674. *Amino-acids and Peptides. Part V.* Determination of* L-*Glutamic Acid by the Isotope Dilution Method.*

By C. C. Barker, I. W. Hughes, and G. T. Young.

The accuracy of the isotope dilution method for the determination of L-glutamic acid has been examined by using synthetic mixtures of amino-acids. Individual results are within 0·7% of the theoretical, whilst the mean is 99·7% of the theoretical value. The procedure has been used to determine the L-glutamic acid formed on hydrolysis of a sample of gliadin.

Of the general methods available for the analysis of amino-acid mixtures, the " isotope dilution " procedure offers important advantages. Developed by Schoenheimer, Ratner, and Rittenberg (*J. Biol. Chem.*, 1939, **130**, 703) and by Ussing (*Nature*, 1939, **144**, 977), this method involves the addition of a known weight of isotopic amino-acid to the mixture being analysed; a pure sample of this amino-acid is then recovered by any suitable means and the change in isotope concentration is determined, from which the proportion of the amino-acid in the original mixture can readily be calculated. Normally, the isotopic amino-acid contains ^{15}N in the amino-group, in which case the isotopic concentrations are assayed in the mass spectrometer. The difficulties of quantitative isolation are avoided by this method, which should be capable of high accuracy.

Many such determinations of amino-acids in protein hydrolysates have been reported (for reviews see, *e.g.*, Shemin and Foster, *Ann. N.Y. Acad. Sci.*, 1946, **47**, 119; Tristram, *Adv. Protein Chem.*, 1949, **5**, 83). The main object of the work presented here was to examine the accuracy of this method for the determination of L-glutamic acid, by the analysis of known synthetic mixtures.

The accuracy of the isotope dilution method depends primarily on the purity of the added and the isolated samples and on the accuracy of the mass-spectrometric assays. The establishment of the purity of an amino-acid is not easy by conventional means, since a contaminant is likely to be of similar composition or even isomeric. In the past, crystallisation has been repeated until the isotopic concentration is unchanged, and such constants as nitrogen content and specific rotation have been used for confirmation. The difficulties involved are illustrated by the fact that a sample of L-glutamic acid hydrochloride, isolated from the hydrolysate of human serum albumin and recrystallised until the ^{15}N : ^{14}N ratio became constant, was found still to be impure by optical-rotation measurements, containing an appreciable amount of L-cystine dihydrochloride (Shemin, *J. Biol. Chem.*, 1945, **159**, 439). The danger of co-precipit-

* Part IV, *J.*, 1951, 1745.

ation of nearly related amino-acids has been emphasised by Keston, Udenfriend, and Cannan (*J. Amer. Chem. Soc.*, 1949, **71**, 249), and there are cases where specific rotations do not differ sufficiently to enable small amounts of impurities to be detected.

We therefore developed the vapour-pressure method for the differential-solubility test of chemical purity (Hughes and Young, *Nature*, 1950, **164**, 503; Hughes, Williams, and Young, *J.*, 1951, 1279), by which contamination of one amino-acid by another, or by its enantiomorph, may be detected in amounts of the order of 0·2%. Experiments with synthetic mixtures enable a limit to be placed on the amount of amino-acid impurity which can be present in a given sample, and errors from this source may be evaluated. Application of the method to the sample of L-[^{15}N]glutamic acid hydrochloride used in this work showed that the sample could not contain more than 0·2% of L-aspartic acid hydrochloride. Although the results of the test are conveniently expressed in this manner, it is likely that part at least of the vapour-pressure difference is due to occluded hydrogen chloride, to which the test will be highly sensitive.

Three synthetic mixtures were prepared and analysed: (*A*) L-glutamic acid hydrochloride, DL-glutamic acid hydrochloride, and L-aspartic acid hydrochloride; (*B*) as above, with the addition of DL-alanine and L-lysine hydrochloride; (*C*) as in (*B*), with the further addition of glycine and L-tyrosine. The relative amounts approximated to those found in insulin. After addition of isotopic L-glutamic acid hydrochloride, basic amino-acids (if present) were precipitated with phosphotungstic acid, and the acidic amino-acids were removed on " Amberlite " IR4—B " anion-exchange resin. L-Glutamic acid was finally isolated as the hydrochloride. Recrystallisation was continued until the differential solubility test gave a difference in pressure corresponding to the presence of less than 0·2% of L-aspartic acid hydrochloride. A portion of the material was then converted into ammonium chloride by the Kjeldahl method and thence into nitrogen by the action of sodium hypobromite. The recrystallisation of the L-glutamic acid hydrochloride was then continued, and samples were converted into nitrogen at each stage.

