CCVI. SYNTHESIS OF *i*- β -HYDROXYGLUTAMIC ACID.

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WITH A NOTE ON THE DISSOCIATION CONSTANTS.

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THE synthesis of β -hydroxyglutamic acid described by Dakin [1919] leaves much to be desired in the matter of the yields obtainable. Since the isolation of this amino-acid from natural sources also offers very considerable difficulties, the compound has not been available in sufficient amount to permit of an extended examination of its chemical and physiological properties.

According to Dakin [1918] β -hydroxyglutamic acid occurs in caseinogen to the extent of about 10 %. In view therefore of its importance as a constituent of protein and still more owing to the recent claim of Dakin, West and Howe [1930] to have isolated from liver a compound, active in curing pernicious anaemia, which yields on hydrolysis one molecule of hydroxyproline and one of β -hydroxyglutamic acid, the latter compound is of considerable biological interest and it was thought to be desirable to re-investigate the possibilities of its synthesis by a more practicable method.

Among the numerous possible syntheses explored by Dakin [1919] the simplest and most attractive from the theoretical point of view consisted in the reduction of the ethyl α -isonitrosoacetonedicarboxylate of Pechmann [1891] which is an easily accessible substance. Dakin's own attempt to effect this reduction, using sodium amalgam under a variety of conditions met with failure, owing probably, as he himself suggests, to secondary reactions on the part of the aminoketo-ester which would be formed during the first stage of the reduction; nor could any more satisfactory results be obtained by the reduction of ethyl α -isonitroso- β -ethoxyglutaconate.

It occurred to us that catalytic methods of reduction might find an application in this problem. It is true that the catalytic reduction of the *isonitroso*- to the amino-group and still more that of the carbonyl to the secondary alcoholic group have, until recently, been regarded as difficult of execution. The activating effect of small amounts of iron on the catalytic reduction of carbonyl com-

pounds observed by Carothers and Adams [1923] and by Faillebin [1923] has, however, largely removed the difficulties in this case, and it has further been shown by Hartung and Munch [1929] that the reduction of an *isonitroso*-compound to the corresponding primary amine is favoured by the presence of excess of hydrogen chloride in the reaction mixture. The latter authors have indeed achieved the direct reduction of several α -*isonitroso*ketones of mixed aliphatic-aromatic type to the corresponding amino-alcohols, the reaction being carried out in alcoholic solution containing three molecular equivalents of hydrogen chloride and the catalyst employed being palladinised charcoal. It is to be noted that Hartung *et al.* [1930] met with exceptional difficulties in the case of *isonitroso*acetophenone, which was readily reduced to the amino-ketone but could be reduced to the alcohol only after substitution of water for alcohol as the solvent and introduction of fresh catalyst.

When the reduction of ethyl α -*isonitroso*acetonedicarboxylate was attempted by the method of Hartung and Munch [1929] it was found that hydrogen was rapidly absorbed up to 2 mols., *i.e.* in amount corresponding with the reduction of the ester to ethyl α -aminoacetonedicarboxylate. That the latter was indeed the product of the reaction at the stage under discussion was proved as follows: when boiled with 20 % hydrochloric acid it lost alcohol and carbon dioxide and the residue, when treated with alkali and mercuric chloride and steam-distilled, gave a satisfactory yield of 2 : 5-dimethylpyrazine; furthermore the partial reduction product gave, when heated with *p*-nitrophenylhydrazine in excess of dilute sulphuric acid, the characteristic *p*-nitrophenylosazone of methylglyoxal.

The reduction could not be carried beyond this stage at a reasonable rate under the conditions already described. If, however, the solution were evaporated and the residue re-dissolved in water, the remaining stage of the reduction could be accomplished in presence of the original catalyst activated by the addition of chloroplatinic acid and a trace of iron. Even so, the second stage (reduction of the carbonyl group) was much slower than the first (reduction of the *isonitroso*-group). When the uptake of hydrogen finally ceased the primary amino-nitrogen in the solution represented only about 40 % of the total nitrogen. It was apparent therefore that some ring closure had occurred, probably to give a pyrrolidonecarboxylic acid derivative; in any case the whole of the nitrogen could be converted into the amino-condition by boiling with 20 % hydrochloric acid, a process which simultaneously removed the ester groups.

