Advance in Analytical Method of Heavy Nitrogen in Japan

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Historical background

^{15}N has been widely used in the research of agricultural and biological sciences in Japan since 1947 when Dr. Nishina, a famous physicist, began to produce heavy nitrogen for the study of "the application of heavy nitrogen for agricultural science and technology". His laboratory could begin to supply 5.67 atom % excess of ^{15}N to several eminent scientists from 1948, and served to analyzing the ^{15}N abundance with its self-made mass-spectrometers.

The first tracer experiments using ^{15}N were "Utilization of ^{15}N as tracer element in the studies of nitrogen transformation in the soil"[12] and "Studies on nitrogen metabolism of rice plant with use of isotopically labelled ammonium sulphate".[15]

In 1955 a CEC21-201 mass-spectrometer was imported from USA and began to be used for analyzing ^{15}N. It increased the accuracy of measurement and decreased the experimental cost to ca. 1/10 compared to the preceding experiments. Subsequently ^{15}N tracer experiment for the evaluation of nitrogen fertilizers in the field increased in number and facilitated reasonable recommendations for improvement of fertilizer and fertilization practice.[17] As the supply of high concentration of ^{15}N-compounds increased, physiological and biochemical studies using ^{15}N as a tracer began to be reported.[1,5,6,13,18,20,23,24] In these researches, ^{15}N was determined with a mass-spectrometer, and in order to obtain accurate results avoiding contamination effect, several milligrams of nitrogen as ammonium was required. The requirement for such large amounts of nitrogen was a main reason for retarded ^{15}N use in plant physiological and biochemical researches compared to radioisotopes such as ^{14}C, ^{32}P etc.

Development of the emission spectrometric analysis of ^{15}N

During the last decade an analytical method for ^{15}N based on emission spectrometry has been developed rapidly and come into practical use.[2,3,11,19]

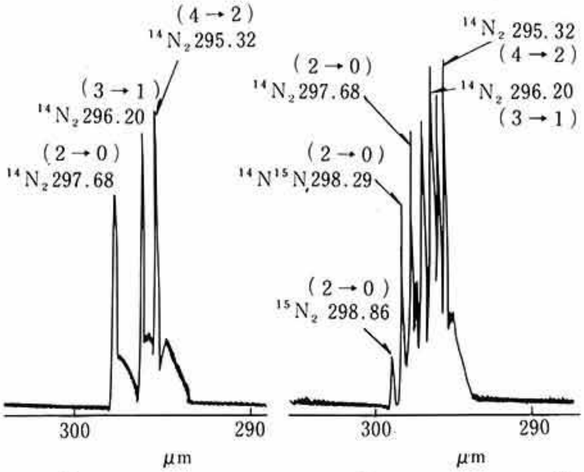
Main advantages of ^{15}N emission spectrometry compared to mass spectrometry are a small size of sample, low running cost, simple analytical procedure and easy handling and maintenance of equipment. The minimum amount of sample required for the measurement is as small as 1 microgram N.

The main disadvantage of emission spectrometry is its low accuracy compared to mass spectrometry. The standard error of the result was 0.5–1.4% for the sample of ^{15}N between natural abundance and 1.5 atom %.[10]

Then usually it is not possible to use it for the researches of comparing the ^{15}N value among those as low as natural abundance. Therefore a great deal of efforts were devoted to improve the method including sample preparation procedure and background estimation.

1) *Principle and development of ^{15}N analyzer*

Spectroscopic measurement of the ^{15}N abundance is based on the measurement of the intensity ratio of the bandheads of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ molecules, between the wavelengths of 297 and 299 μm, which correspond to the 2–0 transition of the second positive system in the nitrogen emission spectra (Fig. 1).



