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Measurement of the bacterial nitrogen entering the duodenum of the ruminant using diaminopimelic acid as a marker

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1. A technique for the separation and colorimetric estimation of 2,4-diaminopimelic acid (DAP) using automated ion-exchange chromatography coupled with an acid ninhydrin detection system is described.

2. Only traces of DAP were found in rumen protozoa and no DAP was detected in rumen fluid prepared by ultracentrifugation or dialysis.

3. The concentration of DAP in rumen bacteria from sheep on a constant feeding regimen, and the ratio of nitrogen to DAP for these bacteria were found to be constant over a 3-month period.

4. The method has proved suitable for the estimation of bacterial N in the duodenal digesta of ruminants.

5. The contribution of bacterial N to the total N leaving the abomasum of a lactating cow fitted with a permanent re-entrant cannula in the duodenum was found to be 50%.

In ruminants a substantial proportion of dietary protein is fermented in the rumen, and the degradation products are utilized for microbial protein synthesis. The protein which becomes available for digestion and absorption beyond the rumen includes unchanged feed protein and microbial protein. The process serves to upgrade dietary protein of low biological value into microbial protein of reasonably good biological value (McNaught, Owen, Henry & Kon, 1954; Bergen, Purser & Cline, 1967) but clearly the degradation of feed protein of high biological value is disadvantageous. This latter consideration has led to many attempts to 'protect' certain dietary proteins from ruminal degradation by a variety of chemical and physical techniques (see (Chalmers, Cuthbertson & Synge, 1954; Whitelaw, Preston & Dawson, 1961; Sherrod & Tillman, 1962; Tagari, Ascarelli & Bondi, 1962; Chalmers, Jayasinghe & Marshall, 1964; Leroy, Zelter & Francois, 1964; Tagari, Henis, Tamir & Volcani, 1965; Zelter, Leroy & Tissier, 1970), and the treatment of casein with formaldehyde has proved particularly useful (Ferguson, Hemsley & Reis, 1967; Reis & Tunks, 1969). The effectiveness of these procedures may be assessed direct by measuring the amounts of feed protein reaching the duodenum in animals prepared with duodenal fistulas, but in only a few instances has it proved possible to assay feed protein in the digesta. Values have been obtained for zein by making use of its solubility in ethanol and lack of lysine (McDonald, 1954; Ely, Little, Woolfolk & Mitchell, 1967), and casein, which was assayed on the basis of its phosphorus content (McDonald & Hall, 1957). Most workers have relied on indirect methods based on the measurement of the contribution of microbial protein to the total protein reaching the duodenum.

Various methods have been used to measure the microbial contribution to nitrogenous materials in ruminant digesta. The potential use of nucleic acids has recently been reviewed by Smith (1969); vitamin B_{12} was used by Weller, Gray & Pilgrim

(1958); ^{35}S has been used to assess protein synthesis and the rate of microbial cell synthesis in vitro (Hendrickx, 1962; Walker & Nader, 1968) and to estimate bacterial protein synthesis in vivo (Roberts & Miller, 1969). Rumen synthesis of methionine in intact cows has also been estimated with ^{35}S (Conrad, Miles & Butdorf, 1967). The presence of diaminopimelic acid (DAP) in bacterial cells (Work, 1950; Work & Dewey, 1953) and its absence from protozoa (Virtanen, 1967; Work & Dewey, 1953; Weller *et al.* 1958) and plant material (Synge, 1953) has led to its use as a marker for bacterial nitrogen. Although Synge (1953) and Purser & Beuchler (1966) have shown that amounts of DAP relative to bacterial N vary between different species of rumen bacteria, it has been demonstrated that on fixed dietary regimens the N:DAP ratio is reasonably constant (Weller *et al.* 1958) and may be used to estimate the bacterial contribution to ruminal and duodenal nitrogenous constituents. This paper describes an improved method for estimating DAP using ion-exchange chromatography and an acid ninhydrin reagent based on the procedure of el-Shazly & Hungate (1966). The method has been used to assess the daily contribution of bacterial N to the total N passing into the duodenum of a lactating dairy cow.

