THE ROLE OF PROTOZOA IN RUMEN FERMENTATION

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SUMMARY

Present knowledge of the fermentative activities of different types of rumen ciliate protozoa is reviewed, and an attempt is made to assess their significance to the host.

It is a source of continuing amazement to the writer that the existence of protozoa in the rumen was not realized until a little over a hundred years ago. Surely, one thinks, such vigorous, motile creatures, large enough to be seen without an elaborate microscope, and living in such a common medium as the rumen content of cattle and sheep, must have been seen by Antony van Leeuwenhoek, who turned his microscope on such a bewildering variety of materials? But it was not until 170 years after his discovery of protozoa that a description of those which inhabit the rumen was published (Gruby and Delafond, 1843). During the following century, many observers described the various types of protozoön to be found in this habitat, and established their taxonomic positions fairly clearly.

Until recently nothing was known of their physiology and relationship to the host, although almost every worker expressed some hypothesis, and the postulated roles of the rumen ciliate protozoa ranged from that of harmful parasite to that of essential symbiont. Erroneous ideas were also held about the way in which they spread from animal to animal. Many attempts were made to culture them *in vitro* in the way that had proved so successful with bacteria; all these attempts failed. In more recent times (*i.e.*, 1925 onwards), it was shown that these organisms are passed between animals by direct passage from mouth to mouth, there being no protective form passing through the intestine or reaching the animal by way of its food. The first attempts at counting the population of protozoa in the rumen were made, and the variation of numbers with the feeding of the ruminant was followed. A little of their physiology was discovered—*e.g.*, observation of iodine-stained suspensions showed that some of the protozoa swallowed and digested plant starch granules, and at the same time produced particles of a starch-like reserve substance.

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The view persisted until quite recently that the protozoa can play no significant part in the rumen fermentation. In part, this view reflected the ignorance of the biochemistry which prevailed at the time, but it also appeared to be supported by the results of the experiment of Pounden and Hibbs (1950). These workers were able to "defaunate" a calf—*i.e.*, they removed all the protozoa from the rumen without appreciably altering the bacterial flora. That the defaunated calf throve as well as the normal control animal was erroneously taken as evidence that the protozoa were mere idle passengers in the rumen.

The elevation of the rumen ciliate protozoa from this lowly position has taken place in the past twelve years, and has resulted from experiments undertaken in a very small number of laboratories throughout the world. (One of these is the Plant Chemistry Division of the D.S.I.R. at Palmerston North.)

Before these experiments are described, it would perhaps be helpful to describe the organisms briefly. The ciliate protozoa commonly seen in rumen content belong to a handful of genera; none of them are found anywhere but in the rumen. In size they vary from 20μ to 200μ. The numbers, both of the different types, and the total, present in the rumen are dependent on a number of factors, the chief of which is the diet of the animal. In general, they are numbered in tens of thousands per millilitre, and their total mass is comparable to that of the much more numerous, but much smaller bacteria. They are unusual among members of the animal kingdom in being anaerobic. Two groups of ciliate protozoa may be discerned, the holotrichs, in which the cilia occur over the whole of the body, and the oligotrichs, in which the cilia are localized.

METHODS OF STUDYING PROTOZOA

The methods used in studying the rumen protozoa are of five kinds; these will be briefly described, and examples mentioned.

(1) Direct observation of the protozoa in rumen content, for example, in counting, and in morphological and taxonomic work such as that of Dogiel (1927). This technique is of limited value when the function of the protozoa in the rumen is under investigation.

(2) Preparation of suspensions of living protozoa by special sedimenting and washing procedures. This technique was

first used by Oxford (1951), and has been widely used since then in biochemical studies. By using these suspensions, which can be made almost free from bacteria by the use of antibiotics, it can be discovered which substances in the animal's food can be decomposed by the protozoa, and what products are formed. Such information, essential to an understanding of what the protozoa do in the rumen, was unobtainable so long as they could not be separated from the mass of bacteria also present.

