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The enzymatic method for the determination of ammonia with glutamate dehydrogenase has been adapted to the AutoAnalyzer. The automated method was applicable for NH₃ measurement in sheep rumen samples, swine gut fluid samples, and bovine plasma. Results were compared with those of the Conway diffusion method and the manual enzymatic method. The automated method allows 30 samples per h to be analyzed routinely. Values were comparable with those with the manual method, but lower than those with the Conway diffusion method.

The specificity of the enzymatic determination of ammonia (NH₃) has been previously described (2, 3, 4). The sensitivity of this determination has been increased at least fivefold by monitoring the change in reduced nicotinamide adenine dinucleotide (NADH) concentration fluorometrically rather than spectrophotometrically (5). This increased sensitivity permits measurement of NH₃ concentrations found in various biological fluids. The desire to measure NH₃ production rates in swine gut fluid contents prompted us to automate the NH₃ determination.

We describe here the use of AutoAnalyzer equipment to measure NH₃ concentrations in rumen fluid, swine gut fluid, and bovine blood plasma.

MATERIALS AND METHODS

Apparatus. A single-channel, AutoAnalyzer continuous-flow analytical instrument (Technicon, Tarrytown, N.Y.) was used with a Turner fluorometer model III (G. K. Turner Associates, Palo Alto, Calif.). The fluorometer had a flow-through door, a 7-60 (360 nm) excitation filter, and a no. 8 (485 nm) emission filter. The Technicon sampler was set at 30 samples per h, with a sample-to-wash ratio of 2:1. The manifold diagram is shown (Fig. 1).

Reagents. Ammonia-free water was used to prepare all reagents. Potassium phosphate buffer (0.1 M, pH 7.8) was used to make up both the stock NADH (2.5 × 10⁻³ M) and the stock substrate solutions. The stock substrate solution contained α-ketoglutaric acid (3 × 10⁻³ M), adenosine diphosphate (1 × 10⁻⁴ M), and glutamate dehydrogenase (GDH) (ammonium sulfate free) (EC 1.4.1.3) (75 IU per ml), all from Calbiochem (LaJolla, Calif.) plus ethylenediaminetetraacetic acid (10⁻⁴ M). The substrate cocktail for samples in the range of 3 to 30 μmol/ml was prepared by mixing two parts of stock NADH solution with 1 part of stock substrate solution immediately before use. This solution was kept on ice during the assay and was protected from light. The substrate cocktail was consumed at a rate of about 10 ml/h. Sample blanks were determined by mixing 2 parts of stock NADH with 1 part of phosphate buffer. Immediately before use, 1 ml of Triton X-405 (Sigma Chemical Co., St. Louis, Mo.) wetting agent was added to each liter of phosphate buffer.

For samples in the range of 0.1 to 5 μmol/ml, the stock NADH was diluted to 1.25 × 10⁻⁵ M before mixing with the stock substrate solution. The sample line was also changed from 0.0075 inch (ca. 0.19 mm) to 0.025 inch (ca. 0.64 mm) ID.

Standards. Anhydrous ammonium chloride was used to make the standard solutions in the range of 0.1 to 30 μmol/ml.

Samples. Rumen fluid was obtained from a fistulated sheep, strained through cheesecloth, and centrifuged at 16,000 rpm in a refrigerated centrifuge for 15 min. A portion was immediately analyzed, and the rest was divided into 2-ml cups and stored at −70°C.

Swine gut content samples obtained at autopsy from the proximal colon, the distal colon, and the cecum were incubated anaerobically. At specified times, portions of the samples were taken, and the incubation was stopped by plunging the sample into an ice bath; the sample was centrifuged at 16,000 rpm, and the supernatant was stored at −70°C until analyzed.

Bovine plasma samples were obtained from a normal steer. Each heparinized blood sample was centrifuged immediately, and a portion was analyzed immediately. The rest was divided into 2-ml cups, stored at −70°C, and used later for precision studies.

Procedure. Analysis was begun by pumping phosphate buffer through all lines and setting the fluorometer base line for zero fluorescence with the zero adjust knob on the fluorometer. The fluorescent NADH containing substrate cocktail was then pumped through.
its line, and the fluorescence reading was set to full scale by adjusting the slit and holes on the excitation side of the sample compartment. The conversion of NADH to NAD decreased fluorescence intensity.

The following parameters were investigated in efforts to obtain maximum sensitivity of the system with minimum sample carry-over and analysis time: (i) NADH concentration; (ii) glutamate dehydrogenase activity; (iii) dialysis time; and (iv) incubation time.

