Rumen Holotrich Ciliate Protozoa

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INTRODUCTION

The ruminant is dependent on the fermentation of its food constituents by the rumen microorganisms. The microbial community is accommodated in a complex forestomach, the ruminoreticulum, which provides a highly specialized anaerobic environment. The temperature is maintained close to 39°C and the rumen contents are well buffered, with salivary secretions and the absorption of acidic metabolites keeping the pH close to neutrality. The physical environment and an excellent supply of nutrients support an extremely diverse microbial community which contains specifically adapted bacteria, protozoa, and fungi.

The rumen protozoa are highly specialized for growth in the rumen ecosystem. The majority of the protozoa are ciliates (10^5 to 10^6 protozoa per ml), although flagellates (e.g., *Monocercomonas* spp. and *Trichomonas* spp.) are found in both the rumen and the cecum and are more numerous in animals lacking ciliates (45, 136). The role of flagellates in the rumen has not been established and estimates of population size must be regarded as unreliable following the redefinition of three of the species listed by Hungate (136) as fungal zoospores (199). Two groups of ciliates, the entodiniomorphid (oligotrich) and the holotrich protozoa, usually occur in the rumen. They depend on the host for their food supply, but they also transform an array of plant and bacterial constituents to cell components and metabolites that are used by the host. The protozoa, unlike the bacteria, are difficult to cultivate in vitro for extended periods. Therefore, most research has emphasized the bac-

terial community. The role and biochemical capabilities of the protozoa are less fully understood. Under certain dietary conditions protozoa account for approximately half of the ruminal biomass and, therefore, substantially contribute to total volatile fatty acid (VFA) production. VFA is the product of rumen microbial fermentation that provides most of the carbon and energy required by the animal and is essential to its nuritional well-being. The two ciliate groups, however, occupy different metabolic niches, as the holotrichs primarily utilize soluble carbohydrates, whereas the entodiniomorphid ciliates ingest and ferment particulate material. The entodionomorphid ciliates are usually more numerous in the rumen and have been better characterized biochemically (53, 54, 56). However, recent studies of the holotrichs have provided information on the pathways of intermediary metabolism leading to product formation, and studies of the metabolic consequences of environmental oxygen have led to a reassessment of the role of a group of organisms historically described as obligate anaerobes. The purpose of this article is to examine the occurrence, role, and metabolic activities of the rumen holotrich ciliate protozoa.

RUMEN HOLOTRICH CILIATES

Genera

The holotrich ciliates in the rumen environment occur in two taxonomic orders of the subclass *Holotricha*. Their classification is often not consistent and recommendations for revision have been made (e.g., see reference 99). The classification of this group of protozoa has been detailed by other reviewers (45, 136, 179) and is summarized as follows:

ORDER	*Prostomatida*	*Trichostomatina*	
FAMILY	*Buetschliidae*	*Isotrichidae*	*Blepharocorythidae*
GENUS	*Buetschlia*	*Isotricha*	*Charonina*
	Parabundleia	*Dasytricha*	
		Oligoisotricha	

The three principal holotrich species in the rumen are the completely and uniformly ciliated *Isotricha intestinalis*, *I. prostoma*, and *Dasytricha ruminantium*. Other species that have been reported and occur infrequently are *D. hukuokaensis*, *Butschlia* (syn. *Buetschlia*) *parva*, *Charonina ventriculi*, *C. equi*, *Blepharocorys bovis* (syn. *C. ventriculi* [265]), *Parabundleia ruminantium*, and *Oligoisotricha bubali* (45, 135, 137, 139, 142). The close relationship of *Charon* (syn. *Charonina*) *ventriculi* to members of the genus *Blepharocorys* was also recorded by Jameson (146) in his original description of *C. ventriculi*. There is, however, no information on the metabolism of these latter organisms or their contribution in the ecosystem. Morphological characteristics and features of value in species recognition are reviewed by Hungate (136) and Ogimoto and Imai (179).

Ultrastructure

The holotrichs are regarded as primitive ciliates (99; T. P. Buckelew and D. M. Kontir, Proc. Pa. Acad. Sci. **51**:95, 1977). However, morphological studies have revealed complex ultrastructural characteristics (102, 228) and surface structures (202). The endoplasm and ectoplasm are separated by a continuous double-layered fibrillar system (240, 241). Polysaccharide storage granules are located in the endoplasm which also contains the adjacent and membrane-

enveloped micro- and macronuclei. Ingested food particles and bacteria occur in vacuoles which are widely distributed throughout the cytoplasm. Many unidentified electron-dense bodies have been observed in the cytoplasm (228), but there is little information available on the identity and functions of the various subcellular structures (Fig. 1).

Studies on *D. ruminantium* and *Isotricha* spp. (269, 270) confirmed that polysaccharide depolymerase and glycoside hydrolase enzymes were sedimentable during differential centrifugation of cell homogenates and were present in fractions containing membrane-bound vesicles that resembled lysosomes. The subcellular distribution profile of enzymatic activity indicated distinct vesicle populations, one of which may have been derived from a Golgi-like body. Such structures have been reported in the rumen entodiniomorphid ciliates (78). The two holotrich genera have both been shown to possess a granular microbody-like organelle, the hydrogenosome (269, 270). The organelle, approximately 500 nm in diameter, was located beneath the subpellicular layer and contained the same enzymes in both genera. The equilibrium densities of the isolated hydrogenosomes were different, having values of 1.18 g/ml in *D. ruminantium* and 1.23 g/ml in *Isotricha* spp., and thus differed in both enzyme complement and equilibrium density when compared with the organelle in the aerotolerant trichomonad flagellates (170). The hydrogenosome was also detected in certain entodiniomorphid ciliates (268) and is characterised by the presence of enzymes that transform pyruvate to acetate and hydrogen under anaerobic conditions. However, the identity and biochemical functions of various other subcellular structures in the holotrichs are less well characterized.

FACTORS AFFECTING SIZE AND COMPOSITION OF THE HOLOTRICH POPULATION

The generic composition and overall size of the rumen ciliate population is determined by various interacting factors, the more important of which are the type of host, its geographical location, the diet consumed, and protozoal interspecies antagonisms. The total number of ciliates present varies considerably. Diets of high digestibility, which provide readily available sources of nitrogen and energy, produce the largest ciliate populations, although the frequency and amount of food consumed by the host, the diurnal cycle of the rumen, food particle size, salt concentration, and dietary supplementation with antibiotics or other drug additives have all been shown to influence the protozoal population (45, 56, 136). However, an understanding of the biological significance of the protozoa in the rumen ecosystem can only be gained from knowledge of their total biochemical input, and this necessitates information on both population size and metabolic activities.

Distribution of Individual Holotrich Genera

The genera *Dasytricha* and *Isotricha* occur widely in ruminants. They usually occur together, but there are reports of the occurrence of a single genus or the absence of both from individual animals in a flock (45, 55, 70, 100). The specificity of these ciliates for particular ruminant hosts has been examined with conflicting results. Although *Dasytricha* failed to survive cross-inoculation between cow and buffalo calves (175), Dehority (Proc. First Int. Reindeer/Caribou Symp., Biol. Sci. Publ. **18**:241–250, 1975) was unable to detect any host specificity for either genus in different Alaskan ruminants. Further studies (71, 77) confirmed that
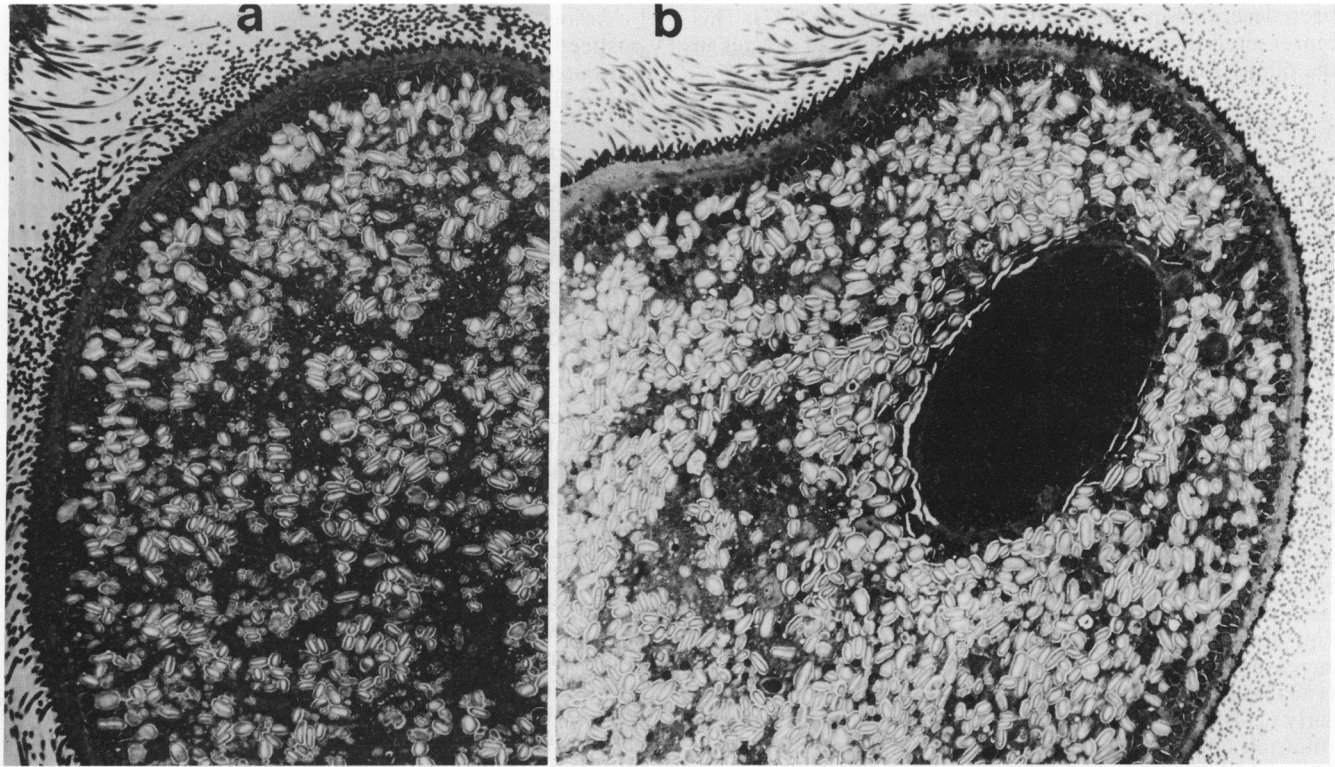
FIG. 1. Electron micrographs of sections of (a) *D. ruminantium* (from reference 269) and (b) *Isotricha* sp. (from reference 270). Panel a is reprinted by permission of the *Biochemical Journal*, vol. 200, p. 365–372, copyright 1981, The Biochemical Society, London, and panel b is reprinted with permission from *Comparative Biochemistry and Physiology*, vol. 74B, p. 357–364, copyright 1983, Pergamon Press, Oxford, England.

holotrich establishment was diet related and suggested that following transfaunation the protozoa altered the environment to facilitate their retention. The occurrence of entodiniomorphid ciliates in various hosts is well documented (42, 136, 179). In young grazing animals the holotrichs establish after the entodiniomorphid population, although *Isotricha* spp. apparently may establish earlier in calves than in lambs (88).

The holotrichs occur in largest numbers when soluble carbohydrates are readily available in the diet (42, 136, 236). The number of holotrichs typically observed in cattle and sheep range up to approximately 10^5 per ml of rumen fluid (136, 247); on forage diets the holotrich ciliates have been estimated to represent approximately 20% (12 to 40%) of the total ciliate population (42). When both genera are present, *D. ruminantium* is frequently more numerous than *Isotricha* spp.

Surveys of the occurrence of the ciliates in various hosts (45, 179) indicate that the genera *Dasytricha* and *Isotricha* are more common in domesticated than in wild ruminants. There are geographical variations in distribution, as exemplified by studies on the protozoal fauna of water buffalo (*Bubalus bubalis*) from Brazil (72), Taiwan (140), Japan (143, 144), and Korea (108). Holotrichs were not detected in the animals studied in west Asia (180). The variety of ciliates is larger in grazing than in browsing animals in Europe (100), but there is a greater variety of ciliate species in browsing animals in Africa (238). Thus, in European ruminants the holotrich species predominate in grazers. In Africa *Dasytricha* and *Isotricha* spp. occur more regularly in browsers (238).

Other holotrich genera occur in low numbers and reports of their occurrence are infrequent. *Butschlia parva* has been reported on a limited number of occasions (45, 69) and had a population density in sheep (69), musk ox (70), and cattle (42) of approximately 10^3 to 10^4/ml, representing 0.2 to 0.6% of the total ciliate population, although values of 2 to 3% were reported in cattle (42). The concentration of *Butschlia parva* in the rumen follows a diurnal cycle similar to that of other holotrichs (69). *Charonina* (formerly *Charon*) spp. are infrequently observed (45, 76, 85, 265), although when present they can be relatively numerous. *C. ventriculi* was present in 17% of the Taiwanese water buffalo examined by Imai et al. (140). Although accounting for approximately 15% of the ciliate population in a Brazilian animal, the genus was absent from the other three beasts examined (72). The population size is affected by diet (76) and *Charonina* sp. was reported to represent over 30% of the protozoal population of a cow receiving a grass hay ration and over 50% of the total rumen ciliate population in sheep fed grass silage (231). *C. equi* constituted up to 12% of the ciliates in grass-fed cattle (42). The concentrations of *Charonina* spp. in the rumen follow a diurnal cycle more closely related to that of the entodiniomorphid ciliates (76).

