Table IV shows results obtained for a variety of yeast cultures. Closest agreement between ergosterol determined by analysis and purified ergosterol isolated occurs when the 230 mμ–281.5 mμ ratio is low. A low ratio indicates that the relative proportion of 24(28)-dehydroergosterol is low and fewer recrystallizations are required to give purified ergosterol.

Discussion

The method described is applicable to samples containing 5 to 100 mg. of yeast solids, and 0.05 to 2.0 mg. of ergosterols. Because of its simplicity, it is useful in obtaining reliable results on large numbers of yeast samples. By making use of the correction for 24(28) - dehydroergosterol, values for ergosterol content are indicative of the amount of ergosterol that can be isolated.

In developing the method, the use of strong alkali in digestion of the yeast was found desirable, as at alkali concentrations of 10% or less low values were obtained. Optimal range of alcohol concentration during the yeast digestion was from 20 to 80%. In partitioning the alcoholic alkali digestion mixture with *n*-heptane, the alcohol concentration was found to be more critical, and it was necessary to adhere closely to the relative amounts of alcohol and water prescribed.

Acknowledgment

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RUMEN MICROBIOLOGY

Characteristics of Free Rumen Cellulases

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By using the viscometric assay for cellulase with a carboxymethylcellulose substrate, the presence of soluble cellulases in rumen ingesta has been demonstrated. At least three enzymes appear to be involved. These enzymes are relatively thermostable and insensitive to oxygen, and have a pH optimum of about 6.5. Some samples of rumen ingesta contain a cellulase inhibitor. In other samples, no evidence of inhibitor is seen, but an activator is present.

THE ENZYMATIC HYDROLYSIS OF CELLULOSE by enzymes from aerobic fungi has been investigated actively in recent years, largely as a result of economic pressures. In the paper, wood, and textile industries, methods of preventing or controlling the process have been the ultimate goal. In the microbiological disposal of cellulosic wastes, and in ruminant nutrition, enhanced degradation of cellulose has been the long-range objective. In the balance of organic matter in soil, cellulose hydrolysis is an integral part of the humification process. A considerable volume of literature has accumulated from study of the cellulases of aerobic fungi, but aside from the recent reports of Kitts and Underkofler (*6*) and Cason and Thomas (*2*) on rumen "cellulase" and of Conchie (*3*) on rumen β-glucosidases, detailed reports on rumen enzymes are not available.

Current understanding of the cellulose-decomposing enzymes of aerobic microorganisms has been critically reviewed by Reese (*11*). From the viewpoint of comparative biochemistry, the process of cellulose hydrolysis in the rumen would be expected to resemble the process in aerobic cultures in principle, but with differences in detail. On the basis of this assumption, the cellulase activity of the rumen would probably result from a mixture of several predominantly extracellular enzymes (*4, 8*) which are relatively thermostable (*7*) and capable of attacking carboxymethylcellulose derivatives of degrees of substitution less than 1.0 (*12*).

Whether the sites of attack on the cellulose chain are random or not appears to depend on the selection of organisms. The major cellulase of *Myrothecium verrucaria* appears to cleave cellulose at random glycosidic linkages (*11*). In contrast, Nisizawa and Kobayashi (*10*) have described the cellulase of *Irpex lacteus* as yielding only cellobiose as a hydrolytic product. Such data suggest that the cleavage here is reminiscent of that seen with β-amylases, which degrade amylose from the nonreducing end yielding only maltose. Whether both

α- and β-cellulases (using nomenclature based on the analogous amylases) exist commonly remains an open question.

In this investigation the authors have used the viscometric procedure for detecting cellulase with a carboxymethylcellulose (CMC-70-H) substrate, in contrast to the reducing sugar assay used by Kitts and Underkofler (*6*). The viscometric method has been adequately described and evaluated by Levinson and Reese (*7*). Its chief advantage is that it has greater sensitivity to α-cellulases than most reducing sugar procedures. This enhanced sensitivity allows the brief incubation times dictated by the relative instability of most enzymes. Because the enzyme activity measured is really the summation of the effects of several different enzymes, probably possessing different specificities and different thermal and pH optima, no attempt to develop a true assay has been made. It has been possible, however, to demonstrate the presence of free cellulases in rumen ingesta and to describe certain of the basic characteristics of these enzymes.

