Phenylpropanoic Acid: Growth Factor for *Ruminococcus albus*

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Phenylpropanoic acid accounted for part of the stimulatory effect of rumen fluid on the rate of growth and of cellulose digestion by cultures of *Ruminococcus albus* strain 8 grown on a chemically defined medium. As little as 3 μM concentration gave maximum response.

A nutritional role for a number of branched and straight-chain volatile fatty acids has been well established through the studies of Bryant and his associates (1–3). Numerous strains of *Bacteroides succinogenes, Ruminococcus albus*, and *Ruminococcus flavefaciens* require one or more of the following acids: isobutyric, isovaleric, 2-methylbutyric, and *n*-valeric.

Many strains of rumen bacteria have been grown on a chemically defined medium containing B vitamins, balanced salts, volatile fatty acids, and a fermentable carbohydrate (4). In addition to these nutrients, the type strain 69 of *R. albus* (8) requires an additional factor in rumen fluid, shown by D. W. Fletcher (Ph.D. thesis, Washington State University, Pullman, 1956) to be a nonvolatile acid. D. J. Clark (M.S. thesis, Washington State Univerisity, Pullman, 1959) found that the methyl ester of the acid has a retention time in a gas chromatograph similar to that of methylmyristate, but myristic acid did not satisfy the rumen fluid requirement.

Because of the advantages of a defined medium in purifying enzymes from cultures of *R. albus*, we studied the nature of the stimulatory factor(s) in rumen fluid and found that part of the stimulation is due to phenylpropanoic acid.

MATERIALS AND METHODS

Culture procedures. Since the type strain 69 is no longer available, a new strain was isolated. Rumen fluid cellulose agar roll tubes (6) were inoculated with serial dilutions of rumen contents from an alfalfa-hay-fed Jersey heifer provided with a rumen fistula. A selected colony, strain 8, was purified by subculture into rumen fluid cellobiose agar roll tubes, returned again to cellulose agar, and stored at −80°C.

The composition of the rumen fluid cellulose agar was one third (vol/vol) deionized or Pyrex-distilled water containing the following ingredients (in g/liter), in the final medium: NaCl, 1.0; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 0.5; $CaCl_2$, 0.1; $MgSO_4$, 0.1; resazurin, 10^{-3}; agar (Difco Laboratories), 12.0; one third a 3% (wt/vol) suspension of Whatman no. 1

filter paper pebble-milled for 24 h in a 1-gal. (ca. 3.785 liters) porcelain jar with flint pebbles; and one third rumen fluid filtered through cheesecloth lined with absorbent cotton. The aqueous portion was first boiled to dissolve the agar, and then the cellulose portion was added. The mixture was boiled again to drive off dissolved oxygen, and then a stream of CO_2 (freed of O_2 by passage over hot reduced copper) was introduced to exclude any air. When the medium had cooled to approximately 55°C, the rumen fluid was added along with 5 g of $NaHCO_3$ per liter and 0.2 g of cysteine hydrochloride.

The agar medium was tubed anaerobically in 3.5-ml amounts in Bellco screw-capped tubes (16 by 125 mm) and stoppered with no. 00 butyl rubber stoppers. After the stoppers were firmly inserted, the protruding ends were cut off with scissors, and the screw caps were applied to hold the stoppers in place. The tubes were autoclaved, and sterile 3% $Na_2S \cdot 9H_2O$ was added to each tube by syringe to give a final concentration of 0.02%. Broth medium was similar except that agar was omitted, the volume was 6 ml, and the cellulose concentration was reduced to 0.3% (wt/vol). The stock culture was carried initially on this cellulose rumen fluid broth, with a 1% inoculum subcultured every 24 h, at which time most of the cellulose was digested.

Growth factor assay. Various crude or fractionated preparations from rumen fluid were tested for growth-promoting activity by addition to 0.3% cellulose broth containing the ingredients listed above except for agar and rumen fluid. Water replaced the volume of rumen fluid and part of the cellulose suspension and, in addition, the following trace minerals were added (mol/liter): H_3BO_3, 10^{-6}; $ZnCl_2$ and $MnCl_2$, 5×10^{-7}; $CoCl_2$, 4×10^{-7}; Na_2MoO_4, 1.5×10^{-7}; Na_2SiO_3, Na_2SeO_3, and $NiCl_2$, 10^{-7}; Na_2WO_4, 10^{-8}; and $Al_2(SO_4)_3$, 10^{-11}. Volatile fatty acids *n*-valeric, isovaleric, isobutyric, and 2-methylbutyric were added, each 1.5×10^{-4} M, along with sodium acetate, 1.2×10^{-2} M. Vitamins were (μg/liter): pyridoxamine, 150; folic acid, riboflavin, and thiamine, 50; *p*-aminobenzoic acid, 30; biotin and cobalamin, 10. The riboflavin and thiamine were filter-sterilized and added after autoclaving. With 0.02% $Na_2S \cdot 9H_2O$, this constituted the basal defined medium cellulose broth to which were added the nutrient supplements to be tested.