O. bubali was observed in water buffalo (86, 139, 140, 143) and cattle (74). The protozoon was in 20 to 40% of the buffalo examined (140, 143). Concentrations in domesticated cattle ranged up to 35% of the protozoal population in unweaned calves to as high as 72% in mature feedlot animals (74). Unlike other holotrich genera, the proportion of *O. bubali* in the population increased when the animals were fed a corn silage concentrate ration. Another holotrich ciliate that has

been described in Zebu cattle is *P. ruminantium* (142). This represented the first description of the genus and species in the family *Buetschliidae*.

Factors Influencing Size of the Protozoal Population

Changes in the composition or physical characteristics of the diet and the level and frequency of feeding produce changes in the rumen ciliate population. Different genera are encouraged by different feeds, and these effects have been reviewed in detail elsewhere (45, 136, 248). The number of holotrich protozoa is generally increased in animals receiving diets high in available carbohydrates (32, 206, 236), although in some seaweed-eating sheep the proportion of *D. ruminantium* in the ciliate population was 36.5% (201). There are reports of the holotrich population being increased by dietary constraint (77), and *Isotricha* spp. were one of the more persistent species in mildly acidic conditions ensuing from starch fermentation (89). Fluctuation in holotrich numbers did not correlate with rumen fluid osmalality (77). The pattern of variation of holotrich numbers in the rumen does not necessarily parallel the changes in the entodiniomorphid population; the proportion of holotrichs can increase under certain conditions in a declining protozoal population (90). The dietary additives aureomycin and tylosin, however, increase the total protozoal population, and the limited data available indicated that both groups of ciliates reacted similarly (214). Diethylstilboestrol supplementation favored the holotrich ciliates (138). Concentrations of monensin above 800 ng/ml are toxic to holotrichs, although metabolic effects are evident at concentrations of 100 to 200 ng/ml where the molar proportions of acetate and lactate formed increased while formate, butyrate, and hydrogen production all decreased (124). The holotrichs are particularly sensitive to the effects of antiprotozoal surfactant detergents (37).

Diurnal Variations in the Holotrich Population

The diurnal variation in population size of the holotrich and entodiniomorphid ciliates in the rumen differ. The entodiniormorphid population decreases for up to 16 h after feeding and then increases to the prefeed level (163, 247, 249). The holotrich population declines for a period of 12 to 20 h after feeding when dividing forms are absent (163, 213, 249). The population recovers to its original level within 4 to 6 h (161, 163, 166, 213, 249). The numbers of *Isotricha* spp. and *D. ruminantium* begin to increase prior to (0.5 to 2 h) the commencement of feeding, although the point at which the population decline begins is not consistent in the studies reported. In some studies, in animals fed once daily (161, 163, 213, 247), the holotrich numbers began to fall once the animal had been fed. In other studies the population increase commenced before feeding and continued during the feeding period so that the population was maximal in the immediate postfeed period and declined when feeding ceased (3, 69, 76, 84, 173). An approximately threefold increase in numbers of *Dasytricha* and *Isotricha* spp. was observed in the 2-h postfeed period in cattle fed on red clover (43, 44) or sugar cane (236). *Butschlia parva* followed a diurnal cycle similar to that of the other holotrich protozoa present (69), whereas *C. ventriculi* followed a distribution pattern more closely related to the entodiniomorphid protozoa (76). In animals fed more than once daily the protozoal population increases (3, 250) and the diurnal variation typical of animals fed once daily is repeated in a shorter time period at each feeding (166, 250). The diurnal variation in grazing animals resembles that of animals fed once each day (251).

Various explanations have been proposed to explain the postfeed decrease in holotrichs. The phenomenon has been attributed to the increased dilution rate associated with food intake (166, 249), protozoal disruption as a consequence of an overaccumulation of storage polysaccharide (43, 44), or settlement of the protozoa in the rumen (168, 236). More recently, sequestration of the protozoa has been implicated. *Isotricha* spp., for example, have a specialized attachment organelle (202) and adhere to plant particles after feeding (203). The sequestration theory was extended by Abe et al. (3) to explain a fourfold increase in holotrich numbers within 1 h after feeding and the ensuing abrupt decline. They proposed that the holotrichs sequestered on the reticulum wall and then migrated into the rumen for a few hours after feeding. Large numbers of holotrichs were associated with the reticulum wall (3). The migration into the rumen may represent a response to a chemical stimulus or the contraction of the reticulum during food intake in cattle or during the anticipation of feeding in sheep and goats (6). Glucose entering the rumen shortly after feeding has been shown to stimulate the migration of the holotrich protozoa into the rumen (173).

Fluctuations in the protozoal community are normally monitored through changes in protozoal concentrations. However, when the holotrichs represent as little as 5% of the ciliate community they still account for some 35% of the total volume or 40% of the protozoal nitrogen (74, 128). Therefore, studies on the ciliate contribution to specific aspects of rumen fermentation should consider the size (mass) of members of each genus as well as concentrations (47).

IN VITRO CULTIVATION OF HOLOTRICH PROTOZOA

Many entodiniomorphid ciliates have been cultivated for extended periods in vitro (50, 53), whereas the holotrichs have only been maintained for short periods of 20 to 60 days (46, 103, 215, 230). The media used for cultivation of the ciliates were complex and contained rumen fluid and extracts (plant, bacterial, protozoal) of unspecified composition and function, although trace element and vitamin requirements were apparent. The ciliates cannot be grown axenically and derive some of their nitrogenous requirements from ingested bacteria (105).

Cell division is impaired in cells containing excess storage polysaccharide (46). To prevent excessive polymer formation and overgrowth of saccharolytic bacteria, sugar addition to cultures is intermittent and daily transfer of the protozoa to fresh medium is essential (46, 103, 230). Population densities of the order of 10^3 cells per ml have been obtained (46).

The rumen ciliates have also been grown successfully in continuous-culture (artificial rumen) systems with minimal agitation, end product removal, and an inert matrix (4, 66, 129, 252), although holotrich ciliates have been maintained in a less complex, gas-mixed system with no dead space (164, 165). Specific modifications to enhance protozoal retention include dialyzing systems (176) and varied inert matrices (5, 66); holotrich numbers approximating 10^4/ml have been reported (5). The concentration of protozoa in the free fluid associated with the solid substrate was consistently higher than that observed in the free liquid (66, 177), indicating that sequestration among the particulate digesta was an important factor in maintaining the protozoal concentration. Our studies (K. Hillman, D. Lloyd, and A. G. Williams, unpublished results) on the survival of single holotrich species and mixed ciliate populations in an artificial rumen system

(RUSITEC) (65) confirmed that metabolically active protozoa can be maintained in the laboratory. Protozoal retention was highest at pH 6.5 to 7.0, with a dilution rate of 0.65 per day and a matrix of barley straw or predigested ryegrass hay. The sugar requirement was satisfied by a solid feed of hay and sugar beet pulp each day. Niacin supplementation of the artificial saliva infusion was beneficial (83). In mixed ciliate populations holotrich numbers declined slowly and small *Entodinium* spp. eventually predominated.

The potential of in vitro culture systems in protozoal studies has not yet been fully realized. They have only been used to evaluate the effects of nutritional factors on protozoal survival (176, 227) and the relationship between the composition of the feed and the total protozoal population (64, 162, 174) or protozoal groups, including the holotrichs (2, 31). However, they have not been exploited to examine the effects of environmental or nutritional parameters on the metabolic activity of monocultures or mixed cultures of protozoa that have been maintained for several cell generations under strictly controlled conditions. Chemically defined media for the in vitro continuous culture of the rumen ciliates, including the holotrich genera *Dasytricha* and *Isotricha*, have been described (216).

Although the holotrichs can be maintained in in vitro culture for short periods of approximately 1 to 2 months, there are still no satisfactory methods for long-term, large-scale cultivation that would generate sufficient quantities of actively growing individual species for detailed biochemical studies. In consequence, studies on the metabolism of the holotrich ciliates have used cells isolated from conventional animals or animals containing species specifically reinoculated after protozoal removal with chemical defaunating agents.

TECHNIQUES FOR ISOLATION OF HOLOTRICH CILIATES FROM RUMEN CONTENTS

The holotrich ciliates synthesize a storage polysaccharide, and the increased cell density imparted by this material has been used to aid cell separation. Protozoa were separated from rumen liquor by sedimenting strained rumen contents (119) or by repeatedly washing the residue remaining after rumen contents had been filtered through surgical gauze (205). Glucose was included to encourage polysaccharide formation by the holotrichs and facilitate a more rapid sedimentation (119). *Isotricha* and *Dasytricha* spp. were subsequently recovered by differential sedimentation of the mixed holotrich preparation in buffer (119) or gradients containing rumen liquor (103) or glycerol (169). Other methods used included sucrose density gradient centrifugation (157). The tendency of the protozoa to adhere to the inner walls of the glassware used in the initial sedimentation was also used to aid separation (103). Cell preparations obtained by these methods were treated with antibiotics to reduce bacterial contamination and starved for 48 h to deplete the storage material in the cells.

These older methods have many drawbacks and separation of the holotrich genera by differential sedimentation is frequently incomplete. The preparations are contaminated by bacteria and entodiniomorphid protozoa. Methods relying on cell adhesion are also unreliable and produce poor yields and have been replaced by filtration procedures that use sintered-glass filters (258) or, more effectively, defined aperture nylon and polyester textiles (262). Filtration techniques are particularly useful in the preparation of protozoal suspensions for metabolic studies owing to the sensitivity of these anaerobic organisms to excessive manipulation and

because the metabolic status of the cell is not affected. Preliminary fermentation (119) or starvation (118) procedures are unnecessary, and thorough washing of the cell preparation with sterile anaerobic buffer ensures negligible contamination by free bacteria. Pretreatment of rumen contents in a mechanical homogenizer or blender to release protozoa from the particulate material should be avoided, as the holotrich ciliates are damaged by these procedures (73).

METABOLISM OF HOLOTRICH CILIATES

The ciliate protozoa transform an array of plant and bacterial constituents to cell components and metabolites that are utilized by the host ruminant. The importance of these activities, however, is still controversial. In the absence of protozoa, an efficient bacterial fermentation continues, and the respective contributions of the bacterial and protozoal communities are difficult to assess. Furthermore, biochemical studies of the ciliates are complicated by the presence of viable ingested and attached bacteria in the protozoal samples. The effects of bacterial contamination of the protozoal preparations can be minimized by effective preparative procedures and the use of selective cell breakage techniques, which do not disrupt bacteria. Disruption can be achieved mechanically by homogenization (269, 270), by ultrasound in an ultrasonic cleaning bath (59), or by chemical disintegration with indole (20). Other agents including skatole (91), infusoricidal (92), and surface-active agents (37, 267) and the sugars mannose, glucosamine, and galactosamine (230, 258) induce cell lysis but have not been used to effect selective cell breakage. Unbroken bacterial cells are removed from the lysate by centrifugation, although enzymes released from ingested bacteria may retain activity for short periods until degraded.

There have been no biochemical studies on *Charonina*, *Blepharocorys*, *Butschlia*, *Oligoisotricha*, or *Parabundleia* spp. and the following section on metabolic activities is, therefore, restricted to the two principal genera, namely, *Isotricha* and *Dasytricha*.

Carbohydrate Metabolism

Soluble sugar utilization. By using cultural (230) and manometric (103, 118, 133, 212, 239, 258) techniques, it was shown that the holotrichs utilize the monosaccharides glucose, fructose, and galactose and soluble oligomers and polysaccharides composed of one or more of these sugars. The range of saccharides fermented is genus dependent. *Isotricha* spp., unlike *D. ruminantium*, will utilize starch of a suitable grain size (103, 133, 274; B. H. Howard, Biochem. J. **67**:18P, 1957), whereas maltose and cellobiose are not effective substrates for *Isotricha* spp. (103, 133, 212; B. H. Howard, Biochem. J. **89**:8P, 1963), although it possesses α-glucosidase, cellobiase, and β-glucosidase activities (255, 261).

D. ruminantium readily ferments sucrose, fructose, D-glucose, raffinose, and inulin. Fructose-containing carbohydrates are utilized most rapidly (239, 258). Certain glucose-containing di- and trisaccharides and galactose are also metabolized (258), whereas L-glucose, glucose phosphates, pentoses, uronic acids, hemicellulosic polysaccharides, and cellulose derivatives did not enhance gas or metabolite formation rates (239, 258). The rate of carbohydrate utilization and the nature of the products formed are substrate dependent. Failure to determine the full range of products has resulted in incorrect conclusions on substrate utilization. For example, both galactose (118) and lactose (239) have

been reported not to be utilized by *D. ruminantium* through their failure to stimulate gas production (galactose) or amylopectin deposition (lactose). However, the protozoon has β-galactosidase activity (261) and both carbohydrates effectively stimulate the production of one or more of the principal fermentation end products (258).

Early studies on the carbohydrate metabolism of *Isotricha* spp. were performed with mixed preparations of *I. intestinalis* and *I. prostoma* (103, 133). However, Prins and Van Hoven (212) were able to examine *I. prostoma* separately, as this species was the only representative of the genus in the animal used. These authors confirmed that glucose, fructose, and fructose-containing carbohydrates (sucrose, raffinose) were metabolized most rapidly. Contrary to earlier results (133; Howard, Biochem. J. **67**:18P, 1957), the monomer galactose and the galactose-containing dimer melibiose were utilized, but, as with *D. ruminantium* (133, 258), at a slower rate than glucose. As with *D. ruminantium* pentoses and poly- and oligouronides are not metabolized (133).

The preferred substrates of *D. ruminantium*, as determined by incorporation rates, were sucrose > fructose > glucose > galactose, and uptake rates of *Isotricha* spp. were some twofold higher (256, 258). Sugar uptake by both genera was not affected by the presence of bactericidal levels of antibiotics included to suppress bacterial contamination (212, 258). There was no evidence for diauxic sugar utilization by *D. ruminantium* as the protozoon was not selective in the uptake of available carbohydrates and did not regulate sugar entry by preferential or sequential utilization. In conseqence, co-utilization of all available substrates occurred such that a maximum monosaccharide uptake of 25 to 30 μmol/mg of protein per h occurred (256). The presence of a readily assimilated substrate also resulted in increased uptake of galactose.

