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Control and Modification of Methods for Determination of ^{15}N in Biological Material

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Modifications of available methods for determination of ^{15}N in biological material are described, including needle-biopsy samples of human muscle tissue. The modifications include special precautions for ammonia distillation, technical improvements of the mass-spectrometer, and the mass-spectrometric technique and corrections for air contamination.

About 30 years ago, Schoenheimer and Rittenberg and their cooperators described in detail their methods for determination of ^{15}N in urine and animal tissues. They clearly pointed out the limitations of the mass-spectrometers available at that time and the importance of methodological pitfalls, particularly of cross contamination of the samples with ^{15}N from previous samples. They also emphasized the errors caused by organic impurities with mass numbers within the range to be examined. A much higher resolution, sensitivity and reliability of service can be achieved with modern mass-spectrometers than with those available some thirty years ago. The possibility of solving problems concerning nitrogen metabolism has increased with the improved techniques, but so has the influence of contamination of the samples.

In this laboratory, protein and amino acid metabolism has been studied for several years. Special attention was paid to the incorporation of administered ^{15}N into the amino acids of muscle protein of man and experimental animals. The method of obtaining samples from muscle tissue by needle biopsy in man [1] has opened new possibilities to study the metabolism of this tissue which constitutes about 40 % of the human body. Samples weighing 5 to 100 mg can easily be obtained repeatedly. On the other hand, preparation of this limited material for isolation of protein and protein-free fractions and the quantitative transformation of the organic nitrogen into nitrogen gas require that every step in the procedure is carefully controlled. For this reason, some previously described methods have been improved to suit not only minute quantities of material but also recently developed mass-spectrometers. The improvements concern the treatment of muscle tissue, plasma and urine, isolation of tissue fractions and of amino acids from hydrolyzed protein,

oxidation of amino nitrogen to free nitrogen, and mass-spectrometric technique. Since the methods may be of use also in other types of ^{15}N studies on biological material, they will be recorded here. Since several papers of our group refer to this communication for ^{15}N determinations, the routine procedures are also briefly mentioned.

A. TREATMENT OF BIOPSY MATERIAL

Samples of muscle tissue in man were obtained by needle biopsy after ^{15}N administration. Except for studies in which different muscles were compared, samples were taken from the same or from the contralateral muscle, because different types of muscle may differ from each other in chemical composition.[1] The quadriceps femoris muscle was most commonly studied. When larger samples were needed – for instance when ^{15}N was to be determined in isolated amino acids – surgery was sometimes employed.

B. TRANSFORMATION OF ORGANIC NITROGEN INTO AMMONIA (WITH SIMULTANEOUS KJELDAHL DETERMINATION)

1. Muscle tissue (and other soft tissues). As soon as possible after the muscle sample was taken it was carefully dissected and all connective tissue removed.[2] Carelessness in this respect has caused serious errors in other laboratories, since the sample is not otherwise representative of clean muscle tissue. During dissection, the clean muscle tissue (5 – 100 mg) was divided into two to five pieces. Since the material loses weight by evaporation, each piece was weighed three to four times on a Cahn electric balance in less than 8 min. The weights at different times after performance of the biopsy were noted. The weight of each piece at zero time was then found by extrapolation.[1,3] One of the pieces was reserved for determination of water content, electrolytes, extractable fat and fat-free solids (FFS), which can be used as a reference. Recently, it has turned out that the DNA content or the total concentration of potassium in the tissue may be preferable for reference. For analysis of larger, surgical, samples, the weight loss by evaporation was not measured, since this material was used only for determination of ^{15}N excess in the nitrogen of isolated amino acids with known nitrogen content. All connective tissue was, however, removed in this material too.