At this point in the earlier experiments hydrochloric acid was removed as far as possible by evaporation of the solution under diminished pressure and the amino-acid was isolated from the residue through its silver salt as described by Dakin [1918]. Subsequently, however, the favourable properties of the hydrochloride (see below) have been utilised to obtain the bulk of the product directly as a crystalline derivative. In either case the total yield of the amino-acid amounted to about 60 % of the theoretical calculated on the ethyl α -*isonitroso*acetonedicarboxylate employed.

The free β -hydroxyglutamic acid obtained in the earlier experiments, being finally isolated by precipitation of its concentrated aqueous solution with alcohol, formed an amorphous powder apparently similar to the product obtained by Dakin. On one occasion, however, the mother-liquor, which contained more water than usual, deposited a little of the amino-acid after long standing in the form of crystalline crusts, and since that time no difficulty has been experienced in obtaining the acid in a crystalline condition from concentrated aqueous solution.

Since β -hydroxyglutamic acid contains two independent asymmetric carbon atoms it is evident that the synthetic product may consist of either one of two racemates or of a mixture. We have at present no satisfactory evidence to offer as a basis for a decision between these alternatives. Although we have at times observed the separation of the acid in crystals of apparently different forms, these forms have not shown any marked differences of solubility, nor have we been able to standardise the conditions of their separation. The more usual type of crystals obtained consists of stout prisms containing water of crystallisation which show no tendency to separate into different components when fractionally crystallised.

The constitution of the synthetic acid was proved by analysis of the acid itself, of its hydrochloride and of various metallic salts; also by the formation of malic semialdehyde as the result of oxidising the acid with 1 mol. of chloramine-*T*. Unfortunately we have so far been unable to isolate the amino-acid from natural sources, so that no direct comparison between the natural and synthetic products has been possible. It is indeed unlikely that any such comparison would be useful for purposes of identification, since for stereochemical reasons the physical properties of the naturally occurring active acid may well be profoundly different from those of the synthetic material. That some such marked difference does indeed exist is indicated by certain contrasts between the behaviour of our product and that of the amino-acid isolated by Dakin.

i- β -Hydroxyglutamic acid crystallises from concentrated aqueous solutions in well-formed prisms containing three molecules of water of crystallisation; it is extremely soluble in hot water but only moderately so in cold. Under certain circumstances, *e.g.* crystallisation from very concentrated solutions at a high temperature, the acid may separate in the anhydrous condition. The hydrated acid melts at 75°, re-solidifies and finally decomposes at 185°; the anhydrous compound melts sharply with decomposition at 198°. This is in marked contrast with the behaviour described by Dakin [1918] whose product had no sharp melting-point, but even at 105° lost water with ring closure to a hydroxypyrrolidonecarboxylic acid. No such ring closure takes place when the dry synthetic acid is heated at 110° even in a vacuum over phosphoric oxide. On the other hand, ring closure does occur when the amino-acid is boiled in aqueous solution, and at p_H 4 the process is considerably more rapid than with glutamic acid under similar conditions.

The synthetic amino-acid yields a well-formed hydrochloride somewhat

similar in character to glutamic acid hydrochloride although naturally more soluble than the latter. No mention of such a derivative is made by Dakin either in connection with natural or synthetic β -hydroxyglutamic acid. We now suspect, however, from some observations which we have made ourselves on the character of the crude precipitate of glutamic acid hydrochloride obtained from caseinogen in the usual manner, that unsuspected losses as the hydrochloride may account in part at least for the many failures to isolate β -hydroxyglutamic acid. In this connection the claim of Engeland [1922, footnote p. 136], based on methylation experiments, that a commercial preparation of glutamic acid hydrochloride contained as much as 50 % of β -hydroxyglutamic acid is of interest.

Finally as to colour reactions. Dakin described two principal colour reactions as characteristic for β -hydroxyglutamic acid, (a) a red colour with sodium diazobenzenesulphonate in presence of excess of sodium hydroxide, which deepened in intensity on warming, and (b) a green fluorescence on warming a solution of the acid with excess of sulphuric acid and a trace of β -naphthol. The pure synthetic amino-acid gives no colour whatever with sodium diazobenzenesulphonate and sodium hydroxide in the cold; on boiling the solution a bluish-red colour with no trace of yellow tinge is obtained¹. The reaction is not, however, very sensitive. The green fluorescence with β -naphthol and sulphuric acid is given by the synthetic amino-acid but again not with great intensity. In any case we do not regard either of these reactions as of great value. Traces of histidine entirely obscure the result of the former test, and there appear to be substances present in protein hydrolysates which give the β -naphthol-sulphuric acid reaction with even greater intensity than β -hydroxyglutamic acid itself.

