0099-2240/78/0035-0024$02.00/0
APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1978, p. 24-31
Vol. 35, No. 1
Printed in U.S.A.

Relative Significance of Exogenous and De Novo Synthesized Fatty Acids in the Formation of Rumen Microbial Lipids In Vitro†

D. I. DEMEYER,* C. HENDERSON,†† AND R. A. PRINS†††
Laboratorium voor Voeding en Hygiëne, Rijksuniversiteit Gent, B-9230 Melle, Belgium

Received for publication 18 July 1977

Mixed rumen microorganisms (MRM) or suspensions of rumen Holotrich protozoa obtained from a sheep were incubated anaerobically with [1-^{14}C]linoleic acid, [*U*-^{14}C]glucose, or [1-^{14}C]acetate. With MRM, the total amount of fatty acids present did not change after incubation. An increase in fatty acids esterified into sterolesters (SE) and polar lipids at the expense of free fatty acids was observed. This effect was intensified by the addition of fermentable carbohydrate to the incubations. Radioactivity from [1-^{14}C]linoleic acid was incorporated into SE and polar lipids with both MRM and Holotrich protozoa. With MRM the order of incorporation of radioactivity was as follows: SE > phosphatidylethanolamine > phosphatidylcholine. With Holotrich protozoa, the order of incorporation was phosphatidylcholine > phosphatidylethanolamine > SE. With MRM the radioactivity remaining in the free fatty acids and that incorporated into SE was mainly associated with saturated fatty acids, but a considerable part of the radioactivity in the polar lipids was associated with dienoic fatty acids. This effect of hydrogenation prior to incorporation was also noted with Holotrich protozoa but to a much lesser extent. Small amounts of radioactivity from [*U*-^{14}C]glucose and [1-^{14}C]acetate were incorporated into rumen microbial lipids. With protozoa incubated with [*U*-^{14}C]glucose, the major part of incorporated radioactivity was present in the glycerol moiety of the lipids. From the amounts of lipid classes present, their radioactivity, and fatty acid composition, estimates were made of the amounts of higher fatty acids directly incorporated into microbial lipids and the amounts synthesized de novo from glucose or acetate. It is concluded that the amounts directly incorporated may be greater than the amounts synthesized de novo.

Detailed studies have clearly established the presence of linoleic acid (*cis,cis*-9,12-octadecadienoic acid) in the structural lipids of both rumen bacteria (18) and Holotrich protozoa (19). In the latter organisms it is found predominantly in the β position of the phospholipids, together with the branched-chain fatty acids. The presence of linoleic acid in rumen microbes may affect the ruminant requirement for essential fatty acids (29) and is due either to microbial biosynthesis or direct incorporation of dietary linoleic acid into microbial lipid.

It has been observed repeatedly that the flow of total fatty acids into the ruminant duodenum is in excess of the quantities of fatty acids consumed, suggesting synthesis de novo of microbial fatty acids in the rumen (1, 20, 27). In sheep fed a synthetic lipid-free and protein-free diet, Viviani and Lenaz (30) found that linoleic acid was present in the rumen in amounts above the level expected from its content in the saliva. Incorporation of [1-^{14}C]acetate or [*U*-^{14}C]glucose radioactivity into microbial octadecadienoic acids was observed using whole rumen contents (10, 21) and rumen protozoa (Viviani and Borgatti, cited by Viviani [29]). Viviani and Borgatti found negligible incorporation of [1-^{14}C]acetate radioactivity into octadecadienoic acids, using mixed rumen bacteria, in line with the widely accepted inability of bacteria to synthesize polyunsaturated fatty acids (17). Emmanuel (9) concluded that rumen protozoa in an anaerobic environment incorporate 1-^{14}C-labeled acetyl coenzyme A into higher fatty acids, octadecenoic acids being produced by direct desaturation of saturated fatty acids. Polyunsaturated fatty acids, however, do not arise by this mechanism. Higher fatty acid desaturation has, however, been observed occasionally in vitro using rumen

† Dedicated to J. Martin on the occasion of his retirement.

