separations which indicate the presence of at least three protein species in the soluble portion of rumen ingesta which catalyze the hydrolysis of carboxymethylcellulose. This multiplicity of enzymes does not necessarily mean that three different bacteria were responsible for elaboration of the three enzymes observed. Miller and Blum (*8*) and Hash and King (*5*) have observed a number of cellulases in culture filtrates from pure cultures. Apparently in this regard also the cellulases of the rumen microflora as a whole resemble those of other cellulose-degrading microflora.

The enzyme-time studies require further investigation. Presumably, some factor other than (or in addition to) ration, animal, or time of sampling, determines whether inhibitor, activator, or neither is indicated by the enzyme-time type of experiment. Should inhibitors of rumen cellulase occur relatively frequently, a clear understanding of their nature, mode of action, and origin might lead to improved digestive efficiency of ruminants. Similarly, if an activator is involved, its identity, origin, and mode of action, if understood, might lead to improved fiber digestion.


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### Literature Cited


(1) Baker, F., *Nature* **143,** 522 (1939).
(2) Cason, J. L., Thomas, W. E., Paper 34, 50th annual meeting, Am. Dairy Science Assoc., 1955.
(3) Conchie, J., *Biochem. J.* **55,** xxi (1953).
(4) Gilligan, W., Reese, E. T., *Can. J. Microbiol.* **1,** 90 (1954).
(5) Hash, J. H., King, K. W., *Federation Proc.* **15,** 555 (1956).
(6) Kitts, W. D., Underkofler, L. A., J. AGR. FOOD CHEM. **2,** 639 (1954).
(7) Levinson, H. S., Reese, E. T., *J. Gen. Physiol.* **33,** 601 (1950).
(8) Miller, G. L., Blum, R., *J. Biol. Chem.* **218,** 131 (1956).
(9) Nelson, N., *Ibid.*, **153,** 375 (1944).
(10) Nisizawa, K., Kobayashi, T., *J. Agr. Chem. Soc. Japan.* **27,** 241 (1953).
(11) Reese, E. T., *Appl. Microbiol.* **4,** 39 (1956).
(12) Reese, E. T., Siu, R. T. G., Levinson, H. S., *J. Bacteriol.* **59,** 485 (1950).
(13) Somogyi, M., *J. Biol. Chem.* **160,** 61 (1945).



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**TOXIC PLANT PRODUCTS**

# Colorimetric Determination of Beta-Aminopropionitrile in Mature Legume Seeds


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β-Aminopropionitrile (BAPN), the toxic principle found in *Lathyrus odoratus* seeds, when incorporated into the diet of different animal species, has been shown to produce marked changes in several tissues of the body. A rapid and convenient method for the determination of this substance in legume seeds has been developed on the basis of a reaction between β-aminopropionitrile and ninhydrin, which produces a green color. Concentrations of β-aminopropionitrile, as low as 50 p.p.m. in the sample analyzed, can be directly detected by this method. *L. latifolius, L. sylvestris,* and *L. splendens* have been reported to be toxic to rats, but showed no detectable β-aminopropionitrile by this method; therefore, toxic substances other than this are present in these seeds.


THE ACTIVE PRINCIPLE in certain toxic legume seeds, particularly *Lathyrus odoratus*, has been found within the past 2 years to be β-aminopropionitrile (BAPN) (*1, 3, 10*). When fed to rats at 0.2% of the ration, β-aminopropionitrile causes the bony deformities and connective tissue breakdown characteristic of odoratism (*9*). As ordinary foods might contain traces of this substance, it seemed desirable to develop a convenient method for determining it in biological materials.

β-Aminopropionitrile on paper chromatograms, treated with ninhydrin, produced a green spot in contrast to the red shades characteristic of most amines. The same color reaction could be produced in dry butanol, and a quantitative relationship between color intensity and β-aminopropionitrile content was worked out. To make this reaction applicable to biological samples, it was first necessary to extract and hydrolyze the naturally occurring β-(*N*-γ-L-glutamyl)-aminopropionitrile, I (*6*), and to separate the resulting free β-aminopropionitrile from interfering ninhydrin-positive substances.

