On the role of higher plant and microbial lipases in the ruminal hydrolysis of grass lipids

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1. The galactolipids of heat-treated, ^{14}C-labelled rye grass S24 administered intraruminally to a sheep fed on an autoclaved diet were rapidly catabolized.

2. When grass was homogenized with rumen contents devoid of higher plant lipases the grass galactolipids were rapidly metabolized, but were not metabolized when the rumen contents were boiled to destroy microbial galactolipases.

3. ^{14}C-labelled monogalactosyldiglyceride, digalactosyldiglyceride and triolein were metabolized, with the release of ^{14}C-labelled fatty acids when incubated with a homogenate (100 g/l) of grass or clover in rumen fluid from a starved sheep, but not when the rumen fluid was heat-treated to destroy microbial enzymes.

4. It is concluded that in the sheep the lipases of rumen micro-organisms play a major part in the ruminal degradation of ingested complex lipids of pasture.

It has generally been assumed that the extensive hydrolysis of dietary triglycerides occurring in the rumen is a property of microbial lipases (Garton, Lough & Vioque, 1961). In a recent investigation of the digestion of ^{14}C-labelled grass in the rumen of sheep it was concluded that the ability to decompose grass galactolipids and lecithin was again mainly a function of the microbial lipases rather than that of the plant enzymes ingested in the pasture diet (Dawson & Hemington, 1974). The rate of hydrolysis of galactolipids and phospholipids in rumen fluid from a pasture-fed sheep was found to be much higher than the value calculated from the lipase activity in the pasture content of the rumen fluid; this was calculated from the spectral analysis of chlorophyll and its breakdown products. Since it was also likely that at least some of the pasture lipolytic enzymes would be decomposed after ingestion, this finding suggested that the hydrolysis of herbage lipids was mostly brought about by the enzymes of micro-organisms.

In contrast to this conclusion, Omar Faruque, Jarvis & Hawke (1974) suggested that in pasture-fed cows the lipolysis of triglycerides and galactolipids (monogalactosyldiglyceride) was mainly due to the activity of higher plant enzymes, and that the rumen micro-organisms had a subsidiary role in the hydrolysis of ingested lipid. They showed that some plant lipases retained their activity for at least 5 h in the presence of metabolizing rumen micro-organisms, and, in parallel experiments that the lipolytic activity of rumen micro-organisms metabolizing autoclaved fescue (*Festuca arundinacea mediterranean*)-leaf extract was very low.

In the experiments reported in this paper we have used a number of experimental approaches to study the source of the enzymes responsible for the digestion of grass galactolipids and, to a lesser extent, triglyceride in the rumen of the pasture or hay-fed sheep. Our observations lead us to conclude that under our conditions and with this particular ruminant the lipases of rumen micro-organisms are more important than the plant lipases in the degradation of higher plant lipids in the rumen.

EXPERIMENTAL

In vivo experiments

^{14}C-labelled grass (Dawson & Hemington, 1974) was introduced into Clun Forest sheep with fistulated rumens. The sheep had been fed previously on either pasture to appetite, or were fed once/d 1·2 kg hay–oats (5:1, w/w) which had been autoclaved at 121° for 1 h to inactivate plant lipolytic enzymes. Serial samples were withdrawn from the rumen, the lipids extracted and the radioactivity determined in monogalactosyldiglyceride, digalactosyldiglyceride and β-carotene by methods described previously (Dawson & Hemington, 1974). The amount of each galactolipid remaining was calculated from the ratio, radioactivity in galactolipid:radioactivity in β-carotene, the latter being a carotenoid which is not metabolized in the rumen.

In vitro experiments

Mixed pasture grass was homogenized with rumen fluid (100 g/l) using an Ultra Turrax homogenizer (Camlab, Cambridge). These proportions of grass and rumen fluid correspond to the sheep eating 500 g pasture grass assuming a rumen volume of 5 l. The rumen fluid was removed through a fistula and very coarse food material was removed by straining through 'butter' muslin; when appropriate the strained rumen fluid was heated at 100° for 8 min to inactivate lipolytic enzymes.