†† Permanent address: Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland.

††† Permanent address: Laboratorium voor Zoötechniek, Rijksuniversiteit Utrecht, Utrecht, The Netherlands.

contents (L. Felinski and M. Kurpios, Proc. FEBS Meet., Warsaw, 1966, p. 177), rumen bacteria (32), and rumen protozoa (3). Sklan et al. (24) showed clearly the synthesis of linoleic acid by desaturation of more saturated fatty acids, using a supernatant fraction prepared by centrifugation of rumen fluid from young calves. Later, they showed such desaturation to be an aerobic process (25). Incorporation of linoleic acid into microbial cell material was demonstrated using washed cell suspensions of rumen holotrichs and bacteria (32), but physical adsorption of the fatty acids to the cell surfaces may have been involved (11). Incorporation of [1-^{14}C]stearate and [1-^{14}C]palmitate by esterification into rumen microbial lipids was reported by Patton et al. (21). Hawke (12) reported the incorporation of [1-^{14}C]linolenic acid radioactivity into microbial lipids using rumen fluid and mixed rumen bacteria. The esterified radioactive fatty acids were considerably less hydrogenated than the radioactive free fatty acids (FFA). The experiments reported in this paper are an attempt to estimate the relative importance of de novo synthesis of long-chain fatty acids and direct incorporation of extracellular unsaturated fatty acids in the formation of rumen microbial lipids.

Part of this work has been presented previously (Henderson and Demeyer, Proc. 2nd World Congr. Anim. Feed, Madrid, 1972, vol. V, p. 33; Demeyer, Henderson, and Prins, Proc. FEBS Spec. Meet., Ind. Aspects Biochem., Dublin, 1973).

MATERIALS AND METHODS

Rumen microorganisms. Before the morning feeding, samples of rumen contents were obtained from an individually penned, rumen-fistulated wether, fed hay (400 g) and concentrates (200 g) at 9 a.m. and 4 p.m. daily. Samples were filtered through stainless-steel wire gauze (16 mesh) and 20-ml samples of rumen fluid were transferred anaerobically (CO_2 flushing) to incubation flasks. Suspensions of Holotrich protozoa consisting primarily of *Isotricha* were prepared from the same animal, as described by Prins and Prast (22).

Incubations. Table 1 summarizes the salient characteristics of all experiments. Mixed rumen microorganisms (MRM) and Holotrich rumen protozoa were incubated with either [1-^{14}C]linoleic acid or [*U*-^{14}C]glucose and [1-^{14}C]acetate. The labeled linoleic acid was used to estimate incorporation of higher fatty acids; labeled glucose and acetate were used to estimate synthesis de novo.

MRM. In a first experiment, 20 ml of rumen fluid was incubated with 5 ml of buffer (2) containing 28 μCi (1.36 μmol) of [1-^{14}C]linoleic acid (Radiochemical Centre, Amersham, England) and 100 μmol each of D-(+)-glucose, D-(−)-fructose, D-(+)-cellobiose, and D-(+)-maltose (Merck Co., Darmstadt). In a second experiment, 500 μmol of D-(+)-glucose and 125 μmol each of D-(+)-cellobiose and D-(+)-maltose were added as carbohydrates (experiment 2a), and a simultaneous incubation without carbohydrates (experiment 2b) was included. The [1-^{14}C]linoleic acid was added to the buffer solution by evaporation, under N_2, of a hexane solution. When examined by thin-layer chromatography in solvent 1 (see below), it was found that only about 90% of the radioactivity moved as FFA; the rest remained at the origin. In experiments 2a and b, hexane solution of the linoleic acid was shaken with silicic acid wetted with distilled water, giving a purified substrate with 98% of the radioactivity moving as FFA. In experiments 1 and 5 the linoleic acid as received was used. As part of the second experiment two further incubations without [1-^{14}C]linoleic acid but with either 20 μCi (500 μmol) of [*U*-^{14}C]glucose (experiment 3) or 20 μCi (250 μmol) of [1-^{14}C]sodium acetate (experiment 4) (Radiochemical Centre, Amersham, England) were included. Incubations were done at 39°C under CO_2 for 4 h in closed flasks permitting gas sampling (8), and the reactions were stopped by injection of 1 ml of H_3PO_4 A.R. (Merck Co., Darmstadt). Zero-time blanks were obtained by immediate injection of acid.