### Reagents

**β-Aminopropionitrile Standard Solution.** Redistilled β-aminopropionitrile (*2*), boiling point 79–81° C. (16 mm.) was dissolved in purified 1-butanol and diluted with the same solvent to a concentration of 100 γ per ml. This solution was kept at 0° C. and was freshly prepared each month.

**Ninhydrin Solution.** Reagent grade ninhydrin (Eastman) was dissolved in 1-butanol and diluted to 20 mg. per ml. This solution was not used when it was more than 24 hours old.

**Sodium Hydroxide Solution.** One-half gram of C.P. sodium hydroxide was dissolved in 20 ml. of 1-butanol and the solution filtered after 4 to 5 hours. The solution should be perfectly clear and should be used within 24 hours.

**1-Butanol.** Technical grade 1-butanol was dried over solid sodium hydroxide for 10 to 12 hours, then distilled.

**Ether.** Reagent grade.

### Recommended Procedure

**Extraction and Hydrolysis.** Place an amount of the finely ground sample (60 to 80 mesh) estimated to contain at least 100 γ of β-aminopropionitrile (1 to 2 grams of β-aminopropionitrile containing lathyrus pea seeds) in a 50-ml. flask with 20 to 30 ml. of water and a drop of antifoam agent (Dow-Corning Antifoam A). Add several porcelain boiling chips and reflux the mixture 40 to 42 hours. Filter the flask contents through cotton, cloth, or glass wool in a small Büchner funnel and wash the residue three times with 2- to 3-ml. portions of water. Dilute the combined filtrate and washings with water to a definite volume in the range of 25 to 35 ml. (Solution A).

**Ether Extraction.** Place a 15- to 25-ml. aliquot of Solution A in the sample tube of a continuous liquid-liquid extractor. The internal diameter of this tube should be such that the solution fills it to a depth of 8 to 10 cm. Saturate the solution with solid potassium chloride, adjust to about pH 10 with anhydrous potassium carbonate, and extract with ether at a reflux rate of 2 to 4 drops per second for 24 hours. Evaporate the ether extract on a steam bath, take up the residue in 1-butanol, and dilute with this solvent to 25 ml. (Solution B).

**Standard Curve and Color Development.** Place duplicate 0.2-, 0.4-, 0.6-, 0.8-, and 1.0-ml. aliquots of the β-aminopropionitrile standard solution in two series of matched colorimeter tubes (the Evelyn colorimeter with 620 mμ Evelyn filter was used), add 1 ml. of the ninhydrin solution to each, and bring the total volume in each tube to 12 ml. by the addition of 1-butanol. Prepare a reagent blank in the same way with no β-aminopropionitrile present.

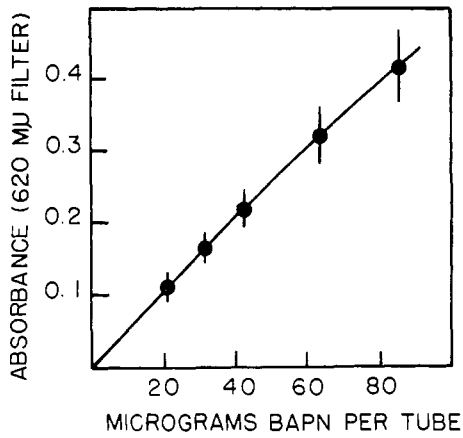


**Figure 1. Standard curve. Vertical lines at each experimental point represent extreme variations in 10 individual standard curves**

Place duplicate 0.5-, 1.0-, 2.0-, and 5.0-ml. aliquots of Solution B in colorimeter tubes and treat in the same manner as the standard series. Close all the tubes with rubber stoppers bearing an 8-cm. length of 6-mm. glass tubing to serve as air condensers, and heat in a boiling water bath for 20 minutes. Cool the tubes for 2 minutes in 10° to 20° C. water (or until the internal temperature drops to 20° to 25° C), add 0.05 ml. of the sodium hydroxide solution to each, reheat all tubes in boiling water for 4 minutes, and finally cool again for 2 minutes. This procedure develops the characteristic green color of the ninhydrin–β-aminopropionitrile reaction product.

