Stomach Fermentation in East African Colobus Monkeys in Their Natural State

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The microbial fermentation in the stomachs of two monkeys, Colobus polykomos, collected in Kenya, was studied. The gas accumulated within the stomach contained H_2 but no CH_4. Volatile fatty acid concentrations were high, but accumulated acid prevented determination of the fermentation rate in untreated, incubated stomach contents. Upon addition of bicarbonate, a very rapid rate could be demonstrated. Some D- and L-lactate were in the stomach contents. Starchy seeds or fruits rather than leaves appeared to have been consumed. Microscopically, the most prominent microorganisms seen were large, very refringent cocci, possibly Sarcina ventriculi, and various smaller cocci and rods. The 28 cultured strains of bacteria included 14 Staphylococcus, 2 Streptococcus, 10 Propionibacterium, and 2 Peptostreptococcus. The culture count constituted 10 to 20% of the direct count. No protozoa or cellulolytic bacteria were found.

An active microbial fermentation in the stomach of leaf-eating monkeys has been inferred (14) from the large amount of digesta in the stomach, from production of methane, and from the relatively high concentration of volatile fatty acids in the stomach contents (8). Measurements of fermentation in the langur monkey, Presbytis cristatus (3), and in Procolobus and Presbytis (14) indicate rates comparable to those reported for stomach contents of domestic (6) and wild ruminants (12).

A brief field trip in Kenya in September 1969 afforded an opportunity to obtain two individuals of Colobus polykomos living on the north slope of Mt. Kenya and to culture the stomach contents and measure the rate of fermentation.

MATERIALS AND METHODS

Animals. Two male specimens were collected immediately adjacent to the Landrover containing the equipment used in the study. The first animal, no. 263, was in the Timao Forest on the north slope of Mt. Kenya at an elevation of about 3,200 m; the second, no. 264, was on the Burguret River on the lower edge of the timberline at an elevation of about 2,000 m.

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Each was weighed and dissected as rapidly as possible. For monkey 263, samples of the gas in the stomach were removed 27 min after the animal was killed. For monkey 264 the gas was sampled at 21 min, and the pH was sampled after 26 min.

The contents of the glandular portion of the stomach of 263 were almost white in color and were finely comminuted. The contents of the saccular portion were about as well comminuted but had a slightly green color, presumably due to ingested leaves. Leaves appeared to be a minor component of the diet. The stomach contents of 264 contained almost no green material. A number of large whole seeds (about 1 cm in diameter) were observed in 263. They were somewhat more abundant in the glandular than in the saccular portion of the stomach.

Material from the saccular stomach of each animal was used for the culture and rate experiments. A sample of the contents from the saccular stomach of each animal was fixed with approximately 1 volume of acid-free Formalin. Direct microscopic counts of the bacteria in these samples were made with a Petroff-Hauser counting chamber and corrected for the dilution by the Formalin. Some of the contents of the glandular portion of the stomach of colobus 264 was Formalin fixed and later examined microscopically.

Field equipment. Transportation by air and in the field necessitated miniaturization of equipment and materials.

A special autoclave was made by turning a stainless-steel cylinder to dimensions of 44 by 160 mm and fitting it with an adjustable safety valve. An easily portable supply of carbon dioxide was contained in a

small cylinder (50 by 265 mm) provided with a needle valve outlet. The valve was threaded into a 15-mm opening at one end of the cylinder. To replenish the gas supply, the needle valve assembly was unscrewed and the cylinder was filled with powdered dry ice. The assembly was then screwed tightly into the cylinder, the valve was opened, and carbon dioxide was allowed to escape for 5 or 10 min. This washed out other gases. The CO_2 was used untreated from the cylinder; facilities for scrubbing traces of oxygen were not feasible in the field.

Small Pyrex culture tubes (10 by 75 mm) were provided with special butyl rubber stoppers (Jenkyns Rubber Co., London, England) (8 mm in diameter at the small end and 11 mm at the large end, 20 mm long). A small hole was drilled in the center of the upper surface, extending two-thirds through the length of the stopper to permit easier penetration by a syringe needle. Prior freezing in dry ice facilitated boring the hole with an ordinary drill press. The tubes were gassed out with carbon dioxide, stoppered, and sterilized before being taken into the field.

Monoject 1-ml sterile disposable syringes, graduated in 0.01-ml divisions, fitted to 1-inch (about 2.5 cm), 20-gauge disposable needles (Becton Dickinson and Co.), were used for quantitative dilutions and for subcultures. In the latter case, the inoculum in only the dead space of the syringe and needle was transferred. Disposable 10-ml syringes were used for filling the tubes and for measuring out media.

