Lipid Metabolism of Rumen Ciliates and Bacteria

I. Uptake of Fatty Acids by *Isotricha prostoma* and *Entodinium simplex*

J. Gutierrez, P. P. Williams, R. E. Davis, and E. J. Warwick

Animal Husbandry Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland

Received for publication June 4, 1962

ABSTRACT

Gutierrez, J. (U.S. Department of Agriculture, Beltsville, Md.), P. P. Williams, R. E. Davis, and E. J. Warwick. Lipid metabolism of rumen ciliates and bacteria. I. Uptake of fatty acids by *Isotricha prostoma* and *Entodinium simplex*. Appl. Microbiol. **10**:548–551. 1962.—Washed suspensions of the ruminal ciliates, *Isotricha prostoma* and *Entodinium simplex*, concentrated C^{14}-labeled oleic, palmitic, stearic, and linoleic acids within the cells during short incubation periods. Radioautographs demonstrated that oleic acid-1-C^{14} was hydrogenated to stearic acid by *I. prostoma*, and Warburg manometric data showed that the sodium salts of oleic, valeric, caproic, and acetic acids, and methyl myristate, methyl laurate, and the triglyceride tributyrin stimulated fermentation of *I. prostoma*. The total lipid and free fatty acid contents of *I. prostoma* were determined.

The metabolism of fatty acids, such as oleic, stearic, and palmitic, by ruminal protozoa is of interest since the utilization of fatty compounds ingested with the feed can be of significance in the nutritional economy of the host. Impure mixtures of ruminal ciliates have been reported to hydrogenate unsaturated lipids, with linoleic acid being converted mainly to stearic acid. The protozoan suspension included both holotrichs and oligotrichs, with the latter species being mostly *Epidinium* (Wright, 1959). C^{14}-labeled tripalmitin, soybean oil, and ethyl laurate have been incubated in vitro with ruminal fluid; hydrolysis of soybean oil was linear for the first 24 hr and then decreased. Fractionation of the ruminal fluid indicated that the enzyme was associated mainly with the protozoa (Hill et al., 1960). Lipolysis of ingested glycerides by ruminants indicated over 75% of the total lipid was recovered in the form of free fatty acids (Garton, Hobson, and Lough, 1958). Oleic and stearic acids have been shown to increase the growth of *Paramecium* in a semidefined medium (Miller and Johnson, 1960). This report describes respiration and metabolism experiments of fatty acids by single species of ruminal ciliates, with information on their cellular lipid contents.

Materials and Methods

Ruminal protozoa were obtained directly from the ingesta of animals harboring large populations of *Isotricha prostoma*, washed in buffer solution until free of debris and most of the bacteria, and suspended in 0.1% $NaHCO_3$, 0.5% NaCl, 0.01% $MgSO_4$, 0.01% CaCl, and 0.1% KH_2PO_4. The gas phase was 5% CO_2 in nitrogen, and the temperature was 39 C. The sedimentation method of separation used to obtain suspensions containing only one species of protozoa from the ruminal ingesta harboring different types of ciliates has been described (Williams et al., 1961). Suspensions of *Entodinium simplex* were obtained from the ruminal ingesta of a 10-week-old calf reared in isolation and inoculated with the single species of protozoa.

Washing techniques yielded protozoa free of most bacteria (Williams et al., 1961). Manometric experiments were conducted with the conventional Warburg apparatus, and gas production was measured by comparison with an endogenous vessel without added substrate. Dihydrostreptomycin sulfate and procaine penicillin G were each added to the protozoal suspensions at a level of 0.5 mg/ml.

Hydrogen production was estimated by absorption with palladium. Fatty acids were neutralized with 0.1 N NaOH before being used in tests for substrate utilization. Tracer experiments using the labeled fatty acids, linoleic acid-1-C^{14}, oleic acid-1-C^{14}, palmitic acid-1-C^{14}, and stearic acid-1-C^{14} (purchased from Applied Science Laboratory, State College, Pa.) were conducted to test the uptake of the fatty acids by washed suspensions of *I. prostoma* and *E. simplex*. The protozoa in the buffer solutions were usually incubated with 0.5 μc isotope for 90 min in a water bath at 39 C. Radioactive solutions to be tested were added to rubber-stoppered test tubes, which were kept anaerobic with 100% CO_2, and the remaining isotope solutions were stored at 5 C for later use. Samples (2 ml) of protozoan cells (59,000 to 134,800/ml) were added to the fractions. Initial samples at zero time, and samples for the later time intervals, were prepared by withdrawing 100 μg onto the planchets from the suspension while it was being vigorously bubbled with CO_2. The disturbance gave a uniform distribution of the cells throughout the fluid. The protozoa were placed on a Millipore pad and washed four times with 2-ml samples of distilled water. Pads with samples were glued with rubber cement to the planchets to prevent curling, and dried under an infrared lamp. Counting was done with a windowless gas-flow counter tube and a Nuclear-Chicago scaler.