Determine the percentage light transmittance of each tube as compared to the reagent blank with a suitable photoelectric colorimeter or spectrophotometer at or near 640 mμ. Plot the absorbance values of the standard series against the corresponding β-aminopropionitrile contents to obtain a standard curve (Figure 1). Prepare such a standard curve for each assay.

**Calculation of Results.** Estimate the β-aminopropionitrile content of each tube containing Solution B by interpolation on the standard curve. Use only those values which fall on the steeply rising part of the curve, from about 20 to 80 γ of β-aminopropionitrile per tube, in subsequent calculations. Calculate β-aminopropionitrile content per ml. of Solution B for each tube in the usable range, and examine the results for agreement between replicates at the same level, and between replicates at different levels. Replicates should agree within ± 10%. The results at increasing levels often show greater variations, but are considered satisfactory if the extreme deviations do not exceed 10% of the mean and if no consistent trend in either direction is evident. If at least three replicates falling in the proper range of the standard curve give satisfactorily concordant values for the β-aminopropionitrile content of Solution B, average the values and use the average for calculating the content per gram of the original sample.

### Conditions Affecting Color Development

**Wave Length of Maximum Absorption.** The absorption spectrum of the green colored solution was determined with a Cary recording spectrophotometer, and showed a single maximum in the visible at 640 mμ (Figure 2). The solution used was prepared from 86γ of β-aminopropionitrile as described above, except that the initial heating period was 10 rather than 20 minutes.

**Time of Heating.** Two series of tubes containing 60 and 179 γ, respectively, of β-aminopropionitrile per tube were carried through the color development procedure, with various tubes being heated for 10, 20, 30, or 40 minutes. In each series, maximum color was present at the end of 20 minutes of heating, and on longer heating the color gradually decreased (Table I).

**Effect of pH.** Acids were found to destroy the green color with the formation of tan to brown shades in the test solutions. Alkali on the other hand intensified the green color severalfold (Table II). When the sodium hydroxide was added before heating, a white precipitate formed in the butanol solution. However, no such turbidity appeared if the alkali was not added until after the tubes had been heated and then cooled to 20° to 25° C.

**Ninhydrin Concentration.** The effect of variations in the ninhydrin concentration was first studied before the influence of alkali on the development of the green color had been discovered. In the absence of added alkali little increase in color intensity resulted when more than 10 mg. of ninhydrin was used per tube. Consequently 20 mg. per tube was adopted for the recommended procedure, and the analyses reported in this paper were carried out at this ninhydrin level. However, when alkali was used, still higher ninhydrin concentrations gave

**Table I. Effect of Heating Time on Color Development**

| γ BAPN per Tube | Heating Time, Min. 10 | 20 | 30 | 40 |
|---|---|---|---|---|
| | Absorbance[a] | | | |
| 60 | 0.092 | 0.208 | 0.194 | 0.168 |
| 179 | 0.310 | 0.377 | 0.310 | 0.276 |

[a] 20 mg. ninhydrin per tube, but no alkali.

**Table II. Effect of Alkali on Color Development**

| γ BAPN per Tube | Absorbance: No alkali | Absorbance: 1 to 2 mg. NaOH per tube |
|---|---|---|
| 12 | 0.0066 | 0.135 |
| 24 | 0.0121 | 0.252 |
| 35 | 0.0166 | 0.392 |
| 47 | 0.0233 | 0.509 |
| 71 | 0.0235 | 0.724 |