MATERIALS AND METHODS

Experimental animals

A mature Clun Forest wether with a rumen fistula, given 1 kg hay and 100 g rolled oats once daily, was used as a source of rumen bacteria when samples were required to assess the variation in N:DAP ratio over a 3-month period for an animal on a constant feeding regimen.

A 4-year-old lactating Ayrshire cow in the 8th month of lactation and yielding 8·3 kg of milk daily was used for the estimation of bacterial N entering the duodenum. This animal had a rumen fistula and a re-entrant cannula in the proximal duodenum about 7 cm from the pyloric sphincter. The maintenance requirement of the cow was supplied by barley straw and a protein balancer offered at 11.00 and 17.00 hours. Production concentrates (400 g/kg milk) were given at 7.00 and 16.00 hours. The total intake of nutrients was slightly in excess of the minimum requirements recommended by the Agricultural Research Council (1965). Paper impregnated with chromic oxide (72·2 g chromic oxide/200 g paper) was added to the rumen of the cow once daily as a marker in the measurement of digesta flow.

Collection and preparation of digesta

Rumen contents were strained through four layers of surgical gauze, and about 400 ml of strained liquor were centrifuged at 100 *g* for 1 min to remove protozoa and dense food particles, and to yield a bacteria-rich supernatant fluid. This bacterial fraction was centrifuged at 22 000 *g* for 10 min to yield clarified rumen liquor (CRL) and a precipitate of bacterial cells, which was washed twice with 0·9% (w/v) NaCl solution and twice with distilled water. The bacterial cells and the CRL were freeze-dried and stored over P_2O_5 in a vacuum desiccator.

Strained rumen liquor (1 l) was allowed to stand in a cylindrical separating funnel for 1 h at 40°, when feed particles accumulated at the surface and protozoa separated out. The lower 800 ml were run off and made up to 1 l with warm water (40°) and

again the protozoa were allowed to separate. The precipitated protozoa were washed three times with water, freeze-dried and stored in a vacuum desiccator over P_2O_5.

Dialysed rumen fluid was obtained by placing inside the rumen four sacs prepared from Visking dialysis tubing (22 mm) each half-filled with 30 ml of salt solution, prepared just before use according to the procedure of Wright & Hungate (1967). The sacs, which were attached by nylon thread to a 200 g metal sinker, were withdrawn after 2 h and their contents were removed. The dialysed rumen fluid (DRF) was freeze-dried and stored in a vacuum desiccator over P_2O_5.

During 24 h collections of duodenal contents the re-entrant cannula of the cow was disconnected and digesta leaving the abomasum was collected. As each 2 l of digesta flowed from the abomasum it was replaced by 2 l of digesta from the same animal at 40° which had been collected 1 week previously and stored at −20°. At the end of each hour the total sample of digesta was homogenized and a 200 ml portion was weighed; the surplus digesta was added to the donor digesta pool. At the end of the collection period the twenty-four portions of digesta were mixed according to the weight of digesta collected each hour to give a sample representative of the daily digesta flow. This material was freeze-dried and stored over P_2O_5 for subsequent analysis.

Hydrolysis of test materials

Freeze-dried material (1 g) was hydrolysed with 450 ml of 6 N-HCl under N by refluxing at 137° for 17 h. The cooled hydrolysate was filtered (Whatman 541 or 540) to remove humin and made up to 500 ml with distilled water. The filtrate (200 ml) was evaporated to dryness under reduced pressure at 45° in a rotary evaporator, and the residue extracted three times with 3 ml 0·1 N-HCl and the volume made up to 10 ml.

Assay of DAP

Reagents. All reagents used were Analytical Reagent grade. Standard solutions were prepared by dissolving amino acids either in sodium citrate buffer, pH 2·2, or in 0·1 N-HCl. Acid ninhydrin reagent was prepared weekly by dissolving 25 g ninhydrin in 600 ml glacial acetic acid. The resulting solution was made up to 1 l with 0·6 M-orthophosphoric acid, and the reagent was stored in amber glass bottles.