(a) ^{15}N 0.365 atom %   (b) ^{15}N ca. 28 atom % (290~300μm)[2)]

Fig. 1. Emission spectra of nitrogen (290-300 μm)

The intensity of this spectrum is proportional to the number of corresponding molecules and the proportionality constants for each isotope may well be taken as approximately equal. Then concentration of ^{15}N (atom %) can be determined by measuring the intensities of spectra concerned as follows:

$$^{15}N\ (\text{atom }\%) = \{2(^{15}N_2) + (^{14}N^{15}N)\} / 2\ \{(^{14}N_2) + (^{14}N^{15}N) + (^{15}N_2)\} \times 100$$

In many ordinary cases, $(^{15}N_2)$ is too low compared with others. In such cases the value of ^{15}N is evaluated on the assumption that the N_2 gas in the discharge tube is produced from the ^{14}N and ^{15}N atoms combining according to probability: when the following equilibrium is established

$^{14}N_2 + {}^{15}N_2 = 2{}^{14}N^{15}N$, and the following relation exists:

$$K = (^{14}N^{15}N)^2/(^{14}N_2)\ (^{15}N_2) = 4$$

Then from the above equation, by substitution and rearrangement

$$^{15}N\ (\text{atom }\%) = 100/\{2(^{14}N_2)/(^{14}N^{15}N) + 1\}$$

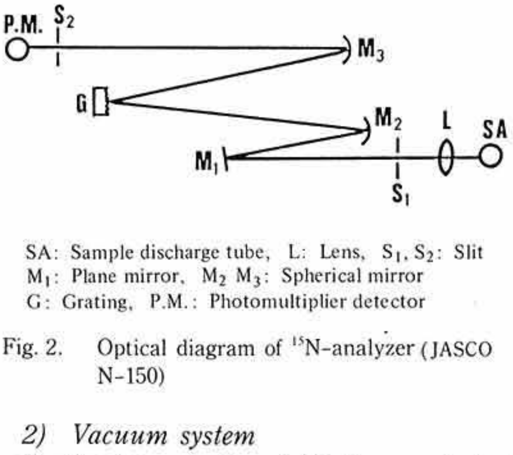
With the notation $R = (^{14}N_2)/(^{14}N^{15}N)$

$$^{15}N\ (\text{atom }\%) = 100/(2R + 1)$$

It is difficult to evaluate directly exact values of ^{15}N because in actual spectra there is a little interaction among adjacent spectra. At lower ^{15}N concentrations the skirt of the $^{14}N_2$ peak overlaps with the $^{14}N^{15}N$ peak so as to cause the ^{15}N value to increase, whereas at higher ^{15}N concentrations the shadow of the $^{14}N^{15}N$ affects the $^{14}N_2$ peak so as to cause the ^{15}N value to decrease. Then it is more generally practical to measure ^{15}N of standard samples, and make a calibration curve of ^{15}N (measured)-^{15}N (theoretical). The measured value can be corrected by this calibration curve. This calibration curve is almost linear in the range of ^{15}N atom % 1.0-15.0, but departs from linearity at both lower and higher concentrations, forming an S-shaped curve overall.

The first commercially available ^{15}N analyzer was STATRON NOI-4 produced in 1968 by Isocommerz GmbH, D.D.R. followed by NOI-5 and automatic Isonitromat 5200. They have a SPM-1 mirror monochrometer (NaCl prism 56) and a 23.12 MHz, 50 W high frequency generator.

In Japan JASCO NIA-1 was produced in 1970 by Japan Spectroscopic. Co., Ltd. followed by N-150. They have a Czerney-Turner mount monochrometer (grating : 1200 lines/mm, dispersion 30A/mm) and a 13.56 MHz, 30 W high frequency generator (Fig. 2).



SA: Sample discharge tube, L: Lens, S_1, S_2: Slit
M_1: Plane mirror, M_2 M_3: Spherical mirror
G: Grating, P.M.: Photomultiplier detector

Fig. 2. Optical diagram of ^{15}N-analyzer (JASCO N-150)

2) Vacuum system

For the determination of ^{15}N, the sample to be measured must be sealed in the discahrge tube under the vacuum below 10^{-4} torr, and the special vacuum device shown in Fig. 3 is widely used.

In Fig. 3, ① is a rotary vacuum pump, ② oil diffusion pump, ③ terminal of vacuum gauge, ④ Pilani vacuum meter (10^{-4} - 20 torr), ⑤, ⑤′ Dewer's bottles, ⑥ Ritterberg's tube, ⑦, ⑦′ glass tubes for discharge tube, ⑧, ⑧′ adaptors, ⑨ twine cocks for gas sampling, ⑩ mixed gas of He and Xe, and ⑪ trap.

