DDPT is thus a suitable reagent for the determination of the amino-acid sequence in peptides by a process of step-wise degradation from the *N*-terminal end of the chain, and has in fact been successfully applied for this purpose. The conditions under which the *N*-terminal amino acid splits off as a substituted DDP-thiohydantoin are so mild that other peptide bonds are not split. The yields of the thiohydantoins vary between 80 and 90 % according to the experimental conditions and the type of the thiohydantoin. We found that the yield of the residual peptide (subject to the next step in the sequence determination) is practically equivalent to the yield of the thiohydantoin. Consequently a small but calculable fraction of the DDP-thioureido-peptide is lost during the process of the elimination of the substituted *N*-terminal group.

SUMMARY

1. A deep-orange *isothiocyanate*, 4-dimethyl-amino-3:5-dinitrophenyl*isothiocyanate* (briefly called DDPT) was synthesized.
2. It was found that this *isothiocyanate* reacts with amino acids or peptides dissolved in water: acetone (1:3, v/v) at the apparent pH 8-9, giving orange thiocarbamyl derivatives (briefly called DDP-thioureidoamino acids or DDP-thioureido-peptides, e.g. DDP-thioureidoglycine) in practically quantitative yields.
3. By the treatment of the DDP-thioureido-amino acids with glacial acetic acid (in the case of DDP-thioureidoglycine) or with water:acetic acid (in the case of other DDP-thioureidoamino acids)

the corresponding substituted 2-thiohydantoins (briefly called amino acid-DDP-thiohydantoins, e.g. glycine-DDP-thiohydantoin) are formed in good yields (80-90 %).

4. By treatment of DDP-thioureido-peptides with water:acetic acid, the *N*-terminal amino acid residue splits off as an amino acid-DDP-thiohydantoin which can be isolated in a pure state by adsorption chromatography; the residual peptide can be treated with DDPT and the process repeated to determine the sequence of amino acids in the peptide.

5. As an example the sequence determination on alanyl-glycylglycine is described.

6. The amino acid-DDP-thiohydantoins can be hydrolysed to the parent amino acids and these can be identified by the use of paper chromatography.

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The Extent of Conversion of Food Protein to Microbial Protein in the Rumen of the Sheep

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Interest in the role of ruminal micro-organisms in the protein metabolism of ruminants stems from the hypothesis of Zuntz (1891) that these organisms might utilize dietary non-protein nitrogen (N.P.N.) for their growth, with resultant synthesis of protein which would become available to the host. Numerous experiments have established the validity of this view (for a review of the extensive literature in this field see McNaught & Smith, 1947); indeed, Loosli, Williams, Thomas, Ferris & Maynard (1949) were able to maintain growth in sheep on a diet in which virtually all the nitrogen was supplied by urea.

In spite of the activity in this field, comparatively little attention has been given to the possibility of microbial digestion of food protein in the rumen. In normal ruminant diets most of the nitrogen occurs in the form of protein. The facts that the salivary glands do not occur in the rumen, that the saliva contains no proteolytic enzyme (Wegner, Booth, Bohstedt & Hart, 1940) and that the rumen contents are strongly proteolytic (Sym, 1938) suggest that ruminal organisms play an active part in the digestion of protein. Pearson & Smith (1943) observed breakdown of protein by rumen liquor *in*

vitro, and McDonald (1948*a*, 1952) showed that soluble protein was extensively degraded in the rumen with the formation of ammonia.

The extent to which ruminal microbes convert fodder protein into the proteins of their own structures has been the subject of several speculations, though little experimental effort has been directed to the problem.

Schwarz (1925) concluded that the 'greatest part' of the protein requirement of cattle was met by the digestion of microbial protein derived from fodder protein. However, his data, confined to analyses of rumen contents, do not warrant such a conclusion, since he found on the average 60% of the total nitrogen of rumen contents in the fodder residues and 32% in the micro-organisms. His views were opposed by Mangold & Schmitt-Krahmer (1927), but the evidence they adduced was equally unconvincing. Mitchell & Hamilton (1929) criticized these speculations and pointed out that it was not enough to perform analyses on the rumen contents of slaughtered animals, but that it was necessary to estimate the daily production of microbial protein in the rumen.

Ferber & Winogradowa-Federowa (1929) calculated that rumen protozoa provided 2% of the protein requirements of the sheep; this estimate was based on their observation that at any given time an average of 7% of the infusoria of rumen contents were in process of dividing, and they assumed that this was also the daily reproductive rate. Hungate (1942) considered this value too low; he found that, in culture, the protozoan *Eudiplodinium neglectum* had a division rate of about once per day and hence calculated that the protozoa provided about 20% of the host's daily protein requirements.