The mass-spectrometric determinations on the nitrogen samples were carried out at the Atomic Energy Research Establishment, Harwell. The ^{15}N concentrations were calculated from the relative intensities of the peaks at mass numbers 28 and 29 ($^{15}N^{14}N^+$); this calculation assumes equilibrium to be established in the reaction $^{14}N^{14}N + {}^{15}N^{15}N = 2\,{}^{14}N^{15}N$, and the equilibrium constant $\frac{[^{14}N^{15}N]^2}{[^{14}N^{14}N][^{15}N^{15}N]}$ to be 4·00. The relative intensities at mass numbers 32 (O_2^+) and 40 (A^+) were also recorded, in order to determine contamination by air; appropriate corrections were then applied to the peaks at masses 28 and 29. The relative intensity at mass 44 was used to determine carbon dioxide, and a correction (for CO^+) was similarly

TABLE I.

Synthetic mixture	L-Glutamic acid hydrochloride present, g.	L-[^{15}N]Glutamic acid hydrochloride added, g.	No. of crystns. of isolated sample	L-Glutamic acid hydrochloride found, g.	%	
A	1·1294	0·1208	5	1·1305	100·10	
			6	1·1311	100·14	Mean 100·1
			7	1·1291	99·97	
B	0·4012	0·0488	5	0·3985	99·33	
			6	0·3977	99·13	Mean 99·3
			7	0·3990	99·45	
C	0·9064	0·0923	5	0·9036	99·69	
			6	0·9022	99·54	Mean 99·7
			7	0·9057	99·92	

applied to the peaks at masses 28 and 29. The relative intensity of the peaks at masses 28 and 32 for a sample of air was 5·4 : 1. The relative intensity of the peaks at masses 44 and 28 for a sample of carbon dioxide was 10 : 1.

The results are summarized in Table I. It will be seen that individual assays are within 0·7% of the theoretical, whilst the mean of the three (99·7%) is considerably closer.

Professor A. C. Chibnall kindly made available a sample of gliadin from a batch in which the L-glutamic acid had previously been determined both by the gravimetric method and by the specific decarboxylase procedure (Chibnall, *J. Int. Soc. Leather Trades' Chem.*, 1946, **30**, 11; Gale, *Biochem. J.*, 1945, **39**, 46). We hydrolysed a portion in the standard manner and determined the L-glutamic acid in the hydrolysate by the isotope dilution method. The results are shown in Table II: our mean value (23·7) for the L-glutamic-acid-nitrogen, expressed as a percentage of the total-protein-nitrogen, agrees remarkably closely with the values given by

Chibnall (23·7) and Gale (23·4). These figures do not of course take into account the possibility of destruction or racemisation of the L-glutamic acid during hydrolysis, and attempts are now being made to investigate this aspect of the problem.

TABLE II.

Experiment	Gliadin, g.	L-[^{15}N]Glutamic acid hydrochloride added, g.	No. of crystns. of isolated sample	Atoms-% excess ^{15}N	L-Glutamic acid found, g. per 100 g. of gliadin *	L-Glutamic acid found, N, % of protein-N
i	1·3030	0·0710	4 (*a*)	1·503	44·04	23·70
			(*b*)	1·490		
			5 (*a*)	1·490	44·23	23·81
			(*b*)	1·490		
			6	1·498	43·99	23·67
				Mean	44·1	23·7
ii	0·7744	0·1668	4 (*a*)	4·576	43·92	23·63
			(*b*)	4·574		
			5 (*a*)	4·567	44·22	23·79
			(*b*)	4·539		
			6	4·576	43·90	23·62
				Mean	44·0	23·7

* Calc. on a dry, ash-free basis.

EXPERIMENTAL.

L-[^{15}N]*Glutamic Acid Hydrochloride.*—Reduction of α-ketoglutaric acid by hydrogen and palladium black in the presence of ammonia containing approximately 15 atoms-% excess of ^{15}N, according to Schoenheimer's method (*J. Biol. Chem.*, 1939, **127**, 301), gave DL-[^{15}N]glutamic acid hydrochloride (80—85%). Resolution was effected by the fractional crystallisation of the strychnine salts of the N-benzoyl derivative (Fischer, *Ber.*, 1899, **32**, 2468). Subsequent hydrolysis and repeated recrystallisation gave L-[^{15}N]-glutamic acid hydrochloride [Kjeldahl-N, 9·5%. Differential solubility test (see Part III, *loc. cit.*): 0·1 g. and 1·0 g., respectively, in water (0·1 c.c.) at 45° gave a vapour pressure difference of 5 mm. of oil (d^{50}, 0·80)].