For quantitative analysis of the lipid content of the protozoa, the following methods were used. *I. prostoma* cells were collected, washed in a column of saline as previously described (Gutierrez, 1958), rinsed with distilled water, and air-dried over a boiling-water bath. The cells were placed at 70 C for 1 hr in petri dishes, removed, and ground in a mortar and pestle. The protozoa were then dried to a constant weight and collected as two samples with weights of 2.287 and 4.448 g. Dried protozoal samples were refluxed in 100 ml of chloroform-methanol (2:1) for 16 hr under oxygen-free nitrogen. The solutions were evaporated over a steam bath with oxygen-free nitrogen, reducing the volume from 100 to 10 ml; 2.0 ml of 0.74% aqueous KCl were added per 10-ml lipid sample, which was then placed in a separatory funnel and stirred. Lower layers were collected, dried to a constant weight under oxygen-free nitrogen, and taken as the total lipid fraction. The upper layer contained nonlipid contaminants and was discarded (Folch, Lees, and Stanley, 1957). Total lipid fraction samples were weighed, saponified by dispensing into 50 ml of 2 N KOH-methanol, and refluxed for 3 hr in an atmosphere of oxygen-free nitrogen. After evaporation, 100 ml of 2 N H_2SO_4 were added. Fatty acids were extracted six times with petroleum ether (bp 60 to 70 C), and the combined extracts were evaporated to constant weight after drying with anhydrous sodium sulfate. The extracts were weighed and taken as the free fatty acid fractions. A sample of the fatty acids (about 100 to 200 μg in chloroform) was chromatographed.

For fatty acid chromatography, the following methods were used. The solvents were (i) chloroform-acetic acid-paraffin oil (50:130:10) and (ii) acetic acid-paraffin oil, prepared by shaking 100 ml of 90% (v/v) acetic acid with 1 ml of paraffin oil in a separatory funnel. After the solution was shaken, the lower layer was used. Whatman no. 1 filter paper was pretreated by dipping through a 10% solution of paraffin oil in ether, and the volatile solvent was allowed to evaporate with a cold-air draught. Samples (25 to 50 μg) of fatty acid solutions were spotted and dried at 70 C for 30 min. Papers were run descending for 18 to 24 hr at room temperature. Later, the mercuric salts were formed by immersing the air-dried chromatogram for 15 min in a 0.1% mercuric acetate solution containing 0.5 ml of acetic acid per liter. Excess mercuric acetate was removed by washing the chromatograms in running tap water for 45 min. After air drying, the chromatograms were sprayed with a 0.2% solution of diphenyl carbazide in 95% ethyl alcohol (Buchanan, 1959). Both unsaturated and saturated fatty acid spots were purple against a white background. For the identification of the unsaturated fatty acids, the air-dried chromatograms were either dipped in 0.001 M potassium permanganate and rinsed in tap water or exposed to iodine vapor. With both reagents, brown spots resulted from the unsaturated fatty acids.

For the preparation of autoradiographs, acetic acid-paraffin or chloroform-acetic acid-paraffin solvent was used with Whatman no. 1 filter paper pretreated with ether-paraffin. A known mixture of oleic, stearic, and linoleic acids with radioactive counts of 500/min was spotted in 25 μg on the chromatograms beside the unknown. For the experiments testing the conversion of oleic to stearic acids, *I. prostoma* in counts of approximately 75,000/ml in 15 ml of bicarbonate-saline solution and 1 μc of oleic acid were incubated under 5% CO_2-95% N_2 at 37 C for 3.5 hr. The cells and supernatant were filtered through paper (Schleicher and Schuell, sharkskin grade), and the protozoa were washed twice with distilled water. Both cells and filtrate were treated with chloroform-methanol (2:1) and saponified as previously noted. The final free fatty acids in both filtrate and cellular extracts were then concentrated to 0.5 ml in chloroform and spotted in 25 μg. In the experiments where stearic acid was tested for conversion to other products, 2 μc of the substrate were used. Chromatograms were placed in a tank and run for 22 hr at room temperature and air-dried. X-ray film strips were placed on the chromatograms and exposed for 2 to 9 days, developed, and fixed. R_F values and spots were then determined.

Results

Washed suspensions of the ruminal holotrich, *I. prostoma*, which were incubated with C^{14}-labeled fatty acids, were able to concentrate the C^{14} within the cells. The results are presented in Table 1. All sample counts were made on 100 μg from a 5-ml total liquid volume. C^{14} from stearic, oleic, linoleic, and palmitic acids was concentrated within *I. prostoma*. Washed suspensions of the small oligotrich, *E. simplex*, were able also to assimilate C^{14} of the long-chain fatty acids. The results for the entodinia are given in Table 2.