The mechanism of sugar uptake by the holotrichs is unknown. The reduced rate of the uptake of a sugar by *D. ruminantium* in the presence of another sugar (i.e., an alternative substrate) indicated that several sugars may be transported by the same route in view of the concomitant, albeit decreased, uptake that was observed with various sugar combinations (256). Competition by monosaccharides for a common carrier has been reported for carrier-mediated processes in other species (220). Glucose and maltose are taken up by different mechanisms by the entodiniomorphid ciliate *Epidinium ecaudatum caudatum* (58), whereas the glucose concentration determines whether active or passive uptake mechanisms operate in *Entodinium caudatum* and *Entodinium simplex* (49, 51).

Galactose may, however, be taken up by a different mechanism in *D. ruminantium* (256). Galactose uptake was increased in the presence of a readily assimilated substrate, suggesting the existence of an energy-dependent uptake mechanism. It is not known whether dimers and higher oligomers are taken up undegraded or as the component monosaccharides as exocellular carbohydrases are produced by *D. ruminantium* (255).

The ecological role of the holotrichs appears to be the rapid assimilation of soluble sugars. The rate of sugar utilization by *D. ruminantium* was influenced not only by the nature and concentration of the substrate but also by various environmental factors, including pH and temperature. During the diurnal cycle of the rumen, however, the rate of glucose uptake was not affected by the presence of reserve polysaccharide within the cell (258). Glucose uptake was, however, adversely affected in the presence of D,L-lactic acid (259). Uptake was reduced by approximately 30% in an

incubation containing 10 mM lactate and the inhibition increased to 70% at a lactate level of 44 mM (259).

Certain carbohydrates, however, have adverse effects upon the cell. In cultural studies mannose, glucosamine and galactosamine were shown to be toxic to the holotrich ciliates (230). The rate of uptake of mannose by *D. ruminantium* was low and extensive cell lysis occurred in incubations with this monosaccharide (133, 258). The formation of other fermentation products were also impaired. The protozoon was unable to control sugar uptake, and the presence of readily assimilated monosaccharides did not preclude the uptake and subsequent effects of the toxic monomers (256). Glucose metabolism by *Isotricha* spp. is also adversely affected by mannose (212, 230) and it would therefore seem that this related protozoon is also similarly unable to control sugar entry. *I. prostoma*, like *D. ruminantium*, proved unable to form amylopectin and fermentation products from mannose, even when glucose was available (212). The mechanism of hexosamine and mannose toxicity in the holotrichs is not known, but hexosamines have also been shown to be hepatotoxic (218, 244).

Since a concomitant uptake of carbohydrates occurs, regardless of molecular size or eventual metabolic effects, the presence of readily fermentable substrates does not prevent the uptake of toxic monomers. Although nonmetabolizable substrates do not impede the uptake or metabolism of effective substrates, maximum metabolite production rates are not maintained in the presence of slowly utilized substrates. However, this absence of selectivity in sugar uptake should enable the microorganism to compete more effectively for substrates in the rumen. The protozoon is thus attracted by chemotaxis to soluble sugars (203) and is able to assimilate them as soon as they become available in the immediate environment.

Products of carbohydrate fermentation. The principal acid end products formed by *Isotricha* and *Dasytricha* spp. during the fermentation of soluble carbohydrates are lactic acid, butyric acid, and acetic acid (103, 118, 133, 212, 239, 258) with traces of propionic acid (103, 118, 258; Howard, Biochem. J. **89**:8P, 1963). At higher fermentation rates some formic acid may also be formed (239; Howard, Biochem. J. **89**:8P, 1963). Hydrogen, CO_2, and a storage polysaccharide are also produced. Early estimates of product formation from glucose indicated that H_2 and CO_2 were produced in an approximate ratio of 1:1 to 2:1 and that the average acid production by *D. ruminantium* and *Isotricha* spp. was 0.062 and 2.35 nmol of acid/cell per h, respectively (103).

The average distribution of the fermented glucose-carbon recovered in the metabolic end products formed during in vitro incubations of *D. ruminantium* (at an initial substrate concentration of 27.8 mM) were, after correction for endogenous activity, as follows: acetate, 1.2%; butyrate, 2.5%; lactate, 19.8%; CO_2, 4.5%; and storage polymer, 73% (258). The rate of production was affected by the concentration of glucose (Table 1) (239, 258), with maximum rates of VFA and lactate formation occurring at pH 7 and at a temperature of 39 to 40°C (258). Glucose uptake and product formation rates were similar in cells isolated at various stages in the diurnal cycle of the rumen.

Although the range of products is not affected by the nature of the carbohydrate substrate, the rate of gas and acid production and the proportions of products formed by *D. ruminantium* are substrate dependent (258). The carbohydrates metabolized most rapidly are glucose, fructose, sucrose, raffinose, and inulin; other oligosaccharides are less effective substrates (255, 256, 258). Although galactose is

TABLE 1. Metabolite formation by *D. ruminantium* and *I. prostoma*

Organism	Substrate	Metabolite formation rate (pmol/cell per h)[a]								Reference
		Acetate	Butyrate	Lactate	Formate	Amylopectin	Gas	CO_2	H_2	
D. ruminantium	Endogenous	5.4	4.9	3.4	ND	−11.0	30.2	15.0	15.2	239
	Endogenous[b]	3.2	2.2	2.8	NA	NA	10.0	NA	NA	258
	Galactose (9.8 mM)	4.0	4.4	11.0	2.7	7.0	NA	8.0	NA	239
	Glucose (5 mM)	11	8	65	1	105	NA	NA	28	239
	Glucose (24 mM)	19	5	105	10	115	NA	NA	18	239
	Glucose (27.7 mM)[b]	6.0	5.3	32.2	NA	57.4	39.7	NA	NA	258
I. prostoma	Endogenous	27	26	14.5	NA	−52	142.3	71.5	70.8	212
	Galactose (9.66 mM)	28	36	78	NA	120	NA	6	NA	212
	Glucose (5.3 mM)	90	86	101	NA	390	471	232	239	212
	Glucose (10.6 mM)	175	145	186	NA	611	869	412	457	212

[a] Cells were kept in vitro in the presence of antibiotics for 20 h prior to recovery for assay. A minus value is the rate of utilization. ND, Not detected; NA, not assayed.

[b] Calculated assuming 6.6 ng of protein per cell (103); average value determined over a 24-h diurnal cycle.

taken up more slowly, the range of products is the same as for glucose (133, 239, 258). Heald and Oxford (118) failed to detect galactose fermentation manometrically, but other workers (133, 239, 258) were able to measure fermentation products, although the formation rates were consistently lower than the rates of formation from glucose (Table 1). Studies on gas and acid production by *D. ruminantium* when more than one substrate was available did not indicate preferential or sequential substrate utilization (diauxie), and the presence of readily assimilated substrates did not preclude the uptake and effects of less effective, nonmetabolizable or toxic compounds (256). Product formation rates were not maintained when toxic (e.g., mannose) or poorly utilized (e.g., galactose) substrates were available together with readily assimilated sugars.

Hydrogen, CO_2, acetate, butyrate, lactate, and amylopectin are the major products of glucose metabolism by *I. prostoma* (212) (Table 1). At high rates of fermentation, lactate is the principal acidic metabolite, whereas at lower rates acetate and butyrate predominate. The lactate formed from glucose is mainly the L-isomer (70%), although racemic lactate is formed by the endogenous fermentation of amylopectin (212). *D. ruminantium* likewise forms L-lactate (239, 258); the D-isomer is produced when the sugar concentration exceeds 20 mM (239).

The rates of formation of acetate, lactate, H_2, and CO_2 by *I. prostoma* all increase with increasing glucose concentration (3 to 24 mM), whereas the formation of butyrate is apparently independent of the initial substrate concentration (212). Galactose is metabolized at a lower rate than glucose (Table 1) and *I. prostoma* is also not able to ferment mannose. The products derived from other carbohydrates by *I. prostoma* and the metabolic potential of *I. intestinalis* have not been determined.

Production and utilization of reserve carbohydrate material. (i) Synthesis. An important feature of the holotrich ciliates is their ability to synthesize and store an intracellular reserve polysaccharide during the limited periods when sugars are available in the rumen ecosystem. Some 75 to 80% of the sugar taken up by the holotrichs can be converted in an energy-consuming process to the polymer (103, 258).

The polymer, originally described as glycogen (Certes, quoted by R. E. Hungate [136]) is an α,1-4-linked homoglucan with an average net chain length of 22 glucose units that are united by α,1-6 linkages (97). The highly branched structure is closely similar to that of amylopectin; the amylose content is negligible.

A wide range of glucose and fructose-containing carbohydrates support amylopectin deposition by the holotrichs (158, 205, 212, 239, 256, 258). All substrates that will stimulate hydrogen production by *I. prostoma* will also serve as precursors for amylopectin formation (212). Acetate is a precursor for amylopectin deposition by *D. ruminantium* (258). Simultaneous breakdown and synthesis of amylopectin occurs with effective substrates in both genera (118, 212). Intracellular polysaccharide levels in the rumen are maximal some 2 to 4 h after feeding and subsequently decline, whereas soluble carbohydrate levels are highest 1 to 2 h postfeed (205, 258). The maximum rates of amylopectin storage determined for *D. ruminantium* and *I. prostoma* are 130 and 1,300 pmol of amylopectin-hexose/cell per h, respectively. Over 90% of ^{14}C derived from ingested [U-^{14}C]glucose in a 1-h incubation was found in the polysaccharide fraction of *D. ruminantium* (258).

The rate of amylopectin deposition by *D. ruminantium* increases up to a glucose concentration of 13 mM but is inhibited slightly at 24 mM (239). Galactose is a less effective substrate for amylopectin synthesis in both holotrich genera (212, 239, 256, 258). Only 69% of the ^{14}C from [U-^{14}C]galactose ingested by *D. ruminantium* in a 60-min incubation was located in the polysaccharide fraction, with 28% remaining in the metabolite pool (256). Synthesis rates from galactose (9.7 mM) were considerably lower, being only 7 and 120 pmol of amylopectin-hexose/cell per h for *D. ruminantium* and *I. prostoma*, respectively (Table 1). Approximately 30% of the energy available through galactose fermentation was used by *D. ruminantium* in the synthesis of amylopectin, whereas approximately 90% of the adenosine 5'-triphosphate (ATP) formed in the fermentation of glucose could be used by the protozoon in amylopectin synthesis (239). Holotrich starch is synthesized by the phosphorylase mechanism common to plants and animals (169).

(ii) Amylopectin breakdown. *Isotricha* and *Dasytricha* spp. possess amylolytic polysaccharide depolymerase and glycoside hydrolase enzymes (169, 255, 261; D. L. Mould and G. J. Thomas, Biochem. J. 67:18P, 1957). α-Amylases specific to *D. ruminantium* and *Isotricha* spp. have been resolved (169; Mould and Thomas, Biochem. J. 67:18P, 1957). The optimum pH for the isotrich amylase was 4.8, whereas the dasytrich amylase had dual optima at pH 5 and 6 (169). The amylases are inhibited by maltotriose, but both genera will degrade the lower malto-oligosaccharides (e.g., maltose, maltotriose, isomaltose), although *D. ruminantium* is more effective than the *Isotricha* spp. (169).

The products formed from the metabolism of storage polysaccharide are lactate, acetate, butyrate, H_2, and CO_2 (118, 212, 239, 256). In the absence of substrate a constant utilization of storage amylopectin occurs (239, 258), with maximum breakdown rates of 12 and 85 pmol/cell per h for *D. ruminantium* and *I. prostoma*, respectively (212, 239, 258). Approximately equal amounts of acetate and butyrate are formed from the endogenous fermentation of storage polysaccharide by *D. ruminantium* (118, 239, 258), whereas the proportion of lactate formed is more variable. Formate is not produced and CO_2 and H_2 are produced in approximately equimolar amounts. Thus, Williams and Harfoot (258) estimated that the proportions of end products were as follows: acetate, 24%; butyrate, 23%; lactate, 34% (i.e., 1:1:1.4); and CO_2, 19%. In contrast, Van Hoven and Prins (239) found the ratio of the acidic metabolites to be 1:0.9:0.6. These variations in proportions are reflected in the endogenous formation rates (Table 1) which are some 2- (acetate, butyrate) to 10-fold (lactate, gas) lower than the rates determined with a rapidly assimilated substrate (239, 258). *D. ruminantium* only forms L-lactate from amylopectin, whereas *I. prostoma* produces a racemic mixture of both isomers during endogenous fermentation.

Acetate and butyrate are the principal endogenous metabolites formed by *I. prostoma* with lesser amounts of DL-lactate (212). The endogenous fermentation rates of *I. prostoma* (Table 1) (212) are approximately five times higher than those of *D. ruminantium*.

Simultaneous deposition and utilization of amylopectin occurs (118, 212), but in the presence of an effective substrate the apparent amylopectin breakdown rate is reduced due to concomitant synthesis (212, 239). At certain concentrations (e.g., galactose or glucose, 20 mM; fructose, 2 to 5 mM) no net loss of amylopectin occurs. However, lactate, which is not a precursor for amylopectin synthesis, inhibits endogenous amylopectin breakdown in both *D. ruminantium* and *I. prostoma*. Endogenous metabolic activity was also reduced in *D. ruminantium* isolated from the animal immediately prior to feeding. The holotrich contained depleted levels of storage material (258). Activity was maintained in cells isolated at intervals up to 9 h after feeding when cell carbohydrate content had not declined appreciably. However, there is limited information (212) to indicate that the rate of amylopectin fermentation in *I. prostoma* was affected by its level in the cells. Abnormalities in polymer utilization have been reported in cells maintained at temperatures 8 to 12° below normal rumen temperatures (93).

