SUMMARY

1. The culture filtrates of each of four strains of *Alternaria tenuis* auct., grown on Czapek-Dox medium, have been shown to contain one or more of five metabolic products or groups of products.

2. One group, alternariol and alternariol methyl ether, has previously been shown to occur in the mycelium of this species; the remaining compounds have not previously been described.

3. A second group, the altenuic acids, consists of three closely related isomeric colourless substances, $C_{15}H_{14}O_8$, containing one carbon-methyl and one methoxyl group. Altenuic acid I, m.p. 183–184°, and altenuic acid II, m.p. 245–246°, are readily converted into altenuic acid III, which has a variable melting point and gives a strong purple colour with ferric chloride in aqueous ethanol.

4. Thirdly, a colourless reducing compound, altenusin, $C_{15}H_{14}O_6$, m.p. 202–203°, has been isolated from two of the strains. It is oxidized by ferric chloride to the yellow dehydroaltenusin, $C_{15}H_{12}O_6$, m.p. 189–190°, which has a deep-brown ferric reaction. Both compounds contain one carbon-methyl and one methoxyl group.

5. Fourthly, altertenuol, $C_{14}H_{10}O_6$, buff-coloured needles, m.p. 284–285°, has been isolated in small amounts from two strains. It contains one methoxyl group, but no carbon-methyl group, and gives an intense green ferric colour.

6. Lastly, two strains produce tenuazonic acid, $C_{10}H_{15}O_3N$, b.p. about 117°/0.035 mm., $[\alpha]_{5461}^{20} - 132^\circ$ in chloroform, which has not been obtained as a solid. On long standing, or more rapidly in boiling aqueous alkali, it isomerizes partly to isotenuazonic acid, $[\alpha]_{5461}^{22} + 23^\circ$ in chloroform, which forms colourless needles, m.p. 61–63.5°. Both compounds are ketonic, and give a strong orange-red ferric reaction.

7. Characteristic derivatives of all these new compounds are described.

8. The molecular formulae indicate some relationship between all these metabolic products except tenuazonic acid.

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The Conversion of Casein into Microbial Proteins in the Rumen

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In an earlier paper McDonald (1954*a*) reviewed the problem of determining the extent to which the microbes of the rumen of the sheep were capable of utilizing the nitrogen of the host's diet for the synthesis of their own proteins; the technical difficulties were discussed in some detail and results given for the fate of the ethanol-soluble protein, zein, in the rumen. For reasons given, it was considered that zein would be less effectively utilized by the microbes than a more soluble protein. An effort was therefore made to devise a procedure for the study of casein, as it had already been shown that this protein was readily attacked in the rumen (McDonald, 1952; Chalmers, Cuthbertson & Synge, 1954).

The characteristic feature of casein as a phospho-protein has been exploited to yield a direct assessment of the fraction of the dietary casein that escapes digestion in the rumen, and hence an assessment of the synthesis of microbial protein in the rumen.

MATERIALS AND METHODS

Animals. The experimental sheep were prepared with fistulae into the rumen and duodenum. The reasons for collecting abomasal contents immediately after passage into the duodenum have previously been recorded (McDonald, 1954*a*). The duodenal fistula was prepared by McDonald's (1953) modification of the surgical technique of Ward, Young & Huffman (1950) and Young (1951), and the rumen fistula by the procedure of Phillipson & Innes (1939). The fistulae were closed with pliable cannulae made from polyvinyl chloride (Welvic Paste P.B. supplied by Imperial Chemical Industries Ltd.).

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Collection of ingesta. From the duodenal cannula the fluid was allowed to flow freely. Phillipson (1952) noted that flow of ingesta from the abomasum may cease for periods as long as 30 min.; this has been repeatedly confirmed in the present work. When the flow ceases there may be a back flow of bile and succus entericus from the duodenum; for this reason any fluid which trickled out from the cannula in the interval between gushes of ingesta from the abomasum was discarded. In practice this proved quite simple, as the bile and succus entericus flow slowly and are mucoid, in contrast with the rapid flow and the watery consistence of the abomasal contents.