The grass homogenates were incubated at 39° under nitrogen–carbon dioxide (19:1, v/v). After the required period of incubation, 10 vol. acetone were added and the mixture heated to boiling for 2 min. After cooling, the acetone suspension was filtered through glass-wool, and the residue washed with 1–2 vol. acetone.

Re-extraction of the residue with chloroform–methanol (2:1, v/v) indicated the presence of a small amount of residual galactolipid ($< 5\%$) but this second extraction was not done routinely. The combined acetone extracts were taken to dryness under reduced pressure and the residue extracted into 5 ml chloroform–methanol (2:1, v/v). After adding 1 ml calcium chloride solution (0·5 g/l) and shaking, the mixture was centrifuged and the lower chloroform-rich phase analysed for glycolipids. Portions (0·03 ml) of the chloroform phase were applied to a thin-layer plate coated with silica-gel (Kieselgel 60 F-254; E. Merck AG, Darmstadt, Germany), which was developed first with chloroform as the developing solvent to separate neutral lipids and pigments, which moved near the solvent front, and after drying the plates, they were developed in the same direction with the solvent system chloroform–methanol–acetic acid–water (85:15:10:4, by vol.) which separated the more polar lipids including the galactolipids. Finally the thin-layer plates were developed with diethyl ether as the solvent; this separated the monogalactosyldiglyceride from a polar pigment band. The separated lipids were located by spraying with Vaskovsky & Kostetsky's (1968) reagent. Areas corresponding to phosphorus-containing lipids were marked, and the thin-layer plates were then baked in an oven at 105° for 40 min to char all lipid-containing areas of silica gel. Mono- and digalactosyldiglycerides were well separated from other lipids (Fig. 1) and the intensity of charring gave an approximate measure of galactolipid breakdown.

The amounts of galactolipid were estimated by exposing the thin-layer plates to iodine vapour and marking the appropriate areas of silica gel. After volatilization of the I_2, the area of silica gel corresponding to galactolipid was transferred to a centrifuge tube. Galactolipids were estimated by a method based on that described by Roughan & Batt (1974). An aqueous solution of phenol (20 g/l) (1 ml) was added to each tube, followed by 4 ml concentrated sulphuric acid. The solution was mixed, allowed to cool for 15 min at room temperature and then the extinction at 480 nm measured. Determinations were carried out