(iii) Importance of amylopectin. The carbohydrate content of rumen protozoa is influenced by the nature of the feed carbohydrate and reaches its maximum level 2 to 3 h after feeding (258). The extent to which protozoal carbohydrate contributes to the total carbohydrate (i.e., undegraded dietary and microbial carbohydrate) entering the small intestine of the ruminant is dependent upon the rate of removal of protozoa from rumen. However, considerable sequestration of the protozoa occurs in the rumen and only a small proportion (6 to 30%) of the protozoal dry matter enters the small intestine from the rumen (252). The endogenous fermentation of the storage polysaccharide by the sequestered protozoa extends and stabilizes the postfeed period of VFA formation in the rumen (see section, "Role in Rumen Metabolism").

Polysaccharide deposition was also indirectly implicated in the onset of bloat in animals (44). Bloat is a condition in which the animal is unable to eruct the fermentation gases and is characterized by a potentially fatal increase in pressure in the rumen. In the presence of excess carbohydrate the holotrich protozoa were observed to degenerate (207, 230). This bursting under natural conditions after feeding in vivo was used to explain the postfeed population decline of the holotrichs. Since the cell contents of burst holotrichs have foam-stabilizing properties, which could influence the foaming properties of rumen ingesta, bursting in vivo was suggested as a cause of bloat (44).

It was proposed that rumen holotrich protozoa are unable to control the intracellular deposition of amylopectin, and thus in the presence of excess substrate continued to synthesize polymer until an overaccumulation resulted in cellular deterioration both in vitro (207, 230) and in vivo (43, 44). Williams and Harfoot (258) also observed cellular degeneration in controlled in vitro incubations and concluded that *D. ruminantium* lacked a mechanism for controlling the entry of substrate into the cell. Subsequently, Van Hoven and Prins (239) demonstrated that a definite amount of amylopectin was stored per cell and claimed that lysis was due entirely to the accumulation of acidic end products, notably lactate. Therefore, they discounted the premise that bursting was related to excess polymer storage through an inability to control substrate entry. However, it is apparent from the studies of Williams (256) that *D. ruminantium* is unable to control substrate entry and that a concomitant uptake of all available carbohydrates occurs irrespective of metabolic consequences. The cause of lysis of the holotrichs, indicated by the latter studies, would appear to be twofold. The protozoon is unable to selectively control the entry of sugars into the cell; however, this excess substrate does not lead to an uncontrolled synthesis of amylopectin as originally proposed (43, 44, 207, 230) but to a detrimental intracellular buildup of acidic fermentation products.

All studies thus far on the metabolism of soluble carbohydrates by the holotrich ciliates have used protozoal preparations in which bacterial activity has been minimized by preparation procedures or suppressed by antibacterial agents. However, in situ the protozoa are likely to directly interact with other microorganisms, as exemplified by the interspecies association of methanogens and certain entodiniomorphid ciliates (152, 229, 243). The metabolic consequences of the interactions between the holotrichs and specific functional bacterial groups (e.g., hydrogen utilizers) warrant further examination so that more realistic metabolic profiles for the organism may be determined.

Metabolism of plant structural and storage polysaccharides. (i) Association with plant material. The ciliate protozoa may be responsible for some 30 to 40% of microbial fiber digestion in the rumen (80). It is generally believed that the particle-ingesting protozoal species are involved in the degradation of the plant structural polymers, whereas the holotrichs and small *Entodinium* spp. are more involved in the utilization of storage polymers and soluble sugars (200).

The overall contribution of the protozoa to the degradation of plant material in the rumen has also been assessed by using electron microscopy. Rapid colonization occurred and large numbers of the entodiniomorphid ciliate *Epidinium* spp. were associated with the damaged regions of the plant material (12, 13, 23, 25). There was extensive degradation of the thin-walled tissues mesophyll, parenchyma, bundle sheath, and epidermis. In the absence of *Epidinium* spp. the plant material was rapidly, and heavily, colonized by *D. ruminantium* (24). The principal colonization site was the pith. Although the plant cells were not further degraded, the organism was able to deeply penetrate damaged parenchymatous tissue (24).

I. intestinalis and *I. prostoma* also attach to particulate plant material in the rumen. It was estimated (203) that, in an animal fed once daily, up to 80% of the *Isotricha* population became attached to plant particles 2.5 h after feeding. These ciliates are attracted by chemotaxis to certain soluble sugars (e.g., glucose, fructose, sucrose) and attach to particulate sources of these carbohydrates by means of a specialized organelle on their anterior dorsilateral surface (203). The organelle is a longitudinal ridge 24 to 37 μm long and approximately 5 μm wide that terminates at or close to the anterior end of the cell (202; C. G. Orpin and F. J. Hall, Proc. Soc. Gen. Microbiol. 4:82–83, 1977).

Large numbers of holotrichs may populate plant fragments in the rumen and thus maintain a close proximity to available sources of soluble carbohydrates. Sequestration within the particulate digesta will, irrespective of growth rates, aid their retention and survival in the rumen (252). Determinations of the holotrich population in the rumen should, but unfortunately rarely do, include an estimate of this particle-associated population.

(ii) Polysaccharide degradation. Studies on the distribution of polysaccharide-degrading enzymes in the rumen ecosystem confirmed that the protozoal fraction possessed activity against both structural and storage polysaccharides (260). The specific activities of the hemicellulolytic enzymes were similar to those in the bacterial population, whereas α- and β-glucanase activities were markedly higher in the protozoal cells. In this section the activities of the holotrich ciliates are considered.

(a) Storage polysaccharides. The principal plant storage polymers are α-glucans and fructans. The amylolytic activity of the holotrichs was discussed above and will not be considered further.

Inulin is a reserve carbohydrate found in the tubers of the *Compositae* family. It is an essentially linear glucofructan in which chains of (2→1)-linked β-D-fructofuranose residues are terminated by an α-D-glucopyranose residue attached as in sucrose (16). A major component of the soluble carbohydrate of ryegrass is also a fructosan (245); the predominant fructofuranan is composed of frucotofuranosyl residues linked 2→6 and terminated by a glucosyl residue (234). Plant fructosans and fructose-containing oligomers are rapidly fermented by the holotrich ciliates (230, 233, 258). Studies on the holotrich invertase indicated that this one enzyme was responsible for the hydrolysis of both sucrose and fructosan. It acted in an exomanner and liberated single fructose units from the polymer (233). Grass fructosan was hydrolyzed four to five times more rapidly than inulin, with *Dasytricha* being the more active of the two holotrich genera.

(b) Cellulose. Although the protozoal fraction of the rumen microbial community degraded cellulose, Halliwell (107) concluded that the protozoa had a minor role in cellulose degradation. However, the protozoal fraction con-

tains appreciable cellulolytic activity (260) and a survey confirmed its widespread occurrence within the entodiniomorphid ciliates (57). However, the data relating to the holotrichs are equivocal.

Carboxymethyl cellulose caused an initial stimulation of gas production by *D. ruminantium* (258). The rate, however, rapidly returned to the endogenous value and endogenous acetate, butyrate, and lactate levels were not significantly enhanced upon incubation with carboxymethyl cellulose (239, 258). However, cell-free extracts of *D. ruminantium* and *Isotricha* spp. release reducing sugar from carboxymethyl cellulose (A. G. Williams and G. S. Coleman, Curr. Microbiol., in press). The activity was very low and may reflect nonspecific action of another polysaccharide depolymerase of glycoside hydrolase, or may originate from bacterial contamination. However, the preparative procedures produced minimal bacterial contamination and disruption, and the subcellular distribution of the activity following differential centrifugation, although similar, was not identical to that of β-glucosidase and β-cellobiosidase. The activity occurred primarily in fractions sedimenting at 10^4 g-min and 10^5 g-min and the fraction nonsedimentable after centrifugation at 6×10^6 g-min. The origin and nature of this enzyme requires further examination.

The occurrence of other β-glucanases in the holotrich ciliates has not been examined in detail, although the holotrichs in the rumen of seaweed-eating sheep were found to store amylopectin when the rumen liquor was incubated with laminarin (87). It was not established whether the holotrichs degraded the seaweed polysaccharide or merely scavenged the glucose and laminaribiose arising from its breakdown by the enzymes of other rumen microorganisms. The entodiniomorphid ciliate *Epidinium ecaudatum*, for example, degrades both β,1-3- and β,1-4-glucans (18), and although both *Dasytricha* and *Isotricha* are able to degrade the β-linked disaccharides cellobiose (β,1-4) and laminaribiose (β,1-3) (134, 255; Howard, Biochem. J. 67:18P, 1957), their activity against the corresponding polysaccharides (e.g., laminarin, lichenan) is not known.

(c) Hemicellulose. Initial manometric studies indicated that xylan and arabinan were not utilized by the holotrich ciliates (7, 212, 239, 258). These findings substantiated those of Bailey et al. (17) who, using paper chromatographic analyses, failed to demonstrate degradation of hemicellulose by holotrich extracts. However, low levels of hemicellulase activity were detected in partially purified holotrich extracts by the colorimetric measurement of reducing sugar formation from isolated hemicelluloses (115, 217). However, the glycoside hydrolases involved in hemicellulose breakdown (α-L-arabinofuranosidase and β-D-xylosidase) are present in both *D. ruminantium* (255, 261) and *I. intestinalis/I. prostoma* (261). The ability of both genera to depolymerize ryegrass (*Lolium perenne*) hemicellulose B and oat arabinoxylan to alcohol-soluble oligomers and the monosaccharides has been confirmed (257). The rate of hemicellulose depolymerization to alcohol-soluble fragments was 5- to 10-fold higher than the rate of reducing saccharide accumulation, confirming that these ciliates possessed endo-acting depolymerases that rapidly generate oligosaccharide fragments. The specific activity of the mixed *Isotricha* spp. preparation was higher (three- to sevenfold) than that measured in the *D. ruminantium* preparations.

These activities were approximately 5- to 10-fold lower than the activities of the larger cellulolytic entodiniomorphid genera, but were higher than those detected in the *Entodinium* spp. examined (257). These hemicellulases, fol-

TABLE 2. Survey of carbohydrases detected in the rumen holotrich ciliates *Dasytricha* and *Isotricha* spp., using di- and trimeric carbohydrate substrates[a]

Carbohydrate substrate	Carbohydrase activity references[b]			
	D. ruminantium		*Isotricha* spp.	
	Detected	Not detected	Detected	Not detected
Maltose	21, 134, 169, 255		134 (t), 169	
Maltotriose	21, 169, 255		169	
Isomaltose	255			
Trehalose	134 (t)	21, Abstr. 1		134, Abstr. 1
Lactose	255 (t)	134, Abstr. 1		134, Abstr. 1
Melibiose	134 (t), 255 (t)	Abstr. 1		134, Abstr. 1
Sucrose	21, 134, 233, 255, Abstr. 1–3		134, 233, 255, Abstr. 1–3	
Raffinose	134, 255, Abstr. 1		134, Abstr. 1	
Melezitose		134		134
Xylobiose		134, Abstr. 1		134, Abstr. 1
Cellobiose	21, 134, 255, Abstr. 1		134 (t)	Abstr. 1
Laminaribiose	134, Abstr. 1		134, Abstr. 1	
Amygdalin	134		134	
Esculin	134		134	
Arbutin	134		134 (t)	
Salicin	134		134 (t)	

[a] Glycoside hydrolases active against glycosides containing a variety of pentoses, hexoses, hexosamines, and hexuronic acids have been detected with chromogenic *p*-nitrophenyl-linked substrates (255, 261, 269, 270).

[b] (t) Low levels of activity detected. Abstr. 1 is Howard, Biochem. J. 67:18P, 1957; Abstr. 2 is Christie and Porteous, Biochem. J. 67:19P, 1957; and Abstr. 3 is Carnie and Porteous, Biochem. J. 73:47P–48P, 1959.

lowing differential centrifugation of lysates, occur principally in two subcellular locations. Approximately 60% of the activity is sedimentable (4×10^5 g-min) in membrane-bound vesicles. The remainder is nonsedimentable after a centrifugation period of 6×10^6 g-min (i.e., 100,000 × g for 1 h) (Williams and Coleman, in press).

The holotrich ciliates thus have the enzymatic capability to degrade hemicellulosic arabinoxylans, but the manometric studies indicate that they are unable to further utilize the component sugars, arabinose and xylose (212, 230, 239, 258).

(d) Pectin. Pectin, but not polygalacturonic acid, oligogalacturonides, or galacturonic acid, is fermented by *D. ruminantium* (239, 258) and *I. prostoma* (212). Pectin supports the formation of gas, acid end products, and amylopectin. Cell extracts of *D. ruminantium* (8, 61) and *Isotricha* spp. (8) possess pectin esterase and polygalacturonase activities. Both pectolytic enzymes were also detected in the extracellular culture supernatant (8). Coleman et al. (61) estimated that *D. ruminantium* formed approximately 11 pmol of reducing sugar (as galacturonic acid)/cell per h from polygalacturonic acid. The production of an unsaturated product confirmed that the enzyme was a lyase type (61). Abou Akkada and Howard (8), however, detected products typical of polygalacturonase (polygalacturonic acid hydrolase) action. Activities were higher in the *Isotricha* spp. preparation and had pH optima in the range pH 8.5 to 9.0 (8). Oligomers were the principal degradation products of *D. ruminantium*, whereas *Isotricha* formed the monomer. Methanol was a product of pectin hydrolysis by both genera. It would appear that the products were not metabolized further. The holotrichs are thus able to depolymerize both pectic and hemicellulosic polysaccharides, but are unable to metabolize the degradation products. These activities may enable the protozoa to penetrate the plant cell wall and gain access to energy sources within the cell contents. Alternatively, the inability to metabolize these carbohydrates may represent an evolutionary degenerative loss of an enzyme or pathway.