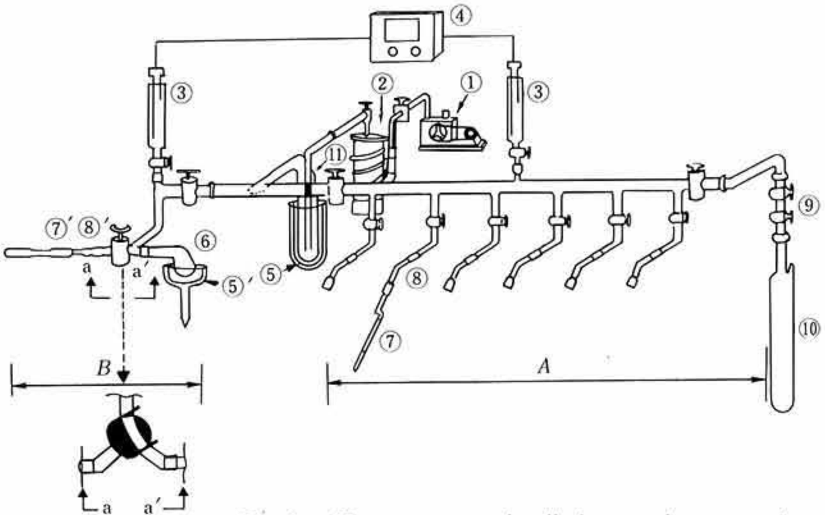


Fig. 3. Vacuum system for discharge tube preparation

The trap ⑪ is used to trap water or carbon dioxide with liquid nitrogen, which is useful to get high vacuum rapidly, but it is not so difficult to get 10^{-4} torr with a completely dried sample without using the trap.

The vacuum system must be air-tight as to maintain under 10^{-3} torr of vacuum at least in 15 min after closing the cock connecting with the oil diffusion pump.

The adaptor ⑧ can be changed in size according to the size of the glass tube used. For example, the glass tube of outer diameter 4, 6, 8 or 12 mm is often used.

Introduction of noble gases is useful to analyze an extra small size of sample of 0.1-1 micrograms N.[4,8]

Pyrex glass (Iwaki Code 7740) tube is used to make the discharge tube.[7] The softing point of Pyrex glass is ca. 820°C and easily handed with the burner of town gas and oxygen mixture, but cannot be heated above 600°C in the oven. It is necessary to clean the glass tube with chromic sulfuric acid mixture and water, heat at 560°C in the oven and keep carefully in dry condition without any contamination of the nitrogenous compounds before use.

3) *Quantity of sample and effect of noble gases*

Nitrogen gas enclosed in the discharge tube can give the good emission light when its pressure is maintained in a range of 2-6 torr.

If the pressure of nitrogen gas is too high, the emission becomes unstable or stops. Co-existence of He can help the emission to stay in stable condition in wide range of pressure. Usually 10 torr of He is recommended to be introduced in the sample tube, when the quantity of sample nitrogen is difficult to estimate.

If the pressure of nitrogen gas is too low, the duration time of emission becomes short due to the adsorption or penetration of nitrogen gas on or into the glass wall. The new method to prevent the loss of nitrogen gas in the tube with the use of noble gases such as Xe and He has been developed.[4] Practically the mixture of 0.20 torr Xe and 10 torr He is introduced into the tube before sealing. Now it has become possible to analyze ${}^{15}N$ content in the trace amount of sample such as 0.2 μg N using the small glass tube (i.d. 2 mm, length 100 mm).

4) *Condensation of ammonia*[22]

If the concentration of ammonium sulfate solution is low, the ammonium must be concentrated.