Köhler (1940) attempted to assess the daily output of microbial nitrogen from the rumen of cattle by comparisons of bacterial numbers or weight in the contents of the rumen and the duodenum, but found his methods inadequate to yield acceptable results.

Thaysen (1945) estimated that a minimum of 180 g./day of microbial protein passed from the rumen of cattle. He based his calculations on analyses of centrifuged rumen liquor and on the assumption that 100 l. of rumen liquor flowed from the rumen to the abomasum each day. McNaught & Smith (1947) used the data from the *in vitro* experiments of Pearson & Smith (1943) to estimate that 100–150 g. of bacterial protein were formed/day in the rumen of cattle; alternatively, using the data of Schwarz (1925) and an assumed rate of flow of digesta from the rumen of the ox of 40 kg./day, they calculated that 75 g. of bacterial protein would be formed/day.

Moir & Williams (1950) observed, in sheep, a constant increase in the numbers of ruminal micro-organisms with increasing protein intakes, suggest-

ing that a constant proportion of the food protein was converted into bacterial protein; they calculated that approximately 50% of the dietary protein was converted into bacterial protein, but subsequently recognized (Moir, 1951) that such a calculation could not be made from their data.

Indirect evidence for the contribution of micro-organisms to the protein metabolism of the host animals has accrued from estimates of the biological value of protein for ruminants. Johnson, Hamilton, Mitchell & Robinson (1942) and Miller & Morrison (1942) noted a relative constancy in the biological values of the proteins they examined. It was suggested (Johnson, Hamilton, Robinson & Garey, 1944) that, according to the capacity of the rumen microbes to utilize nitrogen, all feeds would have the biological value of microbial protein (namely about 60%) whilst any protein not utilized by the micro-organisms would have a biological value approximately the same as that found for non-ruminants. The later observations on biological value made by Lofgreen, Loosli & Maynard (1947) and by Hamilton, Robinson & Johnson (1948) suggest that the proportions of dietary and microbial protein which actually become available to the host might vary considerably on different diets.

A survey of the literature thus reveals that it is likely that the growth of micro-organisms (bacteria and protozoa) in the rumen results in a significant degree of conversion of the animal's food nitrogen into microbial protein, but that the extent of this conversion and the factors influencing it are not evident. The present work comprises an attempt to develop procedures for direct experimental approach to the problem. A preliminary account of this work has previously been published (McDonald, 1948*b*).

EXPERIMENTAL

Methods

The determination of the quantity of microbial protein which passes from the reticulum into the abomasum, and thence through the remainder of the intestinal tract presents two major requirements: the collection of digesta received by the abomasum, and the analysis of the digesta for microbial proteins and/or the undigested food protein.

The collection of the digesta presents numerous difficulties. No means have yet been devised for sampling digesta at the entrance to the omasum or as it leaves this viscus to reach the abomasum. No difficulty is entailed in collecting the digesta from the abomasum via a permanent fistula, but this approach has not been favoured as it was found necessary to collect the whole of the contents of the organ in order to obtain satisfactory sampling, and it seemed probable that the artificial emptying of the abomasum would influence the rate of flow of digesta from the rumen and reticulum, and the rate of secretion of gastric juice. On these grounds it appeared desirable to collect the abomasal digesta immediately after it passed through the pylorus.

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

(For this test abomasal contents were taken from a sheep receiving a diet in which casein replaced the zein of the experimental diet. The excess recovery of zein is due to the presence of traces of nitrogen, probably lipid N, soluble in 80% ethanol but not in 5% trichloroacetic acid.)

Abomasal contents (ml.)	Zein N added (mg.)	Total N found (mg.)	Total N after removal of zein (mg.)	Zein N by difference (mg.)	Zein N recovered (%)
20	0	7.2	—	—	—
20	5.25	12.3	6.8	5.5	105
20	10.5	17.7	6.8	10.9	104
20	15.75	23.0	6.8	16.2	103
20	21.0	28.2	6.9	21.3	101

Theoretically, any difference between the proteins of the food and of the ruminal microbes could be used as the basis of an analytical method for the determination of the proportions of microbial protein and food protein in the digesta. In practice, the task presents many problems, the most important of which are the mixed nature of the microbial proteins and the difficulty in providing suitable diets in which a single protein is the sole source of nitrogen. In these preliminary experiments, therefore, a diet based on the protein zein was used and the analyses were based on the solubility of zein and the insolubility of microbial proteins in aqueous ethanol. In a single experiment, the fact that zein contains no lysine was used as the basis of analytical determination of food protein in the digesta.