Carbohydrase (glycoside hydrolase) enzymes of the

holotrichs. There have been several studies of the range of carbohydrase enzymes formed by *D. ruminantium* and *Isotricha* spp., and these are summarized in Table 2. The holotrichs contain invertase (21, 134, 233, 255; Howard Biochem. J. 67:18P, 1957; A. O. Christie and J. W. Porteous, Biochem. J. 67:19P, 1957; J. A. Carnie and J. W. Porteous, Biochem. J. 73:47–48P, 1959) with optimum activity occurring at pH 5 to 5.5 in crude and purified preparations (14, 233, 255; Christie and Porteous, Biochem. J. 67:19P, 1957). Lineweaver-Burk plots for sucrose in the range of 2 to 50 mM gave K_m values of 4.17 and 2.9 mM for *D. ruminantium* and *Isotricha* spp., respectively (255). The enzyme has a wide substrate specificity for fructose-containing carbohydrates. In addition to sucrose, raffinose and fructosans are degraded (233, 255), although melezitose (*O*-α-D-glucopyranosyl-[1-3]-*O*-β-D-fructofuranosyl-[2-1]-α-D-glucopyranoside) was not broken down (134). The invertase possesses transglycosylase activity and transfers fructosyl moieties at higher sucrose concentrations, producing trisaccharides; tetra- and higher saccharides are not produced (134; Christie and Porteous, Biochem. J. 67:19P, 1957; Carnie and Porteous, Biochem. J. 73:47–48P, 1959).

Glycosidase enzymes have a dual role in carbohydrate metabolism. They remove side chains from the main polysaccharide backbone and also degrade the oligomeric fragments arising from the depolymerizations effected by the polysaccharide-degrading enzymes. Glycosidase activity is essential if complete breakdown of polysaccharides is to occur. However, irrespective of their obvious importance, relatively little information is available on protozoal glycosidases, although enzyme activity may be inferred from studies on carbohydrate utilization. A wide range of α-and β-glycoside hydrolases have been detected in extracts of the rumen ciliate population (79, 261). The removal of galactose side chains by protozoal α-galactosidase is an essential prerequisite to the β-mannanase-catalyzed hydrolysis of the guaran heteromannan polymer (19). Likewise, in the absence of α-L-arabinofuranosidase activity, the arabinose side

chains attached to the plant cell wall xylan impede the action of protozoal xylanase (17, 18).

Initial studies on the holotrich ciliates, using disaccharide substrates, demonstrated the presence of maltase (α-1,4-glucosidase), cellobiase (β-1,4-glucosidase), laminaribiase (β-1,3-glucosidase), and invertase (β-fructofuranosidase) in both holotrich genera (21, 80, 134; Howard, Biochem. J. 67:18P, 1957). More recent surveys with p-nitrophenyl derivatives (261, 269, 270) and disaccharidic substrates (255) have shown that both genera have activity against glycosides of pentoses, hexoses, hexosamines, and hexuronic acids. Certain of these enzymes (e.g., uronidase, mannosidase, xylosidase, arabinofuranosidase, aminidase) generate monomers that either are not utilized or are toxic to the protozoa. Thus far there have been few detailed studies on the holotrich glycoside hydrolases, the exceptions being invertase and α- and β-glucosidase.

The maltase of *D. ruminantium* has maximum activity at pH 5.5 and 50°C, retaining activity to 60°C, whereas the maltases of the entodinomorphid ciliates *Epidinium ecaudatum* and *Entodinium caudatum* are most active at rumen temperatures (38 to 40°C), with considerable loss in activity occurring above these values. Their pH optima are also markedly different, occurring at pH 5.7 to 6.1 (*Entodinium*) and 6.7 (*Epidinium*) (21). The maltase of *D. ruminantium* is a specific α-glucosidase that has some activity on maltotriose. It also possesses transferase properties when incubated with maltose at concentrations of >1%, forming maltotriose and panose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose]. The *D. ruminantium* α-glucosidase was more active than preparations obtained from *Isotricha* spp. (169, 261).

Although *Isotricha* spp. are unable to ferment cellobiose (103, 133, 212; Howard, Biochem. J. 89:8P, 1963) and lack cellobiase (134; Howard, Biochem. J. 67:18P, 1957), they possess low levels of β-glucosidase (134, 255, 261, 270); however, *D. ruminantium* ferments cellobiose (239, 258) and has both cellobiase and β-glucosidase (134, 255, 261; Howard, Biochem. J. 67:18P, 1957). The glucosides esculin (escutilin-6-β-D-glucopyranoside), amygdalin (D-mandelonitrile-β-D-glucosido-6-β-D-glucoside), arbutin (hydroquinone-β-D-glucopyranoside), and salicin (2-hydroxymethylphenyl-β-D-glucopyranoside) are degraded by *D. ruminantium* extracts (134). The β-glucosidase of *D. ruminantium* is considerably more active than that of *Isotricha* spp. (134, 261; Howard, Biochem. J. 67:18P, 1957) and has an optimum at pH 5.5 to 6.0 and increasing activity to 60°C. The K_m of the enzyme for p-nitrophenyl-β-D-glucopyranoside was 2.17 mM (255). Identical pH and temperature optima were obtained with cellobiose as the substrate (255).

Approximately 70 to 80% of the acid hydrolase activity in the holotrich ciliates is sedimentable upon centrifugation, although the distribution of the enzymes in the subcellular fraction indicated at least two separate populations of acid hydrolase-containing organelles (269, 270). Acid phosphatase and N-acetyl-β-D-glucosaminidase were located in vesicles sedimenting at 4×10^5 g-min, whereas the other hydrolases occurred in fractions sedimenting at 10^4 and 10^5 g-min (Williams and Coleman, in press). Very small particles sedimenting at 6×10^6 g-min that were enriched in β-D-glucuronidase were also isolated from the holotrichs and may have been derived from Golgi vesicles (269).

The holotrich ciliates thus contain an array of glycoside hydrolases, and there is some evidence to indicate that environmental conditions can affect the range and level of activity (255). However, it is apparent that further studies on the factors controlling their formation and activity are needed.

In addition to pectolytic activity (8), exocellular carbohydrase activity has also been found in these protozoa, and the kinetic characteristics of the holotrich invertase and β-glucosidase, although having interspecies variations, were the same for the intra- and extracellular form of the enzyme (255). The properties of the invertase in cell-free rumen contents resembled those of the exocellular enzymes formed by the holotrichs. These organisms would appear to be an important source of free invertase in rumen contents. The range and level of carbohydrase was affected by the metabolizable substrates available. The increased exocellular activity associated with starved cells or when a poorly utilized substrate was present suggests that these enzymes may be actively secreted in conditions of nutrient depletion to degrade extracellular polymers. Orpin and Letcher (203) suggested that the isotrich attachment organelle would function in the absorption of nutrients and the excretion of extracellular enzymes. It is logical to conclude that the attachment to plant particles and production of extracellular enzymes to release bound carbohydrates would be advantageous to the protozoa in the rumen.

Nitrogen Metabolism

Protein metabolism. The ability of the rumen protozoa to assimilate both dietary and microbial proteins is important in the nitrogen economy of the host ruminant. However, relatively little is known about the metabolism of amino acids and protein by the holotrich protozoa. Some information has been gained by using mixed protozoal preparations that contained both holotrich and entodiniomorphid ciliates. The metabolic capabilities of the entodiniomorphid protozoa have been reviewed by Coleman (54, 56).

Mixed protozoal preparations containing holotrich ciliates have been used in studies on the nature and properties of rumen protozoal protease (96, 187, 222–224) and metabolism of specific amino acids including lysine (190), tryptophan (95), arginine, proline, ornithine (196, 198), citrulline (198), threonine (197), and methionine (160, 197). Mixed populations have also been used to examine the range of amino acids taken up (11, 186), the factors affecting this incorporation (10, 120), and de novo amino acid synthesis (225, 232). The rumen protozoal fraction has a range of transaminase activities (27, 28, 131) and will incorporate exogenous urea added to the culture medium (181). The evidence regarding ureolytic activity is contradictory. Bonhomme-Florentin observed urea digestion by a ciliate-containing preparation (30), but Onodera et al. (194) were unable to demonstrate ureolysis with a washed protozoal suspension. Thus, although a considerable amount of information on the nitrogen metabolism of holotrich-containing mixed protozoal preparations has been amassed, the particular contribution and capabilities of the holotrich genera are not obvious.

In addition to ammonia, other products of nitrogen metabolism of mixed ciliates that are released from cells include amino acids (e.g., alanine, proline, glutamate, lysine, valine), small amounts of peptides (184, 188, 195), and the breakdown products L-pipecolic acid (185), δ-aminovaleric acid (235), and 2-aminobutanoic acid (193). Heald and Oxford (118) were the first to observe the loss of soluble polypeptide or protein from the holotrichs. As much as 25% of the cellular nitrogen was excreted into the medium by

Isotricha spp. in a 24-h period (113), one-third of which was amino acids (alanine, glutamate, aspartate, proline, ornithine) and ammonia. The excretion was not affected by the presence of exogenous amino acids but was inversely correlated with incubation time and represents the degradation of ingested nitrogenous materials. Exogenously supplied amino acids are not degraded by *Isotricha* spp. but are incorporated directly into protein without modification (112, 246). High levels of amino acids had an apparently toxic effect (112).

Analysis of cell components confirmed that radioactivity from ^{14}C-labeled glucose (258), galactose (256), and acetate (114, 258) is incorporated into the cellular protein of *D. ruminantium*. *Isotricha* spp. are able to incorporate ^{14}C from acetate and sodium carbonate into cellular proteins (109, 110, 114; J. Harmeyer and H. Hill, Radioisotopes Anim. Nutr. Physiol. Proc. Symp., p. 108–109, 1964). Analysis of the protein fraction from both genera indicated that the label was widely distributed in amino acids, confirming de novo amino acids synthesis and incorporation. Protein synthesis by a polysome-containing cell-free extract of mixed rumen protozoa consisting mainly of the genus *Isotricha* has been demonstrated (41). ATP, guanosine 5'-triphosphate, and an energy source were necessary for amino acid incorporation, which was influenced by the concentration of the cations Mg^{2+}, K^+, and Mn^{2+}. Amino acid incorporation was partially inhibited by chloramphenicol (41). The characteristics of protein synthesis by cell-free preparations of *Entodinium* spp. are similar (132).

The holotrichs are thus able to both synthesize amino acids and utilize preformed amino acids released by proteolysis from ingested bacteria or plant material. Stern et al. (228) observed the ingestion and vacuolar digestion of chloroplasts and suggested that chloroplast amino acids were incorporated into protozoal protein. Although Harmeyer (112) was unable to demonstrate free amino acid uptake by mixed *Isotricha* spp., Wallis and Coleman (246), using ^{14}C-labeled amino acids, conclusively demonstrated that free amino acids were taken up from the immediate environment by *Isotricha* spp. The acids were taken up singly and from complex mixtures at a constant rate over a 24-h period. Incorporation rates were amino acid specific and varied from 4 to 5 μg/2 × 10^4 protozoa per day [glycine, (iso-)leucine, serine] to approximately ≥ 0.1 (aspartate, glutamate, alanine). Lysine and methionine were taken up at an intermediate rate of 1 to 2 μg/2 × 10^4 cells per day (246). The amino acids were assimilated into cellular proteins and were not interconverted or metabolized further.

Bacterial Ingestion

Bacteria are attached to the external surfaces of the holotrich ciliates; Imai and Ogimoto (141), depending upon the microscopic technique used, estimated that there were approximately 10 to 20 bacteria per 200 μm^2 of the surface of *I. prostoma* and *I. intestinalis*. It appeared that the bacteria were trapped among the cilia and growing around the body of the ciliates. However, although the holotrichs are hydrogen formers, no externally associated methanogens have been observed (243).

Bacteria are present in digestive vacuoles within the protozoal cell (104, 105, 228, 246) but the evidence for the presence of intact viable bacteria within the protozoal cells is less conclusive than for the entodiniomorphid ciliates (104, 253). Nouzarede, however, reported division and sporulation by a bacillus-like bacterium in the endoplasm of *I.*

prostoma (178). Holotrich suspensions maintained in the presence of antibiotics for 72 h did not contain culturable bacteria (255; N. Yarlett, Ph.D. thesis, University of Wales, 1982). Rickettsia-like organisms have been observed in the macronucleus of *I. intestinalis* (33).

Bacteria, however, have an important role in holotrich nutrition. Although the ciliates failed to engulf *Lactobacillus bulgaricus* (230), both *Isotricha* and *Dasytricha* spp. ingested rumen (104, 105) and nonrumen bacteria (246) and the protozoa cannot be cultured successfully in the absence of viable bacteria (46, 105; Yartlett, Ph.D. thesis, 1982). *D. ruminantium* selectively ingested small cocci (0.5 to 0.8 μm in diameter) which agglutinated at the mouth and were gradually ingested. Small rod-shaped organisms (1 to 1.5 μm in size) were also observed in vacuoles, and the growth of the protozoon was stimulated by these bacteria (105). *I. prostoma* selected and ingested rod-shaped organisms from mixed rumen bacteria, and several strains of bacteria were isolated into pure culture from recently fed protozoal cells (104). Three of the rod-shaped isolates were shown subsequently to be rapidly ingested by *I. prostoma* (104). Suspensions of mixed *Isotricha* spp. ingested *Escherichia coli* at an approximately linear rate of 1,000 to 3,000 bacteria per protozoon per h for 24 h. The bacteria were rapidly rendered nonviable after ingestion and after 24 h none of the bacteria were viable. The bacterial cells were extensively digested, with both incorporation and release of material occurring (246). The bacterial cell wall peptidoglycan is extensively degraded by holotrich-containing mixed ciliate preparations with some conversion of diaminopimelic acid to lysine and pipecolic acid (82, 191, 192, 195). The amino acids derived from the bacterial protein by the mixed *Isotricha* preparations were, however, incorporated directly into protozoal proteins unchanged, although the incorporation of a specific amino acid from the bacterial protein was partially inhibited by an exogenous source of that amino acid (246). Coleman (56) estimated that the retained bacterial amino acids when used for protein synthesis would allow cell divisions every 120 h, whereas in culture the holotrichs divide once every 48 h (103). The holotrich ciliates thus derive some of their nitrogen requirements from the digestion of ingested bacteria.