Determination of L-*Glutamic Acid Hydrochloride in Synthetic Mixtures.*—(*A*) A solution of L-glutamic acid hydrochloride (1·1294 g.), DL-glutamic acid hydrochloride (0·003 g.), L-aspartic acid hydrochloride (0·008 g.), and L-[^{15}N]glutamic acid hydrochloride (0·1208 g.) in water (15 c.c.) was made acid to Congo-red with hydrochloric acid. Amberlite IR—4B (analytical grade) was crushed and washed with water (1 l.), 3% hydrochloric acid (500 c.c.), 3% sodium carbonate solution (500 c.c.), and finally water (4 l.). The treated Amberlite (4 g.) was added to the solution of amino-acids, which was left, with frequent shaking, for 1½ hours, after which the solution was barely acid to Congo-red. The resin was removed by filtration, and the filtrate made acid to Congo-red and retreated with fresh Amberlite (5 g.). The extraction was repeated 4 times using 6, 7, 8, and 8 g. respectively of resin. The combined Amberlite samples were then eluted for 2 hours with water (20 c.c.) kept acid to Congo-red with hydrochloric acid. The resin was then filtered off and eluted a further 4 times. The combined acidic liquors were evaporated to dryness at 50—60° under reduced pressure. The resulting syrupy mass was dissolved in the minimum of water, treated with charcoal, filtered, and saturated at 0° with hydrogen chloride. When the mixture was kept overnight in a refrigerator a crystalline mass separated; this was filtered off, washed, and dried (0·85 g.). The crude L-glutamic acid hydrochloride was recrystallised from 5N-hydrochloric acid in all-glass apparatus. The product was filtered off through a sintered-glass funnel, washed repeatedly, and recrystallised. A small sample from each recrystallisation was transferred to a vacuum-desiccator containing sodium hydroxide pellets and calcium chloride. Each sample was tested for purity in the differential tensimeter, and after the third recrystallisation the vapour-pressure difference for successive samples was nearly constant. Crystallisation was repeated 7 times in all. (All differential-solubility tests on isolated samples were carried out with 0·02 and 0·10 g. respectively of material in 0·01 c.c. of water at 45°.)

No. of crystallisations	4	5	6	7
Yield (based on crude wt.), %	66	50	44	24
V. p. difference (mm. of oil)	4	3	3	3

(*B*) The mixture contained L-glutamic acid hydrochloride (0·4012 g.), DL-glutamic acid hydrochloride (0·005 g.), L-aspartic acid hydrochloride (0·08 g.), L-lysine hydrochloride (0·02 g.), DL-alanine (0·03 g.), and L-[^{15}N]glutamic acid hydrochloride (0·0488 g.). A solution of the mixture in water (15 c.c.) was made acid to Congo-red and heated to 70°. Ether-soluble phosphotungstic acid (2·0 g.) in water (1·5 c.c.) was then added. On the mixture being kept overnight, a heavy white precipitate settled out, which was filtered off. The filtrate was again made acid to Congo-red and treated with 5% phosphotungstic acid solution. After 2—3 hours, a faint precipitate appeared, which was removed by filtration. Further addition of phosphotungstic acid solution gave no precipitate.

The solution was then washed with butanol–ether (1 : 1) (6 × 30 c.c.). The aqueous layer was evaporated to dryness under diminished pressure, water (10—15 c.c.) added, and the solution made

acid to Congo-red. The amino-dicarboxylic acids were isolated with Amberlite IR—4B, as previously described. Isolation and recrystallisation of L-glutamic acid hydrochloride proceeded as before (crude yield, 0·39 g.).

No. of crystallisations	4	5	6	7
Yield (based on crude wt.), %	65	52	45	39
V. p. difference (mm. of oil)	6	4	4	4

(*C*) The mixture consisted of L-glutamic acid hydrochloride (0·9064 g.), DL-glutamic acid hydrochloride (0·003 g.), L-aspartic acid hydrochloride (0·08 g.), L-lysine hydrochloride (0·02 g.), DL-alanine (0·04 g.), glycine (0·02 g.) and L-[^{15}N]glutamic acid hydrochloride (0·0923 g.). L-Glutamic acid hydrochloride (0·755 g.) was isolated as described for mixture (*B*) and recrystallised 7 times.

No. of recrystallisations	4	5	6	7
Yield (based on crude wt.), %	74	62	47	35
V. p. difference (mm. of oil)	4	3	3	3

Preparation of Nitrogen Samples from L-Glutamic Acid Hydrochloride.—Each sample of L-glutamic acid hydrochloride (*ca.* 25 mg.) was converted into ammonia by the modified Kjeldahl procedure and then oxidised to nitrogen with sodium hypobromite according to Rittenberg's method (" Preparation and Measurement of Isotopic Tracers," Edwards, Ann Arbor, Michigan, 1947). We have found it essential to take great care to remove dissolved gases from the solutions before allowing them to react.

Samples of normal nitrogen were similarly prepared from " AnalaR " ammonium chloride. The mass-spectrometric determinations were carried out by Mr. Palmer and his colleagues at the Atomic Energy Research Establishment, Harwell.

