The Biochemistry of Rumen Protozoa

5. THE NITROGEN METABOLISM OF *ENTODINIUM**

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Blackburn & Hobson (1960*a*) reported that when stained particles of casein were added to sheep rumen liquor incubated *in vitro*, oligotrich protozoa could be seen to swallow and digest these particles. One of the most numerous species of such protozoa is *Entodinium caudatum*. Suspensions of this organism containing no other protozoa and almost free from bacteria were used by us in a study of its carbohydrate metabolism (Abou Akkada & Howard, 1960). It was shown that this organism satisfies its carbohydrate needs only by swallowing and digesting starch grains. It seemed of interest to see whether casein particles could also serve as food for the entodinia. Preliminary experiments showed that stained casein particles were readily swallowed and digested by clean, washed suspensions of the entodinia. The work described below is concerned mainly with the decomposition of casein by *E. caudatum* and with the properties of the enzymes involved, together with some other aspects of nitrogen metabolism by this protozoan.

MATERIALS AND METHODS

Protozoa and substrates

Suspensions of living *E. caudatum* and cell-free extracts were obtained as previously described, with the same sheep as source (Abou Akkada & Howard, 1960). The casein was used as the freeze-dried sodium salt, prepared by Blackburn & Hobson (1960*b*), and the casein hydrolysate was supplied by Allen and Hanburys Ltd., London, W. 1. *N*^α-Benzoyl-L-arginineamide hydrochloride and *N*^α-benzoyl-L-arginine ethyl ester were synthesized and given by our colleague, Dr G. Jones. The peptides were all supplied by L. Light and Co. Ltd. They were DL-alanyl-DL-alanine, glycyl-L-alanine, glycylglycine, glycylglycylglycine, glycyl-L-leucine, glycyl-DL-phenylalanine, glycyl-L-tyrosine, glycyl-DL-valine, L-leucylglycine and DL-leucyl-DL-leucine.

Incubation of suspensions of entodinia

Incubation of protozoa and of enzymes was at 38° throughout this work. It was found convenient to carry out all incubations of living entodinia in Warburg manometers, even when no manometric measurements were to be made. Conditions were, in general, the same as in previous experi-

ments (Abou Akkada & Howard, 1960). The bicarbonate buffer solution normally used was replaced by acetate buffer in experiments in which α-amino N and peptide N were to be determined. The main compartments of the flasks contained protozoal suspension (2 ml.) containing about 2 mg. of total N. The side arms contained buffer solution (0.5 ml.) in which the substrates were dissolved at the following concentrations (% w/v): casein, 3.5; casein hydrolysate, 5; urea, 0.5. At the end of the experiments the flasks were removed from the water bath, and the contents were immediately transferred to centrifuge tubes and made up to 5 ml. Centrifuging at 600g for 3 min. yielded a clear supernatant solution, which was removed for analysis. The sediment of protozoa was washed with water (1 ml.) in the tube.

Analytical methods

The total N and protein N in cell-free extracts were determined by a micro-Kjeldahl method as described by Howard (1959). Casein N was determined by mixing the supernatant solution (1 ml.) from a centrifuged suspension of protozoa with 10% trichloroacetic acid (4 ml.). After 1 hr. the sediment was collected by centrifuging and washed with the trichloroacetic acid solution, and the total N was then measured. Non-protein N was determined by precipitating the protein as just described, filtering through Whatman no. 42 paper, and measuring the total N in the filtrate. Ammonia N was determined by the microdiffusion method (Conway, 1957) with the dilute solution of boric acid indicator described by Warner (1956*a*). Amide N was found by determining NH₃ N before and after hydrolysis in 3*N*-H₂SO₄ at 100° for 3 hr. (Conway, 1957). α-Amino N was determined by the method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) after precipitating protein with 0.1*N*-HCl (Warner, 1956*b*). Peptide N was determined similarly after hydrolysis of the 0.1*N*-HCl filtrate in 5.5*N*-HCl at 100° for 16 hr. in a sealed tube. The HCl was removed from the hydrolysate by repeated evaporation of an aqueous solution in a vacuum desiccator over KOH. A rough measure of the small quantity of amino acids excreted by the entodinia in the absence of added substrates was obtained as follows. The supernatant obtained by centrifuging a suspension of entodinia after incubating for 6 hr. was adjusted to pH 10 by the addition of 2*N*-NaOH. The solution was evaporated to dryness at room temperature in a vacuum desiccator over H₂SO₄, and the NH₃-free residue was dissolved in water. Portions of this solution were heated with ninhydrin at pH 4 and pH 5.6 according to Lampson & Singher (1960), and the intensity of the colour formed was compared with that given by leucine. Urea was determined by measuring the increase in NH₃ brought about by incubating with jack-bean urease solution (Conway, 1957).