Metabolism of Nucleic Acids

There are no data available on the synthesis or degradation of purine and pyrmidines by holotrich ciliates (145). The entodiniomorphid ciliates have not been shown to synthesize purines or pyrimidines de novo. They utilize ingested bacterial nucleic acids which are degraded to the nucleotide prior to incorporation into protozoal nucleic acid, and they also assimilate exogenously supplied free bases, nucleosides, and nucleotides (54). The limited information on their interconversion and catabolism by the entodiniomorphid ciliates has been reviewed (54). It is probable that, as with the entodiniomorphid protozoa, the holotrich ciliates obtain the nucleotides needed for nucleic acid synthesis by the degradation of the nucleic acid material of ingested bacteria. The importance of nucleic acid material from dietary sources is not known.

Lipid Metabolism

Composition of holotrich lipids. The lipid fraction of a mixed holotrich preparation consisted of 70% phospholipids and 30% nonphospholipids (Fig. 2). The major fatty acid in all lipids was palmitic acid ($C_{16:0}$) with lesser amounts of

Lipid

Phospholipid (70%)   Nonphospholipid (30%)

Phosphatidylcholine (28%)
Phosphatidylethanolamine (21%)
Phosphatidylethanolamine plasmalogen (22%)
Unknown phospholipid (29%)

Waxes + Hydrocarbon (3.8%)
Alcohol (6.8%)
Monoglyceride (4.8%)
Diglyceride (10.1%)
Unknown glyceride (3.8%)
Hydroxy acid (6.8%)
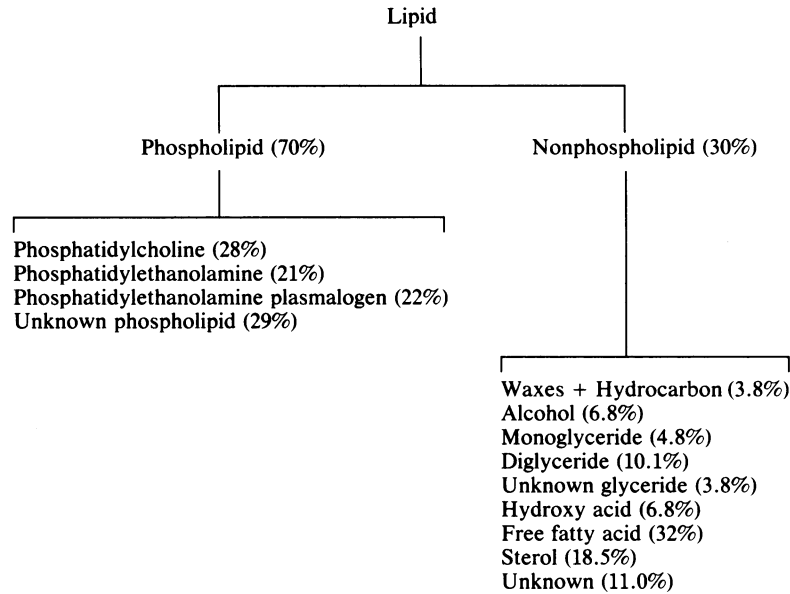Free fatty acid (32%)
Sterol (18.5%)
Unknown (11.0%)

FIG. 2. Composition of major fractions in extracted holotrich lipid. Data were compiled from references 148 and 149.

stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$) (148, 263). A small proportion of the branched-chain fatty acids $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$ was also present, although further characterization of these acids was not undertaken (263). The phospholipids are similar to rumen bacterial phospholipids. They contain plasmalogens and branched-chain fatty acids in the β position of the phospholipid. The principal phospholipids identified were phosphatidyl-ethanolamine, phosphatidylethanolamine plasmalogen, and phosphatidylcholine; a component of the phospholipid fraction was not identified (148) (Table 3). A more restricted range of fatty acids was present in the α(1) position. The β(2) position was occupied by a higher proportion of branched-chain and polyunsaturated fatty acids (148) (Table 3).

The unusual amino acid 2-aminoethylphosphonic acid, which contains a carbon-phosphorus bond, was identified in a mixed protozoal preparation in which it was linked to ceramide in a complex called ciliatine (9). 2-Aminoethylphosphonic acid was subsequently detected in the

TABLE 3. Fatty acid composition in the total lipids and in various lipid classes of holotrich protozoa[a]

Fraction	% of total fatty acids present												
	$C_{14:0}$	$C_{14:0(B)}$	$C_{15:0}$	$C_{15:0(B)}$	$C_{16:0}$	$C_{16:0(B)}$	$C_{17:0}$	$C_{17:0(B)}$	$C_{18:0}$	$C_{18:0(B)}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Total lipid	2.8				37.3				9.1		18.2	10.5	4.0
Free fatty acid	1.3	tr	6.6	3.3	50.1	2.1	2.3	3.9	8.3	tr	5.9	10.2	6.0
Monoglyceride	1.4	tr	3.0	4.4	27.7	2.4	tr	5.1	10.9	tr	18.3	21.5	5.3
Diglyceride	1.3	0.8	4.4	5.9	40.3	2.1	1.4	4.4	10.5	tr	10.0	13.9	4.7
Phosphatidylethenolamine													
Position 1 (FA)	tr	tr	6.2	tr	80.3	1.8	2.5	tr	9.1	ND	tr	ND	ND
Position 2 (FA)	1.1	1.8	1.3	16.9	7.2	10.3	tr	18.6	0.8	ND	9.9	20.1	11.4
Phosphatidylcholine													
Position 1 (FA)	1.1	ND	6.6	ND	74.0	tr	3.0	tr	15.2	ND	tr	ND	ND
Position 2 (FA)	1.5	0.9	2.2	8.5	8.9	2.0	0.8	3.8	1.4	ND	17.9	34.8	17.1
Phosphatidylethanolamine plasmalogen													
Position 1 (fatty aldehyde)	7.3	13.9	6.7	27.5	17.5	2.2	tr	2.6	3.4	ND	10.6	2.9	ND
Position 2 (FA)	tr	tr	2.1	tr	59.5	4.1	tr	13.5	6.9	ND	8.6	5.3	tr
Nonphospholipid components													
Unknown glyceride													
Fatty acids	3.1	0.9	4.3	4.9	32.3	2.0	1.1	4.2	7.7	1.7	14.9	15.2	7.7
Aldehydes	3.5	2.2	7.4	14.5	40.5	2.3	tr	3.4	8.4	ND	15.2	2.4	ND
Alcohol	6.0	5.7	12.1	19.5	32.9	4.2	tr	3.3	8.5	ND	2.9	ND	ND
Diglyceride	1.3	0.8	4.4	5.9	40.3	2.1	1.4	4.4	10.5	tr	10.0	13.9	4.7
Monoglyceride	1.4	tr	3.0	4.4	27.7	2.4	tr	5.1	10.9	tr	18.3	21.5	5.3
Free fatty acids	1.3	tr	6.6	3.3	50.1	2.1	2.3	3.9	8.3	tr	5.9	10.2	6.0

[a] Compiled with information from Katz and Keeney (148) and Williams and Dinusson (263). ND, not detected; (B), branched chain; FA, fatty acid. $C_{14:0}$, Myristic acid; $C_{15:0}$, pentadecanoic acid; $C_{16:0}$, palmitic acid; $C_{16:1}$, palmitoleic acid; $C_{17:0}$, heptadecanoic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid.

phospholipids of *Entodinium caudatum* (67, 130), and varying amounts of the amino acid (26 to 223 mg per g of total N) have been determined in preparations of mixed *Isotricha* spp. (9, 254), although the nature of the 2-aminoethylphosphonic acid-containing lipid component has yet to be established. Similarly, phosphatidyl-*N*-(2-hydroxyethyl)-alanine may be present in the holotrich phospholipid fraction as this phospholipid has been characterized in the lipids of holotrich-containing protozoal preparations (150, 151).

The nonphospholipid components isolated from the holotrich protozoa are summarized in Fig. 2 and their fatty acid composition is given in Table 3. The major sterol was tentatively identified as cholestanol (148).

Lipid synthesis. Both *D. ruminantium* (81, 114, 258) and *Isotricha* spp. (81) incorporate acetate directly into the cellular lipid fraction. Some 20 to 30% of the label in the cell components derived from [1-^{14}C]acetate was recovered in the lipid material (258). There was some incorporation of ^{14}C from [U^{14}C]galactose (256) and [U-^{14}C]glucose (258) into the lipids of *D. ruminantium*. Direct incorporation was low. Only 1.0 and <0.5% of the label derived from galactose and glucose, respectively, was located in lipid material. The major part of the label of incorporated glucose was present in the glycerol moiety of the lipids (81).

Holotrich-containing mixed protozoal suspensions formed structurally related long-chain fatty acids from short-chain precursors (acetate, propionate, butyrate, isoleucine) (94, 219). Fatty acids were elongated by two carbon atoms and shortened by α-oxidation (one carbon moiety) or a restricted β-oxidation (two carbon moieties) (94). Acetate and butyrate were incorporated into fatty acids containing an even number of carbon atoms, principally C$_{16:0}$ (palmitic acid), whereas propionate was incorporated mainly into straight-chain acids having an odd number of carbon atoms, especially C$_{15:0}$ and C$_{17:0}$ (penta- and heptadecanoic acids) (94). Thus it would appear that acetate, propionate and butyrate act as primer molecules for fatty acid synthesis and that malonyl-coenzyme A (CoA) is used to lengthen the carbon chain. Monounsaturated octadecenoic acids were formed by the desaturation of saturated acids, whereas polyunsaturated fatty acids were derived from dietary sources (94). However, Viviani and Borgatti (quoted in reference 242) demonstrated that the holotrich ciliates were able to incorporate [1-^{14}C]acetate into linoleic acid (C$_{18:2}$) and linolenic acid (C$_{18:3}$). The holotrich protozoa are capable of de novo fatty acid synthesis with either glucose or acetate as precursor substrate (81).

Rumen protozoa also form phospholipids de novo from exogenous precursors (36, 208, 219). Detailed studies of phospholipid biosynthesis by the entodiniomorphid ciliate *Entodinium caudatum* have been undertaken (54, 56). *Isotricha* spp. incorporate long-chain fatty acids into cellular lipids and De Meyer et al. demonstrated (81) that linoleic acid was incorporated principally into phosphatidylcholine > phosphatidylethanolamine and sterol esters. Glycerol is incorporated into both diacylphosphoglycerides and their plasmalogen analogs in both *D. ruminantium* and *Isotricha* spp. by a pathway similar to that found in anaerobic bacteria with *sn*-glycerol-3-phosphate, and not dihydroxyacetone phosphate, as the precursor (211).

The principal sterols identified in the entodiniomorphid ciliates include stigmastanol, campestanol, and cholestanol, together with lesser amounts of their 5-β forms and an unidentified component (125). The major sterol in the holotrichs has been tentatively identified as cholestanol, which was assumed to have been derived by modification of

dietary sterols (148). Although the growth of the ciliates is stimulated by exogenously supplied sterols (35, 125, 127, 183), the incorporation of acetate and mevalonate by mixed protozoal preparations suggests that cholestanol may be formed by de novo synthesis. Some direct incorporation of precursors into sterol esters also occurs with *Isotricha* spp. (81). Cholestanol was not formed by dealkylation of C$_{24}$ alkyl sterols (126). However, it would appear that campestanol and stigmastanol arise from the hydrogenation of exogenous unsaturated C$_{28}$ and C$_{29}$ sterol precursors (126).

Although the holotrich protozoa are apparently able to form some lipids de novo from various precursors, it is probable that the lipid requirements of the organisms are met primarily by the direct incorporation of dietary and bacterial lipids (81, 94).

Metabolism of long-chain fatty acids. The holotrich ciliates will take up and incorporate exogenously supplied long-chain fatty acids (81, 94, 101, 106, 264). The relative rates of uptake were stearate > oleate > palmitate > linoleate with *I. prostoma* (106) and palmitate > oleate > linoleate > stearate with *I. intestinalis* (264). The maximum uptake of linoleate by *Isotricha* spp. recorded by Girard and Hawke (101) was 1.6 mg/2 × 10^6 cells per h. Fatty acid uptake by mixed rumen microorganisms is increased by methionine and lysine supplementation (62).

Linoleic acid is rapidly incorporated into sterol esters and polar lipids, although principally into the free fatty acid pool and the phosphatidylcholine phospholipid component (81, 101). Little hydrolysis of phosphatidylcholine occurred in incubations with *Isotricha* spp. (101). However, oleic acid stimulated gas but not short-chain VFA production by *I. prostoma* (106) and *I. intestinalis* (264). Emmanuel (94) demonstrated CO$_2$ release from palmitate and stearate during α-oxidation by mixed rumen ciliates. The triglyceride tributyrin, but not tripalmitin, stimulated the fermentative activities of both *I. intestinalis* and *I. prostoma* (106, 264). Other fatty acids that stimulated manometrically monitored fermentative activity were short-chain fatty acids (C$_2$ to C$_6$), methyl myristate, and methyl laurate (106, 264). The holotrich ciliates ingest chloroplasts (228) and may be involved in the degradation of the galactolipid component of the complex lipids of the plant leaf. The organisms possess galactosidase activity (261) and are also able to transform long-chain fatty acids.