Holotrich protozoa. Immediately after preparation, 1 ml of Holotrich cell suspension (4 × 10^4 to 8 ×

TABLE 1. *Summary of experiments*

Microorganisms	Expt[a]	Substrate added					
		Carbohydrate		Acetate		Linoleic acid	
		μmol	μCi	μmol	μCi	μmol	μCi
MRM	1	600				1.36	28
	2a	1,000				1.36	28
	2b					1.36	28
	3	500	20				
	4			250	20		
Holotrich rumen protozoa	5					0.05	25
	6	100	4				
	7			50	1		

[a] Experiments 2a, 2b, 3, and 4 were carried out simultaneously to compare direct incorporation (2a and 2b) with de novo synthesis (3 and 4). Experiments 5, 6, and 7 were carried out simultaneously for the same purpose, using protozoa.

10^4 cells) was injected through the butyl rubber stoppers of bacterial culture tubes (15) filled with CO_2 and 8 or 9 ml of basal medium (30:70, vol/vol, clarified rumen liquid-anaerobic salt solution [14] containing 0.5% $NaHCO_3$, 0.0001% resazurin, 3 mg of penicillin G-sodium [R.I.T., Genval], 12.5 μg of chloramphenicol [R.I.T., Genval], 3.5 mg of bovine serum albumin [Pentex, BVO 262], and 2.5 mg of cysteine-HCl [B.D.H., Poole, England]. For experiment 5, 0.1 ml of a sterile solution of [1-^{14}C]linoleic acid (0.05 μmol, 25 μCi) in 0.001 M KOH was added. For experiments 6 and 7, either 0.1 ml of a sterile aqueous solution of [*U*-^{14}C]glucose (100 μmol, 4 μCi) or 0.5 ml of a sterile aqueous solution of [1-^{14}C]sodium acetate (50 μmol, 1 μCi) was added. Incubations were done overnight at 39°C. Microscopic observation confirmed that protozoa were still alive at the end of the incubation. Control incubations contained protozoal suspensions killed by boiling for 30 s.

Chemical analyses. (i) Extraction of lipids. In experiments 1, 2, 3, and 4, after analysis of fermentation products, incubation contents were extracted with 36 ml of choroform-methanol (2:1, vol/vol) for 1 min at maximum speed in a Virtis homogenizer (Gardiner, N.Y.). Water and organic phases were separated by centrifugation (20 min, 6,000 × *g*), and the residue between the two phases was refluxed for 1 h with 20 ml of chloroform-methanol (2:1, vol/vol). The extract was separated from the cell residue by centrifugation and added to the first extract. The combined extracts were washed twice with water, made up to 100 ml with absolute ethanol, and dried over anhydrous Na_2SO_4. The washings were pooled with the water phase. Nonextracted fatty acids were esterified by reflux boiling of the cell residues with 10 ml of methanolic H_2SO_4 (230:2, vol/vol) for 1 h, after addition of 0.5 mg of heptadecanoic acid (margaric acid, Merck Co., Darmstadt) as internal standard. The residues finally obtained after filtration and washing were digested with 3 N NaOH (3 h, 80°C) to extract protein and polysaccharide, and the digest was filtered and made up to 25 ml. In experiments 5, 6, and 7, lipids were extracted by three successive treatments with 10 ml of chloroform-methanol (2:1, vol/vol) in a separatory funnel. The residues were heated with 3 N NaOH as described.

