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NITROGEN COMPOUNDS OF CABBAGE. II.  
CHROMATOGRAPHIC ANALYSIS OF THE NON-PROTEIN  
NITROGEN

ROBERT M. ZACHARIUS, EDWARD G. KELLEY, AND J. J. MCGUIRE<sup>a</sup>  
*Eastern Regional Research Laboratory,<sup>b</sup> Philadelphia 18, Pennsylvania*

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In the preceding study of this series (4), the non-protein nitrogen fraction in its entirety was examined in relation to total nitrogen. Using microbiological procedures some ten "essential" amino acids of the non-protein and bulk protein fractions were assayed. However, it became clear that these ten amino acids represent only a small part of the non-protein nitrogen fraction; in fact, arginine might be described as the only "essential" amino acid in the non-protein fraction occurring in major quantities. The Kjeldahl nitrogen provided by these ten amino acids represents 22% of the entire nitrogen of the non-protein fraction.

Little has been published on the qualitative as well as quantitative make-up of the non-protein fraction of cabbage (cf Part I, this series) nor, with the exception of a few specific compounds, has it been examined in detail. Therefore, it was evident that a more complete examination was desirable in order to ascertain the components and quantitative distribution of a large segment of this fraction.

EXPERIMENTAL PROCEDURES

**Materials and preparation of non-protein fraction.** The Wisconsin and Danish strains of Copenhagen cabbage studied were of the same harvest and sampling as that used in Part I (4). Segments of 19 heads of Wisconsin Copenhagen cabbage with a total fresh weight of 1750 g (129 g moisture-free basis) were ground in a Waring blender with a volume of 95% ethanol equal to 3 times the fresh weight of the cabbage. On filtration the ground cabbage residue was reextracted with portions of 70% ethanol until the washings gave only a weak ninhydrin-positive reaction. The residue was further extracted by soxhlet with hot 70% ethanol for 8 hr. All alcohol extracts were combined and concentrated on a rotary evaporator at 40° C under reduced pressure to a volume which represented 7.0 g fresh weight of cabbage/ml or 0.515 g moisture-free basis (MFB)/ml. The Danish strain was treated in like manner. The concentrate was made to a volume equal to 7.0 g fresh weight of cabbage/ml or 0.394 g MFB/ml.

**Methods of analysis.** The extract was analyzed for its amino- and imino-nitrogen constituents by a quantitative paper chromatographic procedure. It was not found necessary to desalt the extract prior to chromatography. The procedure employed was a modified version of the descending two-directional method of Steward and Thompson (12). For convenience, sheets (18¾ x 22½ in) of prewashed Schleicher and Schuell No. 589 blue ribbon filter paper were employed in lieu of acid-washed Whatman No. 1. n-Butanol:glacial acetic acid:water (9:1:2.5) replaced the collidine-lutidine solvent (12) for the separation in the second direction. The compounds were located after development of the color with the ninhydrin reagent (1.0% in 95% ethanol) under the humidified, anaerobic conditions set forth by Steward, Zacharius and Thompson (13). Colored spots were cut out of the paper chromatogram, cut into small rectangular pieces and eluted with 50% ethanol. The color densities were determined on a Beckman DU Spectrophotometer at 570 mμ for all compounds except pipercolic acid

<sup>a</sup> Present address: Food and Drug Administration, Washington 25, D.C.

<sup>b</sup> Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

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and proline; the latter was determined at 440 mmc. Interpretation of density values was made from standard curves prepared in advance for each of the compounds assayed; these standard values were checked at various time intervals.

Pipecolic acid was located on a two-directional paper chromatogram after reacting with 0.05% ninhydrin in 95% ethanol and heating the paper at 70° C for 10 min. The pipecolic acid spot was cut out, cut up into small squares, and reacted further with 4.0% ninhydrin in a test tube and the color density determined at 575 mmc according to the method of Silberstein *et al* (11). No paper blank was found to be necessary. In agreement with Silberstein *et al* this method was not found applicable to the quantitative determination of proline.

The value obtained for histidine can only be considered approximate. Following a uni-directional separation of the extract with the n-butanol-acetic acid solvent, histidine was located from the orange-red color formed with freshly diazotized p-bromoaniline (1) sprayed on the paper chromatogram. The colored area was removed and its density determined with a Lumetron (Photovolt Corporation, New York, New York) after wrapping it around a test tube which fits the adapter. By revolving the tube a maximum reading could be obtained. A constant, K, was established using the formula

$$\frac{\text{mg of histidine}}{\text{maximum density} \times \text{area}} = K.$$

The weight of the paper was taken as a measure of area. A mean value for K was determined in the range 2.5–15.0 mg of histidine and the amino acid values for the plant extracts were obtained using the K value.

Cystine was estimated as cysteic acid after oxidation with 30% hydrogen peroxide and 0.02% ammonium molybdate catalyst after application to the filter paper sheet.

