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Lipolysis and Hydrogenation of Galactolipids and the Accumulation of Phytanic Acid in the Rumen

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By administering ^{14}C-labelled grass to a sheep it has been shown that the ruminal biohydrogenation of the esterified linolenic acid of the galactolipids in chloroplast membranes only occurs after a rapid lipolysis. A slow accumulation of [^{14}C]phytanic acid is presumed to arise by oxidation of phytol residues removed from chlorophylls.

Two important changes occur to complex dietary lipids ingested by the ruminant, first lipolysis which releases free fatty acids and secondly hydrogenation of unsaturated fatty acids. There is now considerable evidence that with triglycerides the hydrolysis of the ester bonds liberating free fatty acids occurs before the hydrogenation (Garton *et al.*, 1961; Hawke & Silcock, 1969). However, triglycerides are virtually absent from the normal herbage diet of the ruminant, and the lipids occurring in the greatest concentration are the glycolipids (Roughan & Batt, 1969). These consist mainly of mono- and di-galactosyl diglycerides whose fatty acids are especially rich in linolenic acid. The latter fatty acid is known to be rapidly converted into stearic acid in the rumen (Dawson & Kemp, 1970). We have therefore carried out an investigation to test whether lipolysis precedes hydrogenation when photosynthetic tissue containing these glycolipids is being digested in the rumen of the sheep.

Perennial rye grass (Aberystwyth strain S24) was grown in a growth chamber similar to that described by Grossbard & Barton (1963) in which the $^{14}CO_2$ in the environment was automatically maintained through the use of a CO_2 monitor coupled with a generator. The grass was harvested after about 3 weeks growth, cut into 1cm pieces, and introduced into the rumen of a sheep through a fistula. The sheep was maintained on grass pasture and allowed to feed after the administration of the labelled grass. Samples (25ml) of rumen contents were withdrawn at intervals and extracted with ethanol (6vol.) at 80°C followed by the addition of chloroform (12vol.): a second extraction with 10vol. of chloroform–methanol (2:1, v/v) at 50°C was carried out. After the combined solvent extracts had been shaken with 0.2vol. of 0.9% NaCl, the lipids in the lower phase were fractionated on a silicic acid column (Mallinkrodt; 20cm×1.8cm diam.). The column was eluted first with chloroform (300ml) and then with acetone (200ml): the former solvent removed the free fatty acids together with non-polar lipids, the latter eluate contained predominantly glycolipids, i.e. the mono-

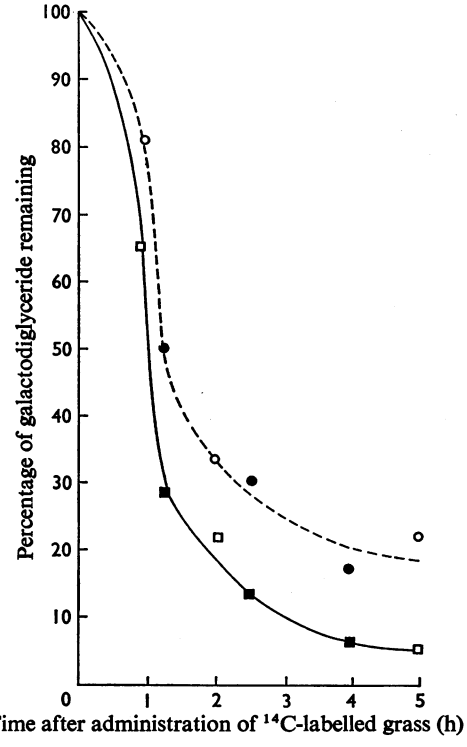

Fig. 1. *Disappearance of grass ^{14}C-labelled glycolipids from rumen*

Details of the experiments are described in the text and in the legend to Fig. 2. The percentage of each galactolipid remaining in the rumen was computed from the ratio of galactolipid radioactivity to β-carotene radioactivity, a pigment which is not metabolized in the rumen during the experimental period (Dawson & Hemington, 1974). This ratio was compared with that in a sample of the original ^{14}C-labelled grass administered. Digalactosyl diglyceride: ●, Expt. 1; ○, Expt. 3. Monogalactosyl diglyceride: ■, Expt. 1; □, Expt. 3.