**Table III. Stability of Color**

| γ BAPN per Tube | Time at Room Temperature, Min. 0 | 35 | 165 |
|---|---|---|---|
| | Absorbance[a] | | |
| 22 | 0.180 | 0.186 | 0.196 |
| 43 | 0.350 | 0.362 | 0.369 |
| 65 | 0.500 | 0.516 | 0.534 |
| 108 | 0.831 | 0.851 | 0.878 |

[a] 0.05 ml. NaOH solution plus 20 mg. ninhydrin per tube.

progressively more intense green colors with the same amounts of β-aminopropionitrile (Figure 3). This effect was not further investigated, except that a few samples were reassayed with 100 mg. of ninhydrin present per tube. The values found on repeated analyses were:

| 20 Mg. Ninhydrin per Tube: Caley peas, mg. % | 20 Mg. Ninhydrin per Tube: L. odoratus peas, mg. % | 100 Mg. Ninhydrin per Tube: Caley peas, mg. % | 100 Mg. Ninhydrin per Tube: L. odoratus peas, mg. % |
|---|---|---|---|
| 21.0 | 153 | 25.1 | 170 |
| 21.2 | 163 | 22.3 | 170 |
| 19.3 | 158 | 17.0 | 172 |
| 15.7 | 164 | 17.2 | 168 |
| 16.8 | 162 | | |

**Stability of Color.** The change in absorbance of the colored solution on standing at room temperature is small during the first 30 minutes and increases slightly with increasing time, as shown in Table III. Off-shade colors (tan to brown) have been observed in the more concentrated solutions (beyond 70 γ of β-aminopropionitrile per tube) after standing longer than 90 minutes. It is recommended that the readings be taken immediately after the final cooling period.

### *Results and Discussion*

The specificity of this method was checked by reacting a number of related substances with ninhydrin under the recommended conditions. The results in Table IV show that, of the compounds tested, only hydrazine and phthalhydrazide produced green shades. As far as the authors are aware, hydrazine derivatives are not of common natural occurrence. Amino compounds in general are well known to give pink or lavender shades with ninhydrin, while proline produces a yellow color. The specificity of the test as a measure of β-aminopropionitrile is further enhanced by the ether extraction step, which is necessary in any event to avoid interference from amino acids and other water-soluble ninhydrin-positive substances that would be expected in the original aqueous extract of the sample.

When the pure Lathyrus factor, I, was carried through the recommended procedure, the β-aminopropionitrile values obtained varied from 93 to 97% of the theoretical. Recoveries of I added to ground legume samples ranged from 86 to 92%. The reproducibility of the standard curve is indicated by Figure 1, where the vertical lines at each experimental point represent the extreme variations encountered in a series of 10 such curves. Repeated analyses of one sample gave values of 163, 157, 158, 164, and 162 mg. %.

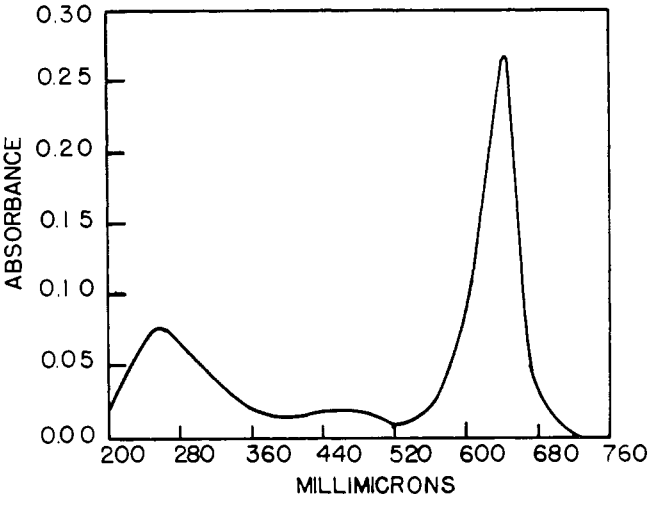


Figure 2. Absorption spectrum in butanol solution of green substance produced by heating BAPN and ninhydrin