The eluent buffer solution was prepared by dissolving 19·6 g sodium citrate in 'deionized' water together with 1 ml aqueous polyoxyethylene lauryl ether (30%, w/v), 5 ml thidioglycol and 0·1 ml caprylic acid; the pH was adjusted to $3{\cdot}30 \pm 0{\cdot}04$ with concentrated hydrochloric acid and the solution made up to 1 l with 'deionized' water. The column was regenerated with 0·2 N-NaOH after each run.

Apparatus. A Technicon automatic amino acid analyser equipped with a 50 cm jacketed column of 0·6 cm internal diameter and maintained at 50° was used for this work. The column was packed with spherical bead resin (Zeo-Karb 225, 8% cross-linked; particle size 9–19 μm) to a height of 20 cm. All fractionations were made at a buffer flow-rate of 80 ml/h. Effluent from the column was sampled at 1·0 ml/min via the sample line with the excess running to waste. Each sample was mixed with acid ninhydrin reagent and diluent (90% acetic acid) in a 12 cm mixing coil (sample 1·02 ml/min, acid ninhydrin 0·92 ml/min, diluent 0·92 ml/min) before passing to a glass reaction coil (5 m × 0·2 cm internal diameter) immersed in an oil-bath maintained

at 90°. The reaction time under these conditions was 5 min, after which the solution was cooled before it passed into the colorimeter cell. The solution was drawn through the cell at 2·5 ml/min leaving approximately 0·3 ml/min to pass to waste at the de-bubbler junction. Optical density was measured at 420 nm.

Quantitative estimation of DAP

The concentration of DAP was determined by direct area comparison with peaks obtained using standard solutions. Peak areas were measured as described by Spackman, Stein & Moore (1958). The range of concentration of standard DAP solutions was 0·5–4·0 mM and the sample size was 0·2 ml.

Recovery of DAP after hydrolysis

Portions (20 μmoles) of DAP (2 ml of standard solution containing 10 μmoles DAP/ml 0·1 N-HCl) were added to 0·5 g freeze-dried bacterial cells of known DAP content and hydrolysed. Five hydrolyses were carried out and the mean recovery of DAP, and its standard error, were determined. This procedure was repeated using freeze-dried duodenal contents of known DAP content. A column load of 0·5 ml concentrated hydrolysate was used and the DAP peak area was compared with that obtained with 0·5 ml standard DAP solution (1 μmole/ml).

Determination of N:DAP ratio for rumen bacteria

Rumen bacterial samples were collected 4 h after feeding, at intervals of approximately 10 d over a period of 3 months, from a sheep on a constant feeding regimen. The N and DAP contents of the freeze-dried bacteria were determined, and the N:DAP ratio for each sample was calculated. The mean N value, DAP value and N:DAP ratio were calculated together with their standard errors.

Estimation of bacterial N entering the duodenum of a lactating cow

To check the feasibility of using DAP to estimate the contribution of bacterial N to the total N entering the duodenum of ruminants, a simple trial was made with a lactating dairy cow. Rumen protozoa, rumen bacteria, CRL and DRF were collected from a cow 1 week before the first duodenal collection was made. A second duodenal collection was made 1 week after the first so that duplicate figures could be obtained. Dry matter of DRF, CRL and duodenal contents was determined by freeze-drying. The N content (micro-Kjeldahl) of rumen bacteria and freeze-dried duodenal contents, DAP in freeze-dried DRF, CRL, bacteria, protozoa and duodenal contents, and chromic oxide in freeze-dried duodenal contents were also determined. A modified version of the method described by Hill & Anderson (1958) was used to estimate chromic oxide.

The total flow of digesta from the abomasum measured during the collection period was corrected to 100% recovery of daily chromic oxide input. From the corrected flow and the contents of dry matter, N and DAP in duodenal digesta, the total amounts of dry matter, N and DAP entering the small intestine were calculated.