(ii) Quantitative determination of lipids. Total lipids and lipid classes were quantified as fatty acids by a combination of thin-layer chromatography and gas chromatography as described by Christie et al. (4). One milliliter of lipid extract was spotted on glass plates (20 by 20 cm) coated with Silica Gel G (Merck, Darmstadt) in 0.5-mm layers, and the plates were developed with hexane-diethylether-acetic acid (75:25:2, vol/vol) (solvent 1). Lipid classes were detected under ultraviolet light after spraying with a 0.1% (wt/vol) solution of 2,4-dichlorofluorescein in ethanol and identified by comparison with standard mixtures (Nu-Check, Prep. Lab, Minn.) containing cholesterol oleate, triolein, oleic acid, and lecithin (standard no. 18-S-A) and monopalmitin, dipalmitin, tripalmitin, and methylpalmitate (standard no. 16-O-A). Sterolesters (SE), methylesters, triglycerides, FFA, mono- and diglycerides, and polar lipids (phospholipids) (PL), in order of decreasing R_f value, were scraped from the plate into glass ampoules (with a constricted neck for sealing) containing an appropriate amount of heptadecanoic acid as internal standard. Preliminary analysis showed that the amounts of heptadecanoic acid normally present in rumen microbial lipids did not exceed 1% (wt/wt) of total fatty acids (see also 7). After 5 ml of methanolic H_2SO_4 (230:2, vol/vol) was added, the ampoules were sealed and heated in a boiling-water bath for 1 h. The digests were diluted to 20 ml with water, the methylesters were extracted three times with petroleum ether, and pooled extracts were washed with water (three times) and evaporated to dryness under N_2 at 30°C.

The residue was dissolved in an appropriate amount of hexane and analyzed by gas-liquid chromatography as described by Demeyer and Henderickx (8) but using Chromosorb WAW DMCS high performance (Hewlett-Packard) as support. Fatty acid composition was determined directly from the ratios of the peak areas, calculated by triangulation. The weight of each individual fatty acid, present in each class, was calculated from the amount of internal standard added, and the total amount of fatty acids present in each class was obtained by addition of the individual amounts. Determination of total fatty acids in an unfractionated extract enables calculation of recovery by summation of the lipid classes determined: an average value of 87.9 ± 3.8% (mean value ± standard error of 10 determinations) was obtained.

(iii) Fractionation of lipids for radioactivity determination. Radioactivity incorporated into neutral lipid classes was determined after thin-layer chromatography in solvent 1, whereas for separation of polar lipids chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, vol/vol) (solvent 2) was used. In the latter solvent neutral lipids migrate with the solvent front, whereas phospholipids are separated into phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lyso compounds in order of decreasing R_f value. PE and PC were identified using a liver PL extract as standard and by the specific spray reagents ninhydrin (PE) and Dragendorff reagent (PC) (31). After detection, spots were scraped off into scintillation vials for radioactivity determination.

Incorporation of radioactivity into SE was confirmed by a separate assay after separating the SE on thin-layer plates, using heptane-toluene (80:20, vol/vol) as solvent (28). SE extracted from sheep blood was used as standard. Fatty acid methylesters were separated according to degree of unsaturation by chromatography on $AgNO_3$-treated plates (23). The distribution of radioactivity between the fatty acid and water-soluble moieties of complex lipids was determined by comparing the activity in the intact lipid with that in the separated methylesters of the fatty acids after transesterification with methanolic H_2SO_4.

Determination of radioactivity. Samples of lipid extracts (evaporated to dryness under N_2), material scraped off thin-layer plates, and samples of water phases were counted in 10 ml of a dioxane-water mixture (26), using a Tracerlab three-channel Corumatic 200 apparatus. Counting efficiency for unquenched solutions was approximately 85%. Quenching by color and/or silica gel lowered efficiencies to minimum values of approximately 70% and was cor-

rected for by the external standard channel ratio method (16).