The role of the holotrich ciliates in biohydrogenation is equivocal. Although rumen content of defaunated animals are only slightly less effective than normal rumen contents in the biohydrogenation of oleic and linoleic acids (68), the presence of protozoa in the rumen markedly increased the plasma-saturated fatty acid levels of sheep receiving cotton seed oil (1). Wright (266) demonstrated hydrogenation of lipids by rumen protozoal suspensions containing principally holotrichs and *Epidinium* spp. However, subsequent studies with the holotrichs have failed to reach a common consensus. Guttierez et al., using *I. prostoma* (106), and Williams et al. with *I. intestinalis* (264) demonstrated the hydrogenation of oleic acid to stearic acid, while De Meyer et al. (81) noted that some hydrogenation of linoleic acid by mixed *Isotricha* spp. occurred prior to fatty acid incorporation. However, in three other reports (1, 39, 101) it was concluded that the holotrichs, alone, possessed little or no ability to hydrogenate added substrates. Although it was suggested that associated bacteria were participating synergistically in the transformations (39, 101), the extent of, and dependence upon, the bacterial involvement was not fully assessed. Contradictory reports have also been made regarding the desaturating

ability of the holotrich ciliates. Whereas Emmanuel (94) proposed that octadecenoic acids were formed by direct desaturation of saturated acids, Abaza et al. (1) concluded that pure suspensions of both holotrich and entodiniomorphid ciliates had no desaturation activity.

Intermediary Metabolism

The variety of substrates ingested and transformed by the holotrichs is well documented. These metabolites are important in the nutrition of the host but, despite this, the intermediary metabolism of the ciliates has not been studied extensively. The pathways leading to product formation and the factors influencing the expression of these activities warrant further examination.

Pathways of product formation. The enzymes involved in the formation of the principal acidic metabolites of the holotrichs have now been detected and in many instances their subcellular locations have been determined (269, 271, 272). The pathways are summarized in Fig. 3. Glucose is metabolized to pyruvate via the Embden-Meyerhof-Parnas glycolytic pathway. In the absence of glucose-6-phosphate dehydrogenase, pathways involving 6-phosphogluconate could not operate (269). The lower specific activities of aldolase and triosephosphate isomerase indicated their role in the control of pyruvate formation (272). Radioactive labeling also suggested the existence of the Embden-Meyerhof-Parnas glycolysis pathway in *Entodinium caudatum* (48). The tricarboxylic acid and glyoxylate cycles are inoperative in *D. ruminantium*, and the absence of formate dehydrogenase indicates that hydrogen is formed from pyruvate by a reaction analogous to that found in saccharolytic clostridia and the flagellate trichomonads (22, 269).

The conversion of pyruvate to acetate and hydrogen in both *D. ruminantium* (269, 271) and *Isotricha* spp. (270) occurs within a microbody-like, subcellular organelle, the hydrogenosome, previously characterized only in certain aerotolerant parasitic flagellates (170). The enzymes associated with the organelle and the regeneration of CoA during acetate formation, however, differ in the holotrich and flagellate (trichomonad) hydrogenosomes. The oxygen-sensitive enzymes pyruvate synthase and hydrogenase were associated with the organelle in the holotrichs, whereas the oxygen-tolerant malate dehydrogenase was nonsedimentable and cytosolic in location, indicating that compartmentation within the hydrogenosome was affording some protection to the more sensitive enzymes (269). The enzymes converting acetyl-CoA to acetate were shown to be acetate kinase and phosphate acetyltransferase (271); the regeneration of free CoA in the ciliate does not therefore involve the succinate thiokinase/acetate–succinate CoA transferase system found in trichomonads (171). The major route of ATP synthesis in *D. ruminantium* occurs via glycolysis and the protozoon obtains at least 4 ATP/glucose from fermentation to pyruvate and the further conversion of pyruvate to acetate (272). The formation of butyrate from acetyl-CoA is essentially the same as that for the saccharolytic clostrida and the rumen bacterium *Butyrivibrio fibrisolvens* (168). In *D. ruminantium* (272) and *I. prostoma* (210) the following enzymes have been detected: acetyl-CoA:acetyl-CoA C acetyltransferase, 3-hydroxybutryl-CoA dehydrogenase, 3-hydroxyacyl-CoA hydrolyase, and 3-hydroxyacyl-CoA reductase. The subsequent production of butyrate from butyryl-CoA occurs via butyryl phosphate and generates ATP, involving the enzymes phosphate butyryltransferase

and butyrate kinase (272). The slow acetyl-CoA-dependent oxidation of reduced nicotinamide adenine dinucleotide (NADH) by cell-free extracts of the ciliates indicated that the acetyl-CoA:acetyl-CoA C acetyltransferase reaction was rate limiting (272).

The activity of the lactate dehydrogenase of *I. prostoma* was not influenced by fructose diphosphate and was maintained over a wide pH range (pH 5.5 to 7.5) (63; G. H. M. Counotte, Antonie van Leeuwenhoek J. Microbiol. Serol. **45**:614, 1979). The K_m values of the enzyme in crude cell extracts for pyruvate and NADH were 1.33 mM and 15 μM, respectively (63). The most important regulatory factors were ATP and the ratio of NADH/NAD plus NADH (63; Counotte, Antonie van Leeuwenhoek J. Microbiol. Serol. **45**:614, 1979).

Studies on the subcellular distribution of all glycolytic enzymes indicated that they were predominantly nonsedimentable by centrifugation at 6×10^6 g-min. There is no evidence for the presence of the subcellular organelle; the glycosome in the holotrichs and this organelle would therefore appear to be confined to trypanosomid protozoa. The enzymes leading to the formation of butyryl-CoA also have a cytosolic location, whereas the activity of the lactate dehydrogenase (269), phosphoacetyltransferase, acetate kinase (271), phosphobutyryltransferase, and butyrate kinase (272) is present both in the cytosol and in association with the hydrogenosome.

The evidence available indicates that identical metabolic pathways lead to product formation in *D. ruminantium* and *Isotricha* spp.

Metabolic consequences of oxygen. Hungate (136) observed that the holotrichs maintained motility in the presence of oxygen longer than other rumen protozoa and that concentrated suspensions of holotrichs remained viable for long periods in the presence of air, although dilute suspensions were unable to survive unless anaerobiosis was maintained (98). Oxygen is present in low concentrations in both the rumen liquid and gas phases and has pronounced effects on metabolic activities (e.g., methanogenesis) (123, 221). The holotrich ciliates are described historically as obligate anaerobes. However, recent studies on their interaction with environmental O_2 suggest that they are aerotolerant anaerobes.

(i) Respiratory activity. *D. ruminantium* remains viable only for a few minutes in the presence of air (O_2; 21 kPa), but it respires and can survive long periods under 1% (1 kPa) O_2 (271) and is as capable as aerobic protozoa of utilizing oxygen at low tensions (155). The apparent K_m values for O_2 determined by bacterial bioluminescence for *D. ruminatium*, *Tritrichomonas foetus*, and *Tetrahymena pyriformis* were 1.70 (25°C), 1.08 (19°C), and 2.43 (21.5°C) μM, respectively (155). The K_m values determined at 39°C, using mass spectrometry, for *Isotricha* spp. and *D. ruminantium* were 2.3 ± 0.5 and 0.34 ± 0.2 μM, respectively (121). Although these oxygen affinities are of the same order, the oxidases involved are probably different from those found in mitochondria of aerobic organisms. Mitochondria are not present in rumen holotrichs and the major organelle of carbon metabolism is the hydrogenosome, which produces hydrogen under anaerobic conditions, but, as in the aerotolerant trichomonad flagellates (38, 172), acts as a respiratory organelle in the presence of oxygen (271). As in *Tritrichomonas foetus* respiration by disrupted organelles was highest in the presence of CoA in combination with pyruvate, suggesting that oxygen uptake accompanied the conversion of pyruvate to acetyl-CoA. Hydrogenosomal respiration of pyruvate was
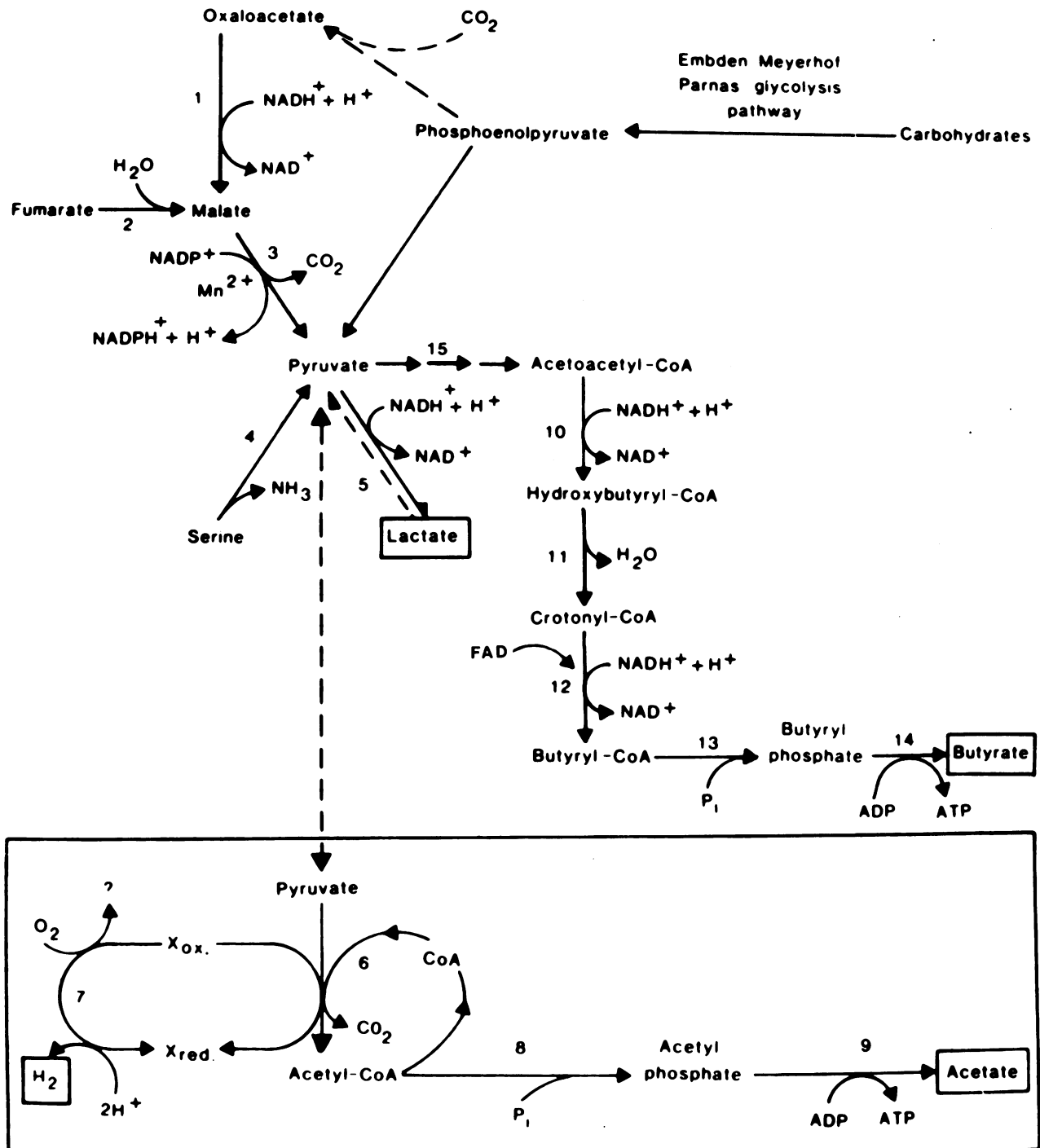
FIG. 3. Proposed pathway of fermentation in *D. ruminantium*. ADP, Adenosine diphosphate; P_i, inorganic phosphate; ox., oxidized; red., reduced. Enzymes are as follows: 1, malate dehydrogenase; 2, fumarase; 3, malate dehydrogenase (decarboxylating); 4, serine dehydratase; 5, lactate dehydrogenase; 6, pyruvate synthase; 7, hydrogenase; 8, phosphoacetyltransferase; 9, acetate kinase; 10, 3-hydroxybutyryl-CoA dehydrogenase; 11, 3-hydroxyacyl-CoA hydrolase; 12, 3-hydroxyacyl-CoA reductase; 13, phosphate butyryltransferase; 14, butyrate kinase; 15, acetyl-CoA:acetyl-CoA C acetyltransferase. Reactions associated with the hydrogenosome are shown in the box.

also stimulated by inorganic phosphate (271), which substantiated the association of phosphoacetyl-transferase and acetate kinase with the organelle. Increased acetate production by *Isotricha* spp. in the presence of O_2 has also been reported (210). The hydrogenosomal fraction isolated from mixed entodiniomorphid protozoa also exhibited respiratory

activity (226; L. Snyers et al., Arch. Int. Physiol. Biochim. **89**:B201–B202, 1982).

The respiration of *D. ruminantium* was unaffected by the mitochondrial inhibitor antimycin A, and this with the electron microscopic evidence of Yarlett et al. (269) confirms that the preliminary descriptions of mitochondria in the

rumen holotrichs were erroneous (98, 102, 210). The organelles observed, on the basis of morphology and location, were probably hydrogenosomes.

D. ruminantium, like *Tritrichomonas foetus* (154), lacks detectable cytochromes (<2 pmol/mg of protein) and the nature of the terminal oxidase is not known. The low sensitivity of the respiratory activity to cyanide and azide (155, 271) with inhibition by chloroquine and quercetin (quinoline derivatives) would indicate the presence of flavoprotein-iron-sulfur-mediated electron transport as detected in *Tritrichomonas foetus* (182).