It appears therefore that marked discrepancies exist between the behaviour of our synthetic product and that of the acid isolated by Dakin from natural sources. Many of these discrepancies could doubtless be explained on stereochemical grounds and it is therefore better not to discuss them further until the resolution of the synthetic acid has been effected and until a reliable method for the isolation of the natural acid has been devised. The first of these problems we hope to take up in the near future; with the second we have been occupied for some time without success. We have repeatedly followed with meticulous care the procedure recommended by Dakin [1918] but have invariably failed to obtain the acid in the manner which he describes. The fraction which should contain β -hydroxyglutamic acid has always consisted of a small amount of gummy material more or less heavily contaminated with glutamic and aspartic acids. That β -hydroxyglutamic acid does indeed occur among the products of hydrolysis of caseinogen we do not for a moment doubt; unequivocal proof of this is afforded by the fact that certain fractions of the

¹ In a private communication to one of us Dr H. D. Dakin agrees that, contrary to his original statement, no colour should be given with sodium diazobenzenesulphonate and sodium hydroxide in the cold.

hydrolytic products yield not inconsiderable amounts of malic semialdehyde when oxidised with chloramine-*T*. We are, however, forced to the conclusion that the separation of β -hydroxyglutamic acid from the other dicarboxylic amino-acids is not by any means so straightforward a process as the original description by Dakin [1918] might lead one to suppose. The mere separation of a synthetic mixture of glutamic and β -hydroxyglutamic acids into its components is a task of considerable difficulty, although our experiments on this point, detailed description of which is reserved for a later communication, have afforded indications of possible lines of attack on the major problem. In the meantime the synthesis of the inactive amino-acid and its properties together with those of some of its simple derivatives are described below.

One of us (C.R.H.) has, during the progress of this work, enjoyed the benefit of consultation by correspondence with Dr H. D. Dakin, concerning the various points which have arisen.

EXPERIMENTAL.

Preparation of ethyl α -isonitrosoacetonedicarboxylate.

The following method has been found more convenient for the preparation of the above compound in quantity than that described by Pechmann [1891].

Ethyl acetonedicarboxylate (20 g.) is dissolved in ether (200 cc.) in a 500 cc. round-bottom flask under a reflux condenser. Hydrogen chloride is passed into the solution at the rate of 2–3 bubbles per second. A solution of ethyl nitrite [*cf.* Thiele and Eichwede, 1900, footnote p. 366] (8.4 cc.) in ether (50 cc.) is added in 10 cc. portions through the condenser. Each addition of ethyl nitrite causes a yellowish-brown coloration and a slight rise of temperature; a fresh quantity of ethyl nitrite is only added when the colour produced by the preceding addition has been discharged. After the final addition of ethyl nitrite, passage of hydrogen chloride is continued for 15 minutes, and the mixture is then set aside overnight. Next day the bulk of the ether is distilled off, and the residue is twice shaken out with excess of dilute sodium carbonate solution. The latter is then acidified with hydrochloric acid and extracted four times with ether. The combined ethereal extracts are dried over calcium chloride and evaporated, finally in a vacuum desiccator over sulphuric acid. The yield of pure ester is almost quantitative.

Reduction of ethyl α -isonitrosoacetonedicarboxylate.

The catalyst was prepared by dissolving 1 g. of palladium chloride in 100 cc. of water with the addition of 1 cc. of concentrated hydrochloric acid, adding 6 g. of "super-norite" charcoal and shaking in an atmosphere of hydrogen until reduction was complete. The product was filtered off, washed with water until free from chloride and dried in a vacuum desiccator. Ethyl α -isonitrosoacetonedicarboxylate (10 g.) was dissolved in absolute alcohol (100 cc.) containing hydrogen chloride (5 g.); 3 g. of the palladium-charcoal were added and

the mixture was shaken in an atmosphere of hydrogen. Absorption of the latter occurred rapidly and continued at a moderate rate until 2 mols. had been taken up when the process became extremely slow; in various experiments this first stage occupied 1.5 to 2.5 hours.