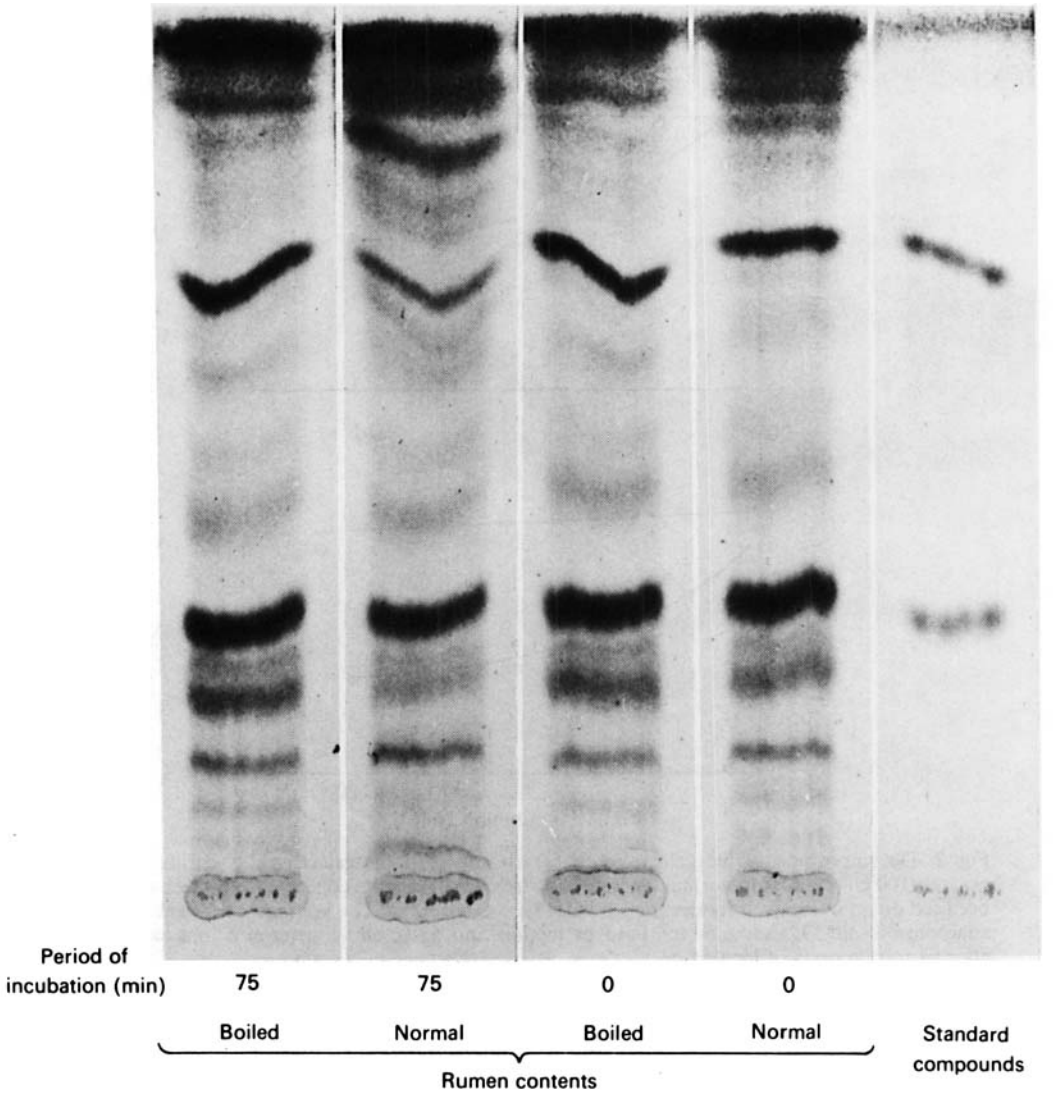

Fig. 1. Chromatogram of the lipids extracted from incubates of homogenates (100 g/l) of grass in normal or heat-treated rumen contents from a sheep which had been fed for 7 d on autoclaved fodder. The homogenates were incubated anaerobically for 75 min at 39°, the lipids extracted and chromatographed on thin-layer silica-gel plates (for details, see pp. 226–228) together with standard compounds. The main lipids seen after charring the thin-layer plates were monogalactosyldiglyceride (fast running) and digalactosyldiglyceride (slow running).

in triplicate with galactose being used as a standard: this gave a linear colour response between 0 and 50 μg galactose.

Radioactive lipid substrates and incubation with homogenates

The preparation of ^{14}C-labelled mono- and digalactosyldiglyceride from rye grass S24 has been described (Dawson & Hemington, 1974). Glyceryl tri[1-^{14}C]oleate was obtained from the Radiochemical Centre, Amersham, Bucks. At the end of each incubation period the incubate was treated first with 20 vol. chloroform–methanol (2:1, v/v) and then, after