Since the material collected is heterogeneous, accurate sampling for analysis is difficult. All samples were measured by weight, large quantities have been used (usually 7–20 g.) and the sample was taken by pouring while the specimen was being stirred. All analyses have been made in duplicate or triplicate.

Diets. The natural diets of ruminants contain a large proportion of roughage, which provides the stimulus to rumination (Phillipson, 1946); this, in turn, greatly stimulates the secretion of saliva (Denton, 1957), which plays an essential role in maintaining the digestive function of the rumen. Purified cellulose, even when fed in considerable quantities, was found to provide an inadequate stimulus for rumination. For these reasons it is very difficult to prepare a satisfactory diet of known chemical composition. The diets used in these experiments were also designed for a study of microbial synthesis of nucleic acids in the rumen (to be reported later); the basic ration was required to be free of nucleic acids and to have a minimum content of other nitrogenous substances. To this end the diet was based on alkali-extracted straw as the roughage. Schmidt & Thannhauser (1945) showed that nucleic acids could be extracted from animal tissues by dilute alkali; after extraction of wheat straw with dilute aqueous NaOH at ambient temperature for 24 hr., no purines could be detected by a procedure (McDonald, 1954*b*) which permitted measurement of purine content in untreated straw. The same extraction served to decrease the content of nitrogen to approx. 0.33%, i.e. about half its original value. It has been shown by Ferguson (1942) that extraction of straw by alkali doubles its feeding value as a source of energy for sheep. These fortunate coincidences have thus enabled the preparation of a partially purified diet, free of purines, and containing only minimal quantities of nitrogenous substances of unknown composition.

The straw was chopped into lengths of about $\frac{1}{2}$ in., extracted for 24 hr. in 1.5% NaOH (w/v) and washed in tap water with automatic siphoning until free from alkali; the bulk of the water was removed in a press and the straw was then dried under a forced draught at a temperature not exceeding 40°.

The composition of the daily ration was: alkali-extracted straw 600 g., rice starch 150 g., sucrose 100 g., casein or casein digest 100 g., molasses 20 g. and salt mixture 30 g. The salt mixture consisted of NaCl 40%, CaCO₃ 17%, Ca₃(PO₄)₂ 20%, MgSO₄·7H₂O 12%, KCl 8%, and purified salts yielding (mg./day) Co 1, Cu 10, Mn 2, Fe 20, and I₂ 1. The ingredients other than straw were made into a suspension with 400 ml. of water and then thoroughly mixed with the straw to give a moist mash. This procedure improved palatability and prevented separation of the fine powders from the straw. The ration was fed once per day and was

usually wholly consumed in 1 hr. Water was allowed *ad libitum*. The casein used for experimental periods was Light White Soluble (British Drug Houses Ltd.); in the intervals between experiments it was replaced by commercial casein (Hopkin & Williams Ltd.). The casein digest was supplied by Allen and Hanburys Ltd.; it was an enzymic digest, completely soluble in 80% (v/v) aqueous ethanol. The molasses contained 0.59 g. of N/100 g., of which 0.13 g. of N/100 g. was non-dialysable and may be regarded as protein N. Vitamins A and D were supplied in concentrated form once weekly.

Nitrogen. This was estimated by the Kjeldahl method according to the procedure recommended by Hiller, Plazin & Van Slyke (1948). In this procedure, if zinc is allowed to accumulate in the distilling flask, due to incomplete removal of the Zn amalgam and excess of Zn powder, the rapid evolution of hydrogen during steam distillation can result in the carrying over of NaOH, in fine droplets, to the distillate. It was found that quantitative distillation of the NH₃ could be achieved most conveniently as follows: the digest was transferred from the Kjeldahl flask to a volumetric flask and made to volume; powdered Zn in appropriate quantity was added to the flask and the contents were thoroughly shaken; amalgamation of the Hg was rapid and duplicate aliquots were then taken for steam distillation of the NH₃ in the usual way.

Protein N (P.N.) was estimated as the nitrogen remaining after exhaustive dialysis as described in the next paragraph; in order to reduce sampling errors, the entire sac with its contents was digested in a Kjeldahl flask; non-protein nitrogen (N.P.N.) was calculated as the difference between total N (T.N.) and P.N.