* Part 4: Abou Akkada & Howard (1961).

Assay of enzyme activities in cell-free extracts of entodinia

Proteinase. A 2% (w/v) solution of casein (0.5 ml.), phosphate-citrate buffer (1 ml.; McIlvaine, 1921) and enzyme solution (1 ml.) were incubated together. Unless stated otherwise, the pH was 6.5 and the incubation period 4 hr. The enzyme action was stopped and residual protein removed from solution by the addition of trichloroacetic acid and filtration as described above. The extent of casein hydrolysis was determined by measuring the intensity of the blue colour produced when the filtrate was incubated at 40° for 30 min. with Folin-Ciocalteu reagent (British Drug Houses Ltd.) (Hull, 1947; Nomoto & Narahashi, 1959). The results are expressed as 'tyrosine' present in solution.

When the effect on the proteinase of added substances was to be determined, the substance was dissolved in the buffer solution and the pH readjusted to 6.5 with alkali. The solution so obtained was used in place of the buffer alone in the method described above. Control tubes containing the added substance with substrate but no enzyme, and tubes containing the added substance with enzyme but no substrate, were incubated at the same time as the test samples in all experiments. When CaCl_2 was to be tested, 0.2M-sodium acetate buffer replaced the phosphate-citrate, and enzyme activity was assayed with the Folin-Ciocalteu reagent as described above. All the controls gave a negligible reading when tested after incubation for 4 hr., and it was also established that CaCl_2 in the concentrations used in the experiment, did not affect the intensity of colour given by tyrosine with the reagent. When cysteine hydrochloride was to be tested the Folin-Ciocalteu reagent could not be used because it gives a colour with cysteine. In this case the difference in casein N content of tubes containing casein, but incubated with and without enzyme, was taken as a measure of activity. The presence of cysteine was shown not to affect the determination of casein N. The experiments with cysteine were carried out under anaerobic conditions by gassing the solutions with N_2 before and after mixing, and by sealing the reaction tubes with rubber stoppers.

Amidase. A 2% (w/v) solution of N^α -benzoyl-L-arginine-amide (0.5 ml.), phosphate-citrate buffer, pH 6.5 (1 ml.), and enzyme solution (1 ml.) were incubated together for 4 hr. The concentration of the amide was thus 12.7 mM. At the end of the incubation period, NH_3 was determined in 1 ml. portions of the solutions. The phosphate-citrate mixture was replaced by sodium acetate if CaCl_2 was present, as described in the preceding paragraph. When testing the effect of CaCl_2 or cysteine hydrochloride, tubes containing substrate with water and three concentrations of the added substance, and a tube containing enzyme without substrate, were included as controls. The NH_3 produced in all these tubes during incubation for 4 hr. was negligible.

Esterase. A solution of N^α -benzoyl-L-arginine ethyl ester in the phosphate-citrate buffer, pH 6.5 (2.5 ml.), and enzyme solution (1.5 ml.) were incubated together for 2 hr. The concentration of the ester was 12.7 mM. The ester remaining at the end of the incubation period was determined by the hydroxamic acid method (Hestrin, 1949).