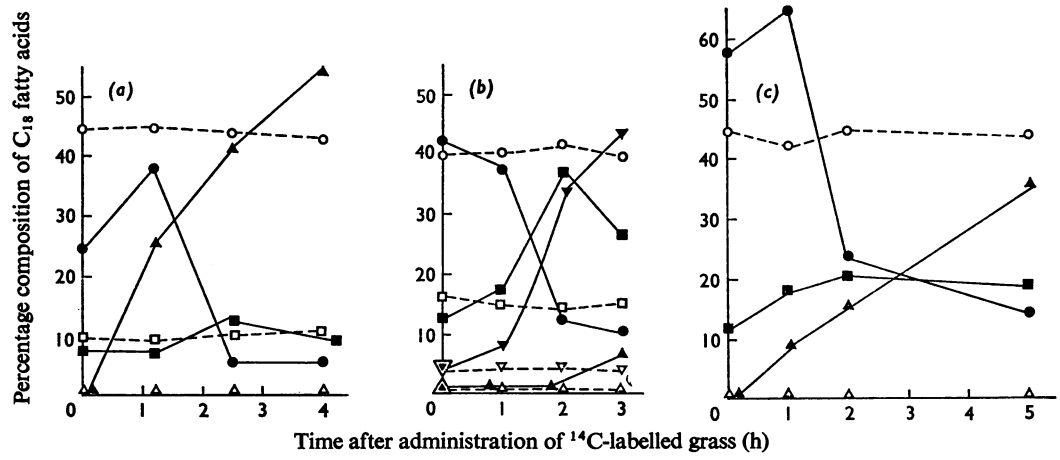

Fig. 2. *Rumen C_{18} fatty acids after the introduction of ^{14}C-labelled grass: percentage distribution of radioactivity in the free fatty acid fraction and the fatty acids of glycolipids in the original grass and in the rumen contents*

The free fatty acid fraction and the galactolipids were isolated from a sample of the original ^{14}C-labelled grass administered and also rumen contents as stated in the text. The fatty acids were analysed by g.l.c. with scanning of the effluent for radioactivity. Glycolipid fraction: ○, linolenic acid (percentage divided by 2); □, octadecadienoic acid; ▽, octadecenoic acid; △, stearic acid. Free fatty acid fraction: ●, linolenic acid; ■, octadecadienoic acid; ▼, octadecenoic acid; ▲, stearic acid. Expt. 1 (*a*): 39g of ^{14}C-labelled grass (8mCi), immediately after cutting, administered to a sheep, which was then given 900g of pasture grass, which was consumed over the initial 75min. Expt. 2 (*b*): 61g of ^{14}C-labelled grass (7.8mCi), cut and deep-frozen for 2 months, administered to a sheep, which was then given 700g of pasture grass, which was consumed between the 1 and 2h points. Expt. 3 (*c*): 94g of ^{14}C-labelled grass (3.6mCi), immediately after cutting, administered to a sheep, which was then given 500g of pasture grass, which was consumed between the 1 and 2h points.

and di-galactosyl diglyceride components of the original grass. The glycolipid fraction was hydrolysed for 1h in 5ml of 6% (w/v) KOH in ethanol–water (19:1, v/v) at 100°C. To the mixture was added 5ml of water and 5ml of hexane and after it had been shaken the hexane layer was rejected. The lower aqueous ethanol layer was acidified with H_2SO_4, and the fatty acids, liberated by the saponification, were extracted into a further 5ml of hexane. The free fatty acids in the original chloroform eluate of the silicic acid column were distributed between hexane–diethyl ether–4% (w/v) NaOH (19:1:20, by vol.), the lower aqueous phase was collected, acidified and the fatty acids were extracted into 0.5vol. of hexane.