After reservation of a piece for other analyses, the remaining material, whether obtained by biopsy or by surgery, was transferred to a Potter-Elvehjem homogenizer for further preparation for ^{15}N determination. The material was homogenized in a volume of 0.85 % saline, which was, in ml, 20 times the weight of material in grams. If the material was not sufficient for ^{15}N determination in the protein (minimum amount of total N about 0.1 mg), the piece already used for determination of FFS *etc.* could be homogenized and added to the other material. The homogenate was quickly precipitated with one tenth of its volume of 50 % (w/v) perchloric (PCA) or trichloroacetic acid (TCA), centrifuged and washed twice with 10 % PCA or TCA.

(*a*) *Protein-free tissue filtrate.* The combined supernatants and washings were digested with sulfuric acid as for Kjeldahl determination, using the "fortified" fluid.[4,5] After digestion, alkalinization and distillation, the nitrogen was titrated in the usual way. After titration, the distillate was acidified with sulfuric acid to prevent loss of nitrogen. Care was taken that oxidation and distillation strictly obeyed the precautions given by Rittenberg *et al.*[4–8] as mentioned later on in this paper (see C). After titration, the distillate was reserved for ^{15}N determination as were all similar distillates here described.

(*b*) *Tissue protein.* The washed protein precipitate was dissolved at about 80°C in 1 ml of the Kjeldahl digestion fluid, digested, distilled and titrated.

2. Blood plasma. Whole plasma, precipitated, washed and redissolved plasma protein, and the protein-free supernatant (including washing fluids) were digested for Kjeldahl determination, distilled and titrated. The distillate was used for determination of ^{15}N.

3. Urine. Total nitrogen, ammonia and urea nitrogen from the 24-hour urine volume were measured. The final solution of ammonia was used for ^{15}N determination.[6,9]

4. Fractionation and preparation of amino acids. The prepared protein from muscle tissue or plasma was freeze-dried. About 10 mg were reserved for the determination of total nitrogen and total ^{15}N, and 30 – 300 mg were hydrolyzed for 30 h at 110°C in 6 N hydrochloric acid.[10] The solution was evacuated to dryness and redissolved in a citrate buffer solution, pH 2.2. The amino acids were separated by preparative chromatography [10] using Zerolite (25 – 40 μ) for ion exchange. The acid and the neutral amino acids were separated in a long column (3.8 × 150 cm) at a constant temperature of 50°C and eluted with a 0.20 M sodium citrate buffer at pH 3.25 and 4.25, passing at a rate of about 260 ml/h, fractions of 25 ml being collected. For the basic amino acids, a short column was used (3.8 × 50 cm) at a temperature of 50°C and eluted with a 0.38 M citrate buffer, pH 5.28, at a rate of 350 ml/h, 30 ml fractions being collected.

From each fraction, a 1 ml sample was reserved for determination of α-amino nitrogen according to Yemm and Cocking.[11]

