate is described.

EXPERIMENTAL.

Preparation of compounds.

*α -Ethyl hydrogen glutamate.*¹ *N*-Benzylcarbonylglutamic anhydride [cf. Harington & Mead, 1935] (20 g.) and alcohol (100 ml.) were heated in a sealed tube for 3½ hours at 125°; the solution was concentrated to low bulk, diluted with ether and extracted with aqueous sodium bicarbonate. The aqueous solution was acidified and extracted several times with ether; the combined ethereal extracts were dried and evaporated leaving an oil which slowly crystallized. After recrystallization from ether the *α -ethyl hydrogen N-benzylcarbonylglutamate* thus formed had m.p. 100°. Yield 25%. (Found: N, 4.58%. $C_{15}H_{19}O_6N$ requires: N, 4.53%. 22.1 mg. required 3.6 ml. *N*/50 NaOH for neutralization to cresol red; $C_{15}H_{19}O_6N$ titrating as a monobasic acid requires 3.57 ml.)

The above *α -ethyl hydrogen N-benzylcarbonylglutamate* (4.5 g.), dissolved in a little 80% alcohol containing HCl (1 equiv.) was reduced with hydrogen and palladium black; the filtered solution was evaporated *in vacuo* and the residue taken up in a little 90% alcohol, adjusted to pH 6.0 with lithium hydroxide and placed in a desiccator at 400 mm. pressure over a dish of absolute alcohol. Crystallization soon set in and was complete in 2 days. Recrystallization in a similar manner yielded *α -ethyl hydrogen glutamate* having m.p. 110°. (Found: N, 7.85%. $C_7H_{13}O_4N$ requires N, 8.01%.) The ester is very soluble in water, aqueous alcohol and aqueous acetone, but only sparingly soluble in absolute alcohol.

¹ Treatment of *N*-benzylcarbonylglutamic anhydride with sodium ethoxide in the cold according to Melville [1934] gives a mixture of α - and γ -esters; thus a sample of the oily product so obtained yielded, on hydrogenation, approximately 30% of γ -ethyl hydrogen glutamate, whilst another sample on long keeping, deposited crystals of *α -ethyl hydrogen N-benzylcarbonylglutamate*; the different treatment described above, which gives a more uniform product, was therefore adopted.

γ-Ethyl hydrogen glutamate was prepared according to Bergmann & Zervas [1933].

Ethyl glutamate hydrochloride was obtained by esterification of glutamic acid in the usual manner and was twice recrystallized.

Glutamic acid was a carefully recrystallized commercial specimen.

Potentiometric methods.

Measurements were made as previously described [Neuberger, 1936]. The liquid junctions which were made inside a cylindrical tube of 3 mm. internal diameter were initially very sharp; the potentials were generally constant for many hours within 0.1 mv. The esters, which were very stable at acid reaction, were hydrolysed with varying ease in alkaline media; in order to prevent local excess of alkali therefore NaOH was added to the mechanically stirred solution of the ester and the mixture transferred to the cell, mixing and transference being effected in an atmosphere of hydrogen freed from traces of CO₂. Readings were taken after suitable intervals; the potentials were plotted against time and extrapolation to zero time was made. The initial drift in potential was about 1 mv. per 10 min. in the case of *pK*₂ of *γ*-ethyl hydrogen glutamate at a *pH* of about 8.8 and 1–2 mv. per hour in the case of ethyl glutamate at a *pH* of about 7.2; *α*-ethyl hydrogen glutamate was quite stable under the conditions of the measurements.

Results.

In Table I the results of the potentiometric measurements are recorded. Column 1 gives the potentials corrected for barometric pressure; in column 2 the potentials are recorded after correction for liquid junction. In column 3, *a* denotes the degree of neutralization, *c* in column 4 gives the total concentration of the acid or base present. The ionic strength was varied by addition of KCl. The apparent dissociation constants were determined as described; the values are generally accurate within ±0.01. In the case of a very low *pK* (*pK*₁ of glutamic acid and of *γ*-ethyl hydrogen glutamate) the large blank correction causes the margin of error to be higher. In the cases of *pK*₂ of *γ*-ethyl hydrogen glutamate and *pK* of ethyl glutamate measurements are also less reliable owing to hydrolysis and/or lactam formation. The value of *pK*₃⁰ of glutamic acid is also only accurate ±0.03, since the correction for ionic strength is larger.