(3) The suspensions of clean protozoa obtained as described can be treated so that the cells are broken, and cell-free extracts obtained. Many enzymes connected with carbohydrate metabolism can be found in these extracts, and, in addition to being of fundamental biochemical interest, they serve to confirm, in most cases, the conclusions drawn from experiments with living protozoa. For example, from the holotrichs, which are well equipped to ferment soluble sugars, such as sucrose, may be obtained potent invertase solutions (Howard, 1959b), and from the oligotrich *Entodinium caudatum*, which feeds on starch but not on soluble sugars, may be prepared a solution containing amylase and maltase but no invertase (Abou Akkada and Howard, 1960).

(4) The cultivation of the rumen protozoa is a difficult task, and attempts to do this have only recently met with success—*e.g.*, Gutierrez (1958) with holotrichs and Coleman (1960) with *Entodinium caudatum*. Gutierrez's success in persuading the holotrichs to multiply in culture by adding suspensions of certain types of rumen bacteria suggests that in the rumen the protozoa may be bacteria-feeders.

(5) Modifications may be made to the protozoal population in the rumen itself; for example, protozoa may be excluded altogether by rearing lambs from weaning in isolation from other ruminants (Eadie, 1962) or the sheep may be partially defaunated by washing out the rumen (Eadie and Oxford, 1957). By using animals so treated the effect of the modification on the ruminant itself or on the other microorganisms in the rumen may be studied, or preparations of single types of protozoa not obtainable from animals containing the normal mixed rumen population may be obtained. Examples of the uses of these animals with a modified rumen fauna will be mentioned later.

FERMENTATION BY PROTOZOA

To date, most success has resulted from experiments of types (1), (2), (3) and (5) above. "Pure cultures", in the ordinary bacteriological sense, of any rumen protozoa have not yet been obtained. For this reason the degradative aspects of their biochemistry are best known, particularly the various ways in which they can decompose carbohydrates. In considering the details of this process, each protozoal type will be dealt with in turn, and the range of substrates decomposed, and the products formed will be mentioned.

The rumen protozoa whose carbohydrate metabolism is most thoroughly explored are the two genera of holotrich, *Dasytricha* and *Isotricha*. These are the most "bacteria-like" in that they live almost entirely on dissolved nutrients. Both genera ferment glucose, fructose, and their related oligo- and polysaccharides sucrose, raffinose, inulin and levan (Heald and Oxford, 1953). The holotrichs will therefore be most numerous in the rumens of animals whose diet contains a high proportion of soluble sugars; at the Rowett Institute, sheep kept as sources of holotrichs are fed wholly on good quality hay.

By using sheep partly defaunated by the procedure of Eadie and Oxford (1957), the writer was able to examine *Dasytricha* and *Isotricha* suspensions separately (Howard, 1959a). The former organism only can ferment galactose, maltose and cellobiose in addition to those already mentioned. The ability of *Dasytricha* to ferment these sugars is rather puzzling, since none of them occurs in the normal diet of a ruminant. Galactose enters the rumen only in small amounts, and then in combined form as galactosides. Since *Dasytricha* appears to be unable to liberate galactose from these compounds, it is difficult to see how the organism can make use of its galactose-fermenting ability in the rumen. The only places where maltose and cellobiose might be encountered by *Dasytricha* are in the close neighbourhood of other micro-organisms which are vigorously decomposing starch and cellulose respectively. Minute amounts of these intermediate hydrolysis products might perhaps diffuse away from the site of amylase or cellulase action; they are never found in rumen liquor as a whole. The larger of the two genera of holotrich, *Isotricha*, in addition to fermenting soluble sugars, can swallow and digest starch grains if they are small. Rice starch has been used in *in vitro* experiments (Sugden and Oxford, 1952), but how far this starch-fermenting ability of *Isotricha* is important in the rumen is unknown.

Another puzzling feature of holotrich biochemistry is their effect on pectin. This polysaccharide, which can form a substantial proportion of the carbohydrates of ruminant diet, is readily decomposed into its constituents (methanol, galacturonic acid, and oligo-galacturonides) both by living holotrichs and by cell-free extracts of them (Wright, 1960; Abou Akkada and Howard, 1961). This decomposition differs from those of other oligo- and polysaccharides by the holotrichs in that the products of hydrolysis are not fermented or otherwise made use of. What advantage these protozoa gain from their synthesis of pectic enzymes is thus obscure at present.