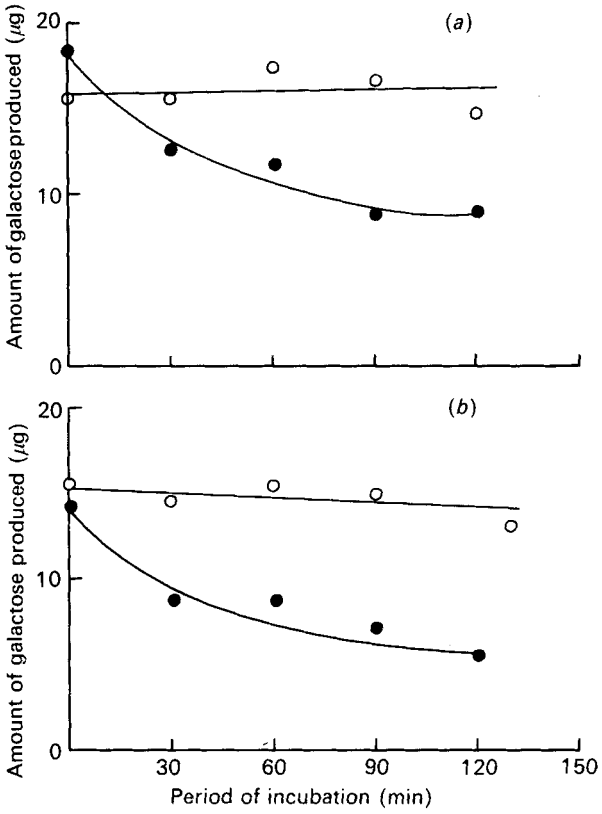

Fig. 2. Decomposition of the galactolipids, (*a*) di- and (*b*) monogalactosyldiglyceride of homogenates (100 g/l) of grass in normal (●) and heat-treated (○) rumen contents from sheep which had been fed on an autoclaved fodder for 5 d before the experiment. The homogenates were incubated anaerobically at 39°. Samples for lipid extraction and galactolipid determinations were taken after various periods of incubation.

shaking, with 1·5 vol. water. A portion of the lower phase was chromatographed on a thin-layer silica-gel (Kieselgel 60 F 254) plate using chloroform–methanol–ammonia (sp.gr. 0·880) (35:10:1, by vol.) as developing solvent to separate galactolipids, or hexane–diethyl ether–acetic acid (70:30:1, by vol.) to separate triolein; appropriate galactolipid and oleic acid standards were applied to each thin-layer plate. The galactolipids and fatty acids were located by exposure to I_2 vapour and the appropriate areas of silica gel transferred to a vial containing scintillation fluid (Unisolve; Koch-Light, Colnbrook, Bucks.), and their ^{14}C content determined using a liquid-scintillation counter (Unilux II; Nuclear Chicago, Illinois, USA).

RESULTS

Fig. 1. indicates the extent of breakdown of galactolipids in homogenates of grass in both normal and heat-treated rumen fluid from a hay-fed sheep. There was marked breakdown of the grass galactolipids incubated with the normal rumen fluid compared with that for rumen fluid in which galactolipases had been inactivated by boiling. Since the sheep had been given autoclaved fodder for 7 d before removal of rumen fluid samples, it can be assumed that the lipases involved in the breakdown were of microbial origin rather than higher plant origin.

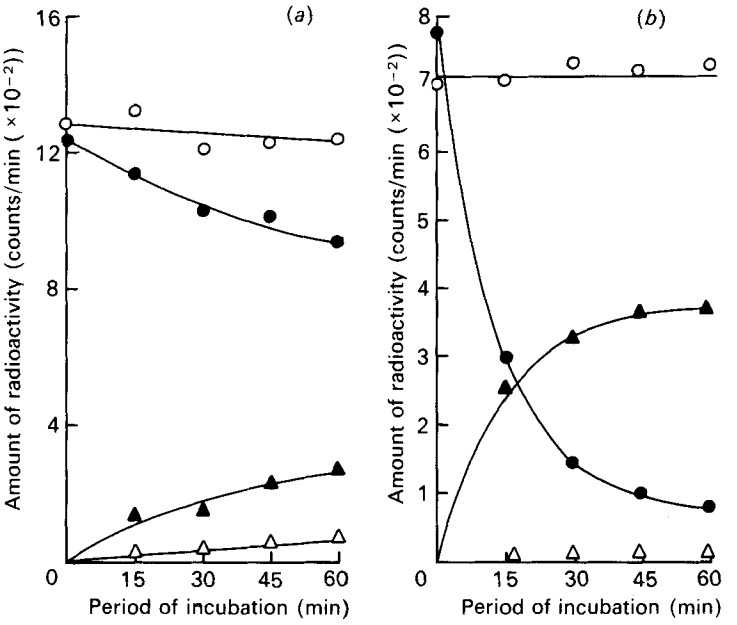

Fig. 3. The degradation of ^{14}C-labelled galactolipids, (*a*) mono- and (*b*) digalactosyldiglyceride, by homogenates (100 g/l) of grass in normal and heat-treated rumen contents from pasture-fed sheep which had been fasted for 24 h before samples of rumen contents were taken. [^{14}C-]monogalactosyldiglyceride or [^{14}C-]digalactosyldiglyceride were incubated with the homogenates and samples removed after various periods of incubation. The samples were extracted with lipid solvents and the amounts of radioactivity remaining in the galactolipid for normal (●) and heat-treated (○) rumen contents, and those amounts accumulating in the free fatty acid fraction were estimated for normal (▲) and heat-treated (△) rumen contents. For details of procedures, see pp. 226–228.