In addition, the daily flow of bacterial N could be estimated from the equation:

$$\text{Bacterial N (g/d)} = R \times \text{DAP (g/d)},$$

where R is the N:DAP ratio determined for bacteria isolated from the rumen of the cow.

RESULTS

Fractionation and detection of DAP

The separation of DAP from amino acids which are likely to be eluted from the column under the conditions described and which give a colour reaction with acid ninhydrin was investigated. Proline, cystine, tyrosine, methionine, lanthionine, cystathionine and djenkolic acid were chosen, this choice being based on the observations of Work (1957), Chinard (1952) and Hamilton (1963).

Initially, the separation of proline, cystine and methionine from DAP was considered. We used a pH 5·5 ninhydrin reagent to locate cystine and methionine because of the weak reaction of these two amino acids with acid ninhydrin. The position of both DAP and cystine in the fractionation pattern was very sensitive to pH changes in the buffer and the best separation of DAP from cystine, methionine and proline was obtained when the pH of the eluting buffer lay between 3·24 and 3·34. Under these conditions the retention time of DAP varied between 23 and 27 min, the order of elution being cystine, proline, methionine and DAP. With buffer pH values below 3·25 the elution of DAP was unduly delayed, and above the upper limit DAP was not separated from methionine.

Lanthionine and citrulline were run with pH 3·26 buffer and it was found that they were well separated from DAP; both were eluted in front of proline. Cystathionine and djenkolic acid were also separated from DAP in this system; both were eluted close to methionine. Tyrosine was eluted considerably later than DAP. With the buffer at pH 3·26–3·34, none of these eight amino acids interfered with DAP estimation.

Fig. 1 shows fractionation patterns obtained with a hydrolysate of ruminant duodenal contents. Because of the extremely low concentration of DAP relative to other amino acids in duodenal contents, it was necessary to concentrate the hydrolysate when DAP was to be measured. Fig. 1(*a*) shows the acid ninhydrin pattern with the sample concentration at about 100 times that shown in Fig. 1(*b*), which is the pattern obtained with pH 5·5 ninhydrin.

A comparison of the two traces shows that DAP was eluted between isoleucine and leucine. To check whether there were any other amino acids which might react with acid ninhydrin and were eluted in this region of the chromatogram, we ran a number of test chromatograms on samples which were unlikely to contain DAP, i.e. feedstuff hydrolysates. The only peaks detected in these tests were those of proline, a number of smaller peaks immediately in front of proline and another one following proline. The pattern was similar to that shown in Fig. 1(*a*) but without the DAP peak. There were no peaks in the region of DAP for feed hydrolysates so we have concluded that the peak eluted between leucine and isoleucine was due entirely to DAP.

Quantitative estimation of DAP

The reaction of DAP with the acid ninhydrin reagent was first investigated manually. The colour intensity was estimated at 420 nm. In these experiments we used pH 3·26 sodium citrate buffer which contained the DAP and which was mixed with equal volumes of reagent and diluent; the pH of the reaction mixture before heating was 1·2. It was found that the reaction was linear at least up to 0·8 μmoles DAP.