Despite the obvious criticism that these experimental techniques could not be expected to reflect accurately the events occurring in the digestion of natural diets, it was felt that preliminary experiments would be worth while as a basis for more extended studies.

The experimental sheep were provided with fistulae into the dorsal sac of the rumen and into the duodenum immediately distal to the pylorus; the fistulae were closed with suitable cannulae. At appropriate times, samples of the rumen liquor and of the abomasal digesta were collected for analysis.

In the first experiment reported, the daily ration comprised: zein 110 g., rice starch 250 g., glucose 80 g., cellulose 230 g., molasses 40 g. and straw chaff 150 g. In this diet, zein supplied 94% of the total N. The diet was supplemented with NaCl, calcium phosphate, and vitamins A and D. The feeds were prepared as a moist mash so that the powdered constituents were held in intimate contact with the fibrous cellulose and straw; the food was given once daily and was usually completely consumed within 2 hr. of feeding.

In the second experiment, the diet consisted of: straw chaff 500 g., starch 100 g. and zein 75 g.; the zein supplied 82% of the total protein in the diet.

Methods of analysis

Total nitrogen was determined by the Kjeldahl method using the procedure of Chibnall, Rees & Williams (1943) or that of Hiller, Plazin & Van Slyke (1948). Protein other than zein was precipitated by dilution of the sample with ethanol to give a concentration of approximately 80% (v/v). After filtration, zein was separated from the ethanolic filtrate by boiling off the ethanol under reduced pressure; the residue was extracted with 5% (w/v) trichloroacetic acid (TCA) and filtered; the nitrogen in the filtrate was designated non-protein nitrogen (N.P.N.) and the zein N was calculated as

the difference between total N of the ethanolic filtrate and N.P.N. In Table 1 are given the results of a test for the recovery of zein added to a sample of abomasal contents.

Lysine was estimated by the decarboxylase method of Gale (1945).

RESULTS

Experiment 1

The results of eight series of analyses of the abomasal contents are given in Table 2, and the average values for all samples taken at a given time after feeding are plotted in Fig. 1. The data were analysed by the *t* test for the significance of difference of means. The analysis revealed no significant differences between the mean values during the first 4 hr. after feeding; the values at 7 and 14 hr. are significantly greater than the pre-feeding levels ($P=0.01$). It is therefore probable that the proportion of zein slowly increases to reach a maximum many hours after feeding and slowly returns to the original value after 24 hr.

It is not possible to use these data for an accurate estimate of the total fraction of zein which leaves the rumen undigested. With certain assumptions, however, an approximate estimate may be made; these assumptions are:

(1) That in the abomasum, none of the zein is digested to fragments sufficiently small to be soluble in dilute TCA. This assumption probably introduces very little error. It has been shown by Laine (1944) that pepsin breaks down zein very slowly. The low values for N.P.N. in the rumen liquor (mentioned below) indicate that the micro-organisms are capable of taking up the products of digestion of the food protein more rapidly than their proteolytic enzymes can degrade the zein; it is probable, therefore, that the digesta contain no appreciable quantity of TCA-soluble fractions of zein derived from microbial digestion in the rumen.

(2) That all the dietary protein leaves the rumen either as microbial protein or as undigested food protein. It has been previously shown (McDonald, 1952) that zein is very much more slowly attacked in the rumen than proteins like casein, gelatin or grass protein, with the result that the concentration of ammonia in the rumen is low when sheep are fed

Table 2. Results of analyses of abomasal contents for total protein N and zein N

Series no.	...	...	1	2	3	4	5	6	7	8
Days on exptl. diet	...	...	10	16	20	26	36	10	29	6
Zein N as % protein N in abomasal contents										
Hr. after feeding										Mean
24			38	26	34	37	48	65	60	44
1			43	26	30	50	42	38	42	40
2			43	36	35	42	46	56	63	47
4			59	51	57	56	34	45	58	51
7			63	62	66	44	68	—	47	59
14			—	—	—	50	62	76	76	66
24			42	35	46	44	49	57	55	47
Mean of all values										49.4

Fig. 1. Graph showing changes in content of zein in abomasal contents.

a zein diet. For convenience of calculation, therefore, it has been assumed that loss of nitrogen from the rumen (as absorbed ammonia) is balanced by the addition of nitrogen to the rumen by saliva.