The extent of the breakdown, determined in similar experiments, is shown in Fig. 2. In a 2 h incubation period the extent of disappearance of both the monogalactosyldiglyceride and digalactosyldiglyceride of the grass was much higher when incubated with normal rumen contents than when the rumen contents were boiled. The rumen contents in the homogenate contained insignificant amounts of galactolipids compared with the grass even though certain rumen micro-organisms contain small amounts of galactolipids in their membranes (Clarke, Hazlewood & Dawson, 1976). In contrast the phospholipids present in the grass were significantly broken down by incubation with the boiled rumen contents although here again there was a much more active degradation in the presence of viable rumen micro-organisms.

Similar results were obtained in experiments using rumen fluid from a pasture-fed animal, in which homogenates containing 100 g grass and normal rumen fluid broke down galactolipids more readily than homogenates where the rumen fluid was inactivated by boiling.

In further experiments samples of rumen contents were taken from a pasture-fed sheep starved for 24 h to eliminate the higher plant lipases from the rumen. The rumen fluid either directly or after heat-treatment was homogenized with grass (100 g/l) and the resulting mixtures incubated with either ^{14}C-labelled mono- or digalactosyldiglyceride. Degradation of [^{14}C]galactolipids or production of ^{14}C-labelled fatty acids was low with grass homogenates which contained heat-treated rumen fluid, compared with the marked decomposition found with the grass homogenates containing normal rumen fluid (Fig. 3). As was found in a previous study (Dawson & Hemington, 1974), the breakdown of pure digalactosyldiglyceride occurred at a faster rate than that of monogalactosyldiglyceride when

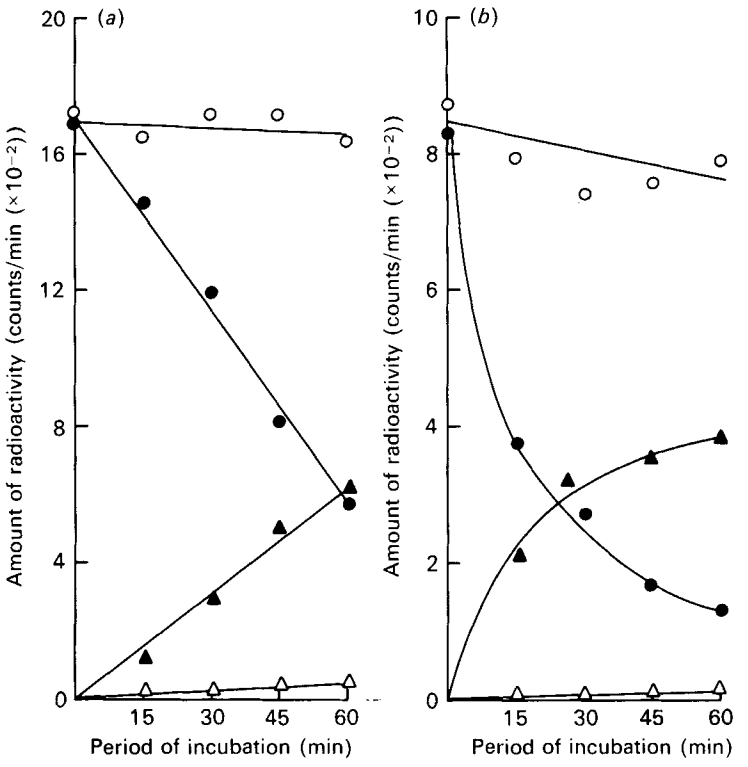

Fig. 4. The degradation of ^{14}C-labelled galactolipids, (*a*) mono- and (*b*) digalactosyldiglyceride, by homogenates (100 g/l) of clover (*Trifolium repens*) in normal and heat-treated rumen contents from pasture-fed sheep which had been fasted for 24 h before samples of rumen contents were taken. [^{14}C-]monogalactosyldiglyceride or [^{14}C-]digalactosyldiglyceride were incubated with the homogenates and samples removed after various periods of incubation. The samples were extracted with lipid solvents and the amounts of radioactivity remaining in the galactolipid for normal (●) and heat-treated (○) rumen contents and those amounts accumulating in the free fatty acid fraction were estimated for normal (▲) and heat-treated (△) rumen contents. For details of procedures, see pp. 226–228.