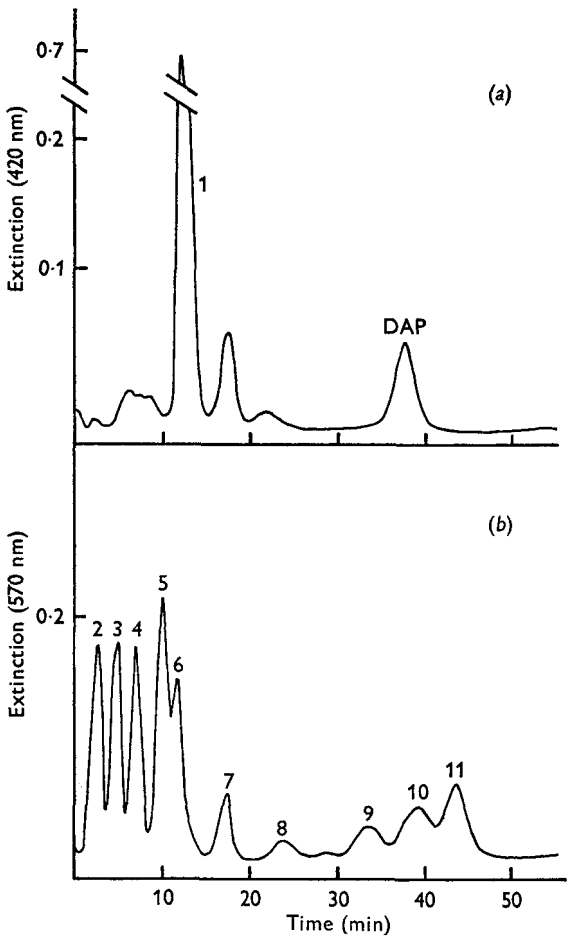

Fig. 1. A comparison between the patterns of amino acid fractionation obtained with (*a*) acid ninhydrin and (*b*) pH 5·5 reagent for a hydrolysate of ruminant duodenal contents. 1, proline; 2, aspartic acid; 3, threonine+serine; 4, glutamic acid; 5, cystine+glycine; 6, alanine; 7, valine; 8, methionine; 9, isoleucine; 10, leucine; 11, norleucine.

Results from a series of chromatograms obtained with a range of DAP standard solutions (0·1046 μmoles–0·8368 μmoles) showed that the C_1 factors (see Spackman *et al.* 1958) varied very little (range 6·23–6·49). These results were all obtained using one set of manifold tubes on the Technicon apparatus and the pump flow-rate was kept constant at 80 ml/h. Experience has shown that any change in tension, or renewal

of tubing, or any variation in flow-rate altered the value of the C_1 factor. Values as low as 4·80 or as high as 7·50 have been recorded. This variation did not affect the feasibility of the method, provided the apparatus was recalibrated daily. Two batches of acid ninhydrin reagent were used; one freshly prepared, the other 1 week old. There appeared to be little variation due to different reagent preparation and this suggested that the acid ninhydrin reagent was stable for at least 1 week.

Recovery of DAP after hydrolysis

The concentrations of DAP in hydrolysates of freeze-dried duodenal contents which had been refluxed for 17, 20, 30 and 40 h were not significantly different and, for convenience, a time of 17 h was chosen for all further hydrolyses. The mean recovery of DAP refluxed in the presence of bacteria was not significantly different from the recovery in the presence of freeze-dried duodenal contents ($P > 0.05$); the total recovery mean (ten samples) was 89% with a standard error of 3%.

Determination of N:DAP ratio of rumen bacteria

The mean values for N (g/100 g dry cells), DAP (g/100 g dry cells), and N:DAP ratio were 8.4 ± 0.4, 0.459 ± 0.037, and 19.1 ± 1.2 respectively for the ten bacterial samples taken at intervals over 3 months from a sheep on a constant feeding regimen. These values, together with their standard errors, are considered by us to show that, provided the feeding regimen is constant and the rumen samples are collected at the same time after feeding, then reproducible estimates of the daily production of rumen bacterial N can be expected.

Table 1. *Estimation of the contribution of bacterial nitrogen to the total N entering the duodenum of a lactating cow*

		Corrected daily flow of digesta (g/24 h)				
Run	Duodenal chromic oxide flow / Chromic oxide intake	Dry matter	Total N	DAP	Bacterial N (= 18 × DAP)	Bacterial N / Total N
1	0·87	5910	264	7·21	130	0·49
2	1·07	6091	274	7·55	136	0·50

Bacterial N entering the duodenum of a lactating cow

No DAP was detected in DRF or CRL. Traces of DAP were found in protozoa but they were too small to measure and were negligible compared with the amounts of DAP in bacteria. They were probably due to the ingestion of bacteria by protozoa or to the contamination of protozoa by bacteria and have not been considered in the calculation of the results. The bacteria from the rumen of the cow had a N content of 8·4% and a DAP content of 0·467% so the resulting N:DAP ratio was 18; this value was used to calculate the results shown in Table 1. From these results it is clear that a reproducible estimate of the contribution of bacterial N to total duodenal N can be obtained with DAP as a bacterial marker. In this particular animal half the N available for absorption at the duodenum was of bacterial origin.