DISCUSSION.

The dissociation constants of glutamic acid.

Several equilibria are involved in the dissociation of glutamic acid and it is necessary to consider these in some detail. Glutamic acid can be regarded as a tribasic acid of which the most acidic form is a cation. The first step consists in the dissociation of H⁺ from the cation according to one of the three reactions:¹

¹ The letters *a*, *b* etc. beneath the equations are arbitrary signs to denote the different ionic species and are used for brevity in the equations developed later in this paper.

	<i>E</i>	<i>E</i> _{corr.}	<i>a</i>	<i>c</i>	<i>μ</i>	<i>pH</i>	<i>pK</i>
			Glutamic acid.				
<i>pK</i> ₁	495.3	493.4	50.0	0.008	0.008	2.683	2.129
	495.8	494.4	50.0	0.008	0.020	2.705	2.146
	497.7	497.5	50.0	0.008	0.084	2.757	2.188
			<i>pK</i> ₁ ⁰ = 2.155 (corrected).				
<i>pK</i> ₂	590.9	589.5	51.0	0.008	0.008	4.313	4.293
	564.5	563.1	25.0	0.008	0.006	3.867	4.301
	590.7	589.4	51.0	0.008	0.014	4.311	4.288
	589.4	588.3	51.0	0.008	0.020	4.292	4.262
	588.0	587.3	51.0	0.008	0.044	4.275	4.244
	585.0	584.7	51.0	0.008	0.084	4.232	4.198
	618.4	617.0	75.0	0.008	0.008	4.772	4.295
			<i>pK</i> ₂ ⁰ = 4.324 (corrected).				
<i>pK</i> ₃	920.4	919.0	55.5	0.008	0.016	9.883	9.807
	893.7	892.3	29.5	0.008	0.012	9.432	9.818
	947.9	946.5	79.8	0.008	0.021	10.348	9.813
	909.1	909.4	55.5	0.008	0.173	9.721	9.641
	912.0	911.8	55.5	0.008	0.096	9.762	9.683
	913.9	913.3	55.5	0.008	0.060	9.787	9.716
	915.6	914.7	55.5	0.008	0.040	9.813	9.741
			<i>pK</i> ₃ ⁰ = 9.960.				
			α-Ethyl hydrogen glutamate.				
<i>pK</i> ₁	562.4	560.9	55.2	0.00725	0.004	3.829	3.881
	562.9	561.4	55.2	0.00725	0.009	3.837	3.888
	563.2	562.3	55.2	0.00725	0.014	3.853	3.905
	563.6	562.9	55.2	0.00725	0.02	3.863	3.915
	565.6	565.2	54.6	0.00725	0.044	3.902	3.946
	563.6	563.4	55.9	0.00725	0.084	3.870	3.931
	590.7	589.3	27.6	0.00725	0.002	4.311	3.876
	534.5	533.1	80.3	0.00680	0.006	3.359	3.871
	535.5	534.1	80.3	0.00725	0.008	3.376	3.886
			<i>pK</i> ₁ ⁰ = 3.846.				
<i>pK</i> ₂	770.8	769.4	73.5	0.00725	0.0058	7.355	7.801
	769.7	768.6	73.5	0.00725	0.02	7.340	7.786
	768.2	767.6	73.5	0.00725	0.042	7.323	7.766
	744.0	742.6	88.8	0.00725	0.005	6.901	7.800
	770.5	769.4	73.7	0.008	0.008	7.354	7.802
	771.4	770.4	73.1	0.008	0.014	7.372	7.806
	769.0	768.7	72.5	0.00725	0.048	7.341	7.760
			<i>pK</i> ₂ ⁰ = 7.838.				
			γ-Ethyl hydrogen glutamate.				
<i>pK</i> ₁	503.4	501.4	31.8	0.01257	0.008	2.823	2.184
	523.3	521.4	15.9	0.01257	0.008	3.151	2.185
	490.4	488.4	47.7	0.01257	0.008	2.605	2.172
	502.4	500.9	31.8	0.01257	0.014	2.817	2.164
	496.4	495.6	50.0	0.008	0.025	2.725	2.180
	497.3	496.8	50.0	0.008	0.044	2.745	2.200
	499.1</						