Fig. 4 shows the concentration device, which has an inner absorption cell in a 30 m*l* flask. A proper amount of 0.1 N HC1 solution is placed in the absorption cell, and the known amount of $({}^{15}NH_4)_2SO_4$ solution in the outer flask. Then ca. 2 m*l* of 40% NaOH solution is poured gently along the wall of the flask, and the rubber cock to which

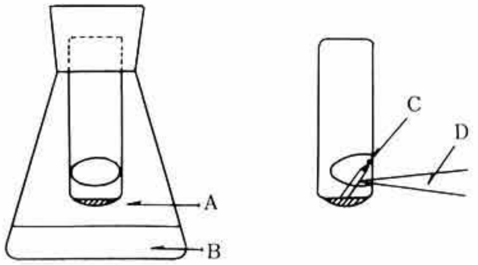


Fig. 4. Reabsorption system of ammonia
A: 1/10 N HCL, B: Ammonium sulfate solution with NaOH, C: Pyrex capillary tube (10 mm), D: Tweezers

the inner absorption cell is fixed is set tightly. After shaking and mixing the sample and NaOH, the evaporated ammonium is absorbed into the HCl solution during ca. 70 hr under 40°C.

The recovery of ammonium into the absorption cell should be complete, otherwise the ^{15}N value becomes smaller because during diffusion process from liquid to gas the $^{14}NH_3$ diffuses faster than $^{15}NH_3$. The volume of the HCl solution in the inner cell should be decided as to get a proper concentration of NH_4^+ for the sampling.

From the HCl solution containing $^{15}NH_4Cl$ in the absorption cell, a small part of the solution is sampled with a Pyrex glass capillary tube (o.d. 1 mm, length 10 mm, inside volume ca. $8\mu l$) and dried carefully under the infra red lamp avoiding any contamination of nitrogenous compounds from atomosphere.

The quantity of nitrogen in the capillary tube must be in a certain range 2 - 6 μg, and be regulated with the amount of original sample nitrogen, volume of HCl solution and length of the capillary tube.

Development of ^{15}N determination method for various kinds of samples

1) Standard procedure to make discharge tube by Dumas method 21)

A piece of solid reaction reagent and a sample are put in a Pyrex glass tube (o.d. 2 mm, length 20 cm). This solid reagent is prepared by compressing a 1:1 mixture of CuO and CaO powder and cutting the resultant solid into small square pillars and heated at 650°C before use3) (Fig. 5).

However because of difficulty of keeping CaO from absorption of CO_2 in a long period, the use of CuO string heated at 650°C and CaO briquet heated at 950°C, both prepared separately, is recommended.

A sample containing N in the range of 0.5 to 5 μg is put in the tube with the above reagent chemicals. A solid sample is used as it is, while a solution sample is used after taken into a Pyrex glass capillary and dried as described above. These samples are placed at about 5 cm apart from the tube end to be closed, and the open end is connected to a vacuum line to produce a vacuum below 10^{-4} torr.

After connecting with a vacuum line and the prescribed vacuum (10^{-4}) has been reached, the reagent and glass except a sample part are heated and the tube is cut off by fusing at a point some 10 cm from the end.

The tube with the reagent and the sample is heated to about 650°C to burn the sample with the generation of N_2 gas. The duration of combustion, though dependent on the kind of sample, is usually about 2-6 hr. When the sample to be analyzed is rich in oxygen as NO_3, a reducing agent

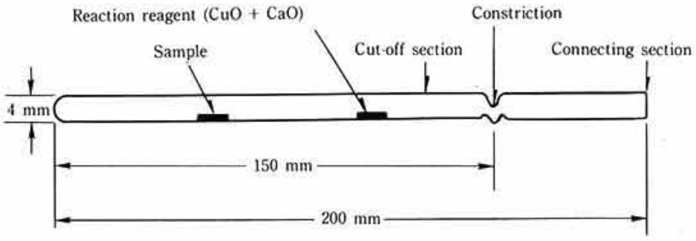


Fig. 5. Standard discharge tube preparation

such as metallic copper should be used.

After taking out from the furnace, the discharge tube is cooled at the room temperature for several hr so that CO_2 and H_2O can be absorbed completely with CaO, and subjected to the analysis by the emission spectrometer.

