DETERMINATION OF N^{15} IN AMINO ACID MIXTURES WITHOUT SEPARATION INTO INDIVIDUAL COMPONENTS

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The determination of excess N^{15} in amino acids presently requires their careful separation from other nitrogen-containing substances, most commonly other amino acids, followed by conversion into ammonia and further into nitrogen (Rittenberg, 1946). The isotope content of the latter is then measured in a mass spectrometer. To obtain results on a complex mixture of amino acids is thus rather laborious and requires about 70 μmoles per amino acid to produce about 1 mg. of nitrogen needed for the measurement. The method is, however, undoubtedly a highly accurate one, if incomplete degradation of the amino acid and contamination of the nitrogen sample with air are avoided.

The availability of amino acids and reagents containing high concentrations of heavy nitrogen makes it frequently possible to design labeling experiments in such a way that only isotope concentrations appreciably above natural abundance are of significance. In such cases, a less precise measurement may suffice and would be of advantage if it could be done considerably faster and with less material.

Based on our studies of the mass spectra of amino acid esters (Biemann, Seibl and Gapp, 1959), we have now developed such a method which requires neither separation of the mixture of amino acids into the individual components nor the degradation of the latter into gaseous products. The mass spectra of ethyl esters of α-amino acids, particularly if obtained with an electron beam of low energy, exhibit an intense peak at the fragment $(R\text{-}CH\text{-}NH_2)^+$ due to loss

of the carbethoxy group from the original molecule. The mass of this fragment, if N^{15}-containing, is one mass unit higher than normally. Consequently, the excess N^{15} can be calculated from the intensity of these two peaks. Since all R-groups present in the common amino acids (with the exception of leucine and isoleucine) differ for more than one mass unit, each such pair of peaks represents one specific amino acid and does not interfere with the others. There are, however, other fragments formed which would interfere. Leucine ethyl ester, for example, besides the strong peak at mass 86 also exhibits one at mass 30 and 44 which coincide with the $(R\text{-}CH\text{-}NH_2)^+$ peaks of glycine (30) and alanine (44). Similarly, a peak at mass 70 in the spectrum of aspartic ester interferes with proline. Even ionization with electrons of low energy does not completely suppress these fragmentations and, because of the difficulty in reproducing the conditions of low energy ionization, cannot be corrected algebraically with the required precision. Fortunately, almost all the amino esters which interfere in such a way differ considerably in their vapor pressure. These interferences can, therefore, be eliminated to a large extent by fractionating the sample into the mass spectrometer.

Table I gives the results on two mixtures of eleven amino acids. One (A) unlabeled and the other (B) with some amino acids containing between 1.9 and 4.3% excess N^{15}, except aspartic acid of 58.7% excess N^{15}, to illustrate the range of applicability.

These values were obtained in the following way. The mixture of amino acids (about 3-5 micromoles of each) is esterified with boiling ethanolic hydrochloric acid, converted to the free esters with dry ammonia in dichloromethane and placed in the sample introduction tube in the manner described earlier for the quantitative determination of amino acids by mass spectrometry (Biemann and Vetter, 1960). The sample is then vaporized in four steps into the mass spectrometer and scanned each time from mass 25 to 145 using an ionization energy of 9.5 - 10.0 eV. With the sample tube at 0° only glycine and alanine ethyl ester vaporize into the instrument.

TABLE I

Amino Acid	Mass of Peaks	Percent Excess of N^{15} Mixture A Found	Mixture B Found		Mixture B Given
Glycine	30/31	0.22	2.13	2.06	2.01
Alanine	44/45	0.17	2.20	1.86	1.89
Proline	70/71	0.19	0.18	0.27	0
Valine	72/73	0.14	2.82	2.83	2.88
Leucine	86/87	0.11	2.76	2.75	2.90
Serine	60/61	0.02	4.44	4.58	4.31
Threonine	74/75	0.17	0.28	0.12	0
Aspartic Acid	116/117	0.08	58.93	58.78	58.67
Phenylalanine	120/121	0.38	4.08	3.90	4.03
Glutamic Acid	130/131	0.05	0.04	0.00	0
Tyrosine	136/137	-0.11	-0.33	0.20	0

When the mixture is warmed to room temperature, valine, proline and leucine ester are introduced. At 150° the esters of serine, threonine, aspartic acid, glutamic acid and phenylalanine are introduced. Tyrosine requires a longer heating time (5 - 10 min.) at that temperature and appears in the fourth step. After scanning the spectrum each fraction is pumped off before the next one is introduced. About two hours of instrument time are required to obtain these four spectra.

The peaks of interest (Table I, column 2) in each spectrum are then measured precisely on the record. The ratio of each pair of peaks (e.g. 30 and 31 for glycine, 44 and 45 for alanine, etc.) is then corrected for the contributions of naturally-occurring H^2, C^{13} and N^{15} and, if necessary, for other amino esters contributing to these peaks. The latter is of significance in this example only for threonine, because the esters of serine, aspartic acid and phenylalanine also contribute to masses 74 and 75. The correction factors are obtained from the spectra of the individual unlabeled amino esters determined under identical conditions.

Column 3 of Table I indicates the accuracy of the method. The percent excess N^{15} can be determined within ± 0.3, and the discrepancies between columns 4, 5 (actual results on the mixture) and column 6 (excess N^{15} given) are of the same order. Since the main source of error is small extraneous

contributions to the peaks for the N^{15}-containing fragments and generally low intensities of the latter, the relative precision increases with increasing N^{15}-content and, up to a certain extent, with increasing sample size; the tyrosine values would be of an accuracy comparable to the others with a larger sample.

In practice the amino acids to be measured are often accompanied by other compounds. The latter must be removed prior to esterification, which can be done most easily by passing the solution through a small ion exchange column (Dowex 50) followed by elution of the acidic and neutral amino acids with 0.2 N pyridine. This procedure also permits the removal of the basic amino acids which would, in certain cases, complicate the N^{15}-analysis. They may then be eluted with 0.2 N ammonia and determined separately. At present we are extending our method to lysine, ornithine and histidine. In these it should be not only possible to determine the average N^{15}-content of each but also to distinguish between the α-amino group and the ε- or ring-nitrogens.

The speed and simplicity of the method described should be of advantage particularly in those studies which require a large number of such determinations on complex mixtures of amino acids but do not need a high accuracy near the natural abundance of N^{15}. Obviously the method is not limited to the amino acids discussed in this paper. The same principle can also be applied to the determination of other stable isotopes in amino acids, unless present in the carboxyl group.

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REFERENCES

K. Biemann, J. Seibl and F. Gapp, Biochem. Biophys. Res. Comm., 1, 307 (1959).

K. Biemann and W. Vetter, Biochem. Biophys. Res. Comm., 2, 93 (1960).

D. Rittenberg in D. W. Wilson and S. P. Reimann, ed., Preparation and Measurement of Isotopic Tracers, J. W. Edwards, Ann Arbor, Mich., 1946, p. 31.