Fatty acids as methyl esters were separated on a Pye 104 gas–liquid–chromatogram apparatus by using a polyethylene glycol adipate column at 185°C and 5% CO_2 in argon at 60ml/min as carrier and counting gas. The emerging fatty acid methyl esters were split for detection by flame ionization and radioactive scanning after oxidation to $^{14}CO_2$ and being passed through a proportional counter. Intact glycolipids in the grass and rumen contents were monitored by running samples of the acetone eluted from the silicic acid column on thin-layer chromatograms of silica gel in a solvent of chloroform–methanol–aq. 35M-NH_3 (35:10:1, by vol.). The plate was examined for radioactivity with a thin-layer radioactive scanner, and mono- and di-galactosyl diglycerides were located by the use of markers.

Decomposition of the ^{14}C-labelled grass galactolipids administered was very rapid in the rumen. There was, however, a somewhat more rapid disappearance of monogalactosyl diglyceride than of digalactosyl diglyceride (Fig. 1). However, the properties of the C_{18} acids in the ^{14}C-labelled galactolipids remaining in the rumen showed little change from the original grass, with esterified linolenic acid constituting approx. 85% of the fraction (Fig. 2). In contrast, the free fatty acid fraction showed evidence in three experiments of the hydrogenation of released linolenic acid through dienes and monoenes to stearic acid (Dawson & Kemp, 1970). There was, in all experiments, an increase in stearic acid and a decrease in linolenic acid. The slower rate of hydrogenation observed in Expt. 2 (Fig. 2) showed a transient rise in octadecadienoic acids, a pronounced rise in octadecenoic acids with rather a sluggish rise in stearic acid compared with the other two experiments. The rates of hydrogenation *in vivo* probably depend on the rumen loading with foodstuffs and the relative numbers and types of hydrogenating micro-organisms present (Dawson & Kemp, 1970).

These combined results indicate that, as with

administered triglycerides, very little biohydrogenation of grass galactolipid linolenic acid can occur in the intact complex lipid. Once lipolysis has occurred there is a very rapid and complete hydrogenation of the liberated linolenic acid to stearic acid. Possibly this is because the isomerase in rumen micro-organisms which converts linolenic acid into *cis–trans–cis*-octadeca-9,11,15-trienoic acid as a preliminary for hydrogenation (Kemp & Dawson, 1968) has a requirement for a free carboxylic acid group on the substrate which is absolute. In this connexion Kepler *et al.* (1970) have shown that the isomerization of linolenic acid to *cis–trans*-octadeca-9,11-dienoic acid as a preliminary to hydrogenation by *Butyrivibrio fibrisolvens* requires a free carboxyl group on the substrate. Thus significant hydrogenation would be prevented until lipolysis had occurred.

In the same experiment it was possible to observe the slow accumulation of a small ^{14}C-labelled fatty acid peak which was subsequently identified by g.l.c. and mass spectroscopy to be phytanic acid. None was observed at 4h, by 6h the percentage was only 1.3%, after 12h it was 4.6%, and after 1 day it was 5.9% of the radioactivity in the total fatty acid fraction. It is known that the chlorophylls in grass chloroplasts are rapidly broken down to phaeophytins and phylloerythrin in the rumen releasing phytyl residues (Dawson & Hemington, 1974). The double bond in phytol can be hydrogenated to yield dihydrophytol (Patton & Benson, 1966), which rumen micro-organisms can oxidize to phytanic acid (Hansen, 1966). Presumably this conversion of phytol into phytanic acid must occur relatively slowly in the rumen, but its eventual absorption from the digestive tract leads to the accumulation of phytanic acid in ruminant tissues (Lough, 1964; Hansen, 1966).

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