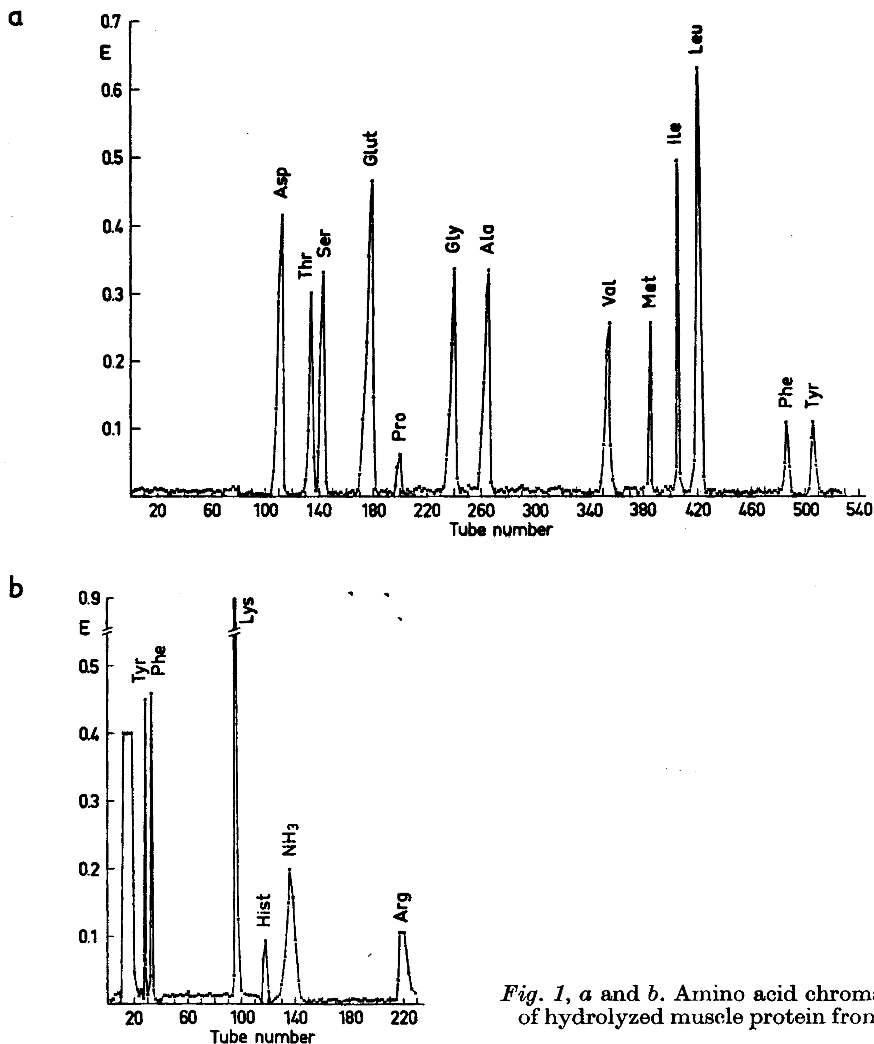

Fig. 1, a and *b*. Amino acid chromatogram of hydrolyzed muscle protein from man.

A typical chromatogram from a preparation of muscle protein is illustrated in Fig. 1, a and b. Table 1 records the recovery from the same preparation.

For nitrogen oxidation, the amino acid fractions were isolated by ion exchange,[12] digested with sulfuric acid, distilled and titrated.

Table 1. Recovery of amino acids from human muscle protein by the method of Spackman *et al.*[10] 100 mg of prepared protein were applied on the columns, corresponding to 116 mg of amino acids after hydrolysis, including hydration. Thus, the recovered quantity, 111.3 mg, corresponds to 96 % of the expected 100 mg protein.

Amino acid	mg AA	mg AA−N	AA %
Long column			
Aspartic acid	11.27	1.156	10.13
Threonine	4.87	0.573	4.38
Serine	5.63	0.751	5.06
Glutamic acid	14.85	1.414	13.35
Proline	6.66	0.810	5.99
Glycine	4.92	0.919	4.42
Alanine	5.95	0.935	5.35
Valine	6.53	0.781	5.87
Methionine	4.56	0.428	4.10
Isoleucine	5.94	0.634	5.34
Leucine	6.14	0.656	5.52
Tyrosine	5.58	0.431	5.00
Phenylalanine	6.70	0.569	6.02
Short column			
Lysine	11.12	2.131	9.99
Histidine	3.23	0.876	2.90
Arginine	6.76	2.173	6.08
Ammonia	0.56	0.461	0.50
Tyrosine	(5.85)	–	–
Phenylalanine	(6.90)	–	–
	111.27	15.698	100

C. PREPARATION OF AMMONIA FOR ^{15}N DETERMINATION

1. Importance of complete oxidation. When the organic nitrogen was transformed to ammonia, it had to be further oxidized to free nitrogen for mass-spectrometry. Hypobromite was used for this final oxidation. However, sulfuric acid digestion does not always transform all organic nitrogen to ammonia.[4,8] Even the "fortified" digestion (special digestion fluid, 280°C, minimum 16 h) according to Sprinson and Rittenberg [4,6] may leave some nitrogen as alkylamines instead of as ammonia. Particularly primary amino nitrogen has a tendency to withstand the oxidation as methylamine. Methylamine and reduced ethylamine are distilled as ammonia and in the Kjeldahl titration in boric or hydrochloric acid they react as cat-ions like ammonia.[13] When treated with hypobromite, however, they are not oxidized quantitatively and then follow the nitrogen gas into the spectrometer. Here, they were a source of trouble. Their mass numbers approach 29, which is also the mass number of $^{14}N^{15}N$. Hence, they cannot be distinguished from heavy nitrogen. These amines have also a tendency to deposit themselves on the tungsten filament.[4,9] Several authors have disregarded these sources of error, which can be avoided only by strict adhering to the description of Rittenberg for ammonia distillation and oxidation.