DISCUSSION

There are essentially three methods of estimating DAP: a colorimetric estimation with acid ninhydrin reagent (Work, 1957), a spectrofluorimetric method (Rogers, Chambers & Clarke, 1967; Sen, Somers & O'Brien, 1968) and a gas chromatographic method (Sen, Somers & O'Brien, 1969). The acid ninhydrin reagent is not entirely specific and may give coloured reaction products on heating with some other amino acids (Chinard, 1952; Newton, Abraham & Berridge, 1953). All the diamino acids investigated by Work (1957) reacted with acid ninhydrin to an extent which depended on the pH and the reaction time. However, absorption spectra and colour intensities were such that conditions could be adjusted so that selective estimation of DAP was possible. The successful application of this technique depends on the presence of a high concentration of DAP relative to those of other reacting amino acids. In ruminant digesta, where the DAP concentration is low, the interference from other amino acids is considerable and this technique is of limited value. A similar conclusion applies to the spectrofluorimetric technique; glucosamine seriously affects the determination (Rogers *et al.* 1967) and there is also some doubt about quantitative aspects of the method (see Sen *et al.* 1968). For ruminant digesta, some form of chromatographic separation of DAP is necessary before its concentration can be measured. The gas chromatographic method described by Sen *et al.* (1969) is attractive, but the number of purification steps make it unsuitable for rapid routine analysis of samples. el-Shazly & Hungate (1966) have described an ion-exchange method for isolating and estimating DAP and we have adapted this method to an automatic system, with considerable saving in time. The procedure has been successfully applied to the determination of the contribution of bacterial N to total N in duodenal digesta. The use of animals with duodenal re-entrant fistulas allowed the measurement of total digesta flow and provided samples of digesta for analysis. In some instances the collection procedure may result in a reduction or an increase in flow (see MacRae & Armstrong, 1969) and chromic oxide has been used as a marker to measure this effect and provide a correction factor.

Assumptions inherent in the use of DAP as a marker for bacterial N are that this amino acid is absent from other nitrogenous components in the rumen, omasum and abomasum, and that the N:DAP ratio determined for the sample of rumen bacteria is truly representative of the total population of bacteria in the rumen. It is also assumed that the isolated bacterial preparation is not contaminated with feed residues with similar sedimentation characteristics, and in support of this assumption there is evidence that rumen bacterial isolates prepared by centrifugation can be practically devoid of feed or plant material contamination (Bergen, Purser & Cline, 1968).

Although the N content of bacterial cells is generally accepted as being approximately 10·5% (see Hungate, 1966), which is higher than the value of 8·4 that we obtained, lower values have been reported for rumen bacteria (McNaught, Smith, Henry & Kon, 1950; McNaught *et al.* 1954; Bergen *et al.* 1968) and it is apparent that the N content of mixed rumen bacteria is very variable. Despite the fact that bacterial N concentrations and N:DAP ratios for different rumen bacterial species vary markedly, Weller *et al.* (1958) showed that the N:DAP ratio for mixed rumen bacterial samples from sheep on a fixed feeding regimen is reasonably constant, and we have confirmed this observation.

In studies on the utilization of non-protein N, and on the protection of dietary proteins from ruminal attack, it is necessary to have information about both bacterial protein synthesis and the amounts of feed protein reaching the duodenum. However, it is not possible to calculate the latter direct by difference from the flow of bacterial N and total N, since the digesta also contain protozoal protein and the endogenous protein secreted into the omasum–abomasum. In order to define fully the N economy of the ruminant, values for the contribution of undigested feed protein, bacterial N, protozoal N and endogenous N to the contents of the duodenum must be available, together with information on the levels and availability of the amino acids present in these nitrogenous fractions.

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