Reactions (1*a*) and (1*b*) lead to zwitterions, whilst reaction (1*c*) produces an uncharged molecule. For electrostatic reasons most of the cations will react according to (1*a*) rather than (1*b*). From what is known of the order of magnitude of the dissociation of the amino-group in amino-acids and their esters and especially from the relative values of the dissociation constants of the esters of glutamic acid given in this paper it can be assumed that the dissociation of the NH_3^+ group is negligible compared with the dissociation of either of the two carboxyl groups. Reaction (1*c*) can therefore be neglected and the second step can be written as follows:

In order to obtain the individual equilibrium constants of the reactions (1*a*), (1*b*), (2*a*) and (2*b*) certain additional assumptions must be made, since actual measurements only give overall constants K_1 and K_2 which may be defined by the equations

$$K_1 = \frac{(b_1 + b_2) h}{a} \quad \text{.....(3),} \quad K_2 = \frac{c \cdot h}{(b_1 + b_2)} \quad \text{.....(4).}$$

In glutamic acid K_1 is only about 100 times larger than K_2 ; the large value of K_1 renders it necessary to perform measurements for the determination of K_1 at *pH* values where reactions (2*a*) and (2*b*) cannot be neglected. The formula generally used for the calculation of dissociation constants from potentiometric measurements therefore leads to incorrect results.

In order to correct for the effect of overlapping of the two steps of dissociation we proceed as follows. To a solution of ionic strength μ containing *A* mol. of glutamic acid we add *B* mol. of HCl or NaOH respectively, *B* having a positive sign if acid is added and a negative sign if alkali is added. Since the solution as a whole is electrically neutral we have then, neglecting OH^- ,

$$a - c + h = B \quad \text{.....(5),}$$

$$\text{and further} \quad -\log h = \text{pH} - \frac{0.5 \sqrt{\mu}}{1 + \sqrt{\mu}} \quad \text{.....(6),}$$

$$a + b_1 + b_2 + c = A \quad \text{.....(7).}$$

By combining (3), (4), (5) and (7) we get

$$A = \frac{(B - h) (h^2 + K_1 h + K_1 K_2)}{h^2 - K_1 K_2}.$$

If we now write for convenience

$$\frac{A}{B - h} = m,$$

we get

$$K_1 = \frac{(m - 1) h^2}{(m + 1) K_2 + h} \quad \text{.....(8).}$$

In order to determine K_1 and K_2 at least two measurements with differing *m* and *h* must be made. If solution I contains *A* mol. of glutamic acid, B_1 mol. of HCl ($B_1 < A$) and h_1 mol. of H^+ while solution II contains *A* mol. of glutamic acid, $-B_2$ mol. of NaOH (absolute value of $B_2 < A$) and h_2 mol. of H^+ , we then have

$$K_1 = \frac{(m_1 - 1) h_1^2}{(m_1 + 1) K_2 + h_1} = \frac{(m_2 - 1) h_2^2}{(m_2 + 1) K_2 + h_2}.$$

From this expression we get finally

$$K_2 = h_1 h_2 \frac{(m_2 - 1) h_2 - (m_1 - 1) h_1}{(m_2 + 1) (m_1 - 1) h_1^2 - (m_1 + 1) (m_2 - 1) h_2^2} \quad \dots\dots(9),$$

and similarly
$$K_1 = \frac{(m_2 + 1) (m_1 - 1) h_1^2 - (m_1 + 1) (m_2 - 1) h_2^2}{(m_2 + 1) h_1 - (m_1 + 1) h_2} \quad \dots\dots(9a).$$

It can be seen that if h_2 is small compared with h_1 equations (9) and (9a) give the formulae for an ordinary acid. The values obtained from (9) and (9a) have to be corrected for activity in order to give the thermodynamic constants. For small ionic strengths it can be assumed that the activity coefficient of the zwitterionic form of glutamic acid can be neglected and that the activity coefficients of H^+ and of the cation of glutamic acid are equal. pK_1^0 is then equal to the negative logarithm of the "concentration constant". pK_2^0 is given by (10):

$$pK_2^0 = -\log K_2 + \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \quad \dots\dots(10).$$

The correction introduced by employing equations (9) and (9a) instead of the formulae for an ordinary acid is under the conditions of the experiments about 0.08 for pK_1^0 and only 0.01 for pK_2^0 .