The method was validated by comparing the AutoAnalyzer results with results with the Conway diffusion method (1) and the manual enzymatic method (4).

The storage stability of the rumen fluid and plasma samples was evaluated by analysis immediately after collection and on days 1, 2, 8, 14, and 22. Each sample was determined in triplicate, and three new cups were thawed for each day's analysis.

RESULTS

The best compromise for the automated test parameters is shown in the manifold design (Fig. 1). The concentrations of reagents that provide the best sensitivity are listed in the methods section. Increasing the NADH concentration decreased the sensitivity but provided a more stable base line. Decreasing NADH concentration increased the sensitivity; the increase was limited by the noise in the base line. The relative fluorescence of the NH₃ standard (9 μmol/ml) increased with increasing GDH activity up to about 60 IU/ml, after which there was no further increase in fluorescence. For a margin of safety, 75 IU of GDH activity was chosen. Doubling the dialysis time of the NH₃ sample (9 μmol/ml) did not increase the fluorescence, but did increase the sample-sample interaction and caused the base line to be more erratic. Increasing the incubation time by passing it through the heating bath a second time did not increase the fluorescence.

A typical run of NH₃ standards is shown in Fig. 2. At the sampling rate of 30 samples per h, the peak height of the NH₃ standard (15 μmol/ml) reached 97% of the steady-state peak height, and there was only a 1.9% sample to sample interaction. The relationship between relative fluorescent peak height and concentration of ammonia was found to be linear over the concentration range of 3 to 30 μmol of NH₃ per ml and over the concentration range 0.1 to 5 μmol of NH₃ per ml. At the higher concentration the line had a slope of 5.59 with an intercept at −2.6. The correlation coefficient was 0.999. For the lower concentration range the slope was 136 with an intercept at −0.89. The correlation coefficient was 0.999.

The plasma samples and the gut fluid samples with NH₃ concentrations below 3 μmol/ml were determined by diluting the NADH reagent by one-half, increasing the sample line size eightfold, and adjusting the sensitivity of the fluorometer. The base line became more erratic, and the coefficient of variation of replicate samples doubled. The limit of sensitivity was 0.05 μmol/ml, which could be differentiated from background.

The precision of the automated method and storage characteristics of the rumen fluid sample pools and of the bovine plasma pools are shown in Table 1. The rumen fluid samples were repeatable and had no detectable change with

![Manifold design for ammonia determination.](http://aem.asm.org)
storage at −70°C for 22 days. Three of the four plasma samples increased in NH₃ concentration with storage, and this increase was most apparent in the sample with the lowest NH₃ concentration.

The comparison between the AutoAnalyzer method, the manual enzymatic method, and the Conway diffusion method is shown for the rumen fluid samples in Table 2. The values found by the manual and automated enzymatic methods agree, but the values found by the Conway diffusion method are higher. Values on swine gut fluid samples were also higher with the Conway diffusion method than with the automated method (Fig. 3). The equation of the line was $y = 0.917 + 1.06x$ with a correlation coefficient of 0.993.

**DISCUSSION**

The automated enzymatic method for NH₃ is rapid, convenient, and precise. It has been applied to rumen fluid and swine gut fluid samples over the range of 3 to 30 μmol/ml without having to change the assay parameters. The values compared favorably with those with the manual enzymatic method, but were somewhat less than those with the Conway diffusion method. Other workers (3) have suggested that the presence of highly alkaline conditions in the Conway diffusion method may cause the release of NH₃ from

![Recorder response for ammonia standards.](image-url)

**TABLE 1. Effect of sample storage on the determination of ammonia in rumen fluid and plasma**

<table>
<thead>
<tr>
<th>Time of storage (days)</th>
<th>NH₃ concn (μmol/ml)</th>
<th>Rumen fluid samples</th>
<th>Plasma samples</th>
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<tr>
<td></td>
<td></td>
<td>1 2 3</td>
<td>1 2 3 4</td>
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<tr>
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<tr>
<td>SDd</td>
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<td>0.9</td>
</tr>
</tbody>
</table>

*a* Each value is the mean of three samples.

*b* The overall mean where $n = 18$ for rumen samples and $n = 12$ for plasma samples.

*c* CV, Coefficient of variation.

*d* SD, Standard deviation.
AUTOMATED AMMONIA DETERMINATION

We believe this method will be useful for determining NH₃ in rumen fluid and intestinal contents under differing dietary conditions, for determining rates of production of NH₃ in microbial cultures, and for determining NH₃ in plasma to monitor toxicity levels.

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LITERATURE CITED