Peptidase. Small-scale qualitative tests were carried out in melting-point tubes (Porter & Hoban, 1954). Approximately equal volumes of enzyme solution and 2% (w/v)

solutions of the peptides in 0.2M-sodium acetate buffer, pH 6.5, were incubated together under toluene overnight, and then spotted on Whatman no. 4 paper. One-dimensional descending chromatograms were run for 7 hr. in the upper layer of butan-1-ol-acetic acid-water (4:1:5, by vol.). Spraying with 1% (w/v) ninhydrin in butan-1-ol and heating gently for 20 min. revealed the amino acids as blue and the peptides as yellow spots.

Quantitative experiments with peptidase used only L-leucylglycine as substrate. The method of Lampson & Singher (1960) was used, in which the conditions allow of reaction of ninhydrin with amino acids only, not the peptide. Tubes containing the peptide (280 μg .), 0.1M-sodium citrate buffer (0.3 ml.) and enzyme solution, in a total volume of 1 ml., were incubated for 4 hr. Control tubes containing the peptide without enzyme, and an equimolecular mixture of leucine and glycine representing completely hydrolysed peptide, were incubated at the same time. The extent of hydrolysis was found by determining the amino acids present in a portion of the solution (0.1 ml.). It was found necessary to replace the 0.05M-phthalate buffer used by Lampson & Singher (1960) by 0.1M-sodium citrate buffer to maintain the reaction mixture at pH 4. In some experiments the effect of metal ions on peptidase activity was investigated by including solutions of the sulphates in the enzyme digests. In these cases, 0.2M-sodium acetate buffers were used throughout. It was shown that none of the metal salts, at the highest concentration tested, interfered with the ninhydrin-amino acid reaction.

Urease. A 0.3% solution of urea in phosphate-citrate buffer, pH 6.5 (1 ml.), was incubated with the cell-free extract of entodinia (2 ml.) for 6 hr. The NH_3 present in a 1 ml. portion of the solution was then determined.

Electrophoretic separation of proteinase and peptidase

Cell-free extract (40 ml.), obtained from the entodinia from approximately 1.5 l. of rumen liquor, was freeze-dried, yielding 93 mg. of fluffy powder which contained 9.3% of N (about 58% of protein). The material (18 mg.) was dissolved in a buffer solution (0.36 ml.) which contained diethylbarbituric acid (0.01M) and sodium diethylbarbiturate (0.05M) and had pH 8.6. The solution was placed along the starting line on a sheet of Whatman no. 3MM paper (20 cm. $\times$ 18 cm.). Electrophoresis took place in the diethylbarbiturate buffer for 18 hr. in an apparatus similar to that of Kunkel & Tiselius (1951); the applied p.d. was 230 v and the current 7 ma. Side strips from the paper, stained with Lissamine Green, showed that the proteins had not separated into bands, so the paper was divided arbitrarily into six strips, each 2.5 cm. wide. Each strip was extracted twice with water (10 ml.), the combined extracts were centrifuged, dialysed, freeze-dried and finally redissolved in water (10 ml.).

RESULTS

Endogenous nitrogen metabolism of Entodinium caudatum

Although the principal aim of this work was to examine the effect of entodinia on added substrates,

the analysis of suspensions which had served as 'no substrate' controls yielded some information on the endogenous metabolism of the protozoa. During incubation for 6 hr. in buffer solution alone, the entodinia were metabolically active and motile, and the loss of nitrogen from the cells was about 6 % (Tables 1-3). No precipitate was seen when trichloroacetic acid was added to the solution at the end of the experiment; the increase in non-protein nitrogen in the solution exactly accounted for the loss of cell nitrogen. In different experiments, 50-70 % of this non-protein nitrogen was ammonia (Tables 1-3), and in a single experiment about 20 % was present in free amino acids (Table 1).