Experimental Methods and Results

Samples of rumen ingesta were obtained *par fistulum* from 3-year-old steers on a maintenance ration, except where otherwise noted. The samples were taken 4 or more hours after feeding, because samples obtained shortly after feeding were frequently of very low activity. After being strained through double cheesecloth, the "juice" was carried to the laboratory. Centrifugation for 15 minutes at 17,000 to 25,000 times gravity in 10-ml. cellulose nitrate tubes yielded a visually clear "supernatant." In some studies the supernatant was passed through a sterilizing filter (Morton or Berkefield) to remove all cells. Hereafter "juice," "supernatant," and "filtrate" refer to these three preparations.

Viscometric cellulase determinations were conducted at room temperature in hand-blown Ostwald viscometers according to the basic method of Levinson and Reese (*7*). The boiled-enzyme controls which are usually satisfactory for enzyme studies are not completely inactivated in the case of many cellulases. In these studies, the boiled-enzyme controls were heated for 15 minutes at 121° C. in an autoclave.

In order to determine whether a significant portion of the detectable cellulases in rumen juice was actually free from cells, the activities of the juice, supernatant, and filtrate preparations from the same sample were compared. Several samples from different animals on different days were used. The results obtained from a typical sample are shown in Figure 1. Figure 2 compares the activity of juice with that of sterile filtrates obtained using Morton and Berkefield filters. With some samples, as in Figure 1, there was no great difference in the activity of the three preparations, but in other samples (see Figure 2) the supernatant and filtrate contained as little as $^1/_3$ of the activity in the juice.

That the changes in viscosity catalyzed by rumen cellulases are roughly indicative of cellulose hydrolysis can be seen in Figure 3. Here simultaneous determinations of change in viscosity and reducing sugar liberation were made on aliquots from the same hydrolysis reaction. Sugar determinations were made using the microcolorimetric procedure of Somogyi (*13*) as modified by Nelson (*9*).

The quantitative effect of temperature on cellulase activity cannot be elucidated accurately using the viscometric procedures, because there is no satisfactory method for comparing curves having widely different initial viscosities (*7*). This is an unfortunate limitation inherent in viscometric studies, which results from the fact that the viscosity of any given carboxymethylcellulose solution falls rapidly as the temperature rises. The effect of temperature on a cellulase, however, can be crudely evaluated viscometrically. The results of such an experiment are shown in Figure 4.

Comparison of the activity of supernatant samples which had been vigorously aerated with tank oxygen at room temperature for 5 minutes with samples held for 10 minutes at 4° C. in the presence of 1 mg. of cysteine per ml., showed no detectable difference in enzyme activity.

The effect of pH on the activity of the crude enzyme recovered in acetone powders from the supernatant fraction reconstituted to the initial rumen concentration was examined by determining the change in fluidity of a solution containing enzyme plus CMC-70-H in phosphate buffers adjusted to pH's from 4.0 to 9.0 in increments of 0.5. No detectable change in pH occurred. The results of a typical experiment are shown in Figure 5. No consistent differences between samples taken from animals on high grain and high hay rations were observed in either the total activity or the pH optima.

Preliminary attempts at fractionation of rumen cellulases by alcohol, acetone, and paper electrophoresis using the supernatant preparation were made. No activity was recovered in protein fractions precipitated in the cold at final ethyl alcohol concentrations between 10 and 80 volume % in 10% increments. The alcoholic supernatants were also inactive. Acetone powders, on the other hand, were active.