The growth response to nutrient supplements was

routinely estimated by visual assessment of the degree to which the volume of the pebble-milled cellulose decreased compared with an unsupplemented control. This method was rapid, convenient, and precise. In a few experiments, the residual cellulose was collected on a small frittered glass filter, dried under vacuum, and weighed. In some experiments cellobiose was used, and the increase in optical density was measured.

Fractionation of rumen fluid. The volatile fatty acids were removed from liter batches of rumen fluid by vacuum distillation after acidification to pH 2 with HCl. A 3-liter round-bottom distilling flask contained the acidified rumen fluid, continuously stirred with a magnetic stirrer-heater. A 2-liter round-bottom receiving flask immersed in ice was connected to the distilling flask and to a vacuum pump. The pump was turned on long enough to remove the dissolved gases and bring the rumen fluid to a rapid boil. At this point, gentle heat was applied to hasten evaporation and keep the rumen fluid at a temperature of 15 to 30°C. No heat was applied when the volume of rumen fluid had diminished below 50 ml. The water, HCl, and volatile fatty acids collected in the 2-liter flask were discarded after tests for growth promotion were negative.

The nonvolatile residue was suspended in 50 ml of water and extracted twice with 150-ml samples of benzene, chloroform, or diethyl ether. These extracts were tested for growth promotion as described below.

The benzene extracts were combined, and 60 ml was evaporated to dryness under a stream of N_2. The residue was dissolved in 2 ml of chloroform-methanol (2/1, vol/vol) and loaded onto a column of Sephadex LH-20 (2.3 by 24 cm) equilibrated with the same solvent. Chloroform-methanol (2/1, vol/vol) was used as the eluent, and 25 10-ml fractions were collected. A 1-ml portion of each fraction was transferred to a culture tube, the solvent was evaporated off, and the tube was filled with CO_2, stoppered, and sterilized. Sterile anaerobic basal defined cellulose broth was injected, and the tubes were inoculated with a rumen fluid-depleted culture of *R. albus* strain 8.

Gas chromatographic and mass spectral analysis. The remaining 9 ml of the growth-stimulating fraction from the Sephadex LH-20 column was evaporated to dryness under a stream of N_2. The residue was dissolved in 0.5 ml of chloroform and was methylated with diazomethane dissolved in ether, prepared from Diazald (Aldrich Chemical Co.). A stream of N_2 was used to evaporate excess ether and diazomethane in a fume hood. A 1.5-μl sample of the methylated fraction was injected into a Finnegan 3200 gas chromatograph-mass spectrograph, equipped with a 30-m SE-54 fused silica capillary column (J & W Scientific) and run from 40 to 280°C at 6°/min. Eluting compounds were ionized at 70 eV, and the mass spectra of the individual peaks were obtained and for identification were computer matched against a library of mass spectral data.

The phenylpropanoic acid content of rumen fluid was estimated by gas chromatography. A defined quantity of acidified rumen fluid was exhaustively extracted with chloroform. The extracts were combined, evaporated down to 2.8 ml under N_2, and methylated with diazomethane. A 10-mg/ml solution of phenylpropanoic acid (Aldrich) in chloroform was also methylated and used as a standard.

RESULTS

The newly isolated strain 8 resembled the type strain 69 of *R. albus* in its colony and cell morphology; rate of growth in cellulose agar; production of H_2, ethanol, and acetic and lactic acids but no succinate; and in its requirement for rumen fluid. Initially, the rumen fluid requirement was strict, and little growth was obtained when it was omitted. A mixture of the sodium salts of acetic, *n*-valeric, isovaleric, isobutyric, and 2-methylbutyric acids improved the growth of cultures lacking rumen fluid. Thiamine, riboflavin, folate, and cobalamin stimulated growth in cultures with 20% (vol/vol) rumen fluid filtered through an Amicon PM-10 filter. *p*-Aminobenzoic acid, biotin, and pyridoxamine were somewhat less stimulatory, and calcium pantothenate was relatively ineffective. A mixture of trace minerals was included in the final assay medium, but the requirements for individual minerals were not examined.

Initial subcultures from stock rumen fluid cellulose broth into the defined broth assay medium without rumen fluid grew about as rapidly as the parallel stock culture on rumen fluid, but after two more transfers growth diminished, and the time for complete disappearance of the cellulose increased to about 4 days as compared with 24 h for the stocks.