Table 1. *Endogenous nitrogen metabolism of Entodinium caudatum*

Cell N at the beginning was 950 and 615 $\mu\text{g./ml.}$ respectively in Expts. (a) and (b). The Table shows gain (+) or loss (-) of N ($\mu\text{g./ml.}$) during incubation under the conditions described in the text. $\times$, Not determined. Amino N was determined with ninhydrin according to Lampson & Singher (1960), with leucine as standard. The same result was obtained when the reaction was carried out at both pH 4 and pH 5.6.

	(a)	(b)
Cell N	-58.0	-49
Non-protein N in solution	+57.8	+47
Ammonia N	+29.7	+30
Amino N	$\times$	+10 approx.

Effect of Entodinium caudatum suspensions on added substrates

When suspensions of entodinia were incubated with casein, a rapid decomposition of the protein occurred. Over 80 % of the casein nitrogen was rendered soluble in trichloroacetic acid during 4 hr. (Fig. 1). A second portion of casein, equal to that first added, was equally rapidly decomposed. During this 8 hr. period the general metabolic activity, as measured manometrically, remained high and constant. Incorporation of the substrate nitrogen into the cells was very slight; the cell nitrogen increased by about 3 % during the incubation period. This increase corresponded to less than 3 % of the casein decomposed (Table 2). In a similar experiment α -amino nitrogen and peptide nitrogen accounted for 22 and 40 % respectively of the non-protein nitrogen in solution at the end of the experiment. Ammonia nitrogen and amide nitrogen accounted for a further 14 and 3 % respectively, and it was calculated from the amino acid composition of casein (Gordon, Semmet, Cable & Morris, 1949) that a further 12 % approximately of the non-protein nitrogen was present in the amino acids and peptides in a form not yielding carbon dioxide with ninhydrin. Thus about 90 % of the non-protein nitrogen could be accounted for. The ratio of peptide nitrogen to α -amino nitrogen was 1.8.

Table 2. *Changes in the distribution of nitrogen during incubation of Entodinium caudatum with casein*

Cell N and casein N at the beginning were respectively 945 and 915 $\mu\text{g./ml.}$ in Expt. 1, and 751 and 1370 $\mu\text{g./ml.}$ in Expt. 2. The Table shows gain (+) or loss (-) of N ($\mu\text{g./ml.}$) during incubation under the conditions described in the text. $\times$, Not determined.

	Expt. 1 With casein	Expt. 2	
		With casein	Without casein
Cell N	+23	+25	-51
Casein N	-825	-1014	0
Non-protein N	+778	+910	$\times$
Amide N	$\times$	-52	0
Ammonia N	+80	+77	+28
Gain of non-protein N + cell N	801	935	.
Recovery (%)	97	92	.
Gain of cell N	as % of original cell N	2.4	3.3
	as % of casein N lost	2.8	2.5

Table 3. *Some changes in the distribution of nitrogen during incubation of Entodinium caudatum with casein hydrolysate*

Cell N and substrate N at the beginning were respectively 745 and 1310 $\mu\text{g./ml.}$ The Table shows gain (+) or loss (-) of N ($\mu\text{g./ml.}$) during incubation under the conditions described in the text.

	Protozoa with substrate	Protozoa without substrate
Cell N	+32	-40
Ammonia N	+34	+28
Gain or loss of cell N	as % of original cell N	+4.3
	as % of casein lost	+2.4

Duplicate portions of a protozoal suspension were incubated with and without casein, and the changes in the distribution of nitrogen in the two suspensions were measured (Table 2, Expt. 2). The ammonia formed in the suspension with casein was equal to the sum of the ammonia formed endogenously and that formed by hydrolysis of the amide groups in the casein. In another experiment, the concentrations of protein nitrogen, amide nitrogen and ammonia nitrogen were determined at different times during the incubation of entodinia with casein for 8 hr. The hydrolysis of amide groups and of the protein appeared to proceed at similar rates; the rate of ammonia production was approximately constant during the experiment (Fig. 2). Deamination of amino acids and incorporation into cell material were also negligible

when the entodinia were incubated with casein hydrolysate (Table 3).