When the absorption had practically ceased the catalyst was filtered off and washed with alcohol; the filtrate and washings were freed from alcohol by evaporation under diminished pressure and the residue was taken up in water (100 cc.). The original catalyst was returned to the solution and in addition there were added 5 cc. of a solution containing 10 % of chloroplatinic acid and 0.5 % of ferric chloride. Hydrogenation was then begun again and allowed to proceed until the remaining 1 mol. had been absorbed. When this had occurred (after 6–8 hours) the solution was filtered. The catalyst was extracted with boiling water and the combined filtrates were evaporated to 100 cc. under diminished pressure.

i-β-Hydroxyglutamic acid.

The solution obtained as described above should theoretically contain ethyl β-hydroxyglutamate. It was found, however, that only about 40 % of the total nitrogen was in the amino condition. Concentrated hydrochloric acid (100 cc.) was therefore added and the whole was boiled under a reflux for 1.5 hours. After this treatment the amino-nitrogen and total nitrogen were identical. Subsequent treatment followed two alternative lines.

(a) The solution was evaporated under diminished pressure, the process being repeated several times after addition of small amounts of water. The residue was taken up in water and treated with 20 % silver nitrate in amount just sufficient to precipitate the remaining hydrochloric acid. The filtrate from the silver chloride was made faintly alkaline to litmus and further silver nitrate solution was cautiously added. The precipitate produced by the first few drops immediately blackened, owing doubtless to the persistence of some reducing material. The addition of silver nitrate solution was carefully continued until a transient white precipitate was produced; at this stage the solution was set aside overnight. Next day the black precipitate was removed by filtration and the amino-acid was completely precipitated from the filtrate by alternate addition of 20 % silver nitrate and 2*N* sodium hydroxide. The silver salt was filtered off, thoroughly washed with water, and decomposed with hydrogen sulphide. The filtrate from the silver sulphide was concentrated to a thin syrup under diminished pressure and poured into excess of alcohol. The precipitate, at first sticky, gradually hardened and was ultimately obtained as a friable powder; it was dried in a vacuum desiccator over phosphoric oxide.

(b) After the properties of the well-characterised hydrochloride had been discovered it was found more convenient to evaporate the hydrochloric acid solution resulting from hydrolysis of the reduction product to a thin syrup under diminished pressure and to saturate the residue at 0° with hydrogen

chloride. After standing overnight in the ice-chest the hydrochloride was filtered off and the remainder of the amino-acid was isolated from the mother-liquor by the method described above. The free amino-acid was obtained in the crystalline condition by addition of 1 equivalent of sodium hydroxide to the concentrated aqueous solution of the hydrochloride, or by dissolving the amorphous preparation obtained as described above in a small amount of hot water and allowing the solution to stand. Occasionally, if the solution be very concentrated and warm when crystallisation commences, the acid separates in the anhydrous condition in stout needles or narrow rhombic prisms. More usually, however, it is obtained as stout prisms which may reach a considerable size and contain $3\text{H}_2\text{O}$; the latter is lost in a vacuum over phosphoric oxide.

Analysis. 0.1554 g. of the air-dried acid lost 0.0378 g. *in vacuo* at 50° over phosphoric oxide. Found: loss of weight 24.3 %; calculated for $\text{C}_5\text{H}_9\text{O}_5\text{N}$, $3\text{H}_2\text{O}$, loss of weight 24.9 %.

The anhydrous acid gave: C, 36.5, 36.8 %; H, 5.6, 5.5 %; N, 8.5, 8.6 %; amino-N, 8.7 %. $\text{C}_5\text{H}_9\text{O}_5\text{N}$ requires: C, 36.8 %; H, 5.5 %; N, 8.6 %.

0.2082 g. required for neutralisation to phenolphthalein 12.48 cc. of 0.099 *N* sodium hydroxide. The acid therefore titrates as a monobasic acid of mol. wt. 168 as against the calculated value of 163.

Behaviour on heating. When heated in a capillary tube the air-dried acid melts partially at about 75° and then re-solidifies; at 102 – 103° obvious loss of water occurs and the whole finally decomposes at 185° . The anhydrous acid melts sharply with vigorous effervescence at 198° . No apparent change occurs when the dry amino-acid is heated at 110° either in the air or in a vacuum over phosphoric oxide. That ring closure can be brought about with moderate ease is shown, however, by the following experiment. 0.5 g. of the acid was dissolved in 5 cc. of water and the solution was boiled under reflux for 7 hours, by which time the ninhydrin reaction had become very faint. Analysis indicated more than 80 % loss of amino-nitrogen. The solution was evaporated to a syrup on the water-bath and the residue was extracted with alcohol. The extract was filtered from unchanged amino-acid and evaporated again to a syrup which slowly crystallised. The product had N, 9.4 % (calculated for $\text{C}_5\text{H}_7\text{O}_4\text{N}$; N, 9.65 %) and yielded only a trace of nitrogen in the Van Slyke apparatus. A comparison between the rates of ring closure of glutamic and β -hydroxyglutamic acids was kindly carried out by Prof. A. C. Chibnall who obtained the following figures (the acids were boiled in aqueous solution at p_{H} 4).