The respiratory system may function to minimize intracellular oxygen tensions as a protection for oxygen-sensitive systems. However, both *D. ruminantium* (269) and *Isotricha* spp. (210, 270) possess functional NADH oxidase and NADH peroxidase activities, which may also have a role in protecting oxygen-sensitive systems from the effects of dioxygen and its reduction products. Low levels of catalase were detected in *Isotricha* spp. by Yarlett et al. (270) but not by Prins and Prast (210). The other recognized protective enzyme, superoxide dismutase, is absent from both holotrich genera (269, 270). The NADH oxidase and NADH peroxidase are located primarily in the cytosol (nonsedimentable cell fraction), although some activity (approximately 35%) was associated with the particulate hydrogenosome-containing subcellular fraction (269, 270). Prins and Prast (210) incorrectly described the subcellular location of the NADH oxidase as mitochondrial but the ferricyanide reductase in the *Isotricha* extracts was similar in many ·characteristics to the mitochondrial enzyme from mammalian sources (210). The NADH oxidase and NADH peroxidase from both *Dasytricha* and *Isotricha* spp. were susceptible to the same inhibitors (e.g., cyanide, azide) (210, 269, 271). Prins and Prast (210) estimated that *Isotricha* spp. would consume approximately 3 pmol of oxygen/cell per min; maximum oxygen consumption by the cytosolic fraction of *D. ruminantium* in the presence of reduced pyridine nucleotide was 10 to 15 pmol of oxygen/cell per h (269). ATP formation occurred during the oxidation of NADH by *Isotricha* spp. (210), although the P/O ratio was low (0.1 to 0.35).

(ii) **Product formation.** The overall significance of oxygen metabolism is unclear. Although the respiratory functions may serve only to protect the protozoa, the respiratory generation of ATP will benefit the organisms. Oxygen uptake accompanies the hydrogensomal conversion of pyryvate to acetyl-CoA, the subsequent formation of acetate generating ATP via substrate-level phosphorylation. Prins and Prast (210) showed that in *Isotricha* spp., in the presence of small amounts of oxygen (<20 μmol per incubation tube), the proportion of acetate formed increased with a concomitant decrease in butyrate and hydrogen formation. The effect of oxygen on hydrogen production has been quantified by mass spectrometry (121, 273). Hydrogen production was reversibly inhibited at oxygen tensions of <2.8 nmol/ml (273). The inhibition of hydrogen production increased almost linearly with increasing oxygen concentrations up to levels typical of the rumen environment (<1.6 μM [221]). Higher oxygen tensions resulted in an irreversible inactivation of the hydrogenase system and hence hydrogen production (121, 273). The apparent K_i of oxygen for inhibition of hydrogen formation by *D. ruminantium* and *Isotricha* spp. was 1.11 ± 0.90 and 1.65 ± 0.47 μM, respectively (121). It is apparent that hydrogen generation in the rumen by the holotrich ciliates will be markedly influenced by the physiological levels of oxygen.

The incorporation of [^{14}C]acetate and [^{14}C]mevalonate into the sterol fraction of mixed rumen ciliates was enhanced in the presence of oxygen (126), and it was suggested by the authors that molecular oxygen was essential for de novo sterol synthesis by the rumen ciliates.

IMPORTANCE OF PROTOZOA IN THE RUMEN ECOSYSTEM

The protozoa contribute to the nutrition of the host not only by their metabolic activities, but also through the postruminal degradation and utilization of the protozoal cells. Although ciliate protozoa are found in all ruminants, numerous studies have been carried out to determine the effects of defaunation on animal production. The results have been contradictory. Furthermore, it is also difficult to quantitatively partition the metabolic contributions of the bacteria and protozoa, although it is accepted that the bacteria are more active on a cell mass basis because of their greater total surface area. There have been no specific studies to assess the importance of the holotrich population per se, and all conclusions relate to the effects of the total ciliate population.

The effects of the protozoal population on ruminant development have been reviewed (29, 56), the majority of studies indicating that either no major differences were apparent or that the average daily weight gain of defaunated animals was lower. However, dietary nitrogen is used less efficiently in the presence of ciliate protozoa, and with low protein diets defaunation was associated with increases in the efficiency of food utilization, growth rate, and wool production (29). Rumen volume (204) and the concentrations of ammonia and VFA in the rumen are frequently, but not always, higher in faunated animals and the relative proportions of the VFA also differ, with faunated animals having increased butyrate or propionate levels. The disparities in the results obtained imply that other factors (e.g., dietary regime, population changes on refaunation, etc.) are also important determinants, and it is apparent that the protozoal involvement in rumen metabolite formation is affected by many environmental interactions.

Protozoa and Nutrition of the Host

The relative importance of holotrich protein, lipid, and carbohydrate in fulfilling the dietary requirements of the host is dependent upon the population size, the outflow of holotrich biomass from the rumen, and the bioavailability of the cellular constituents in the lower digestive tract. It is now well established that protozoa are selectively retained within the rumen and only leave at 20 to 40% of the predicted rate (252). In addition, although the holotrichs are not usually the numerically dominant ciliates present, they are among the larger organisms with mean volumes of approximately 10^5 (*Dasytricha* sp.) and 10^6 (*Isotricha* spp.) μm^3 (47, 52, 210) and will thus contribute significantly to the protozoal biomass leaving the reticulorumen.

The digestion of holotrich carbohydrate, i.e., amylopectin, that enters the small intestine is unlikely to be of nutritional significance to the host (116, 117), although its formation and fermentation by the ciliates in the rumen may be of value (see next section).

Rumen protozoa contain a higher proportion of unsaturated fatty acids than the bacteria and the protozoa thus represent a potential source of lipid for the host animal (148). Some 10 to 20% of the lipid present in the rumen digesta is microbial and approximately 75% of this is protozoal (149).

TABLE 4. Amino acid composition of acid hydrolysates of *Isotricha* spp. and bacteria[a]

Amino acid	Composition (g/100 g, dry wt)	
	Isotricha spp.	Mixed rumen bacteria[b]
Alanine	5.9	7.8
Arginine	4.4	4.9
Aspartic acid	17.4	10.8
Cystine	0.9	0.9
Glutamic acid	12.5	13.7
Glycine	4.5	5.2
Histidine	1.6	1.7
Isoleucine	6.9	6.6
Leucine	8.2	7.5
Lysine	11.4	8.2
Methionine	2.2	2.1
Phenylalanine	2.6	5.3
Proline	3.6	4.6
Serine	4.1	4.7
Threonine	5.3	5.3
Tyrosine	3.4	3.9
Valine	5.1	6.8

[a] Data compiled from Williams and Dinusson (263).

[b] Isolated from defaunated animals.

I. prostoma and *I. intestinalis* have a similar lipid content (7 to 9%), with palmitic acid being the main aliphatic component (106, 264). Girard and Hawke (101) estimated that the *Isotricha* population represented 20 to 25% of the ruminal linoleic acid of a cow on a hay diet, and some of the small amount of polyunsaturated fatty acids which escape from the rumen without undergoing hydrogenation may originate from holotrich lipids. Unsaturated fatty acids are partially protected from hydrogenation by the incorporation into protozoal phospholipids (81, 101, 149).

Holotrich protein is important to the host. Analysis of mixed holotrichs (128) and the two species of *Isotricha* (111, 263) has provided information on the amino acid content of the ciliates (Table 4). Despite the retention of protozoa within the rumen, a significant proportion of the microbial protein available to the host is protozoal in origin (19 to 24%) (55). A dairy cow on a maintenance ration requires 500 g of protein per day. Gutierrez (103) estimated that approximately 33 g of holotrich protein would be available to the host daily from a bovine rumen containing a holotrich population of 3,000 *Isotricha* spp. and 5,000 *Dasytricha* sp. per ml. The estimation is too high because sequestration within the rumen was not considered. It has been speculated that the holotrichs may accumulate and conserve amino acids that are deficient in plants (52). Although the biological value of bacterial and protozoal proteins are similar, the protozoa are more susceptible to digestion (26, 147, 159).

All rumen ciliates engulf and digest bacteria. Bacterial degradation products released by the protozoa are degraded and assimilated by the rumen microflora. The ensuing fermentation of the liberated bacterial amino acids, although stimulating bacterial growth, represents an important loss of amino acids to the host. Coleman (52) calculated that in the rumen 1% of the bacteria were engulfed in 1 min and that a protozoal population of 10⁶/ml would digest 2.5 to 45 g of bacteria in the ovine rumen each day (5 liters of ovine rumen contents contains approximately 50 g of bacteria). Bacterial engulfment by *I. intestinalis* and *I. prostoma* was estimated at 0.5 g each day (52, 246), although Coleman (52) speculated that starved ciliates would engulf bacteria more rapidly.

Role in Rumen Metabolism

In addition to their nutritional role the protozoa make an important contribution to rumen metabolism. The holotrichs not only contribute to short-chain VFA production, but also to some extent control the overall rate at which the acids are formed. Substrate removal by the protozoa prevents a rapid bacterial fermentation to lactic acid. It has been proposed that the protozoal ingestion of starch grains (156, 206) or soluble sugars (118) is beneficial to the host animal because the alternative bacterial fermentation would lead to an accumulation of lactate in the rumen and a detrimental lowering of pH. Starch is ingested actively by *Isotricha* spp. and soluble sugars are ingested by both holotrich genera. On high sugar diets the holotrich protozoa may help to prevent the onset of lactic acid acidosis by rapidly assimilating soluble sugars into amylopectin. The subsequent fermentation of the ingested starch and storage polysaccharide over a prolonged period thus modulates the rate of VFA formation and effectively prolongs and stabilizes the fermentation of ingested food components. Coleman (55) estimated that approximately one-third of the sugars consumed by the animal were converted into amylopectin by the holotrich protozoa. Several authors have attempted to estimate the contribution made by the holotrichs to total VFA production in the rumen (103, 212, 239, 258). Gutierrez (103) calculated that the holotrichs contributed about 10% of the total acids in the rumen. Prins and Van Hoven (212, 239) estimated that in diary cattle on rich pasture 3 to 4% of the VFA were produced by the *Isotricha* spp., with approximately 3% originating from *D. ruminantium*. However, both estimates were determined by using endogenous rates of acid formation. Although soluble sugar levels throughout the diurnal cycle are maintained at a level sufficient to raise fermentation above the endogenous rate for only 2 to 3 h after feeding, the enhanced fermentative activity of the holotrichs during this period may increase their overall contribution. The holotrichs also have the potential to degrade polymeric carbohydrates to free fermentable substrates, and it is apparent that more information is needed on the relationship between diet and metabolite formation rates so that the contribution made by the holotrichs can be ascertained.

In coculture with the holotrich ciliates *Methanosarcina barkeri* continues to form methane when oxygen is present at levels known to be inhibitory to the methanogen in monoculture (Hillman et al., unpublished data). The holotrich ciliates may thus help to sustain anaerobic conditions in the rumen. In animals fed twice daily the oxygen concentration in the liquid phase is approximately 1 to 1.5 μM (221). At this oxygen level, the rate of uptake by both holotrich genera is approximately 500 nmol/ml per h per 10⁵ cells (121), i.e., an oxygen uptake of 11.2 ml of O_2 per liter per h. This represents a total oxygen uptake by *Dasytricha* or *Isotricha* spp., in a bovine rumen of 100 liters volume, of 26.8 liters/day. This is considerably higher than the value of 1 liter/day estimated by Prins and Prast from NADH oxidase activity (210). Using the oxygen consumption rate of rumen contents quoted by these authors (32 ml/liter per h), it can be seen that a holotrich population containing 10⁴ cells of each genus per ml would be potentially able to consume 5.4 liters of oxygen each day. Approximately 7% of the oxygen consumed in the rumen would be utilized by this relatively small holotrich population. In defaunated animals a transient postfeed increase in rumen liquor oxygen levels occurs and this increase was not observed in faunated animals (122).

An unexamined aspect of oxygen scavenging by the

holotrichs relates to their sequestration on the reticulum wall (3). A proposed role for the epithelial microbial population is the utilization of oxygen diffusing across the wall, and unusually high members of facultative organisms have been identified in this specific population (40). The holotrich ciliates would also be effective in utilizing oxygen, and their proposed migration to the wall shortly after feeding may reflect the higher availability of oxygen at this location for the more energetically attractive respiratory generation of ATP during storage polysaccharide utilization. Oxygen levels in the liquid phase of rumen contents fall markedly (≤ 0.25 μM) in the immediate postfeed period (221).

The widespread occurrence of holotrichs in both domesticated and wild ruminants is evidence that these ciliates can compete effectively with the bacterial population and are well adapted to the rumen environment. However, their contribution to the host is still somewhat speculative and a unique protozoal contribution has yet to be established. The protozoa do contribute to both metabolite formation in the rumen and the nutritional well-being of the host ruminant. Recent studies (29, 153, 252) confirm, however, that only a small proportion of the ciliates move down the tract, with approximately two-thirds of the population dying/lysing within the rumen. The value and availability of protozoal cell components to the host are, therefore, in doubt. There is evidence to indicate that defaunation increases the availability of protein to the host, although the effects on energy availability are unresolved (29).

The holotrichs, to some extent, modulate and prolong the fermentation period, and although this may be of value in maintaining an active microbial population in animals receiving a poor-quality diet, such animals would probably benefit from defaunation since a positive response to defaunation occurs when the diet fails to meet the protein requirements of the animal. However, dietary formulations are such that this problem does not arise in intensive animal production systems. There would appear to be no advantage to defaunation unless further studies can demonstrate marked improvements in animal productivity. Furthermore, the defaunating agents currently available are unsatisfactory and some may endanger animal health. The practicalities of establishing and maintaining defaunated herds and their subsequent management to avoid reinoculation by conventional domesticated and wild ruminants should also be considered.

The contribution of the holotrich ciliates to ruminant productivity therefore warrants additional investigation. It is apparent that much has still to be discovered about the biochemistry and behavior of this unique group of microorganisms before their role in the rumen ecosystem is fully appreciated.

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