2) *Preparation of the discharge tube for solid sample or plant powder*[22]

When the amount of the sample is enough to provide the several analysis, a large size discharge tube is prepared as shown in Fig. 6.

The size of the discharge tube is changed in accordance with the amount of sample nitrogen as to give proper pressure of N_2 for the emission.

For several mg of dried plant powder, 0.5 g of CuO powder and 1.0 g of CaO are necessary. In this case, in stead of briquet the gravel size CaO is used.

3) *Amino acids and amides separated with thin-layer chromatography*[22]

The amino acids and amides are separated with 2 dimentional silica gel thin-layer chromatography with phenol-water (4:1, v/v) and butanol-acetic acid-water (4:1:1, v/v). Silica gel to be used should be heated 110°C to remove nitrogen contamination and to be activated. After spraying ninhydrin solution, the central part of each colored spot is taken into the glass tube with silica gel as shown in Fig. 7.

The amount of sample nitrogen is possible to estimate by the density of spot color. If the sampled nitrogen is lower than 0.2 μg, more than 2 plates are used.

After the sampling, each amino acid is eluted with 50% ethanol solution from the silica gel, and dried under low pressure at the temperature below 40°C. Then CuO powder is added, and at a portion above the sample and CuO powder a narrow point is made on which CaO briquet is placed. After making vacuum, CaO and a portion of the tube where the sample is not placed were heated with a burner, then noble gas mixture is introduced and the tube is sealed. The sample tube is heated at 540°C for 6 hr for decomposition.

4) *Rittenberg method*

This method is applicable when the sample is $(NH_4)_2SO_4$ solution.

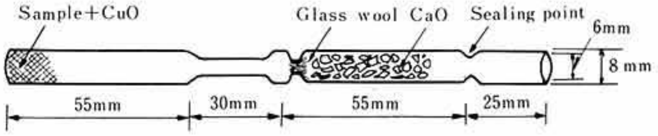


Fig. 6. Discharge tube for plant powder

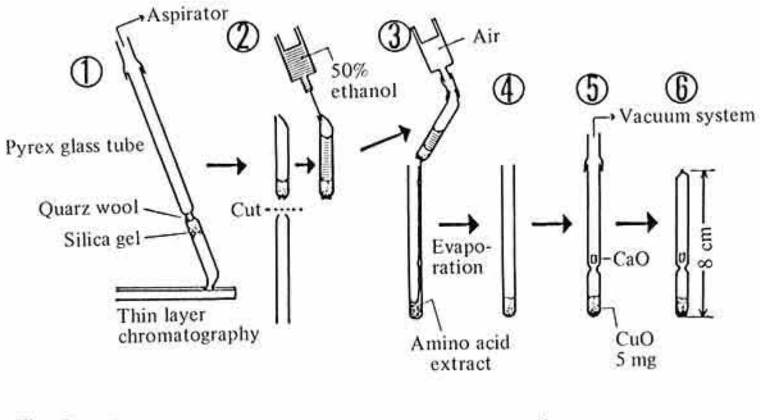


Fig. 7. Preparation procedure of discharge tube for ^{15}N analysis of amino acid and amide

One branch of a 3 way stopcock (B in Fig. 3) is connected with an evacuating apparatus. Other branches are connected with a small Rittenberg tube (about 20 m*l* in volume) and a Pyrex glass discharge tube containing CaO briquet as shown in Fig. 8.

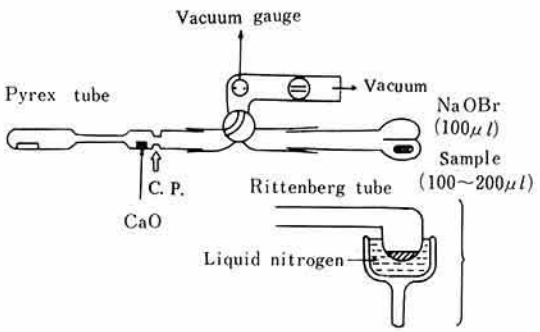


Fig. 8. Discharge tube preparation with Rittenberg method
C.P.: Cut off point

The discharge tube is heated with a burner to expell the adsorbed gases on the inner wall under the vacuum and cooled.