	% of total N as amino-N after		
	1 hr.	2 hrs.	3 hrs.
Glutamic acid	96.9	93.0	84.3
β -Hydroxyglutamic acid	81.2	75.5	62.7

Reactions. The acid, after neutralisation to litmus, gives an intense ninhydrin reaction. With sodium diazobenzenesulphonate and excess of sodium hydroxide it gives no colour in the cold; on boiling a bluish-red colour develops.

When an aqueous solution of the acid is treated with rather more than its own volume of sulphuric acid and the mixture is warmed with a trace of β -naphthol a yellowish solution with a green fluorescence results. The vapours evolved on fusing the acid with zinc dust give an intense pine splinter reaction for pyrrole.

Oxidation with chloramine-T. 0.815 g. of the acid in 5 cc. of water was neutralised with *N* sodium hydroxide (5 cc.) and treated with a solution of chloramine-*T* (1.44 g.) in water (5 cc.). Almost immediate precipitation of toluenesulphonamide occurred. After 30 minutes the reaction was completed by gentle warming, the solution was chilled and the toluenesulphonamide filtered off. The filtrate was treated with a hot solution of *p*-nitrophenylhydrazine (2 g.) in glacial acetic acid and transferred to the steam-bath. Precipitation of the osazone began at once; after 45 minutes the precipitate was filtered off, washed with warm alcohol and dried; it weighed 0.5 g. After recrystallisation from nitrobenzene it formed brownish-red prismatic needles, m.p. 291° (uncorr.). (Found: N, 22.6 %. $C_{16}H_{14}O_6N_6$ requires: N, 22.6 %.)

Derivatives of i- β -hydroxyglutamic acid.

Hydrochloride. This was prepared as described above. It forms colourless prismatic needles, readily soluble in water, sparingly so in concentrated hydrochloric acid. It has m.p. 187° (decomp.). (Found: C, 29.8, 30.0 %; H, 5.0, 5.1 %; N, 7.1, 7.0 %; amino-N, 7.3 %; Cl, 17.7 %. $C_5H_{10}O_5NCl$ requires: C, 30.0 %; H, 5.0 %; N, 7.0 %; Cl, 17.8 %.)

Ethyl ester hydrochloride. β -Hydroxyglutamic acid hydrochloride (7 g.) was suspended in alcohol (70 cc.) and the mixture was saturated with hydrogen chloride. The resulting clear solution was boiled under reflux for 3 hours and then concentrated under diminished pressure. During this process the greater part of the ester hydrochloride crystallised out. This was filtered off (6 g.) and recrystallised from alcohol. m.p. 168.5°. (Found: N, 5.2 %. $C_9H_{18}O_5NCl$ requires: N, 5.5 %.)

Silver salt. Prepared by adding silver nitrate (2 mols.) to a solution of the disodium salt of β -hydroxyglutamic acid (1 mol.) filtering, washing and drying in a vacuum desiccator in the dark. (Found: N, 3.6 %. $C_5H_7O_5NAg_2$ requires: N, 3.7 %.)

The normal *copper* salt had N, 5.9 %, Cu, 28.0 % (calc.: N, 6.2 %, Cu, 28.3 %) and the *calcium hydrogen* salt had Ca, 11.5 % (calc.: Ca, 11.0 %). These salts were prepared according to Dakin [1918] and resembled the products described by him.

Identification of intermediate product in the reduction of ethyl α -isonitrosoacetonedicarboxylate.