Estimation of casein in abomasal contents. It has been shown by Plimmer & Bayliss (1906) and Rimington & Kay (1926) that the phosphorus of casein is liberated as inorganic phosphate by hydrolysis with weak solutions of NaOH at 37°; hence this reaction may be made the basis for an analysis for casein in a mixture which does not contain other alkali-labile phosphorus compounds or large amounts of inorganic phosphate. In the abomasal contents studied here, the concentration of casein was usually so low that conventional precipitation procedures proved inadequate for separation of casein from the inorganic and acid-soluble phosphates; a dialysis technique has therefore been developed for this purpose. Cellophane tubing was cut into 8 in. lengths, washed, knotted at one end and tested for leaks; these were gently inflated to resemble test tubes, stood in a rack and allowed to dry. Into each tube was placed about 0.1 g. of powdered thymol and then a weighed sample (7–10 g.) of abomasal contents; the walls of the tube were thoroughly washed down with 8–10 ml. of water and the upper end knotted in such a way as to include an air bubble under slight pressure. The sac was then clipped on the bars of a frame supported on an axle which carried a large pulley wheel. The frame used could hold more than 30 sacs. The frame was then completely immersed in a tank through which cold tap water was allowed to run continuously, and was rotated at approximately 20 rev./min. by a geared motor via reduction pulleys. As the axis of rotation was horizontal and at right angles to the long axis of the sac, the dialysate (i.e. the material subjected to dialysis) was continuously stirred by the included air bubble; the diffusate, in turn, was continuously stirred by the rotation of the frame. This technique gave very efficient dialysis. After 36–48 hr.

dialysis, the flow of water was stopped and N -NaOH was added to bring the pH of the diffusate to about 10. After 2 hr. further rotation, the sacs were removed, the free ends cut off and measured for length; the sacs were then blotted 'dry', weighed, shaken to mix and immediately emptied into centrifuge tubes. The weight of contents was calculated from the gross weight by use of a previously determined correction for the weight of a known length of cellophan tubing wetted and blotted 'dry'. After centrifuging, the opalescent supernatants were removed and 2 ml. portions were placed in 10 ml. volumetric flasks with 5 ml. of 1.4 N -NaOH-0.3 M -NaCl and hydrolysed for 24 hr. at 37°. After hydrolysis, inorganic phosphate was estimated as described in the following section. The amount of inorganic phosphate before hydrolysis was estimated in each dialysate by treating a measured portion with H_2SO_4 to a final concentration of 0.2 N , at 37° for 24 hr.; under these conditions (as noted by Rimington & Kay, 1926) no inorganic phosphate was liberated from the casein used in this work. The dialysis reduced the concentration of inorganic phosphate to satisfactorily low levels; most values fell between 0.1 and 0.2 mg./100 g. of abomasal contents and the range was 0.007-0.76 mg./100 g.

Proteolytic activity of pepsin and of microbial enzymes was destroyed by heating the abomasal contents to 80° immediately after collection. Thymol was found to be a suitable preservative and the amount used was sufficient to ensure a small excess remaining in the sac at the end of the dialysis. Casein added to abomasal contents could be quantitatively recovered; the results of a test of the method are given in Table 1.

Estimation of casein by this procedure is of course not specific and is influenced by two factors: (a) possible incomplete extraction of residual traces of inorganic phosphate by acid in the estimation of phosphate in the control; (b) presence in the dialysed residues of substances other than casein which yield inorganic phosphate on alkaline hydrolysis. Substances of this nature (possibly phosphoproteins) have been observed in animal tissues; Davidson, Frazer & Hutchison (1951) and Kennedy & Smith (1954) found that inorganic phosphate contributed to the 'phosphoprotein' fraction as defined in terms of extraction procedures of Schmidt & Thannhauser (1945) and Schneider (1945). In the results reported here, it is evident that the errors from these sources are quite small.