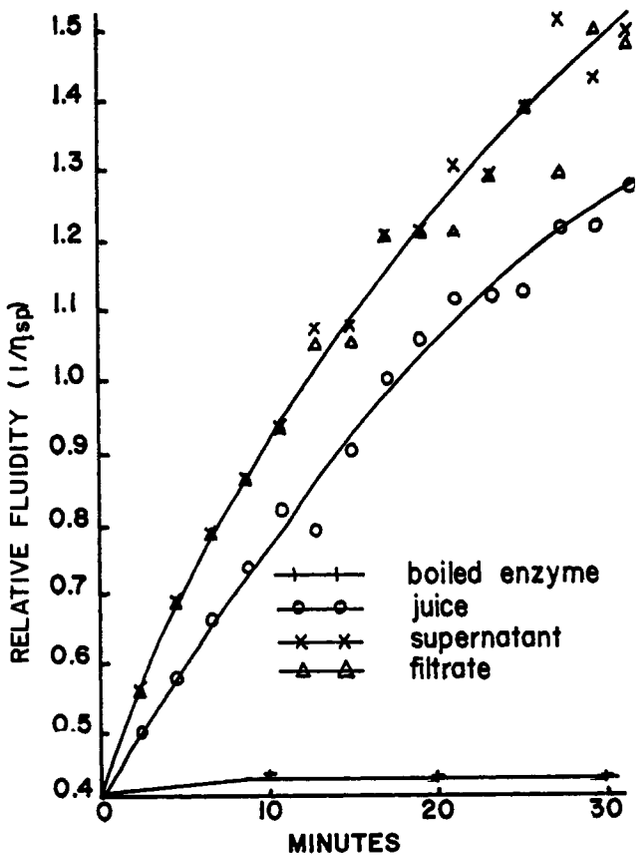

Figure 1. Hydrolysis of CMC-70-H by three fractions of rumen ingesta

System. 3.0 ml. of 0.20% CMC-70-H in water plus 1.0 ml. of enzyme at 24° C.

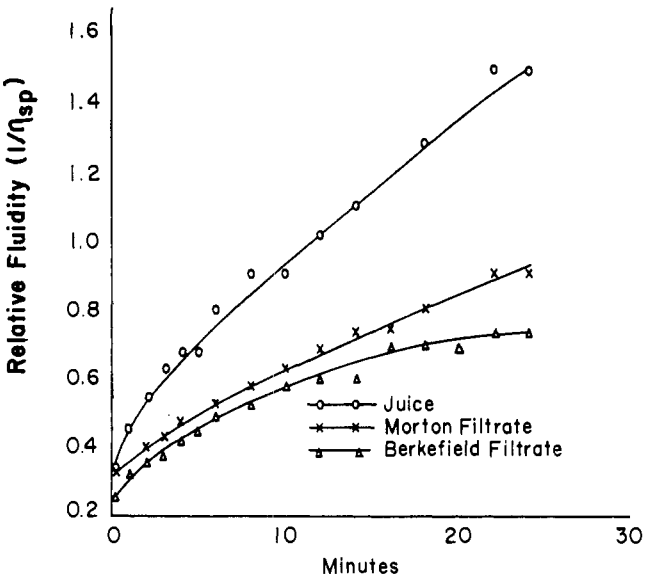

Figure 2. Cellulase activity in rumen juice and two sterile filtrate fractions

System. 3.0 ml. of 0.20% CMC-70-H in water plus 1.0 ml. of enzyme at 37° C.

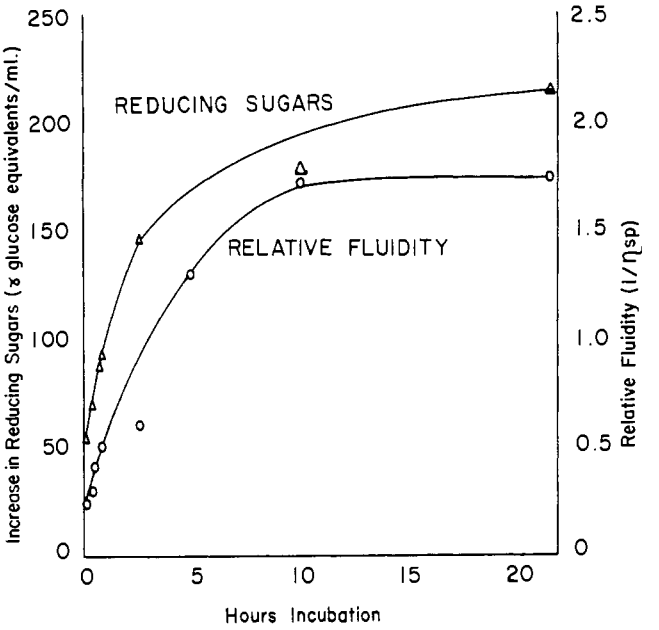

Figure 3. Liberation of reducing sugars and increase in fluidity on incubation of rumen cellulase with CMC-70-H