(3) That the rate of flow of digesta from the abomasum to the duodenum is constant. Phillipson (1952) has discussed the variations which occur in this rate of flow; although complete cessation of flow for periods up to 30 min. were observed, substantial flow of digesta usually occurred whenever collections were made; the rate of flow was not definitely correlated with time of day or with the act of feeding. Since the diurnal variation in the mean percentage of zein in the total protein of the duodenal contents was not great, it seems likely that the assumption of a constant rate of flow would not introduce a large error.

(4) That the protein other than zein consists solely of microbial protein. Evidence has not yet been obtained to enable an estimate to be made of the magnitude of the error introduced by this assumption. There will also be present protein derived from (a) the undigested fragments of dietary straw, which however contributes only 5% of the total protein in the diet; (b) from the gastric juice; the error from this source is probably not

great, as analysis of gastric juice, obtained from an abomasal fistula after emptying the entire stomach, showed a protein content of only 4 mg. N/100 ml.; (c) a small amount of protein may also be derived from undigested protein of the saliva and of desquamated epithelial cells from the mucosa of the stomach. The protein from these various sources will constitute an error increasing the value for microbial protein; this error will, however, be compensated by the fact that some of the microbial protein (and especially that of the delicate protozoa) will be degraded by peptic digestion in the abomasum to fragments soluble in 80% ethanol and hence estimated as N.P.N.

On the basis of these assumptions the following calculation can be made as a first approximation: the area of the graph in Fig. 1 below the mean curve indicates that 56% of total protein nitrogen in the abomasal digesta is comprised of zein; and since zein provided 94% of the dietary protein, it is evident that approximately 40% of the zein had been converted, in the rumen, into microbial protein.

Experiment 2

In this experiment advantage was taken of the fact that zein contains no lysine; hence if the dietary zein were used by the ruminal micro-organisms for growth, they would have to synthesize their own lysine. The difference in lysine contents of the diet and the abomasal contents could therefore be exploited to estimate the output of microbial protein from the rumen. Abomasal contents were collected for periods of 1 hr. and samples of rumen liquor were taken at the same time.

The intake of protein nitrogen was 13.4 g./day, comprising 11.1 g./day of zein nitrogen with 2.3 g. of protein nitrogen in the straw. The lysine present in the straw represented 0.16 g. nitrogen/day so that the percentage of lysine nitrogen in the total dietary protein nitrogen was 1.2%. The proteins of the rumen liquor were taken as a satisfactory approximation to 'microbial proteins' since the preparation of rumen liquor by straining through muslin removed all but traces of straw fragments

and the adherent zein; the absence of zein was demonstrated by the correspondence between the N.P.N. values obtained after precipitation with ethanol and aqueous acid respectively. The microbial proteins of the rumen liquor were found to have a lysine nitrogen content of approximately 7% of the protein nitrogen; this value may be compared with values, calculated to the same basis, of 6.8% (Loosli *et al.* 1949) and 6.1% (Duncan, Agrawala, Huffman & Luecke, 1953) for microbial proteins of ruminants fed on diets in which urea was the sole source of nitrogen. The protein of the mixed abomasal contents, comprising undigested straw-protein, zein and microbial protein exhibited lysine nitrogen contents of 3.9–5.7%. Whilst accurate calculations cannot be made from these data, it is evident, following the same assumptions as previously given, that some 40–50% of the ingested zein must have been converted in the rumen into microbial protein. This result is in accord with the results obtained in the previously recorded experiment.

DISCUSSION

The need for information on the capacity of the ruminal micro-organisms to utilize dietary nitrogen for their growth has been recognized by numerous workers, for this represents an important facet of the nitrogen metabolism of the ruminant. It is clear that the nitrogenous substances absorbed by the host are not merely those of the diet, as in the monogastric animal, but a mixture of dietary constituents, products of microbial metabolism in the rumen and the constituents of the micro-organisms themselves. In this connexion, interest centres chiefly on the proteins which ultimately become available for absorption by the ruminant.

No definite evidence has previously been reported which would indicate the extent to which dietary protein is converted into microbial protein in the rumen and hence the proportions of microbial and dietary proteins which leave the rumen. The present work indicates that a direct experimental approach to the problem is practicable. For this preliminary work, zein was chosen for the analytical advantages it provided; it is desirable, however, that the work should be extended in an effort to study the fate of proteins in ordinary diets and at various levels of feeding. The difficulties inherent in the task are numerous, and it will be clear from the assumptions necessarily made in this paper that much collateral work on the physiology of digestion in the ruminant will be required before a full understanding of the role of micro-organisms in the digestion of protein in the ruminant is attained.