A striking feature of fermentation by the holotrichs is the conversion of a large proportion (often more than half) of the sugar used into a storage polysaccharide (Heald and Oxford, 1953). This material, shown to be amylopectin by Forsyth and Hirst (1953), is laid down in the form of minute starch grains, 2 to 3 μ in diameter. When the supply of external carbohydrate becomes exhausted, the protozoa break down the polysaccharide stored away in these grains. Of what might be termed the "true" fermentation products of the holotrichs, that is, neglecting the starch storage, about half is accounted for by lactic acid, about one-third by roughly equal amounts of acetic acid and butyric acid, and the rest by CO_2 and H_2. Acetic acid, butyric acid and CO_2 are normal constituents of rumen liquor; hydrogen and lactic acid are transient products readily metabolized further by other rumen micro-organisms.

The oligotrich protozoa of the rumen, in contrast to the holotrichs, appear to rely mainly or solely on particulate matter for their carbohydrate needs. Abou Akkada and Howard (1960) were able to make use of a sheep fed on a starchy diet after partial defaunation by the method of Eadie and Oxford (1957), from the rumen liquor of which clean suspensions of the oligotrich *Entodinium caudatum* could be prepared. It was found that this organism uses only granular starch, and is unable to metabolize soluble sugars, or even starch in solution. *Entodinium caudatum* resembles the holotrichs in being able to convert a portion of the carbohydrate digested into granules of storage polysaccharide. A suspension of *E. caudatum* taken fresh from the rumen and stained with iodine will thus show both the large plant starch grains in the gut of the micro-organism and the smaller grains of protozoal starch deposited near the tail. The products of fermentation by *E. caudatum* are qualitatively the same as those of the holotrichs—*i.e.*, acetic acid, butyric acid, lactic acid, CO_2 and H_2—but there is a

quantitative difference in that lactic acid forms a negligible proportion of the whole. The importance of this will be discussed later.

A second type of oligotrich protozoön from the rumen which has recently been studied is *Epidinium ecaudatum*. This organism is particularly numerous in cattle feeding on clover, and it has therefore been studied almost exclusively in New Zealand (Oxford, 1958, 1959; Bailey, 1958). Like *Entodinium caudatum* it is primarily a starch eater, but can also make use of some soluble sugars. *Epidinium ecaudatum* possesses a powerful xylanase enzyme system (Bailey *et al.*, 1962), which enables it to decompose the important hemicellulose group of plant polysaccharides. If the products of hydrolysis, arabinose and xylose, can be fermented by *E. ecaudatum*, this will be the first instance known of a rumen protozoön fermenting any carbohydrate other than the common hexoses and their compounds. The chief products of the fermentation of starch by *E. ecaudatum* are volatile fatty acids, CO_2 and H_2; as with *Entodinium caudatum*, lactic acid is only a very minor product.

It is agreed that one of the main function of the rumen is the digestion of cellulose by the micro-organisms living there. It will have been noted that so far in this paper cellulose has not been mentioned as a substrate for the rumen protozoa. None of the types mentioned so far are ever seen to swallow fibrous material in rumen liquor and the biochemical evidence suggests that they do not possess the enzymes necessary to decompose cellulose. This important material is digested by some of the rumen oligotrichs; they include the largest of these organisms, and belong to the genera *Diplodinium*, *Eudiplodinium* and *Metadinium*. They can be observed to swallow plant fibres, which are then digested. It is not known whether these cellulose-eating protozoa dissolve the cellulose by means of their own enzyme secretions, or whether they rely on cellulolytic bacteria living in their digestive sac. These protozoa are particularly difficult to maintain in a living suspension, and no one has yet succeeded in determining what products are formed by the fermentation of cellulose. Examination of iodine-stained suspensions shows that a portion of the digested cellulose is converted into granules of storage starch.

Considering only carbohydrates, the function of the rumen ciliate protozoa may be summarized as the conversion of the cellulose, starch, fructans and soluble sugars which enter the rumen into volatile fatty acids. All these substrates can be fermented by rumen bacteria of various types, and it may legi-

timately be asked what proportion of the total fermentation is contributed by the protozoa, and how far the ruminant would suffer if the protozoa were not present.