2. Distillation as ammonia. In order to save material, determination of ^{15}N was carried out on the Kjeldahl distillate (after the titration). The distillate was captured in boric,

or (if the content of nitrogen exceeded about 2 mg) in 0.1 N hydrochloric acid. Because of the risk of cross contamination with ^{15}N adhering to the glass walls, the distillation was continued for at least 8 min, the distillation apparatus being washed by distillation of water for eight minutes between each sample.[4] In control experiments, two urine samples with different known ^{15}N excesses were distilled immediately after distillation of standard solutions ($^{15}NH_4Cl$) with different ^{15}N excesses. After each distillation, the glassware and the distillation apparatus were treated either with the recommended precautions or according to routine laboratory hygiene. Table 2 demonstrates that the error by contamination may be very high if samples with high and low excess are distilled alternately.

Table 2. Cross contamination by distillation, using routine and recommended procedures.

Standard ^{15}N exc. %	Test sample ^{15}N exc. %	^{15}N exc. % by use of routine procedure			^{15}N exc. % by use of recommended procedure		
		^{15}N exc. % recovered	Contamination ^{15}N exc. %	Contamination Error %	^{15}N exc. % recovered	Contamination ^{15}N exc. %	Contamination Error %
	0.2158	0.2210	5.2×10^{-3}	2.41	0.2170	1.2×10^{-3}	0.57
3.0057	0.0098	0.0149	5.1×10^{-3}	52.04	0.0109	1.1×10^{-3}	11.22
	0.2159	0.2189	3.0×10^{-3}	1.39	0.2165	6×10^{-4}	0.28
2.0149	0.0097	0.0130	3.3×10^{-3}	34.02	0.0102	5×10^{-4}	~5.15
	0.2158	0.2179	2.1×10^{-3}	0.97	0.2160	2×10^{-4}	–
1.5594	0.0099	0.0119	2.0×10^{-3}	20.20	0.0100	1×10^{-4}	–
	0.2157	0.2166	9×10^{-4}	~0.42	0.2159	2×10^{-4}	–
0.5505	0.0098	0.0106	8×10^{-4}	~8.16	0.0099	1×10^{-4}	–
	0.2158	0.2160	2×10^{-4}	–	0.2157	–	–
0.0503	0.0098	0.0101	3×10^{-4}	–	0.0098	–	–

After titration, the distillate was immediately acidified with sulfuric acid. It was then evaporated to about 10 ml, made alkaline with sodium hydroxide, and again distilled into 10 ml of 0.1 hydrochloric acid, the same precautions as above being taken. The

Table 3. Loss of nitrogen by evaporation and distillation.

Sample No.	N mg/ml after 1 distill.	N mg/ml after evaporation and redistill.	Recovery, %
1	0.2212	0.2254	101.9
2	0.1596	0.1582	99.1
3	0.2744	0.2758	100.5
4	0.1330	0.1274	95.8
5	0.1582	0.1568	99.1
6	0.2226	0.2254	101.3
7	0.2660	0.2618	98.4
8	0.1666	0.1638	98.3
9	1.039	1.020	99.4
10	1.037	1.039	100.0

new acid solution of ammonium chloride was again evaporated at 80°C to a volume of about 1 ml. Table 3 illustrates that the loss of nitrogen by evaporation and redistillation of standard solutions of ammonium sulphate is negligible.