These depleted rumen fluid-free cultures were used as inoculum for assaying the effects of various preparations from rumen fluid. Addition of as little as 0.1 ml of sterile rumen fluid caused a marked increase in the rate of cellulose digestion.

The mixture of volatile fatty acids vacuum-distilled from acidified rumen fluid was not stimulatory but the residue was. The growth-promoting factor was stable to heat sterilization at 121°C for 20 min at pH 2, 7, or 11. Benzene, chloroform, or diethyl ether were all effective in extracting the factor from the residue. It could be removed from these solvents by reextraction with an aqueous alkaline solution. The growth factor was stable to ozone treatment and also to catalytic hydrogenation with 45 lb/in^2 of H_2 in the presence of a platinized asbestos catalyst. These results indicated that the factor was a saturated acid.

The concentrated chloroform-methanol solution of the material extracted from acidified rumen fluid was chromatographed on Sephadex LH-20 and 25 10-ml fractions were collected. One milliliter of each fraction was tested for growth promotion. Fraction 7 was markedly stimulatory, and fraction 6 showed a slight stimulation. Gas chromatographic analysis of the methyl esters prepared from the remainder of fraction 7 yielded four principal peaks, eluting at positions 84, 95, 129, and 179 (Fig. 1).
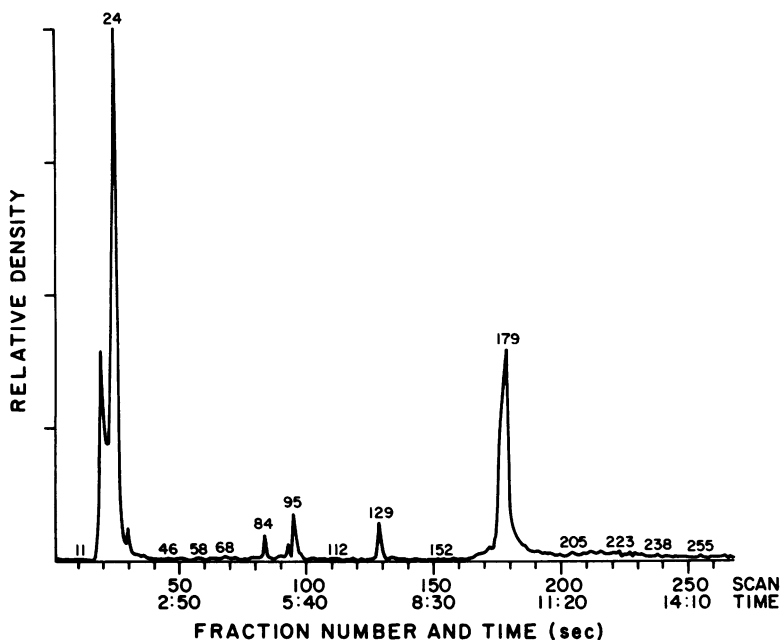
FIG. 1. Results of gas chromatographic analysis of the methyl esters prepared from diazomethane and fraction 7 from the Sephadex LH-20 column. The four principal peaks at 84, 95, 129, and 179 were identified as the methyl esters of cyclohexane carboxylic acid, benzoic acid, phenylacetic acid, and PPA, respectively.

The four peaks were identified by their mass spectra as the methyl esters of cyclohexane carboxylic acid, benzoic acid, phenylacetic acid, and phenylpropanoic acid (PPA), respectively. Pure samples of benzoic, phenylacetic, and phenylpropanoic acids were tested for growth enhancement at 1.5×10^{-4} M. Only PPA stimulated the growth of strain 8, decreasing the time for complete digestion of the cellulose to about 2 days as compared with 4 days in unsupplemented controls.

The concentration of PPA in a sample of rumen fluid was 660 μM.

Visual observations of cellulose disappearance in tubes with various concentrations of

TABLE 1. Effect of PPA concentration on the amount of undigested cellulose after 24 h of incubation

Culture tube no.	Concn of PPA (μM)	Wt of residual cellulose (mg)[a]
1	0	11.0
2	1	9.4
3	2	6.8
4	3	5.3
5	6	5.0
6	12	5.2

[a] Initial cellulose was 18 mg/6 ml.

PPA indicated that as little as 3 μM was as effective as higher concentrations. These observations were confirmed by another experiment in which the residual cellulose was weighed (Table 1). A concentration of 5 μM was selected for the stock culture medium in which the daily transfers had been carried for several months with no diminution in the rate of cellulose digestion.

The maximum optical densities reached by cultures of *R. albus* grown (with PPA) on various concentrations of cellobiose are shown in Table 2. Inspection indicates that maximal optical density is linear with cellobiose concentration only to about 0.15%. At the limiting concentrations of 0.05 and 0.1% cellobiose, the rate of growth was significantly stimulated by PPA at concentrations of 1.5 and 3.0 μM as is shown in Fig. 2 for 0.05% cellobiose.