Zein is not a very satisfactory protein for use as the major fraction of the dietary protein; it is highly insoluble in aqueous solutions and when a suspension

is warmed to body temperature (39° for the rumen) it forms a glutinous, fibrous mass, thus reducing the surface area available for enzymic attack. In addition the protein lacks lysine and tryptophan, and hence these amino acids must be synthesized by the micro-organisms. The effect of these properties is reflected in the finding (McDonald, 1952) that zein is slowly attacked in the rumen and that, in contradistinction to ordinary diets or those containing casein and gelatin, ammonia does not accumulate in significant amounts in the rumen. For these reasons, it seems likely that the observed degree of conversion of zein into microbial protein is probably less than would occur in animals fed on ordinary diets.

The application of these experimental procedures to other dietary proteins is at present being undertaken.

SUMMARY

1. When sheep were fed a partially purified diet to which zein contributed 94% of the total nitrogen, it was found that approximately 40% of the zein was utilized by ruminal micro-organisms for the synthesis of their own proteins.

2. This conclusion was based on the separation of the ethanol-soluble protein, zein, from the microbial protein in the digesta received by the duodenum. The conclusion was supported by the result of an experiment based on analyses of lysine in the foodstuffs, ruminal micro-organisms and abomasal contents.

3. Reasons are advanced for suggesting that, in normal dietary regimes, a higher proportion of food protein would be converted in the rumen into microbial protein.

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Purification and Properties of Phosphoprotein Phosphatase from Ox Spleen

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Harris (1946) detected for the first time, in frog's eggs, an enzyme capable of effecting a specific dephosphorylation of phosphoproteins and accordingly termed it phosphoprotein phosphatase. A similar enzyme was found to be present in rat tissues by Feinstein & Volk (1949) who also studied some of its properties. The enzyme was not activated by metal ions, nor did it lose its activity when dialysed. A characteristic property of the enzyme was its activation by reducing agents. This was, however, contradicted by Norberg (1950) who, in his experiments, could find no activation. On account of the divergent views on this question, and since the previous investigators used crude tissue extracts as the enzymic material in their studies, the problem has been re-investigated with a purer enzyme preparation. The object of the present study was also to establish the existence of phosphoprotein phosphatase in animal tissues as an independent enzyme quite distinct from the phosphomonoesterases. Accordingly a method has been evolved for the purification of the enzyme from ox spleen. The final product obtained represents a 200-fold purification and can be considered to be the purest specimen of the enzyme so far obtained.

EXPERIMENTAL

Materials

Substrates. Casein prepared according to the method of Cohn & Hendry (1930) was used routinely as substrate. Phosvitin and vitellin were prepared from egg yolk according to the methods of Mecham & Olcott (1949) and of Calvery & White (1931), respectively. Phosphopeptone was prepared in the form of its barium salt from a peptic-tryptic digest of casein according to the method of Damodaran & Rama-

chandran (1941). The glycerophosphate employed was the β -isomer obtained from British Drug Houses Ltd. (B.D.H.).

Activator. Thioglycollic acid (B.D.H.) was used as an activator, unless otherwise stated. The enzyme was found to exert its maximum activity in the presence of 0.001M-thioglycollic acid.

Buffer. Michaelis veronal-acetate buffer.

Methods

Measurement of enzyme activity. The test mixture employed for the measurement of the enzyme activity was made up as follows: 1 ml. of the mixture contained 10 μ moles casein P, 1 μ mole activator, 20 μ moles buffer at pH 6 and varying amounts of the enzyme solution. The mixtures were incubated at 37° for 30 min. After deproteinization with trichloroacetic acid (TCA), the activity was followed by the estimation of the liberated inorganic P by the method of Fiske & SubbaRow (1925). Controls with casein and water and blank values with water and sample were run at the same time.

Unit of enzymic activity. This was defined as the amount of enzyme catalysing the splitting of 1 μ g. of inorganic P/min. at 37° and at pH 6 from a test mixture containing 10 μ moles of casein P/ml.

Specific activity. This was expressed in units/mg. protein N.

Determination of protein nitrogen. The protein was first precipitated from the test solution by TCA. When washed free from ammonia the N content was determined by the micro-Kjeldahl method (Pregl, 1945).

RESULTS

Purification of phosphoprotein phosphatase

Ox spleen was preferred as starting material for the preparation of the enzyme on account of its ready availability and relative abundance.

Extraction of the enzyme. Spleens from freshly slaughtered oxen were removed and conveyed from the slaughter house