System. 3.0 ml. of 0.20% CMC-70-H in 0.05M phosphate buffer at pH 6.70 saturated with Dowicide A at 25° C.

Paper electropherograms of 0.01 ml. of the supernatant, after migrating for 6 hours in veronal buffer (pH 8.5, ion strength 0.06) under a potential gradient of 12 volts per cm., indicated the presence of at least two migrating cellulases and a single stationary "β-glucosidase." At the end of the separation the strips were cut into 1-cm. sections, and each section was submerged in 4 ml. of 0.20% carboxymethylcellulose in 0.1*M* phosphate buffer at pH 6.75, saturated with Dowicide A, and incubated at room temperature for 12 hours. The viscosity of the reaction mixture was then determined. Increase in fluidity over the initial value was used in establishing enzyme distribution along the strip. β-Glucosidase activity was estimated by determining the amount of 6-bromo-2-naphthol liberated when 1-cm. sections of duplicate electropherograms were incubated with 4 ml. of $10^{-4}M$ 6-bromo-2-naphthyl-β-D-glucopyranoside in 0.03*M* acetate buffer at pH 3.5 saturated with Dowicide A, for 12 hours by the procedure of Hash and King (*5*). The distribution of cellulases on paper electropherograms is shown in Figure 6. Traces of β-glucosidase activity were apparent near the origin but nowhere else.

Enzyme-time studies on supernatant preparations of ingesta from animals fed either hay or grain only have not been consistent. These studies were conducted using 3 ml. of 0.20% CMC-70-H in 0.05*M* phosphate buffer at pH 6.75 as substrate and 1 ml. of supernatant or supernatant plus water as enzyme. With a given enzyme preparation the results were reproducible, but some

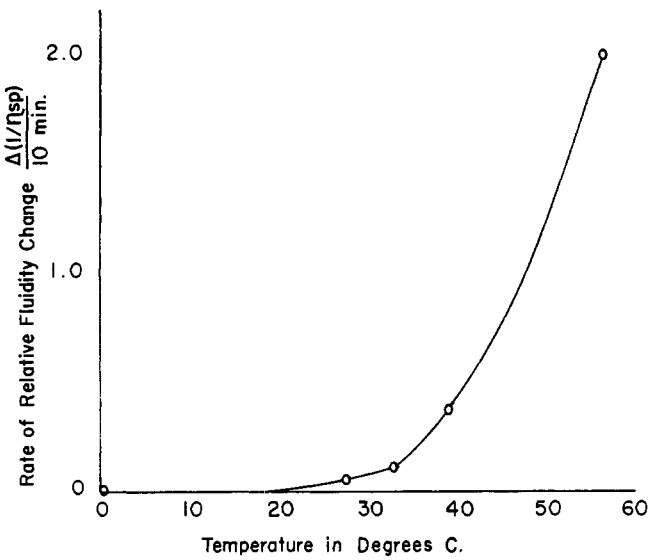

Figure 4. Effect of temperature on CMC-70-H hydrolysis by rumen cellulases

System. 3.0 ml. of 0.20% CMC-70-H in water plus 1.0 ml. of juice, incubated 10 minutes at temperatures shown