Suspensions of entodinia were incubated for 6 hr. with and without urea. The concentrations of urea and ammonia were measured in both solutions at the beginning and end of the experiment. The results are shown in Table 4.

Properties of cell-free extracts of Entodinium caudatum

The cell-free extracts, which contained about 130 $\mu\text{g.}$ of total nitrogen/ml., caused a progressive decomposition of casein, rapid during the first 4 hr. of incubation and falling off after this time. About 40% of the casein was decomposed in the first 4 hr., after which time the ratio of peptide

Fig. 1. Decomposition of casein by *E. caudatum*. ○, Casein N in solution during an 8 hr. incubation period after the addition of casein; ●, casein N in solution during a 4 hr. incubation period after the addition of a second lot of casein 4 hr. after the first; △, endogenous fermentation during incubation for 8 hr. with a single lot of casein, calculated as CO_2 . The buffer solution contained 0.1% of NaHCO_3 , and the gas phase was $\text{N}_2 + \text{CO}_2$ (95:5). Other conditions of incubation are described in the text.

Fig. 2. Changes in the distribution of nitrogen during incubation of casein with *E. caudatum*. ○, Casein N; ●, amide N; △, ammonia N. Conditions of incubation are described in the text. Ordinates represent the proportion of N as a percentage of that present in each form at the beginning (casein N and amide N) or end (ammonia N) of the experiment.

Table 4. Urea as substrate or end product of the metabolism of *Entodinium caudatum*

The concentration of urea at the beginning was about 1 mg./ml. Conditions of incubation are described in the text.

	Ammonia N ($\mu\text{g./ml.}$)		Urea N ($\mu\text{g./ml.}$)	
	Protozoa alone	Protozoa with urea	Protozoa alone	Protozoa with urea
Beginning of expt.	1.4	2.8	0	494
End of expt.	16.5	17.8	1.3	482
Change	+15.1	+15.0	+1.3	-12

nitrogen to α -amino nitrogen in the non-protein nitrogen was 1.4. The proteolytic activity of the extracts was greatest at pH 6.5–7.0 (Fig. 3). In phosphate-citrate buffer, pH 6.5, the activity was markedly increased by cysteine in concentrations above 0.3 mM (Fig. 4a), and in sodium acetate buffer, pH 6.5, Ca^{2+} ions also activated the enzyme (Fig. 4b).

Fig. 3. Variation of activity of some enzymes of *E. caudatum* with pH. ○, Proteinase; ●, amidase; △, peptidase. Assay conditions are described in the text.

Fig. 4. Effect of some added substances on the activities of enzymes of *E. caudatum*. (a), Cysteine hydrochloride; (b), calcium chloride. ○, Proteinase; ●, amidase. Assay conditions are described in the text.

The cell-free extracts were incubated in phosphate-citrate buffer, pH 6.5, for 4 hr. with various amides, each in a concentration of 20 mM. The hydrolysis of the amides, expressed as $\mu\text{g.}$ of ammonia nitrogen liberated by 1 mg. of protein nitrogen in the enzyme solution, was as follows: propionamide, 0; asparagine, 10; glutamine, 11; N^α -benzoyl-L-arginineamide, 145. The activity of the extracts towards the last-named substrate, when the pH was varied, and when cysteine hydrochloride or calcium chloride was present, seemed almost identical with the proteolytic activity (Figs. 3 and 4). When portions of the same enzyme solution acted upon equimolar solutions of the amide and ethyl ester of N^α -benzoyl-L-arginine, the ester was hydrolysed about 12 times as fast as the amide.

All of the ten peptides referred to above were hydrolysed by the extract of *entodinia*. No hydrolysis of the peptides was observed when solutions of the substances were incubated without enzyme. No urease could be detected in the extract (less than 0.6 $\mu\text{g.}$ of ammonia nitrogen/hr./mg. of protein nitrogen).