Kjeldahl N values were obtained by the official A.O.A.C. (7) method using semi-micro Kjeldahl apparatus. Amino-nitrogen determinations were made by the Van Slyke method with a modified auxiliary reaction chamber as described by Doherty and Ogg (3). Free ammonia was determined employing essentially the method of Pucher *et al* (9) modified by the inclusion of a trap between the distillation flask and connecting bulb consisting of an adapter filled with glass helices. The ammonia was collected in 4% boric acid and the titrations carried out with 0.02N HCl with Kjeldahl mixed indicator. Glutamine was determined both by the paper chromatography method described above and according to the ammonia method of Pucher *et al* (9). Asparagine was also determined chromatographically by two different procedures and by ammonia difference according to Vickery and Meiss (15). In an alternate chromatographic procedure for asparagine, the asparagine spot on the two-directional chromatogram was located with 0.05% ninhydrin, cut out and the determination, essentially followed that described by Connell *et al* (2).

## RESULTS AND DISCUSSION

Results of the analysis of the alcohol extractable nitrogen of Wisconsin Copenhagen cabbage are shown in Table 1.

A summation of amino-nitrogen furnished by the individual compounds assayed chromatographically accounts for 76% of the determined Van Slyke nitrogen value and the total nitrogen of these same compounds is equal to 60% of the Kjeldahl value. These figures appear reasonable in view of the fact that we have only assayed for the ninhydrin-positive nitrogen components of the fraction. The total nitrogen of the 10 "essential" amino acids represents 33% of the summed total nitrogen of the compounds determined. In terms of the summed Van Slyke nitrogen only 19% is contributed by these "essential" amino acids.

The major ninhydrin-positive component is glutamine while the amide of aspartic acid is present in less than one-fourth of that amount. It is noteworthy that S-methylcysteine sulfoxide represents both the major amino acid

TABLE 1  
Chromatographic analysis of the non-protein nitrogen fraction of cabbage

| Compound  | Amino acid<br>mg/g<br>(MFB) | Expressed as<br>V.S. amino N<br>mg/g<br>(MFB) | Expressed as<br>total N<br>mg/g<br>(MFB) |
|---|-----------------------------|---|--|
| Aspartic acid.....                              | 2.2                         | 0.22  | 0.22                                     |
| Glutamic acid.....                              | 3.4                         | 0.32  | 0.32                                     |
| Asparagine.....                                 | 2.2 (2.0) <sup>1</sup>      | 0.23 (0.21) <sup>1</sup>                      | 0.45                                     |
| Glutamine.....                                  | 10.0 (10.68) <sup>2</sup>   | 1.7 (1.84) <sup>2</sup>                       | 1.9                                      |
| Serine.....                                     | 2.2                         | 0.3   | 0.3                                      |
| Threonine.....                                  | 1.1                         | 0.1   | 0.1                                      |
| Alanine.....                                    | 3.0                         | 0.47  | 0.47                                     |
| Histidine.....                                  | 0.59                        | 0.05  | 0.16                                     |
| Lysine.....                                     | 0.43                        | 0.08  | 0.08                                     |
| Arginine.....                                   | 5.1                         | 0.41  | 1.6                                      |
| Methionine.....                                 | 0.25                        | 0.02  | 0.02                                     |
| Methionine sulfoxide.....                       | 0.18                        | 0.01  | 0.01                                     |
| S-methylmethionine.....                         | 0.03 <sup>3</sup>           | 0.003   | 0.003                                    |
| Cystine.....                                    | 0.16                        | 0.02  | 0.02                                     |
| S-methylcysteine sulfoxide.....                 | 6.6                         | 0.61  | 0.61                                     |
| Valine.....                                     | 0.67                        | 0.08  | 0.08                                     |
| Leucine + isoleucine.....                       | 1.1                         | 0.11  | 0.11                                     |
| Tyrosine.....                                   | Trace                       | —   | —  |
| Phenylalanine.....                              | 0.68                        | 0.06  | 0.06                                     |
| Tryptophan.....                                 | 0.0                         | 0.0   | 0.0                                      |
| γ-Aminobutyric acid.....                        | 0.36                        | 0.05  | 0.05                                     |
| Proline.....                                    | 0.35                        | 0.00  | 0.04                                     |
| Pipecolic acid.....                             | 0.54                        | 0.00  | 0.06                                     |
| Total.....                                      |                             | 4.84  | 6.66                                     |
| Van Slyke Amino N determination on extract..... |                             |   | 6.34 mg/g (MFB)                          |
| Kjeldahl N.....                                 |                             |   | 11.1 mg/g (MFB)                          |

<sup>1</sup> By a second ninhydrin method of color development.

<sup>2</sup> By Vickery's NH<sub>3</sub> method (9).

<sup>3</sup> Calculated from Winteringham (16).

V.S. = Van Slyke.

constituent as well as the sulfur-containing amino acid of this extract. The value reported here is in close agreement with that reported by Morris and Thompson (8) in a survey of *Brassicas*. The quantity of cysteine and methionine on a comparative basis is extremely small. Arginine is the third major amino compound present and because of its high nitrogen content is second only to glutamine on a total nitrogen basis. These three compounds make up 43% of the Van Slyke nitrogen of the fraction.