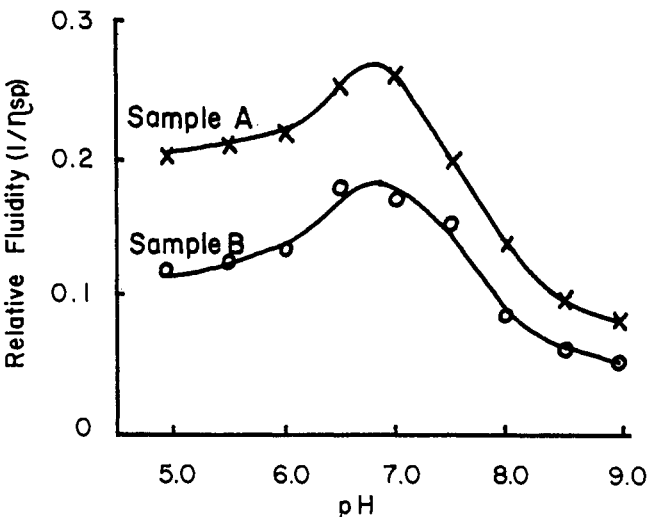

Figure 5. Effect of pH on activity of rumen cellulases

System. 3.0 ml. of 0.05M phosphate buffers containing 0.20% CMC-70-H plus 1.0 ml. of enzyme. Incubation at 23° C. for 30 minutes. Sample A from animal on 100% grain ration for 3 days, sample B from animal on 100% hay ration for 3 days

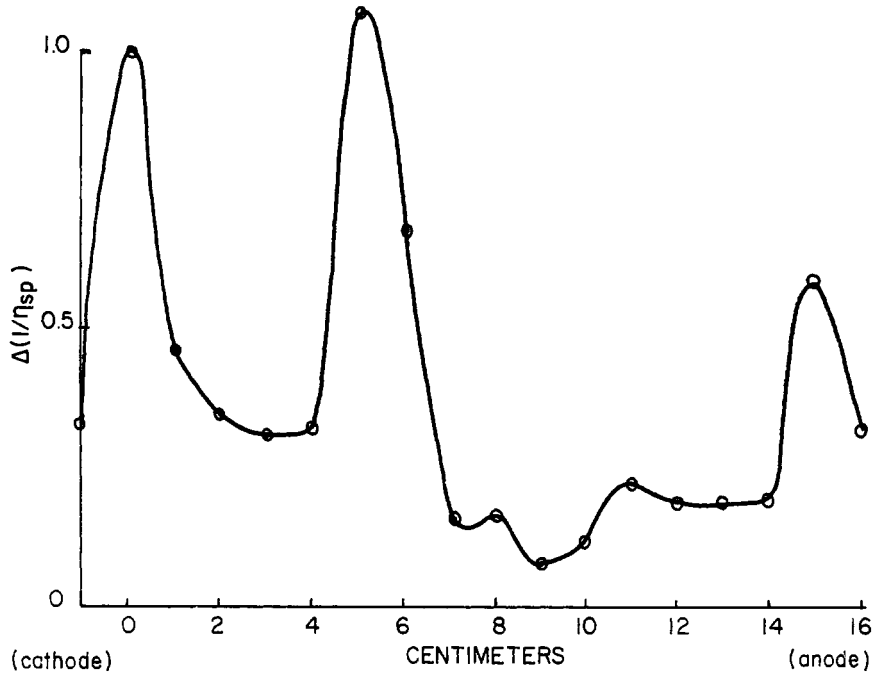

Figure 6. Paper electrophoretic separation of rumen cellulases

samples indicated the presence of an inhibitor (hay fed of Figure 7), while most samples indicated an activator (grain fed of Figure 7). When several dilutions of a given crude enzyme preparation are incubated for varying times selected so that the product of enzyme concentration and incubation time is constant, the amount of reaction catalyzed will be constant if neither cofactors nor inhibitors are present in the enzyme source. The enzyme must, of course, be known to be stable under the conditions of the experiment. This is true regardless of the kinetics of the reaction, because all dilutions pass through identical sequences of reaction order but at rates that are proportional to the amount of enzyme. The presence of an inhibitor in an enzyme preparation can therefore be detected by an increased amount of reaction at the higher enzyme dilutions; activators and cofactors result in decreasing activity as the enzyme is diluted.

Discussion

Certain of the results reported here are in close agreement with those of Kitts and Underkofler (*6*). The unusual temperature stability of the enzyme has been corroborated. This characteristic appears to be common among cellulases. The pH optimum observed here is approximately 6.5, somewhat higher than that previously reported.