Washed suspensions of *I. prostoma* were used in respiration experiments to test whether the fatty acid stimulated gas production when the substrate was added from the sidearm. Usually, 30 mg of the neutralized substrate were added from the vessel sidearm after the initial equilibration period during which 5% CO_2 was passed through the manometers. The amounts of gas produced in the various runs from different substrates are shown in Table 3. In a separate experiment with sodium oleate, the ratio of hydrogen to carbon dioxide produced by *I. prostoma* was approximately 1:1. As a check against bacterial activity, control experiments demonstrated little activity from sodium oleate by supernatant wash water free of protozoa (*see* Table 3).

Normally, fermentation acids, such as acetic, butyric, and lactic, are produced from an energy source such as glucose by *I. prostoma*. However, steam distillation of protozoal cultures provided with 0.1% nonlabeled sodium oleate and 1 μc of oleic acid-1-C^{14} showed that no detectable radioactivity was present in the volatile acid fraction from 80 ml of saline-bicarbonate buffer containing 4,000 to 5,000 protozoa/ml, nor was there significant titratable

acidity in the volatile acid fraction over controls. *I. prostoma* incubated with oleic acid-1-C^{14} and stearic acid-1-C^{14} was solvent extracted, saponified, and degradative cellular products were demonstrated by paper chromatography. When an *I. prostoma* suspension was incubated for 3.5 hr with oleic acid-1-C^{14} and extracted, autoradiographs of the chromatogram showed that oleic acid was converted to a compound with an R_F similar to stearic acid (0.46 with acetic-acid paraffin solvent) and to a second unknown faint spot with an R_F of 0.82. After elution with chloroform, the radioactivity of the two spots was compared by counting. The weak spot had approximately one-third the activity of the stearic acid spot. Thus, hydrogenation of oleic acid appears to occur when the fatty acid is taken into the protozoan cell. Protozoal extracts prepared after incubation of washed suspensions of *I. prostoma* with 2 μc of stearic acid-1-C^{14} were spotted on paper chromatograms. Radiograms showed that most of the stearic acid-1-C^{14} was concentrated into the cellular material of *I. prostoma* as stearic acid or a component possessing an R_F value comparable to stearic acid. In addition to the concentrated spot, a second less dense spot occurred, with an R_F value similar to linoleic acid. In the oleic and stearic acid experiments, no radioactivity appeared in radioautographs prepared from chromatograms in which the supernatant fluid free of protozoa had been spotted.

TABLE 1. *Concentration of C^{14} fatty acids by Isotricha prostoma*

Substrate	Initial culture	Supernatant	Washed cells
	*count/min**	*count/min*	*count/min*
Stearic acid-1-C^{14}	16,980	103	21,508
Oleic acid-1-C^{14}	18,031	803	15,848
Linoleic acid-1-C^{14}	11,390	198	3,707
Palmitic acid-1-C^{14}	6,732	76	5,384

* The initial count is given for 100 μg of fluid taken from a 5-ml culture.

TABLE 2. *Concentration of C^{14} fatty acids by Entodinium simplex*

Substrate	Initial culture	Supernatant	Washed cells
	*count/min**	*count/min*	*count/min*
Stearic acid-1-C^{14}	20,921	4,343	39,358
Oleic acid-1-C^{14}	5,999	4,073	17,511
Linoleic acid-1-C^{14}	4,448	227	2,747
Palmitic acid-1-C^{14}	2,202	1,461	7,670

* The initial count is given for 100 μg of fluid taken from a 5-ml culture.

TABLE 3. *Gas production* from fatty acids and other substrates by Isotricha prostoma*

Substrate	Endogenous	Experimental	Difference	Duration of run
				min
Sodium oleate	70	124	54	100
Sodium valerate	90	179	89	120
Methyl myristate	99	233	134	30
Methyl laurate	113	180	67	30
Sodium caproate	43	75	32	60
Sodium acetate	104	206	102	50
Glycerol	79	167	88	120
Tributyrin	51	127	76	90
Control (supernatant; no protozoa) Sodium oleate	19	21	2	75

* Expressed as mm of pressure.

TABLE 4. *Total lipid and free fatty acid determinations*

Sample no.	Cellular dry wt.	Chloroform-methanol unpurified lipid	KCl purifiep lipid	Free fatty acid*
	mg	*mg*	*mg*	*mg*
1	2,287	212 (9.27%)	173 (7.56%)	245 (53.49%)
2	4,448	—	348 (7.82%)	—

* From 458 mg of pooled purified lipid.

Total lipid and free fatty acid determinations were made on washed suspensions of *I. prostoma*. The values are given in Table 4.