As pointed out above the constants pertaining to the individual reactions (1a), (1b), (2a) and (2b) cannot be obtained from the actual measurements; but the constants of the individual reactions K_1' , K_1'' , K_2' and K_2'' are related to the overall constants K_1 and K_2 by the equations:

$$K_1 = K_1' + K_1'' \quad \dots\dots(11),$$

$$\frac{1}{K_2} = \frac{1}{K_2'} + \frac{1}{K_2''} \quad \dots\dots(11a),$$

[*vide* Wegscheider, 1895; Adams, 1916; Bjerrum, 1923].

From (3), (4), (11) and (11a) we get

$$\frac{K_1'}{K_1''} = \frac{K_2''}{K_2'} = \frac{b_1}{b_2} = q \quad \dots\dots(12).$$

It is well known that the effect of COOH as substituent on the dissociation of another carboxyl group is more or less equal to the effect of COOEt. We may assume therefore that K_1'' is equal to the first dissociation constant of α -ethyl hydrogen glutamate. If this assumption is correct the value obtained for K_1' should be equal to K_1 of γ -ethyl hydrogen glutamate. Actually, we find $pK_1' = 2.164$, whilst the experimental value for pK_1^0 of γ -ethyl hydrogen glutamate is 2.143. From (11a) we get $pK_2'' = 4.315$ and $pK_2' = 2.633$.

It has been pointed out by Wegscheider [1895] that for a symmetrical dibasic acid K_1 , the overall constant must be for statistical reasons twice as large as K_1' , the individual constant of each group. For an unsymmetrical dibasic acid, however, the statistical factor is very small [Bjerrum, 1923]. This is shown very well in the case of glutamic acid, where one carboxyl group is adjacent to NH_3^+ and the second carboxyl removed therefrom by two carbon atoms. Here the statistical factor causes an increase only of about 2% in K_1 .

A solution of glutamic acid contains, apart from several ionic species, two types of zwitterions resembling respectively α - and γ -aminobutyric acid. Their ratio is equal to q which is about 48, i.e. most of the zwitterions are of the α -amino-acid type.

Change of apparent dissociation constant with ionic strength.

The first dissociation constant of glutamic acid should, at low ionic strength, show the behaviour of a cationic acid, since the activity coefficient of the zwitterionic form can be neglected under these conditions; the second constant

on the other hand, should be that of an ordinary acid. Experimental data show that this is approximately the case. The third step in the dissociation is represented by the formula

It may be assumed that in the first ion the two opposite charges, NH_3^+ and $\alpha\text{-COO}^-$, neutralize each other and the ion can be regarded as univalent, whilst the second ion is bivalent. We therefore should have

$$pK_3^0 = pK_3 + \frac{1.5\sqrt{\mu}}{1 + \sqrt{\mu}}.$$

But the experimental data do not fit this formula satisfactorily. This may be due to the fact that the ionic diameter of the bivalent ion is rather large and the simple formula is only approximately correct in the case where the two charges of the bivalent ion are not far apart. If the ionic diameter is assumed to be 6 Å., which is a very probable value, we get excellent agreement as shown in

Fig. 1. Variation of pK_3 of glutamic acid with ionic strength of solution. The curve is drawn according to the equation $pK_3 = 9.96 - \frac{1.5\sqrt{\mu}}{1 + 2\sqrt{\mu}}$; the crosses represent experimental observations.

Fig. 1. The formula used for the extrapolation of pK_3^0 can now be written in the form

$$pK_3^0 = pK_3 + \frac{1.5\sqrt{\mu}}{1 + 2\sqrt{\mu}}.$$

In all other cases the simple formulae for cationic acids and ordinary acids can be applied, since the activity coefficients of the zwitterions can be neglected with the small ionic strengths used.