It is difficult to arrive at an answer to the first question, as it involves counting procedures and estimates of the fermentative ability of the protozoa *in vivo*, both of which can be subject to considerable error. Gutierrez (1955) has estimated that the holotrichs alone contribute more than 10% of the volatile fatty acids in the rumen of cows.

If protozoa are removed from, or prevented from entering the rumen, the ruminant appears to be very little, if any, the worse (Pounden and Hibbs, 1950; Eadie, 1962). Protozoa-free lambs were found to have a larger bacterial population than control animals (Eadie and Hobson, 1962), and this might indicate that the bacteria had "taken over" the activities of the protozoa. When protozoa were later established in one of the previously protozoa-free lambs, the bacterial numbers fell to the normal figure.

In a few minor ways the protozoa may metabolize carbohydrate in a manner more advantageous to the ruminant than do the bacteria. For example, a function of possible value is the conversion of soluble sugars into intracellular starch by the holotrichs and the swallowing of starch grains by *Entodinium* and *Epidinium*. This "locking away" of carbohydrate in more slowly fermentable forms may help to smooth out the fluctuations in fermentation rate in the rumen caused by intermittent feeding.

The low rate of lactic acid production characteristic of *Entodinium caudatum* could be of value to an animal whose rumen contains a high proportion of this organism. The sudden ingestion of large amounts of starch can be harmful to ruminants, because of the rapid production of large quantities of lactic acid, and the consequent fall of pH of the rumen contents to an abnormally low level. The presence of micro-organisms which can not only store away added starch for a more leisurely digestion, but can also, during the course of this digestion, produce insignificant amounts of lactic acid, would clearly be of benefit to the ruminant in preventing the development of this undesirable acidity.

OTHER METABOLIC FUNCTIONS

The role of the ciliate protozoa in ruminal metabolism other than of carbohydrate may be summarized fairly briefly, not necessarily because they are of little consequence in such

processes as protein decomposition, ammonia production and lipid hydrolysis, or in the upsetting of normal metabolism which results in such disorders as bloat, but rather because too little is known of their biochemical activities. Their nitrogen metabolism is much less well understood than that of carbohydrates. This is undoubtedly because the former is more intimately connected with the processes of growth; until growing and multiplying protozoa, free from gross contamination by other microorganisms, are available to the biochemist, their nitrogen needs and excretion products, and their growth factor requirements, will remain largely unknown.

It was suggested many years ago (Mangold, 1929) that the chief importance of the rumen protozoa was that they "ennobled" plant protein into higher quality animal protein which could be more easily digested by the ruminant. Later work has indeed shown that holotrich protein has a high digestibility for rats (McNaught *et al.*, 1954) and that the protozoal protein is rich in essential amino acids (Weller, 1957). Hungate (1955) has estimated that this protein may contribute as much as 20% of that required by the ruminant. On the other hand, what little is known of nitrogen excretion by rumen protozoa suggests that they may waste some of the protein in the diet by excessive deamination of amino acids, thus producing larger amounts of ammonia than can be utilized by the bacteria for re-synthesis into protein. Ammonia was found to be the chief nitrogenous excretory product of *Entodinium caudatum* (Abou Akkada and Howard, 1962), though it is not produced by the holotrichs (Heald and Oxford, 1953).

Lipids are both hydrolysed and hydrogenated in the rumen (see Garton, 1959, for review). The extent to which the protozoa share in the hydrolysis is unknown, but they have been shown by Wright (1959) to hydrogenate both sodium linoleate and the lipid in clover chloroplasts. The significance of this for the ruminant is unknown.

This summary of the part played by the ciliate protozoa in the changes which take place in the rumen reveals many gaps in present knowledge. It may seem to outside observers that but meagre progress has been made in the past decade in studying them both as organisms in their own right, and in their relationship to the host. Those, however, who are familiar with these delicate and elusive creatures will realize the magnitude both of the advances already made, and of the challenge they offer to the investigator.

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