Separation of proteinase and peptidase activities in cell-free extracts of Entodinium caudatum

The six fractions obtained after paper electrophoresis were examined for their total nitrogen content, and for their hydrolytic activities against casein, N^α -benzoyl-L-arginineamide and glycyl-L-leucine. The enzyme tests were carried out as already described except that the incubation period was 18 hr., and toluene was added as a preservative. The results are summarized in Table 5. Fractions 4 and 5, which contained 86 and 84 % of the total proteinase and amidase activity respectively, but no peptidase, were pooled. Fractions 2 and 3, which contained all the peptidase activity and only 12 and 14 % of the proteinase and amidase respectively, were also pooled. The combined fractions 4 and 5, which did not hydrolyse glycyl-L-leucine, also had no chromatographically detectable effect on L-leucylglycine, glycyl-L-alanine, glycylglycine, DL-alanyl-DL-alanine and DL-leucyl-DL-leucine. The effects of cysteine hydrochloride and calcium chloride on the activity of the purified proteinase were determined. The results were identical with those obtained with the unfractionated cell-free extract (see Fig. 4). Sodium chloride (30 mM) did not affect the proteinase activity.

The combined fractions 2 and 3 were used to determine the variation of peptidase activity with pH; the maximum activity was found at pH 5.8–6.0 (Fig. 3). The effect of some metal ions was also studied, by incorporating cobalt sulphate, magnesium sulphate, manganese sulphate, zinc sul-

Table 5. *Separation of enzymes in cell-free extract of Entodinium caudatum by paper electrophoresis*

The conditions of electrophoresis and enzyme assay are described in the text. The specific activity of the proteinase is expressed as $\mu\text{g.}$ of 'tyrosine' liberated by 1 mg. of N in the enzyme solution. The specific activity of the amidase is expressed as $\mu\text{g.}$ of ammonia N liberated by 1 mg. of N in the enzyme solution. +, Peptidase activity detected chromatographically. -, No peptidase activity detected chromatographically. The fractions are numbered from the origin in the direction of the anode. Recovery of N was 1.47 mg. (98% of that applied to the paper, with allowance for that lost in the guide strips).

Fraction no.	N ($\mu\text{g.}$)	Proteinase		Amidase		Peptidase
		Percentage of total activity	Specific activity	Percentage of total activity	Specific activity	
1	270	2.6	228	2.5	25	-
2	270	1.3	108	1.3	12.5	+
3	450	10.5	550	12.6	76	+
4	240	56	5300	57	640	-
5	190	30	3600	27	384	-
6	50	0	0	0	0	-

phate and sodium chloride at concentrations of 0.1, 1 and 10 mM. The magnesium, manganese and sodium salts had no effect at any concentration on the activity of the enzyme. Zinc sulphate had effect only at the highest concentration, when the enzyme activity was increased by 30%. Increases in activity of 12, 24 and 60% respectively were caused by the three concentrations of cobalt sulphate.

DISCUSSION

A thorough investigation of the nitrogen metabolism of any micro-organism should include experiments with growing cells, to discover which substances serve as nitrogen sources for the synthesis of proteins etc. Such experiments are not yet possible with any of the rumen protozoa. Culture of these organisms *in vitro* has been accomplished only in media of considerable complexity which are not suitable for biochemical analysis. In the present work we have used *E. caudatum* cells which, though motile and metabolically active, are not growing or multiplying. In the absence of added substrates the cells excreted nitrogen; when casein or casein hydrolysate was added, little or no conversion of the substrate into cellular material occurred. Our results, therefore, do not indicate with certainty the role of *E. caudatum* in the changes undergone by nitrogen compounds in the rumen. They do, however, suggest which substances might be attacked, and what products might be formed.

When *E. caudatum* suspensions in buffer solution were incubated without added substrate the cells slowly lost nitrogen to the medium. In fresh suspensions about 1% of cell nitrogen/hr. was excreted. Ammonia was the chief product, accounting for 50-70% of the nitrogen excreted in different experiments. This contrasts with the observation of

Heald & Oxford (1953) that ammonia is not an end product of endogenous metabolism of nitrogen by the holotrich protozoa of the rumen, *Isoetricha* and *Dasytricha*.

