288. *Amino-acids and Peptides. Part VIII.* Determination of* D- *and* L-*Glutamic Acid by the Isotope-dilution Method.*

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A new method based on the isotope dilution principle has been developed for the determination of the total (D- + L-)glutamic acid in mixtures of amino-acids. In control experiments with synthetic mixtures the glutamic acid found was 99·2—99·3% of the theoretical amount. The procedure has been applied to the analysis of gliadin.

Accurate determination of both enantiomorphs of an amino-acid in a protein hydrolysate is difficult. Procedures based on partition chromatography (*e.g.*, Moore and Stein, *Ann. N.Y. Acad. Sci.*, 1948, **49**, 265) and on ion-exchange (*e.g.*, *idem*, *J. Biol. Chem.*, 1951, **192**, 663) determine the total of both isomers (D and L), but so far their accuracy appears to be

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rather less than is often desirable for investigations of protein structure. The isotope-dilution principle, avoiding as it does the difficulty of quantitative isolation, is clearly attractive for this purpose. In its most usual application, the L-amino-acid is isolated and therefore this form only is assayed. A modification has already been used by which both enantiomorphs of glutamic acid have been determined (Graff, Rittenberg, and Foster, *J. Biol. Chem.*, 1940, **133**, 745; Wieland and Paul, *Ber.*, 1944, **77**, 34). Isotopic DL-glutamic acid hydrochloride is added to the mixture to be analysed, from which pure L- and DL-glutamic acid hydrochlorides are then isolated; from the isotopic dilution of these two samples, the amount of D(and L)-glutamic acid may be calculated. It is however sometimes difficult to isolate the pure racemate, since it is normally present in low concentration and has a higher solubility than the enantiomorphs, and it is then necessary to determine the composition of the mixture of isomers by measuring its optical rotation.

We have developed an alternative method for the determination of the total (D- and L-)glutamic acid, using the isotope-dilution principle. A known weight of L-[^{15}N]glutamic acid hydrochloride is added to the amino-acid mixture, which is then benzoylated, and the solution is treated with acetic anhydride at room temperature for 48 hours. Under these conditions, benzoylglutamic acid is racemised and after hydrolysis of the benzoyl derivatives a sample of DL-glutamic acid hydrochloride can readily be isolated in the normal way. From the isotopic dilution, the total glutamic acid originally present can be calculated directly. Loss of glutamic acid before racemisation is achieved will affect the determination only if optical isomers are lost differentially, whilst after racemisation such loss will cause no error. Control experiments showed that under the above conditions benzoyl-L-glutamic acid is racemised to the extent of 97·5%; calculation shows that no appreciable error is introduced by this incompletion. For example, if 5% of the glutamic acid is originally present as the D-form and if racemisation is 95% complete, the error is less than 0·03% of the total glutamic acid (if isotopic dilution is 1 : 10).

This procedure has been tested by the analysis of known mixtures of amino-acids, containing both D- and L-glutamic acid. The isolation and conversion of the samples into nitrogen, and the mass-spectrometric analyses (for which we are indebted to the Atomic Energy Research Establishment, Harwell) followed the procedure described in Part V (*J.*, 1951, 3047). The results are shown in Table 1. The total glutamic acid

TABLE 1.

Synthetic mixture	Total glutamic acid hydrochloride present (g.)	L-[^{15}N]Glutamic acid hydrochloride added, g.	Atoms-% excess ^{15}N	DL-Glutamic acid hydrochloride isolated, No. of cryst.	Atoms-% excess ^{15}N	Total glutamic acid hydrochloride found (%)
A	0·7308	0·1036	14·956	5	1·869	99·25
				6	1·869, 1·872	99·20
				7	1·865, 1·865	99·50
						Mean 99·3
B	0·7051	0·0998	14·956	5	1·864, 1·865	99·37
				6	1·863, 1·862	99·48
				7	1·869, 1·866	99·19
						Mean 99·3
C	0·6450	0·1000	14·998	5	2·021, 2·028	99·35
				6	2·030	99·06
				7	2·024, 2·023	99·43
						Mean 99·3
D	0·6870	0·0892	14·998	5	1·737	99·09
				6	1·731, 1·734	99·40
				7	1·736, 1·738	99·13
						Mean 99·2
E	2·0052	0·3485	15·214	4	2·266	99·34
				5	2·266, 2·268	99·26
				6	2·265	99·35
						Mean 99·3