RESULTS AND DISCUSSION

Incorporation of [1-^{14}C]linoleic acid radioactivity. Table 2 shows the recoveries of radioactivity obtained in experiments using [1-^{14}C]linoleic acid as substrate. There were relatively small discrepancies between the recoveries. In the incubations with added substrate there were increases in the radioactivity not extracted by chloroform-methanol. The nature of this bound lipid is unknown. Tables 3 and 4 show the distributions of radioactivity and fatty acids in the different extractable lipid fractions before and after incubation.

In none of the experiments was an increase in total lipid noted, indicating no net synthesis of long-chain fatty acids. From the distribution of fatty acids between the lipid classes (Tables 3 and 4), it is clear that there is an increase in the amount of esterified fatty acids in SE and PL at the expense of FFA when fermentable substrate is added to the incubations (experiments 1 and 2a). A coresponding shift in the distribution of radioactivity from FFA to SE and PL is observed after incubation. In PL the radioactivity is mainly concentrated in PE and

TABLE 2. *Amounts of radioactivity recovered before and after incubation of rumen microorganisms with [1-^{14}C]linoleic acid*

Expt[a]	Radioactivity (dpm × 10^3)					
	Added	Recovered in water phase	Lipids		Cell residue	Total
			Extracted	Not extracted		
Before incubation						
1	62,203	ND[b]	57,008	320 (0.56)[c]	ND	57,328
2a	45,203	141	42,866	283 (0.65)	63 (0.15)	43,353
2b	45,203	162	44,098	432 (0.96)	87 (0.19)	44,779
5	ND	11,060	80,550	ND	226 (0.25)	91,836
After incubation						
1	62,203	ND	52,915	1,108 (2.05)	ND	54,023
2a	45,203	144	40,172	920 (2.19)	748 (1.78)	41,984
2b	45,203	97	45,858	451 (0.97)	135 (0.29)	46,541
5	ND	6,535	73,760	ND	301 (0.41)	80,596

[a] Experiments are described in the text: experiments 1, 2a, and 2b used MRM; experiment 5 used protozoa (see Table 1).
[b] ND, Not determined.
[c] Number in parentheses is percentage of total.

TABLE 3. *Distribution of fatty acids between the lipid classes before and after incubation of rumen microorganisms with [1-^{14}C]linoleic acid*

Expt[a]	Lipid (mg of fatty acid)					
	Extractable[b]					Not extracted[b]
	SE	FFA	PL	Other[c]	Total	
Before incubation						
1	0.22	9.29	1.39	0.53	11.43	0.56
2a	0.32	15.88	3.33	0.52	20.05	1.93
2b	0.27	15.42	3.71	0.69	20.09	2.26
5	0.10	3.30	1.90	4.90[d]	10.20[e]	ND[f]
After incubation						
1	0.36	7.26	3.05	0.39	11.06	0.31
2a	1.19	14.49	3.65	0.37	19.69	0.86
2b	0.32	15.57	3.96	0.36	20.15	0.98
5	0.10	3.30	1.90	4.90[d]	10.20[e]	ND

[a] See footnote, *a*, Table 2.
[b] Extractable or not extracted with chloroform-methanol (2:1, vol/vol).
[c] Methylesters and tri-, mono-, and diglycerides.
[d] Mainly triglyceride.
[e] Mean value of determinations before and after incubations.
[f] ND, Not determined.

TABLE 4. *Distribution of radioactivity between the lipid classes before and after incubation of rumen microorganisms with [1-^{14}C]linoleic acid*

Expt[a]	Radioactivity (% of total)						
	SE	FFA	PE	PC	Other PL[b]	PL (total)	Other[c]
Before incubation							
1	0.2	98.7	0.2	0.1	0.3	0.6	0.5
2a	0.4	96.0	0.5	0.1	2.7	3.3	0.3
2b	0.3	96.4	0.3	0.1	2.5	2.9	0.4
5	0.2	85.9	0.3	0.1	7.2	7.6	6.3[d]
After incubation							
1	7.5	84.0	3.3	2.2	1.9	7.4	1.1
2a	4.6	83.9	3.7	1.0	5.2	9.9	1.6
2b	2.5	92.5	1.2	0.3	3.0	4.5	0.5
5	1.3	64.3	3.7	13.7	9.9	27.5	6.9[d]

[a] See footnote *a*, Table 2.
[b] Lyso compounds and unidentified impurity of the substrate (see text).
[c] Methylesters and tri-, mono-, and diglycerides.
[d] Mainly triglyceride.