D. TRANSFORMATION OF AMMONIA INTO NITROGEN

The concentrated acid sample solution was transferred to one of the branches of a Rittenberg tube, and about 1 ml of the sodium hypobromite solution into the other.[5] Before application, the tube and its content were deep-frozen in a mixture of carbon dioxide and ethyl alcohol.[14]

For a correct determination, it was important that the Rittenberg tube was evacuated to the lowest possible pressure, to avoid contamination with atmospheric nitrogen. For this reason, the evacuation apparatus was reconstructed.[15] The final vacuum should be 10^{-4} mmHg, or lower. Before the oxidation process, the Rittenberg tube was warmed to about 60°C, again deep-frozen, and the evacuation procedure repeated.[14]

Before introduction of the sample nitrogen into the spectrometer, we interposed a ground-in column of about 15 cm length, containing phosphorus pentoxide, between the Rittenberg tube and the gas-inlet of the spectrometer to remove all traces of water.

E. MASS-SPECTROMETRIC PROCEDURE

1. Measuring device. The mass spectrometer used was an Atlas CH 4, modified in several respects,[15] the most important being a switch-over device, allowing sample and standard to be measured successively. By this device, a standard could be repeatedly introduced in a long series of determinations.

The ^{15}N excess was calculated according to the Rittenberg formula

$$^{15}\text{N excess, atom per cent} = 100/(2R+1)$$

where $R = I_{28}/I_{29}$[6,9]
and I = intensity.

The background intensity (I_{29}) was measured at the highest possible vacuum before each determination.

2. *Correction of ^{15}N excess value for air contamination by use of m/e 40 argon.* (*a*) *Procedure.* In spite of all precautions it could not be avoided that traces of air contaminated the contents of the Rittenberg tube. Atmospheric nitrogen may be a source of error in determination of the ^{15}N excess ratio of the sample. Sprinson and Rittenberg [4] corrected this error by measurement of the oxygen peak at mass 32, which was considered as a measure of the leakage of air into the tube. By measuring the $I_{m/e\ 40}$ they calculated the oxygen in the tube originating from atmospheric air. It has been suggested that atmospheric oxygen should be removed by repeated deep-freezing and thawing of the Rittenberg tube.[12,16] We did not consider this method reliable, since some oxygen may emanate also from hypobromite [17] copper hydroxide or peroxide.[4]

Using standard types of mass-spectrometry, however, determination of the small argon-40 peak was time-consuming and difficult.[14] This difficulty can now be avoided.[15] The spectrometer was provided with a built-in switch, allowing rapid changes of the magnetic field current to the m/e 40 range. By means of a potentiometer, the current is then adjusted to the m/e 40 argon peak. Thereby the contamination with air could be exactly checked by the argon content in each sample without disturbing the peak adjustment.

The intensity of the ion current at m/e 40 was determined (expressed as mV) at each determination. At the same time the background intensity was frequently checked at m/e 40, 28, and 29. For correction of air contamination, the excess-argon 40 intensity (after subtraction of the background value) was related to the ^{15}N excess per cent, provided that the background intensities were constant.[4] The amount of excess argon was correlated with

$$\frac{100}{2R\text{p}+1} - \frac{100}{2R\text{s}+1}$$

where Rp and Rs represent the ratio $= I_{28}/I_{29}$ for sample (p) and standard (s).[6,9]

When the ^{15}N excess values found by determination of a series of standards with a constant amount of ^{15}N were correlated with the corresponding argon-40 intensities (which depend on air leakage), a logarithmic function was obtained. From this function the ^{15}N excess per cent value could be extrapolated to zero argon-40 intensity (corresponding to ^{15}N ratio without air). The regression coefficient thus obtained, is, however, valid only for the ^{15}N excess at which it was obtained, since it varied with the ^{15}N excess. This variation (regression coefficient *versus* ^{15}N excess) followed a logarithmic course also, reaching a critical point at a certain argon-40 intensity. This critical point constitutes the limit beyond which the curve could not be used for calculation of corrections, irrespective of the ^{15}N excess. This curve could also be used for calculation of the tolerable air contamination. A new logarithmic curve was obtained if the correction factor found in this way was correlated with the ^{15}N excess per cent within the optimal argon-40 range. This curve illustrated that the percentage correction was high at a low ^{15}N excess and *vice versa* (Fig. 2). The results indicated that the curve should not be used for reading the per cent correction at an argon-40 intensity which is above 40 mV. This value was in good agreement with the highest contamination with air, 3 %, which could be tolerated.[4,5]