At the tested nonlimiting concentrations of cellobiose, growth often failed completely, even when inoculated from a successful culture with the identical initial concentration; or a lag period of a few hours to several days sometimes occurred, yet the maximal optical density was always about equal to that shown in Table 2. At these higher cellobiose concentrations, PPA exerted little effect on the time of initiation or the rate of growth.

The requirements for the individual volatile

TABLE 2. Maximal optical densities of *R. albus* cultured on various concentrations of cellobiose, all with 3 μM PPA

Expt	Maximal optical density with concn of cellobiose (%) of:				
	0.05	0.1	0.25	0.5	1.0
1	0.28	0.55	0.78		
2	0.31	0.55	0.80		
3	0.29	0.56	0.81	0.82	0.78

fatty acids were retested after the defined test medium had been supplemented with PPA. Acetate was still essential, and both isobutyric and 2-methylbutyric acids were required (the concentration could be reduced to 2.5×10^{-5} M), but no stimulation by isovaleric or *n*-valeric acid could be demonstrated.

DISCUSSION

PPA was first identified in rumen fluid by Tappeiner (12). He distilled the volatile fatty acids from 40 liters of rumen fluid and identified crystals forming in the residual concentrated liquor as silver phenylpropanoate. PPA is a metabolite in a number of plants (9) and has been shown to occur in ester linkages in cistacea oils (10).

Lactoste-Bastié (Thèse no. 213, University of Toulouse, Toulouse, France, 1965) found that PPA increased when L-tyrosine diminished in concentration in incubated rumen contents, but did not increase when L-phenylalanine was metabolized. Conversion of tyrosine to PPA was confirmed in isotopic experiments by Scott et al.

(11). These investigators found that PPA was more abundant in rumen fluid, 300 μM, than in ruminant blood, urine, or saliva. We found the PPA concentration in a sample of rumen fluid to be 660 μM. Since the growth of *R. albus* strain 8 was achieved at a 3 μM concentration, several transfers into defined medium without PPA were necessary to demonstrate the requirement.

The amount of PPA in the rumen fluid is significantly higher than can be accounted for by formation from L-tyrosine (11). Since the structure of lignin is based on monomeric units of PPA derivatives, it is tempting to postulate that some of the PPA in the rumen derives from anaerobic breakdown and reduction of these materials. Gaillard and Richards (5) have described soluble low-molecular-weight lignin-carbohydrate complexes in the rumen, and Grbić-Galić and Young (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, O20, p. 199) have demonstrated that PPA is an intermediate product in the anaerobic decomposition of ferulic acid.

There is much variability in the nutritional requirements of bacterial strains classified as *R. albus*. The RAM strain (9) exhibited a slightly different colony type in rumen fluid cellulose agar and could be cultured on a defined medium lacking rumen fluid, as could 10 of 15 strains studied by Bryant and Robinson (3). Some of the remaining five may have required PPA, but the great variability in nutritional requirements of independent isolates of *R. albus* and the fact that growth of strain 8 on PPA-supplemented defined medium was still slower than on rumen fluid suggest that rumen fluid contains additional unidentified nutritional factors for some strains of *R. albus*.
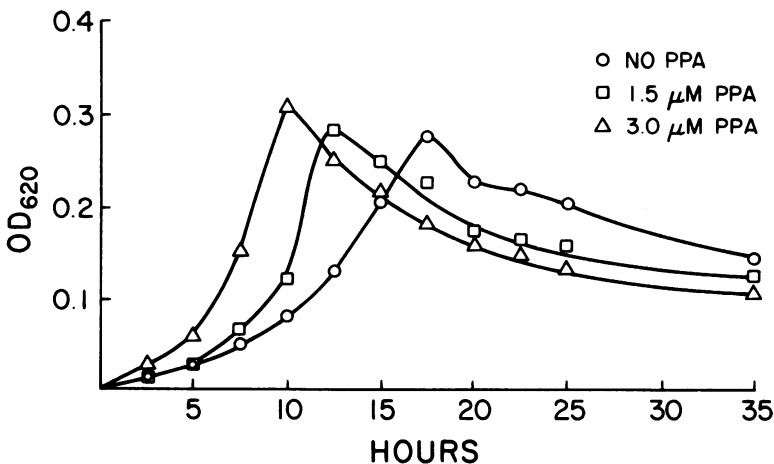

FIG. 2. Effect of PPA concentration on growth of strain 8 on 0.05% cellobiose. OD_{620}, Optical density at 620 nm.

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