PC. A significant part of the radioactivity (2%) not altered by incubation is present in an unknown component (included in "other" PL in Table 4). This component migrates with the solvent front in solvent 2 but is immobile in solvent 1.

The changes in the distribution of radioactivity with incubation are shown in Fig. 1, from which it is clear that the radioactivity associated with FFA is incorporated, in order of decreasing intensity, into SE, PE, and PC in incubations with MRM. In experiment 2a there was a significant incorporation of radioactivity into a group of compounds tentatively identified as lysophospholipids. The Holotrich protozoa incorporated relatively small amounts of radioactivity into SE, indicating a bacterial role for SE in the MRM incubations. Incorporation of [1-^{14}C]palmitate (21) and [1-^{14}C]linolenate (12) into SE has been observed earlier. According to Hawke (12), radioactivity was associated with the fatty acid part of the molecule, whereas Patton et al. (21) found radioactivity also in the sterol part.

With Holotrich protozoa (experiment 5) most of the radioactivity is incorporated into PC, in line with the predominance of PC in protozoal lipid (6).

The distribution of radioactivity between saturated and mono-, di-, and polyunsaturated fatty acids in FFA before incubation and FFA, SE, and PL after incubation is illustrated in Fig. 2. For incubations with MRM it is clear that the major part of radioactivity recovered, after incubation, in FFA and SE is associated with saturated and monounsaturated fatty acids, indicating extensive hydrogenation. As hydrogenation is confined to the FFA fraction (13), the results suggest that the hydrogenation products of linoleic acid are specifically incorporated into SE.

A considerable part of the radioactivity in the

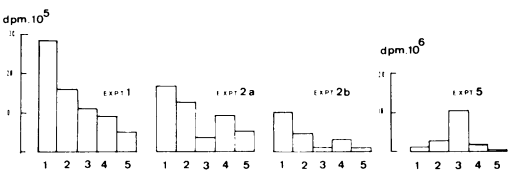

FIG. 1. *Incorporation of radioactivity into lipid classes, after incubation of rumen microorganisms with [1-^{14}C]linoleic acid. Net increase with incubation in radioactivity (dpm/flask) incorporated into: (1) SE, (2) PE, (3) PC, (4) lysophospholipids, and (5) tryglycerides and partial glycerides, shown from left to right in that order. Experiments 1, 2a, and 2b used MRM; experiment 5 used Holotrich protozoa (see Table 1).*

PL is, however, still associated with diunsaturated fatty acids, indicating incorporation of linoleic acid or its conjugated diene homologues and confirming data obtained with [1-^{14}C]linolenic acid (12). It is reasonable to conclude that the fatty acids incorporated were C_{18} acids, since α- or β-oxidation would have removed the label as CO_2 or acetate. The effect of hydrogenation before incorporation was also observed, albeit much less pronounced, in the incubations with Holotrich protozoa. In contrast to earlier results (3), our data do not suggest a complete absence of hydrogenating activity in Holotrich protozoa. The observed hydrogenating activity is not likely to be due to contaminating bacteria present in the protozoal suspension. Microscopic counts of bacteria and protozoa in the suspension used in experiment 5 gave values of 10^3 and 80×10^3, respectively. From these values an approximate surface area ratio for protozoa-bacteria of 5×10^5:1 can be calculated, making it clear that bacterial surfaces are unlikely to compete with protozoal surfaces for fatty acid hydrogenation.