Inorganic phosphate. When casein was hydrolysed as described above, the breakdown products could not be completely removed by suitable protein precipitants; the

addition of molybdate to the 'protein-free' solutions led to the formation of a cloudy precipitate which prevented the use of such methods as that of Fiske & Subbarow (1925). Further, the method of Berenblum & Chain (1938) could not be used owing to the formation of emulsions on shaking the hydrolysates with isobutanol and to adsorption of phosphomolybdate on the precipitate; the modifications of this method by Martin & Doty (1949) and Weil-Malherbe & Green (1951) were used as the basis for the following procedure: The alkaline hydrolysates (containing NaCl) were neutralized with 1 ml. of 7 N - H_2SO_4 and treated with 1 ml. of m - $AgClO_4$ in 20% $HClO_4$ (w/v) (prepared by dissolving Ag_2O in the calculated amount of $HClO_4$). The $AgCl$ thus formed assisted in the removal of interfering substances. The mixture was made to 10 ml., vigorously shaken and then centrifuged; 2 ml. of the supernatant was pipetted into a glass-stoppered tube containing 6 ml. of isobutanol-light petroleum (b.p. 100-120°) (85:15, v/v); 1 ml. of 5% ammonium molybdate (w/v) in 4 N - H_2SO_4 was added, the tube was stoppered, vigorously shaken by machine for 1 min. and then briefly centrifuged. 4 ml. of the clear upper layer was pipetted into a glass-stoppered tube and the blue colour developed by addition of 0.5 ml. of acid $SnCl_4$ (the stock solution contained 43 g. of $SnCl_4 \cdot 2H_2O$ in 100 ml. of conc. HCl ; before use, this solution was diluted 1:400 in 18 N - H_2SO_4). This diluted solution is miscible with the organic solvent and the $SnCl_4$ remains in solution; the final concentrations are 0.01% $SnCl_4$ and 2 N - H_2SO_4 . [In later work it has been noted that, with some batches of $HClO_4$, emulsions are formed on addition of the $SnCl_4$. This can be prevented by diluting the stock solution of $SnCl_4$ 1:400 with a mixture of ethanol and 18 N - H_2SO_4 (3:1, v/v).] The blue colour, which was very stable, was read at 625 $m\mu$. Standards and appropriate blanks were put up with each series of analyses. A convenient standard, diluted from a stock solution, contained 4 μ g. of P in 2 ml. and gave a density of approx. 0.36 at 625 $m\mu$ and 1 cm. light path; hence satisfactory analyses could be made of solutions which contained about 0.3 μ g. of P/ml.

The casein in the abomasal contents was calculated from the relation:

$$\text{Casein N (as \% of total protein N)} = \frac{P.P. \times N/P}{P.N.} \times 100,$$

where P.P. is protein phosphorus (mg. of P/100 g. of abomasal contents), N/P is the nitrogen/phosphorus ratio in the dietary casein and P.N. is total protein N (mg. of N/100 g. of contents).

Table 1. *Recovery of casein added to abomasal contents*

For this test abomasal contents were taken before feeding, from a sheep receiving the casein diet. To three portions of the contents were added 2 ml. of a casein solution containing 66.25 μ g. of protein P/ml. The mixture was dialysed and analysed as described in the text.

Abomasal contents (g.)	Amounts (mg./100 g. of contents)				Casein P recovered (%)
	Casein P added	Inorganic phosphate-P	Protein P	Casein P recovered	
6.63	0	0.71	0.10	—	—
7.00	0	0.69	0.20	—	—
6.96	0	0.72	0.18	—	—
7.15	1.85	0.63	2.12	1.93	104
7.65	1.73	0.73	1.92	1.73	100
7.01	1.89	0.66	2.12	1.93	102

Table 2. *Analyses of abomasal contents*

The figures for casein N (mg./100 g.) are calculated from the preceding column; the casein used contained 13.1% of N and 0.64% of P.

Time after feeding (hr.)	Amounts present (mg./100 g. of contents)				Casein N as % of protein N
	Protein N	Non-protein N	Protein P	Casein N	
Sheep fed on hydrolysate diet					
0	92	36	0.04	0.82	0.9
1	86	55	0.09	1.85	2.2
2	78	63	0.10	2.05	2.6
5	100	57	0.09	1.85	1.8
10	100	47	0.04	0.82	0.8
Sheep fed on casein diet					
0	80	38	0.14	2.87	3.6
1	96	48	1.18	24.2	25.2
2	92	52	1.89	38.8	42.1
5	107	57	0.75	15.4	14.4
10	98	46	0.16	3.28	3.3