Media. Media used for initial cultivation included sheep rumen fluid cellulose broth, sheep rumen fluid without added carbohydrate, sheep rumen fluid cellulose agar (SF), colobus stomach fluid agar (CF), and colobus stomach fluid cellulose agar (CC). The methods of anaerobiosis were essentially as described earlier (10), except that the later modification using syringes and needles (11) was substituted for the earlier dilutions by pipettes and the gassing with glass capillaries.

CF medium was prepared in the field. Some of the stomach contents were diluted with an equal volume of water, mixed, and filtered by squeezing the mixture through several layers of cheese cloth. The filtrate was used as two-thirds of the final medium. The remainder was a mixture of equal parts of mineral solutions A (0.6% NaCl, 0.3% KH_2PO_4, 0.15% $(NH_4)_2SO_4$, 0.06% $CaCl_2$, and 0.06% $MgSO_4$) and B (0.3% K_2HPO_4). Ionagar (Colab laboratories, Inc., Glenwood, Ill.) at a 0.7% concentration was melted in the mineral mixture before addition of the stomach fluid. Resazurin (0.0001% final concentration) was included in all media.

The medium, total volume of 18 ml, was placed in a 30-ml Pyrex Kjeldahl flask with a shortened neck, constricted to take the small stopper used on the culture tubes. After being autoclaved, the medium was cooled to 50 C, and the required amounts of 10% $NaHCO_3$ and 2.5% cysteine hydrochloride were injected and mixed to give final concentrations of 0.5 and 0.025%, respectively. The melted medium was tubed in 1.8-ml amounts and kept in a bucket of water at 50 C until inoculated. The temperature of water baths was maintained by addition of boiling water as needed.

CC medium was similarly prepared except that a pebble-milled suspension of 2% filter paper cellulose composed one-third of the medium, undiluted colobus stomach fluid composed one-third, and equal parts of mineral solutions A and B constituted the remainder.

The sheep rumen fluid broth (one-third each water, sheep rumen fluid filtered through cotton, and mineral solution AB), sheep rumen fluid agar (same as broth but agar added), and SF (2% pebble-milled cellulose suspension substituted for the water) had been prepared in the culture tubes at Cambridge, England, by using rumen fluid from a sheep fed on grass. No additional substrate was added to the rumen fluid agar medium since previous experience with the rumen indicated that the colony count obtained without added substrate was about as high as with substrate and, though the colonies were smaller, longevity was greater, presumably because of decreased metabolite production. The bicarbonate and cysteine were added to the tube after the medium was melted and ready to be inoculated in the field.

The colobus stomach contents were the consistency of a thick paste but were sufficiently liquid that, after mixing, a 1-ml sample could be sucked up into the calibrated portion of a 5-ml measuring pipette broken off squarely at the 5-ml calibration mark and provided with a rubber mouth tube. The sample was transferred to a tube containing sheep rumen fluid broth, the tube was gassed out, and the contents were thoroughly mixed. A 0.2-ml portion was then injected into a second rumen fluid dilution tube and further serially diluted through seven more tubes. From these, 0.1 ml was inoculated into each tube of the various culture series. After removing the inocula from the tubes of the original sheep rumen fluid dilution series, 0.2 ml of 2% cellulose suspension was injected into each tube to test for cellulose digestion that might occur in liquid but not in agar cultures.

After inoculation, the tubes from the first monkey were rolled in ice water, labeled, and incubated in the investigator's pocket. They were taken to the guest house overnight, where they were placed in a vacuum bottle containing water at 45 C. This had cooled by morning, when the temperature was adjusted to 39 C. The cultures from monkey 264 were placed in the vacuum bottle immediately after the agar had solidified, and that same day were transported back to the laboratory at Muguga, where all tubes were incubated at 39 C.

After 8 days, the incubated tubes were examined and the colonies were counted in the higher dilutions showing growth. Individual colonies selected as representative of those observed were subcultured to a similar medium and also to rumen fluid glucose agar. In the higher dilutions containing fewer than 12 colonies, all colonies were subcultured.

Subcultures were made at Muguga with the intent of shipping them immediately to California for further study, but permission to import was not obtained in time and it was necessary to take the cultures to New Zealand, where they were maintained until they could be sent to California. Between the initial subculture and the final characterization, a number of the strains were lost.

Zero time rate measurement. A 40-g sample of the

fresh stomach contents was placed anaerobically in a rubber-stoppered container and immersed in the water bath at 39 C. At 0, 15, 30, and 60 min for colobus 263 and at 0, 21, and 41 min for 264, a subsample of the incubated material was removed to a previously weighed plastic tube containing 1 ml of 10 N NaOH. The subsample was mixed with the alkali, and its weight was determined by difference. The alkaline samples were shipped to Davis by air freight and were analyzed immediately for volatile fatty acids.

Nonvolatile acids in these samples were determined in 1971 after adding water to make up that lost by evaporation during storage.