found in each experiment is 99·2—99·3% of the theoretical amount. From the close agreement with the result of Experiment *E*, in which the L-glutamic acid hydrochloride used in this work was itself analysed by this method, it seems likely that the accuracy may be slightly greater than these figures suggest.

As in the determination of the L-isomer, it is important to establish the purity of the isolated sample. Differential solubility tests (Part III, *J.*, 1951, 1279; Part IV, *ibid.*, p. 1745) will not detect the presence of a slight excess of one enantiomorph in the racemate, since the D- and the L-form dissolve independently, but they will detect other impurities in the normal manner. Optical-rotation measurements will then give satisfactory evidence of optical purity, when the absence of foreign contaminants has been established. It is however a great advantage of this procedure that for most applications even gross contamination of the racemate by the L-isomer has a negligible effect on the analysis. For example, if 5% of the glutamic acid is originally present as the D-form, racemisation is 95% complete, and the isotopic dilution is 1 : 10, it may readily be calculated that the presence in the isolated racemate of 5% of the L-isomer would result in an additional error of only 0·02%.

The total glutamic acid obtained on hydrolysis of a sample of gliadin (kindly supplied by Professor A. C. Chibnall) was determined by this method and the results are shown in Table 2. The mean value of 45·8 g. of dry, ash-free protein compares with the corresponding figure of 44·1 g. for the L-isomer from the same sample (Part V, *loc. cit.*), the difference

TABLE 2.

Experiment	Gliadin, g.	L-[^{15}N]Glutamic acid hydrochloride added, g.	Atoms-% excess ^{15}N	DL-Glutamic acid hydrochloride isolated: No. of cryst.	Atoms-% excess ^{15}N	Total glutamic acid found, G. per 100 g. of gliadin *	N, % of protein-N
(i)	1·5176	0·0598	14·810	4	1·061	45·83	24·64
				5	1·062, 1·060	45·85	24·65
				6	1·061, 1·060	45·87	24·66
					Mean	45·85	24·65
(ii)	1·3332	0·0728	14·810	4	1·435, 1·431	45·76	24·60
				5	1·434, 1·436	45·70	24·57
				6	1·434	45·73	24·59
					Mean	45·73	24·59
				Mean of experiments (i) and (ii):		45·8	24·62

* Calc. for dry, ash-free protein.

(1·7 g.) representing D-glutamic acid in the hydrolysate. This is remarkably close to the estimate (1·85 g.) based on optical-rotation measurements of fractions isolated by the gravimetric method by Chibnall and his colleagues (*J. Int. Soc. Leather Trades' Chem.*, 1946, **30**, 11). This amount may of course arise from racemisation during hydrolysis, a point that is now under investigation.

EXPERIMENTAL

Determination of the Total Glutamic Acid in Synthetic Mixtures.—(*A*) A solution of L-glutamic acid hydrochloride (0·7186 g.), DL-glutamic acid hydrochloride (0·0122 g.), and L-[^{15}N]-glutamic acid hydrochloride (0·1036 g.) in water (80 c.c.) was made alkaline to phenolphthalein paper with 5N-sodium hydroxide. Sodium carbonate (3·27 g.) was added and the solution was cooled to 0—2°. Benzoyl chloride (4·8 c.c.) was added, and the reaction mixture stirred vigorously for 4½ hours, after which the solution still gave a faintly positive ninhydrin test. Further additions of sodium carbonate (0·82 g.) and benzoyl chloride (1·1 c.c.) were made; stirring was continued for another 3 hours, a negative ninhydrin test being then obtained. When the solution had regained room temperature, acetic anhydride (60 c.c.) was added and the mixture was left overnight. A further volume of acetic anhydride (60 c.c.) was then added, and the solution left for 30 hours, after which it was evaporated to dryness below 50° under reduced pressure. The residual white solid was dissolved in water (40 c.c.), made acid to Congo-red with 5N-hydrochloric acid, and again evaporated to dryness *in vacuo*. The residue