RESULTS AND DISCUSSION

Two sheep were used in these experiments; one was maintained on the diet with casein for 2 years and the other with casein digest for 7 months. No difficulty was experienced in maintaining complete consumption of the ration every day once the animals had become accustomed to the diets. The results of three experiments on each animal are summarized in Figure 1. The origins of the data in this figure are illustrated with the protocols of two experiments, one on each sheep, given in Table 2. The curves for the sheep fed with casein digest show quite clearly that only negligible values are obtained for apparent casein N when in fact no casein was fed; these observations give an estimate of the error unavoidably included in the analyses for the other animal. The curves for the sheep fed with casein show a sharp rise in the contribution of casein to the protein of the ingesta, with a peak at about 2 hr. after feeding and a sharp decline later. This decline is in accord with previous observations that casein is rapidly degraded in the rumen of the sheep (McDonald, 1952; Chalmers *et al.* 1954); the decline proceeds so rapidly that, at about 8–10 hr. after feeding, casein makes a negligible contribution to the total protein of the ingesta.

The calculation of the relative contributions of casein and of microbial protein to the total protein received by the small intestine is based on certain assumptions which have been previously discussed in detail (McDonald, 1954*a*). Several further assumptions, implicit in these experiments, are:

(a) That the several component fractions of casein are attacked at the same rate in the rumen and that the casein molecules are degraded in such a way that the ratio of N/P in the molecules of protein, defined in terms of the dialysis used, is unchanged: i.e. that phosphate is not split off leaving protein molecules with a higher N/P ratio or that amino

Fig. 1. Changes in concentration of casein in abomasal contents (●) in three experiments on one sheep. In control experiments (■) the 'apparent casein' is shown when none was fed; the nitrogen of the diet was supplied as casein hydrolysate. The area above the broken line A represents the probable contribution of the indigestible protein of straw in the diet to the total protein N.

acids or peptides are not split off leaving a lower N/P ratio. Hipp, Groves, Custer & McMeekin (1952) showed that casein of cow's milk contains three components, α -, β - and γ -caseins, in the ratios of approximately 75:22:3 respectively, and that these components have phosphorus contents of approximately 0.98, 0.60 and 0.11% respectively. Stirling & Wishart (1932) found that enzymic digestion of caseinogen by pepsin and trypsin yielded products

insoluble in trichloroacetic acid with respectively lower and higher N/P ratios than the original protein.

(b) That all the inorganic phosphate estimated after alkaline hydrolysis is in fact derived from casein. The very low values obtained from the sheep fed with casein digest indicate that this assumption is substantially correct.

(c) That the nitrogen of the straw passes through the rumen and abomasum undigested. This assumption is probably justified since the proteins of straw are known to have a low digestibility in sheep and cattle (Blaxter & Mitchell, 1948) and the material fed had been extracted with sodium hydroxide, which removed a large fraction of the total N. To the degree that the straw protein had been digested in the rumen, the contribution of the microbial protein given above will have been underestimated. The broken horizontal line *A* in Fig. 1 represents the contribution of the straw protein to the ingesta, and for purposes of this calculation it is assumed to be equal to the contribution of the straw to the total dietary N.

In the earlier work with zein it was assumed that the loss of nitrogen from the rumen due to the absorption of ammonia was compensated by the addition of nitrogen to the rumen by the saliva. The diets used in the present experiments contained large quantities of starch and sucrose; McDonald (1952) and Annison, Chalmers, Marshall & Synge (1954) showed that administration of starch caused a steady decline in the concentration of ammonia, and there is good reason to believe that this phenomenon is due to the utilization of carbohydrate and ammonia for bacterial growth. In Fig. 2 are shown the changes in concentration of ammonia N and N.P.N. in the rumen of the sheep fed with the casein digest; this shows clearly that in spite of a high concentration of N.P.N. (chiefly peptides and amino acids) the ammonia concentration rose only during the first hour after feeding and thereafter steadily declined to extremely low values. Since salivary N is of the order of 10–20 mg. of N/100 ml., and salivary secretion is some 4–7 l./day, it is clear that the assumption mentioned above is unlikely to introduce gross error in these experiments.