In addition to ammonia the entodinia also excrete traces of other substances, not volatile at pH 10, which give a purple colour when heated with ninhydrin. By assuming these substances to be amino acids having the same molar colour yield as leucine (Moore & Stein, 1948), a further 20%, approximately, of the non-protein nitrogen may be accounted for. In the ninhydrin test carried out according to Lampson & Singher (1960) the same result was obtained at both pH 4 and pH 5.6, suggesting that no simple peptides were present. The identity of the remaining 10-20% of the nitrogen compounds excreted by *E. caudatum* has not yet been established. Our experiments exclude both protein precipitable by trichloroacetic acid and urea.

E. caudatum suspensions are strongly proteolytic; a rapid loss of casein occurred when this substance was incubated with the protozoa, and at the same time a mixture of peptides and amino acids accumulated. It was noticed in all such experiments that the destruction of protein never went to completion; a little under 10% of the substrate was always recovered in the trichloroacetic acid precipitate (Fig. 1). No explanation for this cessation of proteolysis can be offered. It cannot be that the protozoa were harmed, or the enzymes inhibited, by the products of hydrolysis. The metabolic activity of the protozoa as measured manometrically was remarkably constant throughout the experiment, and when a further portion of casein was added to the suspension, it also was rapidly decomposed (Fig. 1).

When casein is digested in the rumen a large proportion of the nitrogen is converted into

ammonia (McDonald, 1952). No deamination of amino acids could be brought about by our suspensions of *E. caudatum*, however, although it is possible that deaminating activity originally possessed by the protozoa when in the rumen may have been lost in preparing the suspension (Lewis, 1955). The ammonia found after incubating casein with the entodinia could be completely accounted for by that formed from amide groups in the protein by hydrolysis, and by assuming that endogenous production of ammonia proceeded at the same rate in the presence of the substrate as in its absence (Table 2). When casein hydrolysate containing no amide nitrogen was used as substrate only a negligible increase in ammonia production was observed (Table 3).

Urea, which enters the rumen as a constituent of saliva, appears to play no part in the metabolism of *E. caudatum*. The living organisms neither decomposed added urea, nor did they produce it during endogenous metabolism (Table 4). The cell-free extracts of the protozoa contained no urease.

The optimum pH of the proteinase, pH 6.5–7.0, agrees with observations of other workers that the proteolytic activity of various rumen protozoal preparations is near neutrality (Schlottke, 1936; Sym, 1938). Calcium chloride and cysteine hydrochloride both increased the activity of the proteinase; this activation was not caused by the Cl^- ion common to these substances, since sodium chloride, added at a higher concentration, had no effect. In experiments not reported here the activation caused by cysteine was slightly less when anaerobic precautions were not taken.

N^c-Benzoyl-L-arginineamide is widely used as a synthetic substrate in the study of proteinases. This substance was readily hydrolysed by extracts of the entodinia. It was concluded from a number of experiments that the proteinase and amidase activities of the extracts are functions of the same enzyme. The two activities corresponded very closely in their response to change of pH, and to the presence of calcium and cysteine (Figs. 3 and 4). The proportion of the total activity found in each of the electrophoretic fractions was almost the same for the proteinase and amidase (Table 5).

The proteinase of *E. caudatum* shows some similarities to trypsin. It can hydrolyse the amide and ester linkages in the amide and ethyl ester respectively of *N*^c-benzoyl-L-arginine (Bergmann, Fruton & Pollok, 1939). Under comparable conditions hydrolysis of the ester is about 12 times the faster. Schwert, Neurath, Kaufman & Snoko (1948) found the ratio in reaction velocities with trypsin to be about 60. The proteinase also resembles trypsin in being activated by Ca^{2+} ions (Duke, Bier & Nord, 1952). It differs, however, in having a slightly lower pH optimum [pH 6.5–7.0, instead

of the range pH 7.5–9.0 reported for trypsin by Bergmann *et al.* (1939) and Bier & Nord (1951a, b)], and also in being activated by cysteine (cf. Viswanatha & Liener, 1956). In this latter respect the *E. caudatum* proteinase resembles the proteinase of *Clostridium histolyticum* (Weil & Kocholaty, 1937; Ogle & Tytell, 1953), a micro-organism which is anaerobic like *E. caudatum*.