Incorporation of [*U*-^{14}C]glucose and [1-

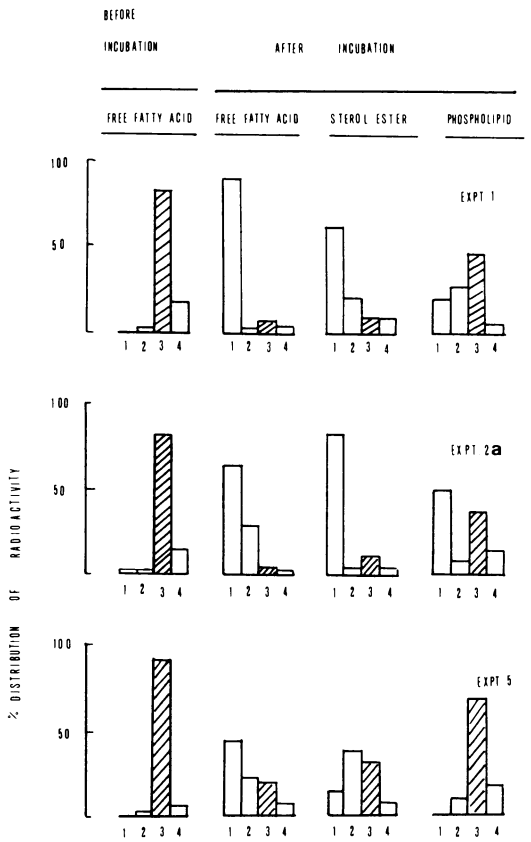

FIG. 2. *Distribution of radioactivity (percent) between the C_{18} fatty acids in the major classes of rumen lipids before and after incubation with [1-^{14}C]linoleic acid. Each rectangle represents the percentage of the total radioactivity in the lipid class which is associated with the different C_{18} fatty acids: (1) saturated; (2) monounsaturated; (3) diunsaturated (hatched rectangles); (4) polyunsaturated. In experiments 1 and 2b MRM were used; in experiment 5 Holotrich protozoa were used (see Table 1).*

[^{14}C]acetate. Table 5 shows the distribution of radioactivity between the various fractions obtained by lipid extraction before and after incubation. Low values for incorporation of radioactivity into lipids were obtained (less than 1% of total radioactivity added). With protozoa using [*U*-^{14}C]glucose as substrate, a considerable part of the incorporated radioactivity is recovered in the water-soluble moiety of the lipids.

Distribution of radioactivity between the lipid classes was determined for incubations with glucose, and net incorporation was calculated by correction of the amounts found after incubation for those found in the blanks. The highest incorporation was found in PL, followed by unidentified compounds not migrating in solvent 1 but migrating with the solvent front in solvent 2.

Estimation of fatty acid incorporation and synthesis. To estimate the incorporation of long-chain (C_{18}) fatty acids into microbial lipid, it was necessary to assume that the added [1-^{14}C]linoleic acid and its hydrogenation products were present in a single pool of FFA. From the distribution of radioactivity between the lipid classes (Tables 2, 3, and 4) and the amounts of C_{18} fatty acids in the FFA pools before and after incubation (Table 6), the specific activities of these pools were calculated. Analyses showed that the proportion of C_{18} fatty acids in the FFA pools changed little with incubation, varying between 84 and 88% of the total fatty acids in MRM and 61 and 62% in the protozoa. The specific activities of the FFA pools before and after incubation are shown in Table 5. Apart from the protozoal experiment, there was relatively little change in specific activity with incubation.

From the net increase in radioactivity associated with complex lipid (Fig. 1) and the mean specific activity of the FFA pool, the incorporation of C_{18} fatty acid was calculated (Table 5). There was considerable incorporation of fatty acids into the complex lipids, the MRM being more active than the protozoal suspensions. It was also clear that the presence of fermentable substrate stimulated incorporation (experiments 1 and 2a). Two factors affect the accuracy of these incorporation values. The differences in recoveries of radioactivity in the extracted lipid (Table 2) cause the values in experiments 1, 2a, and 5 to be underestimated and the value in experiment 2b to be overestimated.