If these assumptions are accepted, the following calculation can be made as a first approximation: in Fig. 1, the areas under the curves for the casein-fed sheep indicate that casein comprised 8.5, 8.7 and 9.2% (mean, 8.8%) of the total protein which passed from the abomasum to the small intestine. Since the straw contributed 13% of the dietary protein, it is evident that only 10% [i.e. $100 \times 8.8 / (100 - 13)$] of the dietary casein reached the small intestine. This estimate is presumably too high, because some 'apparent casein N' appears in the abomasal contents of the sheep fed with casein

hydrolysate; no correction is applied for this because more precise calculation does not appear to be justified. However, it is evident that in the experiments reported here at least 90% of the casein in the diet was degraded in the rumen and resynthesized into microbial protein.

In the three experiments reported, the maximum concentration of casein in the abomasal proteins, at approximately 2 hr. after feeding, averaged 47%. In ruminants the fodder passes, after mastication, into the rumen, from which it passes slowly, over a period of some days, to the abomasum and small intestine (see Balch, 1950). Thus each feed is mixed with the residues from preceding meals. If, in our experimental sheep, the rumen contained, at 24 hr. after feeding, an amount of protein equivalent to that in a day's ration [as observed by Agrawala, Duncan & Huffman (1953) in bovines], the freshly consumed casein would be mixed with an equal quantity of microbial protein derived from the microbial growth of preceding days. Hence it would be expected that the maximum concentration of casein in the ingesta leaving the rumen would be of the order of 50% of the total protein.

Attention has frequently been drawn to the importance in ruminant nutrition of microbial degradation of protein in the rumen and to the compensating protein synthesis due to microbial growth; this subject has recently been critically reviewed by Chalmers & Synge (1954). Although the qualitative aspects have been much studied, the technical difficulties have prevented a direct quantitative approach. McDonald (1954*a*) listed previous attempts at quantitative estimation of microbial contributions of protein and described a

Fig. 2. Changes in the concentration of non-protein N (○) and ammonia N (●) in the rumen liquor of a sheep fed on a diet in which casein digest provided 87% of the total N. Each point represents the average of two observations.

new experimental approach to the problem; it was shown that, when zein was fed as the major nitrogenous component of the diet, some 40 % of the zein was utilized by rumen microbes for synthesis of their own proteins. Since zein was highly insoluble in aqueous media and was but slowly attacked in the rumen (McDonald, 1952), it was predicted that, with more favourable diets, the micro-organisms would make a greater contribution to the total protein finally available for digestion and absorption by the host ruminant; this prediction has now been verified. Light white casein was selected as the dietary protein because it was soluble in rumen fluid, and was rapidly attacked by proteolytic organisms in the rumen (McDonald, 1952; Chalmers & Synge, 1954; Chalmers *et al.* 1954; Annison *et al.* 1954). No information is yet available on the synthetic capacity of rumen microbes when the host is fed on ordinary diets; however, it is apparent that the proteins of fresh leaves are readily susceptible to degradation, since ammonia rapidly accumulates in the rumen of animals at pasture (McDonald, 1948; Johns, 1955); it is possible that under these conditions much of the pasture protein is converted into microbial protein.

One limitation of the present experiments should be stressed: only one level of protein intake (100 g./day) has been studied. This level is well above the minimum required for nitrogen equilibrium but is much below the intake expected for sheep on good pasture. There may be an upper limit to the capacity for growth of the micro-organisms in the rumen—a limit determined by the factors governing the maximum concentration of organisms in the rumen contents and the rate of passage of ingesta through the rumen; the available data are inadequate for calculation of this growth rate.

SUMMARY

1. A partially purified diet, suitable for adult sheep, has been devised. When casein provided 87 % of the nitrogen in this diet, at least 90 % of the casein was degraded in the rumen and utilized for the synthesis of microbial proteins.

2. This conclusion was based on the estimation of casein in the mixed ingesta leaving the stomach. A chemical procedure was developed for determining small amounts of casein in the presence of other proteins; this procedure relies on the removal of inorganic phosphate from the original sample by

dialysis, the alkaline hydrolysis of the phosphate group of casein and the subsequent estimation of the inorganic phosphate so formed.

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