The chloroform-methanol extraction of the protozoal cells gave a higher lipid fraction value than when 0.74% aqueous KCl was used in the extraction (see Materials and Methods). The purified lipid fraction containing the free fatty acids of the protozoa was chromatographed on paper and found to contain three well-separated spots. Most of the fatty acid content of *I. prostoma* was concentrated in a spot with an R_F similar to palmitic acid, and two lesser spots with R_F values similar to myristic and lauric acid, but which showed unsaturated properties when exposed to potassium permanganate.

DISCUSSION

Crude fractions containing mixtures of microorganisms from the ruminal ingesta have been shown to hydrogenate unsaturated fatty acids which are normal constituents of forages (Garton et al., 1958; Wright, 1959). Bypassing the ruminal organisms by feeding linoleic and linolenic acids to sheep through a duodenal cannula has demonstrated that the fatty acids are not hydrogenated, but are deposited in the fat depots of ruminants in the unsaturated form (Ogilvie, McClymont, and Shorland, 1961). Little is known of the microorganisms responsible for the hydrogenation of fatty acids in the rumen, although crude fractions of ruminal ingesta containing mixed protozoan species have been able to hydrogenate unsaturated fatty acids, such as linoleic acid (Wright, 1959), and to hydrolyze ethyl laurate and ethyl oleate (Hill et al., 1960).

In the present experiments, washed suspensions of protozoal species from the two predominant groups of ruminal ciliates, the holotrichs represented by *I. prostoma*, and the oligotrichs by *E. simplex*, showed a high affinity for C^{14}-labeled fatty acids, and, since these are representative species, it is presumed the ability to metabolize long-chain fatty acids is a common characteristic among

ruminal ciliates. Wright (1959) suggested the degree of unsaturated fatty acids in milk fat may be affected by the protozoal population in the rumen. Thus, the biochemical role attributed to the protozoa in the rumen may have to be modified, since they have currently been connected with functions which did not appear to influence the quality of meat and milk production. Among the physiological functions of the ruminal ciliates are the production of fatty acids which are absorbed and yield energy to the host, their use as a source of protein as they leave the rumen, and their aid in the digestion of forages and feedstuffs such as starch and cellulose. Ingestion of associated bacteria has also been demonstrated for several species of ruminal ciliates (Gutierrez, 1958; Gutierrez and Davis, 1959). Pure cultures of the ciliates have now been shown to hydrogenate compounds such as oleic acid, and the fatty acid also increases the rate of endogenous fermentation in *I. prostoma*. The mechanism(s) whereby fatty acids stimulate gas production remains to be studied.

The experiments of Ogilvie et al. (1961) in which linoleic acid was fed through a duodenal cannula bypassing the ruminal organisms illustrate a drastic method for controlling the degree of fat saturation in the tissues of sheep. A second method, which may be useful for the partial control of fatty acid saturation, is defaunation by drenching animals with solutions of $CuSO_4$ (Becker, Schulz, and Emerson, 1930). The contribution of the bacteria to the hydrogenation of fatty acids in the rumen has been reported to be low (Wright, 1959), but quantitative experiments are necessary to evaluate fully the role played by the protozoa and bacteria in hydrogenation and lipolysis of fats.

LITERATURE CITED

Becker, E. R., J. A. Schulz, and M. A. Emmerson. 1930. Experiments on the physiological relationships between the stomach infusoria of ruminants and their hosts, with a bibliography. Iowa State Coll. J. Sci. **4**:215–251.

Buchanan, M. A. 1959. Paper chromatography of the saturated fatty acids. Anal. Chem. **31**:1616–1618.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497–510.

Garton, G. A., P. N. Hobson, and A. K. Lough. 1958. Lipolysis in the rumen. Nature **182**:1511–1512.

Gutierrez, J. 1958. Observations on bacterial feeding by the rumen ciliate *Isotricha prostoma*. J. Protozool. **5**:122–126.

Gutierrez, J., and R. E. Davis. 1959. Bacterial ingestion by the rumen ciliates *Entodinium* and *Diplodinium*. J. Protozool. **6**:222–226.

Hill, F. D., J. H. Saylor, R. S. Allen, and N. L. Jacobson. 1960. *In vitro* lipolysis by rumen ingesta. J. Animal Sci. **19**:1266.

Miller, C. A., and W. H. Johnson. 1960. Nutrition of *Paramecium:* a fatty acid requirement. J. Protozool. **7**:297–301.

Ogilvie, B. M., G. L. McClymont, and F. B. Shorland. 1961. Effect of duodenal administration of highly unsaturated fatty acids on composition of ruminant depot fat. Nature **190**:725–726.

Williams, P. P., R. E. Davis, R. N. Doetsch, and J. Gutierrez. 1961. Physiological studies of the rumen protozoan *Ophryoscolex caudatus* Eberlein. Appl. Microbiol. **9**:405–409.

Wright, D. E. 1959. Hydrogenation of lipids by rumen protozoa. Nature **184**:875–876.