In one section of the Rittenberg tube is put a sample solution of 100 to 200 μl containing about 50 - 100 μg of N and in another section 100 m*l* of the reaction reagent NaOBr solution (obtained by adding dropwise 8 m*l* of Br_2 to 50 m*l* of 40% NaOH with cooling). After having been frozen both the sample and the reaction reagent in liquid nitrogen, the reaction tube and the discharge tube are adequately evacuated, and then the stopcock for the reaction tube is closed. Both the sample and the reaction reagent are melted by removing the liquid nitrogen to evolve any dissolved gas and, after being frozen again, are evacuated by opening the stopcock. This procedure, frozen-evacuation-melting-frozen, should be repeated at least 3 times to expell the disolved gas completely.

The stopcock to the reaction tube is then closed and both the sample and the reaction reagent are melted and mixed each other to make them react with the generation of N_2 gas. After the end of the reaction, the remainder of the reaction mixture is frozen and the N_2 gas is introduced from the reaction tube into the discharge tube. Then the discharge tube is cut off rapidly at the previously prepared cut-off point. The discharge tube prepared in this way must exhibit clear N_2 emission when it is put under the action of a Tesla coil.

Calibration and error

Contaminated gases introduced in a trace amount during the preparation process sometimes give the systematic error on the results. OH of water and CO of carbon dioxide have some spectra near the band peak of $^{14}N^{15}N$, and this effect appears relatively large when the ^{15}N concentration is low.[9] The preparation of the discharge tube with Dumas combution method sometimes induces the interference of CO_2 or O_2 evidently. The contribution of the CO_2 band at the shoulder point of $^{14}N^{15}N$ in its long wave length side, is almost same as that of the peak head of $^{14}N^{15}N$. There is a minimum in a spectral line of CO_2 on the left side of the shoulder of N_2, but at this point the contribution of CO_2 on the N_2 becomes smaller than at the band head of $^{14}N^{15}N$ (Fig. 9).

In this case if the minimum point in the long wave length side of $^{14}N^{15}N$ peak is selected as background for the peak of $^{14}N^{15}N$, unexpected higher results of ^{15}N value is given. Then the author recommends to select the shoulder point

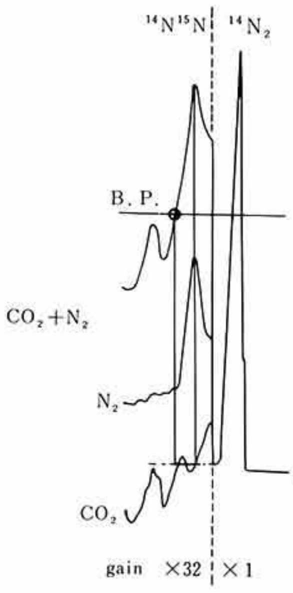


Fig. 9. Effect of CO_2 on the peak of $^{14}N^{15}N$
B.P.: Background point

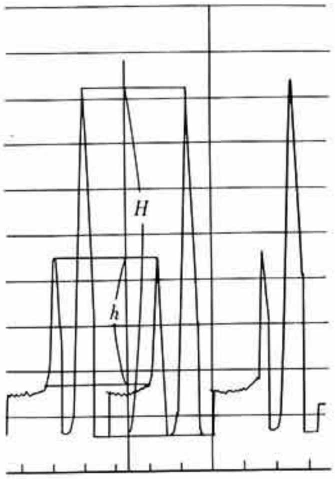


Fig. 10. Chart for the measurement of ^{15}N in low abundance
h: Height of $^{14}N^{15}N$, H: Height of $^{14}N_2$, r: Amplification ratio of h to H, $R = r \times H/h$, ^{15}N atom % $= 100/(2R + 1)$

as background for $^{14}N^{15}N$ as shown in Fig. 10. Accordingly, the calibration curve with standard samples should be made by the same way.

Above 1 atom % of ^{15}N sample the influence of CO_2 contamination is usually low and need not be taken into consideration. O_2 also gives some positive error for the results, so that great care should be paid to avoid the O_2 gas in the discharge tube.

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