(a) Ethyl α -isonitrosoacetonedicarboxylate (5 g.) was subjected to the first stage of the reduction in the manner described above. The residue obtained by evaporation of the alcoholic solution was dissolved in 20 % hydrochloric acid (50 cc.) and boiled under reflux. Decarboxylation set in rapidly and was

complete in 45 minutes. The solution was evaporated under diminished pressure, the evaporation being several times repeated after addition of small amounts of water. The residue was taken up in water (35 cc.) and the solution was added with cooling to 80 cc. of 33 % potassium hydroxide; 10 g. of mercuric chloride dissolved in cold water (100 cc.) were then added and the whole was immediately steam-distilled. After 75 cc. had passed over the base had practically ceased distilling. The distillate was treated with a solution of mercuric chloride which produced a crystalline precipitate of the mercuric chloride double salt with 2 : 5-dimethylpyrazine; after standing overnight the precipitate was collected, washed and dried in a desiccator. It amounted to 5 g. corresponding with a yield of 70.5 % of the theoretical calculated on the ester originally taken. (Found: Hg, 62.2 %. $C_6H_8N_2 \cdot 2HgCl$ requires: Hg, 61.7 %.) The base recovered from the mercuric chloride salt was further identified by conversion into the *picrate*, M.P. 157°. (Found: picric acid, 68.5 %; calculated, 67.9 %), and the *chloroaurate*, M.P. 153°.

(b) The residue from the evaporation of the alcoholic solution of the first stage reduction product from a second quantity of 5 g. of ethyl α -isonitroso-acetonedicarboxylate was treated with a solution of *p*-nitrophenylhydrazine (5 g.) in excess of hot 5 % sulphuric acid and transferred to the steam-bath. Within a few minutes the solution became filled with the characteristic scarlet needles of methylglyoxal *p*-nitrophenylosazone. After 1 hour the precipitate was filtered off, washed with alcohol and dried (1.4 g.). After recrystallisation from nitrobenzene it had M.P. 297° (decomp. uncorr.).

NOTE ON THE DISSOCIATION CONSTANTS.

By GEORGE MAXWELL RICHARDSON¹.

Application of amino-acid dissociation constants to the interpretation of protein titration curves is a recognised procedure which depends upon accurate knowledge of these constants and concise statement of the conditions of measurement. Data are now presented for crystalline preparations of β -hydroxyglutamic acid, accessible to measurement for the first time.

Bjerrum's zwitterion hypothesis, which possesses the cumulative experimental evidence of Borsook and MacFadyen [1930], Michaelis and Mizutani [1925], and Birch and Harris [1930, 1, 2] in support of its more logical interpretation of the behaviour of amino-acid solutions, is adopted throughout the text. Basic dissociation constants are calculated as constants of hydrion dissociation, after Brønsted [1923]. These generally accepted conventions,

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presented more fully by Greenstein [1931], allow an abbreviated formulation of the acid-base exchanges as follows:

Decreasing magnitudes, K_1' , K_2' , K_3' , represent the successive dissociation constants (uncorrected for activity of the various species of A) possible within this scheme. In this paper, however, calculation of the slightly different

Fig. 1. Titration of 0.02 *M* β -hydroxyglutamic acid, 25°.

Calculated titre, uncorrected ————— Corrected - - - - -
 Experimental titre, uncorrected x Corrected o

titration constants of Simms [1926], G_1' , G_2' , G_3' , is made. It is to be emphasised that electrometric titration of any dicarboxylic amino-acid is attended by the usual difficulties of technique and calculation relating to comparatively acid solutions, and that this susceptibility to error will necessarily appear in the values of G_1' .

Experimental data are presented in Fig. 1, the curve representing calculated requirements, the points experimental findings. Table I presents a portion of these data arranged for calculating Simms's titration constants. Experimental titres are corrected throughout for the concentration of uncombined HCl or

NaOH at the stated p_H . Bracketed values represent determinations in poorly buffered regions, or values embodying heavy correction. The Sørensen logarithmic nomenclature (p_{G_1} , etc.) is adopted.

Table I. *Calculation of titration constants.*

25°. 0.02 *M* β -hydroxyglutamic acid, 3H₂O. 0.1012 *M* NaOH. 0.1972 *M* HCl.