The glutamine value as obtained by quantitative paper chromatography was equal to 94% of that found by the ammonia method of Vickery (Table 2). This amide provided 29% of the Van Slyke nitrogen of the Wisconsin Copenhagen strain and 41% in the case of the Danish Copenhagen cabbage.

Efforts to determine asparagine by the ammonia method of Vickery had yielded very large and unreproducible values which were far in excess of the chromatographic ones. This is attributed to destruction by acid hydrolysis of S-methyl-cysteine sulfoxide with the concomitant production of ammonia (8, 14). Thus, whenever the latter sulfur amino acid is present in the sample, ammonia production by even mild acid hydrolysis for the determination of asparagine (or other amides) must be excluded.

**TABLE 2**  
**Free ammonia and glutamine of the non-protein nitrogen fraction of cabbage**

| Sample   | Free NH <sub>3</sub><br>mg/g<br>(MFB) | Glutamine<br>NH <sub>3</sub><br>mg/g<br>(MFB) | Calculated<br>glutamine<br>(from NH <sub>3</sub> )<br>mg/g<br>(MFB) | Calculated<br>glutamine<br>Van Slyke N<br>(from NH <sub>3</sub> )<br>mg/g<br>(MFB) | Van Slyke N<br>mg/g<br>(MFB) |
|--|---------------------------------------|---|---|--|------------------------------|
| Cabbage extract<br>before buffer hydrolysis..... |                                       |   |   |  |                              |
| Strain 1 <sup>1</sup> .....                      | 0.706                                 |   |   |  | 6.34                         |
| Strain 2 <sup>2</sup> .....                      | 0.409                                 |   |   |  | 10.27                        |
| Cabbage extract<br>after buffer hydrolysis.....  |                                       |   |   |  |                              |
| Strain 1 <sup>1</sup> .....                      |                                       | 1.24  | 10.68   | 1.85   | 3.62                         |
| Strain 2 <sup>2</sup> .....                      |                                       | 2.84  | 24.70   | 4.22   | 4.78                         |

<sup>1</sup> Strain 1 refers to Wisconsin Copenhagen cabbage.

<sup>2</sup> Strain 2 refers to Danish Copenhagen cabbage.

Although the Danish strain was found to contain a much higher level of Van Slyke nitrogen in the alcohol extractable fraction than the Wisconsin strain, 60% of this difference is immediately attributed to glutamine as calculated from columns 3 and 5 of Table 2.

It is interesting to note that the quantity of pipercolic acid present exceeds that of its proline analogue—a protein constituent—and is very similar in concentration to many of the “essential” amino acids in the non-protein fraction.

Quantities of pyrrolidone carboxylic acid were found present in the ethanol extract as well as evidence of some unidentified peptides and other simple ninhydrin-positive compounds. Conceivably the ring compound arose as an artifact from glutamine on extraction and storage of the extract. A large amount of an unidentified Erlich-reagent-positive compound, presumably an indole derivative, was also detected in the extract; the presence of tryptophan, however, could not be established.

S-methylmethionine could not be easily assayed by the above method because of its low concentration in the complex mixture wherein many of the components are present in considerably greater magnitude. The figure given is calculated from Winteringham (16). It is rather remarkable that this sulfur amino acid was detected, isolated and identified (6) somewhat earlier than S-methylcysteine sulfoxide (8, 14) when one considers the concentration of each of these compounds.

Contrary to the findings of others (5, 10), the presence of hydroxyproline could not be established in the non-protein nitrogen fraction. Furthermore, in agreement with the microbiological studies in Part I, and contrary to the published analysis of Majumder *et al* (5), arginine represents one of the larger amino acid constituents of the non-protein nitrogen of cabbage.

Any differences between the values reported here and those for the 10 amino acids in Part I (cf Table 3) may be attributed to both differences in preparation of the non-protein nitrogen fraction and methods of assay. Microbiological assay values obtained on the unhydrolyzed cathode appeared to reflect unknown growth factors—possibly attributable to peptides. Chromatographic determinations reported in this paper were made on an unhydrolyzed aqueous ethanol extract. Some of the differences between the latter

values and the hydrolyzed cathode presumably resulted from peptide hydrolysis. Moreover, it was shown (4) that the extraction of free lysine by aqueous ethanol was incomplete when compared with the cathode or hot water extracts.

#### SUMMARY

Results of the chromatographic analysis of the non-protein nitrogen of Wisconsin Copenhagen cabbage are given.

Glutamine represents the major amino-nitrogen compound of the non-protein nitrogen fraction of cabbage. S-methylcysteine sulfoxide is second in concentration in this fraction while arginine represents a third major amino constituent. These three compounds provide 43% of the Van Slyke nitrogen of this fraction. The individual components assayed by the chromatographic method account for 76% of the Van Slyke nitrogen value and 60% of the Kjeldahl value.

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