was extracted with dried acetone, and the extract filtered and evaporated to dryness under reduced pressure, leaving a white solid which was refluxed for 4½ hours with 15% hydrochloric acid (45·5 c.c.). On cooling of the solution, benzoic acid separated and was removed by extraction with benzene. The aqueous layer was evaporated to dryness under reduced pressure; the residue was dissolved in a small volume of water, treated with charcoal, filtered, and concentrated to small bulk. The solution was saturated with hydrogen chloride at 0°. After being kept overnight in the cold, a crystalline mass separated and was filtered off, washed, and dried (0·55 g.); it had $[\alpha]_D^{18}$ +0·45° (*c*, 3·0 in 5% hydrochloric acid).

The crude DL-glutamic acid hydrochloride was recrystallised in all-glass apparatus, the first fraction which should contain any unchanged L-glutamic acid hydrochloride being rejected. The second fraction was filtered off through sintered-glass, washed, and recrystallised. Crystallisation was repeated 7 times and portions from each crystallisation were powdered and transferred to a vacuum-desiccator containing calcium chloride and sodium hydroxide pellets.

Differential-solubility tests for purity were carried out on the later fractions by the vapour-pressure method described in Part III (*J*, 1951, 1279). After the fourth crystallisation the product had no observable rotation (*c*, 1·0 in 5% hydrochloric acid).

No. of crystallisations	4	5	6	7
Yield (based on crude wt.), %	67	53	45	42
V. p. difference (mm. of oil)	4	3	3	3

(*B*) A solution of L-glutamic acid hydrochloride (0·6939 g.), DL-glutamic acid hydrochloride (0·0112 g.), and L-[^{15}N]glutamic acid hydrochloride (0·0998 g.) in water (75 c.c.) was benzoylated and racemised as described for synthetic mixtures *A*. DL-Glutamic acid hydrochloride (0·49 g.; $[\alpha]_D^{17}$ +0·5°, *c*, 2·5 in 5% hydrochloric acid) was isolated and crystallised seven times in all, in the usual manner.

No. of crystallisations	5	6	7
Yield (based on crude wt.), %	66	58	49
V. p. difference (mm. of oil)	3	4	3

(*C*) The mixture contained L-glutamic acid hydrochloride (0·6345 g.), DL-glutamic acid hydrochloride (0·0105 g.), L-[^{15}N]glutamic acid hydrochloride (0·1000 g.), L-aspartic acid hydrochloride (0·08 g.), and L-lysine hydrochloride (0·015 g.).

The mixture was benzoylated and racemised in the usual manner. On completion of the hydrolysis of the *N*-benzoyl-DL-glutamic acid with 15% hydrochloric acid and removal of the free benzoic acid, the aqueous layer was evaporated to dryness, redissolved in water (20 c.c.), made acid to Congo-red, and treated with a solution of ether-soluble phosphotungstic acid (3·5 g. in 2 c.c. of water) at 70° for the removal of basic amino-acids; the amino-dicarboxylic acids were extracted by using the anion-exchange resin Amberlite IR-4B (both procedures were as described in Part V, *J*, 1951, 3047). DL-Glutamic acid hydrochloride (0·45 g.; $[\alpha]_D^{21}$ +0·75°, *c*, 2·0 in 5% hydrochloric acid) was finally isolated and crystallised 7 times from 5N-hydrochloric acid as described in the previous experiments.