The conspicuous difference between these results and those of Kitts and Underkofler (*6*) is the observation that there is free cellulase in rumen ingesta under a variety of conditions and that the fraction of the total activity which is soluble is large, 30 to 90%. The authors have been unable to rationalize the difference. The activity passed two different kinds of sterilizing filters. With the thought that perhaps Berkefield filters which had been used for a number of years might have adsorbed materials which inhibited cellulases or might carry sufficient charge to retain the enzyme on the surface of the filter itself, the activity of filtrates which had been passed through old filters was checked. There was no retention of the enzyme. Probably the difference in results arises from the difference in the analytical methods used. The viscometric procedure and the microcolorimetric procedure for reducing sugars used here allow quantitative detection of hydrolysis which has proceeded only to the extent of 0.01 to 0.02% completion. The lowest level of activity reported as numerically significant by Kitts and Underkofler (*6*) is equivalent to 1% hydrolysis. Presumably, the much greater sensitivity of the assays reported here explains the discrepancy in results.

The partitioning of rumen cellulases among the aqueous phase, plant fiber particles, and the microbial cells themselves undoubtedly varies with such factors as pH, ion strength, temperature, time since feeding, and ration composition. Whether a significant fraction of the total rumen cellulases is an integral part of the bacterial cell surfaces remains an open question. The problem has not yet been approached by use of surface enzyme-inhibiting agents such as uranyl ion, but photomicrographic studies have suggested that the cellulases of many bacteria may be bound to the cell surface (*7*). The present data in no way negate the surface enzyme concept with regard to rumen organisms, but rather demonstrate that an appreciable portion of the rumen cellulases is elaborated in the form of a typical, free, extracellular enzyme system.

The activities reported here are typical of those obtained from the ingesta of three fistulated steers at various times during the day. When these supernatants are assayed, the amount of enzyme is considerably less than that reported in fungal filtrates by Levinson and Reese (*7*).

The idea that rumen "cellulase" is a single enzyme must be viewed with reserve, considering the electrophoretic

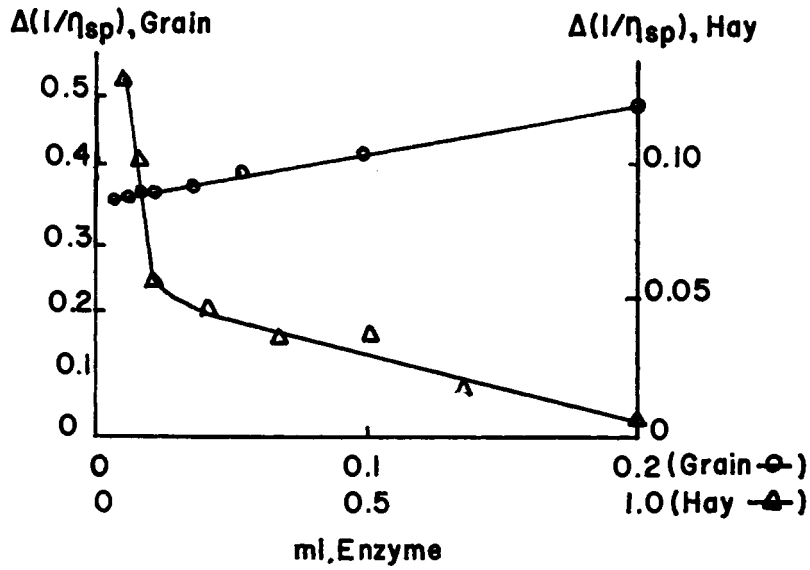

Figure 7. Evidence of cellulase inhibitor and activator in rumen ingesta

System. 3.0 ml. of 0.2% CMC-70-H in 0.05*M* phosphate at pH 6.75 plus 1.0 ml. of enzyme at 22° C. Both samples supernatants.

$$E \times T_{grain} = 1.0 \text{ ml. min.}$$
$$E \times T_{hay} = 3.0 \text{ ml. min.}$$

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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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.