incubated with rumen contents. The reverse situation is found in normal ruminal digestion, and is possibly attributable to the greater dispersion of digalactosyldiglyceride when it is presented as a pure substrate rather than in a plant membrane complex. Similar results were obtained when clover (*Trifolium repens*) (100 g/l) was used instead of grass in the preparation of the rumen fluid homogenates (Fig. 4). Similar results were obtained using grass homogenates and ^{14}C-labelled triolein, with greater amounts of labelled fatty acid being liberated when the rumen fluid was not heat-treated (Fig. 5).

Fig. 6 shows the extent of degradation of galactolipids in the rumen of a sheep after the administration of finely-ground ^{14}C-labelled grass. The clearance of [^{14}C]galactolipids from the rumen of pasture-fed sheep is compared with that of sheep fed on autoclaved fodder before the administration of ground ^{14}C-labelled grass which had been boiled to inactivate lipolytic enzymes. The rate of decomposition of galactolipids, and particularly mono-galactosyldiglyceride, was more rapid in the experiment with pasture-fed sheep; however it was clear that for both feeding regimens, and in experiments in which higher plant lipases were absent, a rapid hydrolysis of the ^{14}C-labelled grass glycolipids occurred and was essentially completed 11 h after administration of the grass.

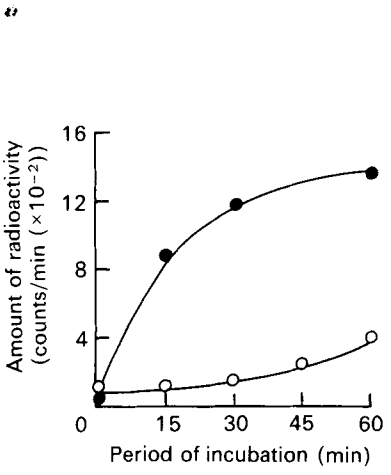

Fig. 5

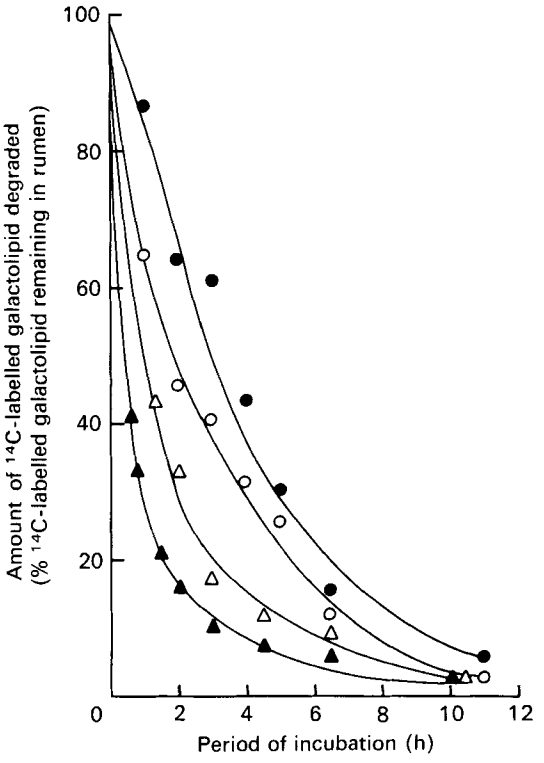

Fig. 6

Fig. 5. The release of fatty acid from [^{14}C]triolein by homogenates (100 g/l) of grass in normal (●) and heat-treated (○) rumen contents from sheep fed on autoclaved fodder for 3 d before samples of rumen contents were taken. After various periods of incubation samples were removed and extracted with lipid solvents and the amount of radioactivity in the free fatty acid fraction was estimated. For details of procedures, see pp. 226–228.