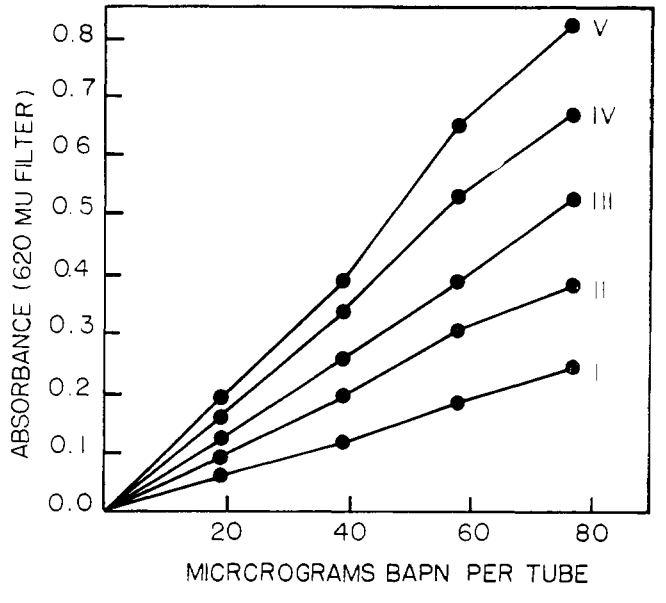


Figure 3. Relation between ninhydrin concentration and intensity of green color developed

I to V. 5, 10, 20, 50, and 100 mg. of ninhydrin per tube, respectively

**Table IV. Ninhydrin Color Reactions of BAPN Analogs and Other Nitrogeneous Compounds**

| Compound | Formula | Color Developed |
|---|---|---|
| Acrylonitrile | $CH_2$:CHCN | None |
| β-Alanine | $NH_2CH_2CH_2COOH$ | Violet-purple |
| Allylamine | $CH_2$:$CHCH_2NH_2$ | Purple |
| Aminoacetonitrile | $NH_2CH_2CN$ | Tan-yellow |
| *Bis*-β-(cyanoethyl)-amine | $NH(CH_2CH_2CN)_2$ | Tan-yellow |
| Ethanolamine | $HOCH_2CH_2NH_2$ | Violet-purple |
| Ethylene cyanohydrin | $HOCH_2CH_2CN$ | Brown-orange |
| Glyconitrile | $HOCH_2CN$ | Purple |
| Hydrazine | $NH_2NH_2$ | Green |
| Lactonitrile | $CH_3CH(OH)CN$ | None |
| Propionitrile, β-acetamido- | $CH_3CONHCH_2CH_2CN$ | None |
| Propionitrile, β-dimethylamino- | $(CH_3)_2NCH_2CH_2CN$ | None |
| Propionitrile, β-(*N*-γ-L-glutamyl)-amino- | $CONHCH_2CH_2CN$ <br> $\vert$ <br> $CH_2CH_2CH(NH_2)COOH$ | Pink-purple |
| Phthal hydrazide | $C_6H_4CONHNHCO$ | Green |

Recovery experiments using an acid or basic treatment to extract and hydrolyze the Lathyrus factor gave low, but variable, results. The low values were attributed to hydrolysis of β-aminopropionitrile to β-alanine, the presence of which was readily detected by paper chromatography.

The mechanism of the reaction between β-aminopropionitrile and ninhydrin, and the nature of the green substance are unknown. Japanese workers (*11*), in a study of the reaction between 2-methyl-4-amino-5-ethylamino pyrimidine and ninhydrin, found that one of the products isolated from the reaction mixture produced a green color in the presence of sodium hydroxide. This uncharacterized compound when mixed with ninhydrin formed a substance, $C_{18}H_{10}O_6$, corresponding to hydrindantin.