(*b*) *Example of calculation*. Standards with different ^{15}N excess and with different argon-40 intensity obtained by different degree of air contamination were read in the mass spectrometer. The regression between the R values and the argon-40 intensities was computed, and the regression coefficients for the respective ^{15}N excesses were calculated. The argon-40 intensity was extrapolated to zero for each ^{15}N excess value. Table 4 illustrates a typical calculation pattern.

Table 4.

Standard No.	^{15}N exc. % by $I_{m/e\,40} = 0$ mV	Coefficient of regression	Coefficient of correlation	Number of determinations n
1	1.503×10^{-2}	1.00×10^{-3}	-0.981	10
2	1.919×10^{-2}	1.14×10^{-3}	-0.926	12
3	3.986×10^{-2}	1.68×10^{-3}	-0.973	10
4	12.721×10^{-2}	3.14×10^{-3}	-0.999	11

When the correction factors expressed as per cent correction at a constant argon-40 intensity (I Ar m/e 40 = 1.0 mV) were calculated, a new logarithmic function was obtained, as recorded in Table 5.

3. *Errors of the method*. Every few months the spectrometer was calibrated with a ^{15}N ammonium chloride standard (Table 6). If sufficient material was available, all

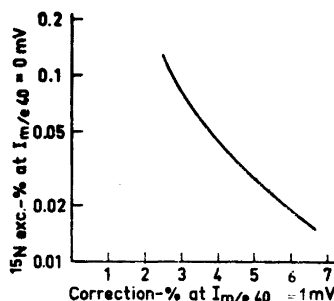

Fig. 2. Changes in correction according to ^{15}N excess.

Table 5.

Standard No.	^{15}N exc. % by $I_{m/e\ 40} = 1.00$ mV	Correction %
1	1.403×10^{-2}	6.65
2	1.805×10^{-2}	5.94
3	3.748×10^{-2}	4.26
4	12.407×10^{-2}	2.47

samples were analyzed in triplicate. The error of the method calculated from 97 duplicate determinations (range of ^{15}N excess per cent 0.0005 – 3.0063) was 5.1961×10^{-4} excess atom per cent.

Table 6. Recovery of ^{15}N from ^{15}N NH_4Cl standards.

Standard No.	Amount ^{15}N μg	Amount ^{14}N mg	^{15}N exc. % theor.	^{15}N exc. % before ^{40}A correction	^{15}N exc. % after ^{40}A correction
1	5.88	414.88	0.0014	0.0011	0.0012
2	5.88	207.44	0.0026	0.0025	0.0026
3	5.88	103.72	0.0056	0.0053	0.0056
4	5.88	51.86	0.0113	0.0114	0.0115
5	5.88	25.93	0.0227	0.0220	0.0228
6	11.76	25.93	0.0453	0.0437	0.0444
7	17.64	25.93	0.0680	0.0661	0.0670
8	11.76	12.965	0.0907	0.0901	0.0917
9	23.52	12.965	0.1814	0.1798	0.1802

By use of the methods described here, the distribution of administered ^{15}N (ammonium acetate, glycine, urea) was examined in healthy volunteers,[18,19] in uremic patients,[20–22] and in surgical cases in the postoperative catabolic state.[23,24]

For repeated determinations of ^{15}N excess in urine, plasma, and muscle tissue for up to 20 days after administration, about 10 mg of ^{15}N per kg body weight were found sufficient. When determination of ^{15}N incorporation in the individual amino acids was required, we recommend a dose of about 15 mg of ^{15}N/kg body weight.

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