Fig. 6. The degradation of ^{14}C-labelled grass galactolipids in the intact rumen of sheep fed on either pasture (●, ○, mono- and digalactosyldiglyceride respectively) or autoclaved fodder (▲, △, mono- and digalactosyldiglyceride respectively). ^{14}C-labelled grass was administered through a rumen fistula to sheep fed on autoclaved fodder, and before administration it was maintained at 100° for 8 min to inactivate plant galactolipases. Serial samples of rumen contents were taken and the lipids extracted and separated and their radioactive content estimated. Because of the difficulty in obtaining uniform samples and the movement of digesta through the rumen, the radioactive content of the galactolipids in each sample was related to the amount of non-metabolized β-carotene present, and expressed on a '% decomposition of the original [^{14}C]galactolipid administered' basis. For details of procedures, see pp. 226–228.

DISCUSSION

The results of the present experiments clearly indicated that micro-organisms of the ovine rumen can rapidly break down the galactolipids present in pasture leaf membranes. This rapid catabolism is shown by the clearance from the intact rumen of the galactolipids administered in ^{14}C-labelled grass to animals which had been fed autoclaved fodder and then given intraruminally the ^{14}C-labelled grass which had been heat-treated to eliminate any intake of higher plant lipases. In addition the breakdown of grass galactolipids or of added ^{14}C-labelled galactolipid or triolein substrates by grass homogenates in rumen fluid is only appreciable in experiments in vitro when the rumen fluid had not been boiled to

destroy the lipolytic activity of the micro-organisms. This finding of microbial breakdown of galactolipids confirmed results of recent work in this laboratory in which a number of rumen organisms containing very active galactolipases have been isolated in pure culture.

It was shown by Sastry & Kates (1964) and confirmed by others (e.g. Helmsing, 1969) that in the leaves of some plants there is an active deacylating galactolipase in chloroplasts and cell-sap cytoplasm fractions. We have shown that both grass membrane galactolipids and ^{14}C-labelled galactolipid substrates could be substantially broken down when grass homogenates in rumen saline were incubated anaerobically at 37° (Dawson & Hemington, 1974). These intrinsic lipases have also been reported by Omar Faruque *et al.* (1974), who used various strains of grass and ^{14}C-labelled triolein or monogalactosyldiglyceride as substrate. However, in this study we have consistently failed to observe this hydrolysis in grass homogenates prepared using rumen fluid and therefore presumably the galactolipase present in the grass is not active in these conditions. Results of preliminary experiments have indicated that when grass is homogenized in bovine saliva (sheep saliva being difficult to collect) then the degradation of grass galactolipids is substantially inhibited and this might therefore account for the inhibitory action of rumen fluid.

Although the present experiments indicate that the microbial lipases are more important than the higher plant lipase in breaking down ingested plant lipids in the ovine rumen, determination of the actual rates of the two processes is difficult. Thus in experiments in vitro with grass homogenates or extracts, it is virtually impossible to reproduce exactly the extent of cell breakage and dispersion caused by mastication and the precise conditions in which the disrupted grass is added to the rumen fluid as a bolus mixed with saliva. We know, however, that in the cow a large proportion of the grass galactolipids remains when the bolus is delivered to the rumen (Dawson & Hemington, 1974). There is no doubt that in the in vivo experiments done using heat-treated, ^{14}C-labelled grass administered through a rumen fistula to an animal given a lipase-free diet, the rate of [^{14}C]galactolipid clearance from the rumen is slower than when the same ^{14}C-labelled grass is given untreated to a pasture-fed animal. However, the presence of higher plant lipases in the rumen of the pasture-fed animal may not be the explanation for the faster rate of galactolipid breakdown. The autoclaved-hay diet would be virtually free from galactolipids (Dawson & Hemington, 1974) compared with the pasture diet, and consequently the microbial population in sheep given the autoclaved diet is likely to have low levels of galactolipases, if these enzymes are adaptively formed in response to dietary galactolipids. Furthermore heat-treatment of ^{14}C-labelled grass may produce physical changes, particularly in denaturing proteins, which could inhibit plant membrane disintegration and digestion (Chalmers, Jayasinghe & Marshall, 1964). At present there is no experimental system which would give a precise measure of relative importance of the two types of digestive process which occur under the normal physiological conditions in the rumen.

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