Because of structural specificity, enzymes of the trypsin type cannot hydrolyse all the peptide bonds in casein; trypsin was found to liberate only 13% of the total amino acids (Van Slyke *et al.* 1941). Liberation of free amino acids from casein in our experiments was 36% by the living cells and 42% by a cell-free extract, and these values are not necessarily the limits of hydrolysis attainable. It was not surprising, therefore, that a strong peptidase activity was found in the cell-free extracts. In veronal buffer, pH 8.6, the peptidase was less mobile in an electric field than the proteinase, so that a considerable degree of separation of the enzymes was achieved by electrophoresis. The peptidase seemed to have a wide range of substrates; all of the dipeptides, and the tripeptide, which we had at our disposal were hydrolysed. It is possible of course that the entodinia may synthesize a number of peptidases, each with a more restricted range of activity. Since any evidence for this is lacking, the peptidase activity is dealt with here as though it were the property of a single enzyme. The *E. caudatum* peptidase had a much lower pH optimum than the proteinase; at a pH which is suitable for the living cells, i.e. pH 6.5–7.0, the activity was only one-half or less of its maximum value. As with the proteinase, so the peptidase of *E. caudatum* has similarities with the corresponding enzymes of *C. histolyticum* (Mandl, Ferguson & Zaffuto, 1957), particularly in its activation by metal ions.

As far as the results of our experiments *in vitro* may be applied to the rumen, they suggest that *E. caudatum* may play an important part in the ruminal hydrolysis of proteins. The peptides and amino acids formed no doubt serve as starting materials for protein synthesis by the organism. *E. caudatum* also contributes to the ammonia that is produced in the rumen.

SUMMARY

1. When suspensions of the rumen oligotrich protozoan *Entodinium caudatum* are incubated in buffer solution, about 1% of cellular nitrogen/hr. is excreted. One-half or more of this is in the form of ammonia, and about one-fifth in the form of amino acids.

2. The *Entodinium caudatum* suspensions rapidly hydrolyse casein. Peptides and amino acids are

the principal products. Ammonia is formed by the hydrolysis of amide groups in the casein, but no deamination of amino acids occurs.

3. Negligible conversion of casein or casein hydrolysate into cellular material takes place during incubation of these substances with *Entodinium caudatum*.

4. Urea plays no part, either as substrate or end product, in the nitrogen metabolism of *Entodinium caudatum*.

5. Cell-free extracts of the entodinia contain a proteinase which, in addition to hydrolysing casein, also hydrolyses the amide and ethyl ester of *N*^α-benzoyl-L-arginine. Activity is greatest at pH 6.5-7.0, and is increased in the presence of cysteine or calcium ions.

6. A peptidase, which hydrolyses a wide range of peptides is also found in the cell-free extracts. Its activity against L-leucylglycine is greatest at pH 5.8-6.0, and is increased by cobalt and to a lesser extent by zinc ions.

7. The proteinase and peptidase can be separated by electrophoresis on paper at pH 8.6.

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Condensed Tannins

12. POLYMERIC LEUCO-FISETINIDIN TANNINS FROM THE HEARTWOOD OF *ACACIA MEARNSII**

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The heartwood of the black-wattle tree (*Acacia mearnsii*) contains, amongst others, a 'leuco-fisetinidin' tannin which apparently corresponds to

the trimeric form of the accompanying (+)-7,3',4'-trihydroxyflavan-3,4-diol (Roux & Paulus, 1961c). The isolation, from the same source, of related tannins of progressively higher molecular weight is described in the present study.

* Part 11: Roux & Paulus (1961c).