Dissociation constants and structures.

Table II gives the dissociation constants of glutamic acid, its esters and of a number of related compounds. The dissociation constant of ethyl hydrogen glutarate has not been measured, but it can be estimated by using the rule

Table II. *Dissociation constants.*

	First COOH	Second COOH	NH ₃
<i>n</i> -Butyric acid	4.820	—	—
Glutaric acid	4.337*	5.420*	—
Ethyl hydrogen glutarate	4.638†	—	—
Alanine	2.340	—	9.870
γ-Amino- <i>n</i> -butyric acid	4.230	—	10.430
Ethyl γ-amino- <i>n</i> -butyrate	—	—	9.710
Glutamic acid	2.164†	4.315†	9.960
α-Ethyl hydrogen glutamate	3.847	—	7.838
<i>iso</i> Glutamine	3.81†	—	7.88†
γ-Ethyl hydrogen glutamate	2.148	—	9.19
Glutamine	2.17†	—	9.13†
Ethyl glutamate	—	—	7.035
Glycine ester	—	—	7.75

* Gane and Ingold [1931].

† See text.

‡ Melville & Richardson [1935].

For other data *vide* Neuberger [1936] and this paper.

discovered by Wegscheider [1895] that *K* of a half-ester of a symmetrical dibasic acid is half as large as the first constant of the acid. Such an estimate which can be regarded as correct within ± 0.04 leads to pK_{ester} of 4.638, and fits quite well into the series of dissociation constants of the half-esters of the paraffin dicarboxylic acids, ethyl hydrogen succinate having pK 4.521 and ethyl hydrogen suberate pK 4.836. For the first pK of glutamic acid the value used in Table II is that of pK_1' and for the second pK that of pK_2'' .

These dissociation constants furnish a very good example of the quantitative relationship existing between ionization and structure. The first pK of glutamic acid and pK_1 of γ-ethyl hydrogen glutamate are practically equal which is to be expected if we assume that the effect of COOEt is equal to that of COOH. Both pK values are decreased by 0.176 or 0.191 respectively as compared with pK_1 of an α-amino-acid such as alanine. This effect is quantitatively the same as that of the second COOH in glutaric acid on pK_1' , this alteration in magnitude being $pK_{\text{butyric acid}} - pK_{1\text{glutaric acid}} - \log 2 = 0.182$. The influence of the carboxyl group on NH₃⁺ is larger; the introduction of a carboxyl in the γ-position into an α-amino-acid ester as in α-ethyl hydrogen glutamate decreases the pK of NH₃⁺ by 0.735 (difference between $pK_{\text{glycine ester}}$ and $pK_{\text{ethyl glutamate}}$) which is similar to the influence of introduction of COOEt into the γ-position of butylamine as in ethyl γ-aminobutyrate. Another interesting point is that pK values of α-ethyl hydrogen glutamate and *isoglutamine* on the one hand and of γ-ethyl hydrogen glutamate and glutamine on the other are nearly identical.

The influence of the charges on the pK values is even more interesting. The difference between pK_2 of α-ethyl hydrogen glutamate and pK of ethyl glutamate is 0.803 and is due to the influence of the negative charge of the carboxyl group in α-ethyl hydrogen glutamate.

This difference should be equal to that between pK_3 of glutamic acid and pK_2 of γ -ethyl hydrogen glutamate; the figure actually found is 0.75.

The influence of the negatively charged γ -carboxyl group on NH₃⁺ should be opposite in sign but equal in absolute value to the influence of NH₃⁺ on the γ -carboxyl group. The difference between pK_1 of α -ethyl hydrogen glutamate and ethyl hydrogen glutarate is 0.791, which is in very satisfactory agreement.

Comparison between pK_2' of glutaric acid ($pK_2 - \log 2$) and pK_2 of glutamic acid leads to the similar value of 0.804.

The influence of the negatively charged α -carboxyl on the γ -carboxyl group can be estimated by comparing pK_2 of glutamic acid and pK_1 of α -ethyl hydrogen glutamate.