The amount of L-glutamic acid hydrochloride (*X*) in the original mixture was calculated from the formula $X = A[C_0/C) - 1]$, where *A* is the amount of L-[^{15}N]glutamic acid hydrochloride added and C_0 and *C* are the atoms-% excess of ^{15}N in the added and the isolated material respectively.

The mass-spectrometer readings for the determination of L-glutamic acid hydrochloride in the synthetic mixtures are recorded in Table III and the calculated results of the analyses in Table I.

TABLE III.

	Relative intensity of peaks.*							
	Observed		Observed		Corrected		Atoms	Atom-%
	mass 28	mass 29	mass 32	mass 44	mass 28	mass 29	% ^{15}N	excess ^{15}N
Synthetic mixture (*A*).								
Normal nitrogen	100	0·7342	0·08	1·16	99·452	0·7298	0·3656	—
L-[^{15}N]Glutamic acid hydrochloride	100	35·449	0·74	0·96	95·908	35·419	15·587	15·221
Normal nitrogen	100	0·7316	0·08	0·54	99·514	0·7279	0·3644	—
L-Glutamic acid hydrochloride isolated :								
5th recrystn.	100	3·6417	0·56	1·20	96·856	3·6185	1·8337	1·469
6th ,,	100	3·6834	0·30	0·92	98·288	3·6706	1·8331	1·469
7th ,,	100	3·6659	0·44	0·54	97·570	3·6481	1·8353	1·471
Synthetic mixture (*B*).								
Normal nitrogen	100	0·7386	0·17	0·86	98·996	0·7310	0·3679	—
L-[^{15}N]Glutamic acid hydrochloride	100	35·710	0·59	1·53	96·661	35·685	15·583	15·215
LGlutamic acid hydrochloride isolated :								
5th recrystn.	100	4·0699	0·36	1·03	97·953	4·0547	2·0278	1·660
6th ,,	100	4·0816	0·33	1·01	98·117	4·0676	2·0308	1·663
7th ,,	100	4·0783	0·30	0·73	98·307	4·0658	2·0261	1·658
Synthetic mixture (*C*).								
Normal nitrogen	100	0·7344	0·15	1·15	99·075	0·7273	0·3657	—
L-[^{15}N]Glutamic acid hydrochloride	100	34·781	0·71	1·30	96·036	34·752	15·321	14·955
L-Glutamic acid hydrochloride isolated :								
5th recrystn.	100	3·4404	0·80	1·00	95·580	3·4080	1·7516	1·386
6th ,,	100	3·4435	0·80	1·60	95·520	3·4104	1·7539	1·388
7th ,,	100	3·4497	0·70	1·10	96·110	3·4211	1·7487	1·383

* The relative intensity of the peak at mass 40 (A^+) never exceeded 0·01.

Determination of L-Glutamic Acid in Gliadin.—The sample of gliadin had 17·69(9)% Kjeldahl-N, corrected for 9·8% moisture and 0·86% ash.

(i) From gliadin (1·3030 g.), hydrolysed with hydrochloric acid, L-glutamic acid hydrochloride (0·62 g.) was isolated and recrystallised in the usual manner.

No. of recrystallisations	4	5	6
Yield (based on crude wt.), %	65	50	32
V. p. difference (mm. of oil)	3	2	3

(ii) Concentrated hydrochloric acid (3·8 c.c.) was added to gliadin (0·7744 g.) and left at room temperature for 16 hours, complete dissolution occurring. Water (2·79 c.c.) was added to the solution which was then heated for 24 hours under reflux.

L-[^{15}N]Glutamic acid hydrochloride (0·1668 g.) was added to the solution which was again heated for 1½ hours. The solution was then evaporated to dryness at 50—60° under reduced pressure and the residual solid dissolved in water (10 c.c.). L-Glutamic acid hydrochloride (0·37 g.) was isolated as already described and recrystallised 6 times.

No. of recrystallisations	4	5	6
Yield (based on crude wt.), %	71	55	48
V. p. difference (mm. of oil)	5	3	3

The samples of L-glutamic acid hydrochloride were converted into nitrogen in the usual manner. The results of the analyses are recorded in Table II; the added L-[^{15}N]glutamic acid hydrochloride had 14·964 atoms-% excess of ^{15}N. The corrections for air and carbon dioxide were similar in magnitude to those shown in detail in Table III.

We thank the Department of Scientific and Industrial Research for continued financial support, the Royal Society for a grant for the purchase of materials, the Director of the Atomic Energy Research Establishment for the mass-spectrometric assays, and Professor A. C. Chibnall for helpful discussions.

Department of Chemistry, Bristol University.
The Dyson Perrins Laboratory, Oxford University. [*Received, July 6th*, 1951.]