Gas analyses. A sample of the gas in the stomach was taken by syringe before the stomach wall was opened. The short sampling needle was replaced with a 20-gauge, 6-in (about 15 cm) needle, and the gas was transferred to the base of a dry culture tube (16 by 150 mm), drawn out to a capillary in its central portion. Some of the gas sample was used to flush out the base of the tube, and the narrow portion was then quickly sealed with a propane torch to enclose the sample in an all-glass container. The samples were sent to Davis and analyzed with a Perkin-Elmer model 154B gas chromatograph, using a silica gel column and N_2 as carrier gas.

Analysis of fermentation products and substrates. The pH of the stomach contents was tested with paper having a range of 5.5 to 7.0. Volatile fatty acids in the stomach contents and in the products of the metabolism of pure cultures were determined on a 700 F and M gas chromatograph with a flame ionization detector and a 6-ft (about 1.9 m) column filled with FFAP on Chromosorb W. Carrier gas was helium at a flow rate of 40 ml/min. Column temperature was 125 C, injection port was 210 C, and detector was 290 C. Membrane-filtered (0.22 μm pore size; Millipore Corp.) acidified supernatant fluid was injected directly into the column, or, in analyses of pure cultures, the volatile fatty acids were first distilled at low temperature in a closed vacuum system with the receiving tube dipped in ice water.

Qualitative examination for nonvolatile acids was performed by spotting acidified stomach contents on a thin-layer chromatographic plate of ECTEOLA cellulose 300 (Macherey, Nagel and Co.), separating the acids by development with ethanol-water-NH$_4$OH (16:3:1) and detecting with a spray of 0.04% bromothymol blue adjusted to pH 8.0.

For quantitative measurements, samples were first made alkaline and evaporated to dryness; they were then acidified and the volatile fatty acids were separated by vacuum distillation. The residue was extracted with three 5-ml volumes of freshly distilled anhydrous peroxide-free ether. Water (0.2 ml) was added, and the acids were titrated. The ether was evaporated off at room temperature, and water was added to a total volume of 1 ml. L(+)-Lactate in this material was determined with the Lactostat Kit (Sigma Chemical Co.). This involved enzymatic oxidation to pyruvate and reduced nicotinamide adenine dinucleotide, the latter being determined spectrophotometrically. D(−)-Lactate also was determined from enzymatic nicotinamide adenine dinucleotide reduction by using D(−)-lactic dehydrogenase (Boeh-

ringer Co., Mannheim). In both reactions, the pyruvate formed was removed by a pyruvate-glutamate transaminase reaction

Total nitrogen was measured by the Kjeldahl method. The anthrone method was used for carbohydrates.

To determine the starch content of the Formalin-fixed stomach contents, a 75-mg sample was dried to constant weight and suspended in 25 ml of water; 1 ml of human saliva was added, and a 10-ml sample was immediately cooled in an ice bath and centrifuged at 4 C. The remainder was incubated for 24 h at 37 C, and a 10-ml sample was similarly centrifuged. The increase in anthrone values of each supernatant after saliva treatment was used as a rough measure of the starch content. Total carbohydrate was estimated by taking 0.1 ml of the above suspension and analyzing it by the anthrone method.

For analyses of the fermentation products of the pure cultures, the strain was inoculated into either peptone beef extract broth (PB) or, for some requiring additional nutrients, into dilute rumen fluid broth. PB contained 4.5 g of peptone (Difco), 2.25 g of beef extract, 0.45 ml of 0.1% resazurin, 210 ml of deionized water, 75 ml of mineral solution A, 75 ml of mineral solution B, 90 mg of cysteine hydrochloride, 11.25 ml of 4% glucose solution, and 33.75 ml of 10% NaHCO$_3$ solution.

The rumen fluid medium differed in containing only 0.135 g of peptone and 0.135 g of beef extract, with 45 ml of rumen fluid clarified by centrifugation replacing 45 ml of water.

To assure identical CO_2 content in all tubes, the medium (except for the glucose and bicarbonate) was prepared in a round-bottom, 1-liter flask, equilibrated at room temperature with 100% CO_2, sealed, and sterilized. The sterilized medium was cooled to room temperature and then opened, with a gassing needle to exclude air from the flask but without bubbling the CO_2 through the medium. The sterile glucose and bicarbonate solutions were then added aseptically and anaerobically and mixed, and 9 ml of the medium was transferred anaerobically to the sterile culture tubes, each containing 1 ml of water. Air was continuously excluded also from the culture tube without bubbling the contents, and the tubes were closed with a recessed butyl rubber stopper. They were inoculated with 0.2 ml of culture, and some were refrigerated as a control. The same media without glucose were inoculated and incubated as a control for fermentation products formed from substrates other than glucose.