		p_{G_2}' (0.1122 g. acid in 25 cc.)						
		Correction (equivs.) for						
NaOH			H ⁺	+A	+A ⁻⁻		a_2	p_{G_3}'
cc.	p_H	μ						
0	3.219	0.00193	0.0299	0.0704	—		0.1003	4.172
0.32	3.368	0.00262	0.0216	0.0510	—		0.1352	4.174
0.65	3.518	0.00347	0.0156	0.0366	—		0.1794	4.178
1.25	3.753	0.00531	0.0093	0.0216	—		0.2756	4.173
2.02	4.019	0.00782	0.0053	0.0119	—		0.4126	4.172
2.50	4.176	0.00936	0.0037	0.0083	—		0.5014	4.174
3.00	4.337	0.0109	0.0027	0.0057	—		0.5957	4.169
3.70	4.598	0.0131	0.0015	0.0032	—		0.7291	4.168
4.24	4.863	0.0147	0.0008	0.0017	—		0.8326	4.166
4.51	5.054	0.0155	0.0005	0.0011	0.0001		0.8845	4.170
4.81	5.381	0.0161	0.0003	0.0005	0.0002		0.9423	{ 4.168 4.196 4.262 }
5.01	5.900	0.0167	0.0001	0.0002	0.0005		0.9806	
5.06	6.240	0.0168	—	0.0001	0.0011		0.9896	
5.108 (= first equiv.)								
							Mean	4.172

p_{G_1}' (0.1089 g. in 25 cc.)			p_{G_3}' (0.1122 g. in 25 cc.)		
HCl			NaOH		
cc.	p_H	p_{G_1}'	cc.	p_H	p_{G_3}'
0	3.221	2.10	5.12	6.881	9.20
0.125	3.106	2.09	5.20	7.485	9.20
0.40	2.886	2.09	5.30	7.817	9.23
0.90	2.595	2.10	5.49	8.126	9.22
0.93	2.582	2.10	5.92	8.474	9.21
1.49	2.357	2.09	6.60	8.801	9.20
2.25	2.149	2.11	7.49	9.119	9.20
3.01	1.989	2.11	8.41	9.423	9.19
4.25	1.803	2.10	9.10	9.686	9.18
6.01	1.634	2.14	9.75	10.057	9.19
9.76	1.418	2.06	10.25	10.553	9.20
13.60	1.299	2.10	10.51	10.860	9.19
			11.10	11.262	—
			11.76	11.497	—
			12.77	11.700	—
			14.02	11.859	—
Mean 2.10			Mean 9.20		

In discussion of the data various points arise. Firstly, the table provides an interesting comparison between the very constant p_{G_2}' , values, where ionic strength and H⁺ concentration are both comparatively low, and the erratic p_{G_1}' , or the decreasing p_{G_3}' , values, where a large correction for free H⁺, or a rapidly increasing ionic strength, respectively, are involved. Secondly, certain evidence for a fourth (hydroxy) dissociation constant at about p_H 13 may be detected in Fig. 1, though the absence of any satisfactory basis for correcting for free OH⁻ precludes definite evaluation of the constant. An attempt to eliminate the effect by larger OH⁻ correction disturbs the terminal p_{G_3}' values and fails to give good alignment of the end-point. Thirdly, preliminary titration of a preparation, dried at 50° over P₂O₅, revealed that titratable

groups about p_H 9 exceeded the more acid titratable groups by about 4 %. A further recrystallisation, however, reduced the extent of the divergence to about 2 %, approaching the limits of possible experimental error. The interfering p_G lay between 9.0 and 9.4. As neither analysis nor method of preparation offers an easy explanation for a defect of $-\text{COOH}$ as against $-\text{NH}_2$ groups, the observation is merely recorded. Fourthly, it may be noted that an air-dried sample of the β -hydroxyglutamic acid containing $3\text{H}_2\text{O}$ was found to titrate with respect to its acid groups as 75 % hydroxyglutamic acid by weight (theory 75.1 %). The anhydrous preparation titrated as 99 % acid.

Table II. *Dissociation constants at 25°.*

	Mol. conc.	$-\text{COOH}$	$-\text{COOH}$	$-\text{NH}_2$	Diff. ($p_{\text{K}_{\text{NH}_2'}}$ - $p_{\text{K}_{\text{NH}_2}}$)	Authors
Glutamic acid	—	2.15	4.22	9.60	—	Various [<i>vide</i> Kirk and Schmidt, 1929]
β -Hydroxyglutamic acid	—	2.32	4.23	9.56	0.04	[Kirk and Schmidt, 1929]
β -Hydroxyglutamic acid	0.02	2.09	4.18	9.20	0.40	This paper
β -Asparagine	0.02	2.02	—	8.80	—	[Chibnall and Cannan, 1930]
β -Hydroxyasparagine	0.01	2.12	—	8.26	0.54	" " "
Aspartic acid	0.02	2.05	3.76	9.85	—	" " "
Hydroxyaspartic acid	0.02	1.95	3.47	9.03	0.82	" " "