The analytical results obtained on a number of Lathyrus species are collected in Table V. *L. latifolius* and *L. splendens*, which have been reported to be extremely toxic to rats (*7*) and *L. strictus*, *L. sylvestris Wagneri* (flat pea), and *L. Tingitanus* showed no detectable β-aminopropionitrile by this method. Likewise two of the Lathyrus species most often implicated as causative agents of human lathyrism—namely, *L. sativus* and *L. cicera* (*8*)—also appear to contain no appreciable quantities of this substance. These results provide further convincing evidence that human lathyrism and the syndrome produced in rats by *L. odoratus* are distinctly different conditions (*9*). Other legume seeds (at least one variety tested), giving negative results are: soybean, garden pea (three varieties), garden bean (three varieties), clover (two varieties), alfalfa (two varieties), mung bean, cow pea, hairy vetch pea, white lupine, and fava bean.

Dupuy and Lee (*4*) independently demonstrated the presence of bound β-aminopropionitrile in *L. odoratus* and *L. pusillus* and its absence in *L. latifolius*, *Vicia sativa*, *Canavalia ensiformis*, *Phaseolus coccineus*, *Trifolium incarnatum*, and *Medicago sativa* by a similar qualitative test with ninhydrin. The legumes were extracted for 12 hours in a Soxhlet extractor with absolute methanol and the methanol extracts chromatographed on Whatman No. 1 paper in a butanol-acetic acid–water system. Upon spraying the paper with 0.15% ninhydrin in acetone, a marine-blue color for β-aminopropionitrile was obtained.

In order to compare the two procedures, a sample of *L. odoratus* was analyzed according to Dupuy and Lee's method. Visual comparison of the color obtained with the color resulting from the present method showed little or no difference in shade. An exact characterization of the color, as obtained by the procedure described herein, is provided by the spectrum shown in Figure 2.

It is estimated that β-aminopropionitrile concentrations down to about 5 mg.% in the original samples would have been detected by the present method. In some cases, red to brown shades developed in the final test solution instead of the usual green. Paper chromatograms of concentrated extracts of the sample were run in each of these cases, and uniformly failed to show any green spot.

**Table V. BAPN Content of Seeds of Various Lathyrus Species**

| Lathyrus Species | Common Name or Variety | BAPN Content,[a] Mg. per 100 G. |
|---|---|---|
| *Odoratus* (flowering sweet pea) | Cuthbertson | 92 |
| | Giant Spencer | 58 |
| | Early Spencer | 160 |
| | Ruffled Spencer | 75 |
| *Pusillus* | Singletary pea | 62 |
| *Hirsutus* | Caley pea | 21 |
| *Odoratus* (Cuthbertson) | | |
| Germinated seed | | 71 |
| Plant (above ground level) | | Trace |
| Roots (below ground level | | 0 |

[a] Air-dried basis.


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## References


(1) Bachhuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., Strong, F. M., *Proc. Soc. Exptl. Biol. Med.* **89,** 294–7 (1955).

(2) Buc, S. R., *Org. Syntheses* **27,** 3–5 (1946).

(3) Dasler, W., *Proc. Soc. Exptl. Biol. Med.* **88,** 196–9 (1955).

(4) Dupuy, H. P., Lee, J. G., *J. Am. Pharm. Assoc., Sci. Ed.* **45,** 236–9 (1956).

(5) Lewis, H. B., Fajans, R. S., Esterer, M. B., Chen, C. W., Oliphant, M., *J. Nutrition* **36,** 537 (1948).

(6) Schilling, E. D., Strong, F. M., *J. Am. Chem. Soc.* **77,** 2843–5 (1955).

(7) Schulert, A. R., Lewis, H. B., *Proc. Soc. Exptl. Biol. Med.* **81,** 86–9 (1952).

(8) Stockman, R., *J. Pharmacol. Exptl. Therap.* **37,** 43 (1929).

(9) Strong, F. M., *Nutrition Revs.* **14,** 65–7 (1956).

(10) Wawzonek, S., Ponseti, I. V., Shepard, R. S., Wiedenmann, L. G., *Science* **121,** 63–5 (1955).

(11) Yamagishi, M., *J. Pharm. Soc. Japan* **74,** 599–601 (1954).



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