The difference in the pK values is 0.468; the difference between pK_1 and pK_2 of glutaric acid is 0.487 after correction for statistical effect. The distances between the different groups in glutamic acid can be calculated by using Bjerrum's formula [*vide* Neuberger, 1936]. The distance between the centres of the two carboxyl groups according to this calculation is 6.5 Å. and the distance between the NH₃⁺ group and the γ -carboxyl group about 4 Å. Applying the formula of Ingold [1931] the distances are 7.2 Å. and 5.2 Å. respectively. These values can only be regarded as approximate, but it can safely be concluded that the distance between NH₃⁺ and the γ -carboxyl group is considerably smaller than the distance between the two carboxyl groups. Such a structure, which is

also made probable by the X-ray analysis of crystalline glutamic acid [Bernal, 1931], explains the ease with which pyrrolidonecarboxylic acid is formed from glutamic acid.

Note on dissociation constants of proteins.

The work described here and in an earlier paper was undertaken partly with the intention of eliciting more information about the relationship of structure and ionization in complex compounds. Proteins are polyvalent electrolytes which according to currently accepted ideas consist of long peptide chains. Their ionization depends mainly on groups derived from trivalent amino-acids, such as the dicarboxylic and the basic amino-acids. It is commonly assumed, in the absence of any decisive evidence, that the γ -carboxyl groups of glutamic acid and the β -carboxyl groups of aspartic acid which are not present in amide linkage are free, whilst the corresponding α -carboxyl groups are in peptide linkage. If these assumptions are accepted, it is possible to predict with some accuracy the order of magnitude of the dissociation constants of the different groups. Effects through the chain of the molecule can be neglected if the substituent is more than 4 carbon atoms removed from the ionizing group, whilst charge effects depend entirely on the absolute distance and not on the distance in the direction of the chain and decrease generally more slowly with distance than do chain effects. A γ -carboxyl group on which no charge effects are acting will have *pK* 4.5–4.6, whilst a β -carboxyl group will have *pK* about 4.4–4.5. A protein molecule even in its isoelectric state carries a great number of positive and negative charges and the actual *pK* of any group will depend mainly on the distribution of positive and negative charges in its neighbourhood. If, for example, a carboxyl group is surrounded by a number of positive charges its *pK* will be much lower than 4.5. Since, in general, dissociation of carboxyl groups occurs over a *pH* range, where the number of positive charges of the protein molecule is larger than that of negative charges the average *pK* of carboxyl groups will be < 4.5. Proteins cannot be regarded as symmetrical polybasic acids; they are on the contrary highly unsymmetrical and the different strengths of the dissociating groups will be mainly responsible for the spreading of the titration curve; statistical factors will generally be of secondary importance. Only in the case of highly symmetrical proteins such as protamines will the spreading of dissociation constants be due to statistical factors. Since the charge effect is of such quantitative importance, a change in the shape of the molecule without any chemical alteration will cause a change in the individual dissociation constants and a shift of the isoelectric point since the distribution of charges will be altered. This may be the possible explanation of the experiments of Margaria [1934], who found that frog's muscle changed its apparent *pH* as measured by indicators during contraction and stretching.

All the methods available at present give somewhat questionable data about dissociation constants of proteins; but any discussion regarding this point is outside the scope of this paper.

SUMMARY.

1. α -Ethyl hydrogen glutamate has been synthesized.
2. Dissociation constants of glutamic acid, of α - and γ -ethyl hydrogen glutamate and of ethyl glutamate have been determined by extrapolating the apparent constants to zero ionic strength. Special formulae have been derived

to accommodate the effect of overlapping in the case of pK_1 and pK_2 of glutamic acid.

3. The different dissociation constants have been quantitatively explained on the basis of the corresponding structures; in particular it has been shown that the distance between NH_3^+ and the γ -carboxyl group in glutamic acid is smaller than the distance between the two carboxyl groups.

4. Principles derived from work on amino-acids have been considered in their application to proteins; it has been shown that dissociation constants and isoelectric points do not depend only on the chemical structure, but also on the shape of the molecules. Further, it has been pointed out that variation of dissociation constants in proteins depends on constitutional rather than on statistical factors.

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