In the MRM incubations it is possible that not all of the FFA associated with plant particles is free to exchange with the pools calculated (Table 6). The specific activity would thus be underestimated and the incorporation correspondingly overestimated.

Similar estimates can be made of the incorporation of glucose and acetate carbon into microbial fatty acids. Maximal estimates are obtained if the pool sizes of glucose and acetate are assumed to equal the total amount of free reducing carbohydrate and acetate, respectively, present at the start of the incubation. It is reasonable to assume that acetate is incorporated as a two-carbon unit and not after degradation to CO_2.

Table 7 shows that such calculations give estimates for incorporation of glucose and acetate, equivalent to the synthesis of <100 μg of stearic acid, protozoa being much less active than MRM. The value for acetate is comparable with estimates for de novo synthesis of rumen microbial lipid made by Czerkawski et al. (5).

Although values thus calculated can only be

TABLE 5. *Amounts of radioactivity recovered before and after incubation of rumen microorganisms with [U-^{14}C]glucose and [U-^{14}C]acetate*[a]

Substrate added	Expt	dpm × 10^3	μmol	Radioactivity recovered in:					
				Water phase	Lipids			Not extracted	Cell residue
					Extracted				
					Fatty acid	Glycerol	Total		
Before incubation									
[U-^{14}C]glucose	3	49,020	500	45,728			38	302	67
	6	10,315	100	10,550			0.2	ND	2.2
[1-^{14}C]acetate	4	19,560	250	ND			155	1	0.3
	7	3,607	50	3,360				ND[b]	
After incubation									
[U-^{14}C]glucose	3			24,747	88	36	124	567	613
	6			5,937	1.2	7.1	8.3	ND	603
[1-^{14}C]acetate	4			ND	166	9	175	4	6.7
	7			2,392			0.4	ND	1.6

[a] Experiments 3 and 4 used MRM; experiments 6 and 7 used protozoa (see Table 1).
[b] ND, Not determined.

TABLE 6. *Estimation of fatty acid (C_{18}) incorporation into microbial lipids*[a]

Expt	C_{18} fatty acids present in FFA pool				Mean sp act of FFA pool	C_{18} fatty acids incorporated	
	Before incubation		After incubation			dpm × 10^3	μg of C_{18} acid
	mg	Sp act[b]	mg	Sp act			
1	8.29	6,787	6.22	7,146	6,966 ± 181	7,725	1,109
2a	14.01	2,937	12.32	2,736	2,836 ± 100	4,753	1,676
2b	13.06	3,255	13.59	3,121	3,188 ± 67	1,850	580
5	2.01	34,424	2.05	23,135	28,779 ± 5644	15,712	546

[a] See Table 1 and footnote *a*, Table 2.
[b] Specific activity: dpm × 10^3 per mg of C_{18} fatty acid.

TABLE 7. *Estimation of higher fatty acid synthesis by rumen microorganisms*[a]

Expt	Substrate added	Pool size				Fatty acid synthesis		
		μmol	mg of C	dpm × 10^3	Sp act (dpm × 10^3/mg of C)	dpm × 10^3	μg of C	μg of stearic acid
3	[U-^{14}C]glucose	649	46.7	49,020	1,050	61	58	76
4	[1-^{14}C]acetate	1,381	33.1	19,560	591	19	32	42
6	[U-^{14}C]glucose	100	7.2	10,315	1,433	1.2	0.8	1
7	[1-^{14}C]acetate	268	6.4	3,607	564	0.4	0.7	1

[a] See Table 1 and footnote *a*, Table 2.

very rough estimates, they clearly indicate that, at least for the conditions of our incubations, where a readily available supply of preformed long-chain fatty acids exists, the rumen microorganisms preferentially utilize these as substrates for cell lipid production. Furthermore, unsaturated fatty acids appear to be partially protected from hydrogenation by their incorporation into microbial phospholipids.

ACKNOWLEDGMENT

One of us (C.H.) is indebted to the Rowett Research Institute and the Agricultural Research Council for financial support, which allowed his participation in this study while visiting the University of Ghent.

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