The comparison of the constants here determined with those of Kirk and Schmidt [1929] reveals definite discrepancy in the case of the third (amino) dissociation constant. It is believed, however, that the synthetic origin and purity of our preparation, and the satisfactory mathematical analysis accorded by the data are sufficient evidence for the greater reliability of the present constants. This belief is further supported by the comparisons of Table II, in which our data are compared with those of Kirk and Schmidt, and with the data of Chibnall and Cannan [1930] for hydroxyaspartic acid and its amides. The influence of an adjacent hydroxyl group on the p_G of the amino-group is to be noted. In particular, the glutamic acids should be compared with the β -asparagines, where there is likewise an α -amino group, a β -hydroxy group, and an almost non-polar γ -group.

Finally, for general reference, the p_G constants are converted to the real dissociation constants by the formula of Simms [1926]. Thus, the successive values of p_K , determined by titration of 0.02 M β -hydroxyglutamic acid at 25°, become 2.09, 4.18 and 9.20.

EXPERIMENTAL.

Titration were conducted in the manner of Cannan and Knight [1927]. Reagents and apparatus were prepared and standardised with the usual precautions. Temperature was maintained closely at 25°. The half-cell adopted was a saturated calomel electrode, standardised against 0.1 M HCl from a constant boiling-point stock. Junction was made at the tip of a goose-neck saturated KCl bridge of the Clark type. The H_2 electrode potential assumed for 0.1 M HCl was 0.0637 v., junction potentials being deliberately ignored throughout.

Care was observed particularly during acid standardisation or titration, to prevent fortuitous junction potentials arising from a dead space in the tip, containing, between the KCl and the external liquid, a solution of some previous p_H . On the other hand, increase of ionic strength of the solution due to KCl introduction was minimised. Potentials were established at duplicate, freshly palladinised, gold-plated platinum wire electrodes, and measured against a Cambridge potentiometer and galvanometer. In no observation did duplicate potentials differ by more than 0.2 mv. and there was generally agreement closer than 0.1 mv. An exception arose, however, with those less pure preparations already mentioned, variations up to 0.4 mv. occurring about p_H 10.

Finally, due mention must be made of the basis upon which observed H^+ activities (p_H) were converted into H^+ or OH^- concentrations. The simplified Debye-Hückel equation, $-\log \gamma = a\sqrt{\mu}$, where a varies with the dielectric constant of the solution and the degree of solvation of the ions, was used. In these calculations, a has been accepted, empirically, as a constant, 0.245, for both H^+ and OH^- .¹

In calculation of μ , the ionic strength, it is assumed for simplicity that a positive and negative charge on the same ion are mutually neutralised in their influence on the ionic atmosphere. Thus $+A^-$ is disregarded and $+A^{--}$ is regarded as a univalent ion [compare Greenstein, 1931]. On this basis, μ increases most rapidly during the reaction $+A^{--} \rightarrow A^{--}$, which would produce just that gradual decrease in p_{G_s} values which Table I reveals. On the other hand, the full assumption is contrary to the conclusions of Borsook and MacFadyen [1930], who wittingly offer no alternative formulation for the modified participation of such ions. On the stated basis, μ is readily shown to equal the sum

$$[+A] + [H^+] + [Na^+] + [A^{--}],$$

where, with negligible error,

$$[+A] = [A] \frac{H^2}{(G_1 + H)(G_2 + H)}, \text{ and } [A^{--}] = [A] \frac{G_3}{(G_3 + H)},$$

$[A]$ representing total amino-acid concentration and H , H^+ activity. OH^- activities were derived from H^+ activities by assuming Lewis, Brighton, and Sebastian's value [1917] for p_{K_w} , 13.995, without modification for altered dielectric constant.

¹ The activity of these two ions in solutions of moderate ionic strength is shown by data of Lewis and Randall to be closely comparable, while the above value of a is of similar order to that adopted by Simms for H^+ (0.28). It differs from the decreasing value of Randall and Breckenridge [1927] for chloride solutions of increasing ionic strength. It was found that the values of these authors gave no concordant data in the calculation of p_{G_1} . The critical survey of Borsook and MacFadyen [1930] is sufficient justification in this case for selecting an empirical constant.

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