No. of crystallisations	5	6	7
Yield (based on crude wt.), %	58	44	39
V. p. difference (mm. of oil)	4	3	—

(*D*) A solution of the mixture containing L-glutamic acid hydrochloride (0·6743 g.), DL-glutamic acid hydrochloride (0·0127 g.), L-[^{15}N]glutamic acid hydrochloride (0·0892 g.), L-aspartic acid hydrochloride (0·08 g.), L-lysine hydrochloride (0·01 g.), DL-alanine (0·03 g.), L-tyrosine (0·01 g.), and glycine (0·01 g.) was treated as described for mixture *C*, and DL-glutamic acid hydrochloride (0·46 g.; $[\alpha]_D^{20}$ + 0·6°, *c*, 3·0 in 5% hydrochloric acid) was isolated and crystallised 7 times in all.

No. of crystallisations	5	6	7
Yield (based of crude wt.), %	60	52	45
V. p. difference (mm. of oil)	5	3	4

(*E*) L-Glutamic acid hydrochloride (2·0052 g.) and L-[^{15}N]glutamic acid hydrochloride (0·3485 g.) were dissolved in water (60 c.c.). The analytical procedure followed that for mixture *A*, yielding 1·5 g. of crude DL-glutamic acid hydrochloride, which was crystallised 6 times.

No. of crystallisations	4	5	6
Yield (based on crude wt.), %	58	52	45
V. p. difference (mm. of oil)	3	4	3

The isolated samples from each experiment were converted into nitrogen by Rittenberg's method (" Preparation and Measurement of Isotopic Tracers," Edwards, Ann Arbor, Michigan, 1947). The mass-spectrometric determinations were carried out by Mr. Palmer and his colleagues at the Atomic Energy Research Establishment, Harwell. The $^{15}N : ^{14}N$ ratio was calculated from the relative intensity of the peaks at mass numbers 29 and 28, as in Part V (*loc. cit.*); corrections were similarly applied for air and carbon dioxide. The total glutamic acid hydrochloride in the original mixture (X) was calculated from the formula $X = A\ [(C_o/C) - 1]$, where A is the amount of L-[^{15}N]glutamic acid hydrochloride added and C_o and C are the atoms-% excess of ^{15}N in the added and isolated materials respectively. The results are shown in Table 1.

Determination of the Total Glutamic Acid in Gliadin.—The sample of gliadin had 17·69(9)% Kjeldahl-N, corrected for 9·8% of moisture and 0·86% of ash. It was from the same batch as that used in Part V (*loc. cit.*).

(i) Gliadin (1·5176 g.) was treated with concentrated hydrochloric acid (8·12 c.c.) at room temperature for 18 hours, complete dissolution occurring. Water (6·2 c.c.) was added and the solution was then boiled under reflux for 24 hours. L-[^{15}N]Glutamic acid hydrochloride (0·0598 g.) was added and the heating was continued for a further hour. After repeated evaporation *in vacuo* at 50° to remove excess of acid, the product was benzoylated and then racemised in the manner described above. DL-Glutamic acid hydrochloride (0·5 g.; $[\alpha]_D^{20}$ + 0·75°, *c*, 2·0 in 5% hydrochloric acid) was isolated as previously and crystallised 6 times in all. After the fourth crystallisation the product had no observable rotation (*c*, 0·8 in 5% hydrochloric acid).

No. of crystallisations	4	5	6
Yield (based on crude wt.), %	65	56	49
V. p. difference (mm. of oil)	4	3	3

(ii) In a duplicate experiment with 1·3332 g. of gliadin, 0·0728 g. of L-[^{15}N]glutamic acid hydrochloride was added; 0·48 g. of crude DL-glutamic acid hydrochloride was isolated and crystallised 6 times.

No. of crystallisations	4	5	6
Yield (based on crude wt.), %	52	47	39
V. p. difference (mm. of oil)	3	3	—

The subsequent procedure and calculation were as described above for the synthetic mixtures. The results are shown in Table 2.

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