After completion of growth at 37 C, the cultures were allowed to come to room temperature and the gas produced was measured. A 1-inch, 21-guage needle, attached to a 20-ml syringe well lubricated with water, was inserted through the stopper, and the contents of tube and syringe were equilibrated by vigorous shaking. The amount of excess gas in the syringe was recorded. Without removing the first syringe, 1 ml of normal HCl was injected and, after equilibration, the total gas volume was again read. The second volume minus the first gave a measure of the residual bicarbonate in the tube. The difference between the second volumes in the incubated and in the refrigerated tubes gave the volume of gas pro-

duced in metabolism, aside from that released from bicarbonate. The difference in residual bicarbonate gave a measure of the amount of acid produced during metabolism. The differences in gas volumes for duplicate cultures differed usually by less than 2%.

One milliliter of 2 N NaOH was then injected, still without removing the initial syringe, and the CO_2 was absorbed. Ten milliliters of N_2 was injected to maintain a gas pressure above atmospheric. This third volume was read, the gas in the syringe was injected back into the tube, and the syringe was withdrawn. The difference between the gas absorbed in the incubated and refrigerated cultures gave the amount of CO_2 produced or used. The gas in the tube was analyzed for H_2 by injecting 0.5 ml into a Perkin-Elmer 154B gas chromatograph with a silica gel column and N_2 as carrier gas. Peak heights were compared with a standard.

Fermentation rate measurements. A volume of mineral solution containing 0.5% $NaHCO_3$ was added to an equal volume of stomach contents in each of three wide-mouth bottles closed with a rubber stopper perforated by a 2-inch (about 5 cm) needle attached to a water-lubricated 10-ml glass syringe (16). An initial interval was allowed for temperature equilibration to 39 C and to check the uniformity in the rate of gas production in the three bottles. Strong acid was then added to one of the bottles as a control to release all CO_2 from the bicarbonate. Differences in residual bicarbonate gave a measure of the difference in acid production. The total gas released during the fermentation minus that due to acid production gave a measure of the amount of gas produced in metabolism.

Before each reading, the contents of each bottle were vigorously shaken by hand to equilibrate the CO_2 between liquid and gas phases. The amounts of gas evolved were corrected to standard conditions. A temperature of 39 C and vapor pressure of 52 mm of Hg were assumed, though the actual temperature and vapor pressure were somewhat lower than this because the measuring syringe was not immersed in the water bath. Barometric pressures of 522 and 564 mm of Hg ($= 6.95 \times 10^4$ and 7.5×10^4 N/m^2) were assumed for the experiments with colobus 263 and 264, respectively. The pressure increase due to the weight of the syringe barrel, 10 g/cm^2 ($= 9.806 \times 10^2$ N/m^2), amounted to less than 1.5% of the total pressure and was neglected. All of these errors made the reported rates of gas production slightly less than the actual.

Determination of guanosine-cytidine percentages. Strains identified as *Staphylococcus* were treated with lysostaphin (Schwartz/Mann, Orangeburg, N.Y.) according to Klesius and Schuhardt (13), and the base composition of the recovered deoxyribonucleic acid was estimated from its buoyant density in a cesium chloride gradient by using a Spinco model E analytical centrifuge.

Tests on Staphylococcus aureus. For production of enterotoxin, strains were grown in sac cultures according to the method of Donnelly et al. (7). The amount of enterotoxin formed was measured according to the microslide method of Untermann (17). Stan-

dard disks were used to measure antibiotic sensitivity. For arsenic and cadmium sensitivity measurements, disks were impregnated with 250 μg of $Na_2HAsO_4 \cdot 7H_2O$ or 3.1 μg of $Cd(NO_3)_2 \cdot 4H_2O$.

RESULTS

Measurements in the field and many of those from the laboratory are shown in Table 1.

The results of analyses of zero time rate samples are shown in Table 2. These were from gas chromatographic analyses completed in the fall of 1969 just after the materials were shipped to Davis. The zero time rate experiments were started 41 min after colobus 263 was sacrificed and 35 min after colobus 264 was sacrificed. The Formalin-fixed stomach contents, analyzed in 1971, contained roughly the same kinds and quantities of fermentation products. The Formalin had been specially purified for fixation of tissues and was free of acids.

In 1971, the zero time rate samples were examined also for nonvolatile acids. The thin-layer chromatography plates did not show any succinic acid but did show a little lactic acid. For neither one did the relative size of the spots indicate that their concentration increased during the incubation period. The amounts of lactic acid are shown in Table 2, including the results of analyses for D- and L-lactate on the Formalin-fixed stomach contents.

Fermentation rate. In the experiment with stomach material from colobus 263, three bottles were incubated, each containing 42 ml of colobus stomach contents (saccular portion) mixed with 42 ml of balanced salt solution containing 0.5% $NaHCO_3$. The samples were

TABLE 1. *Measurements on two colobus monkeys*

Measurement	Colobus 263	Colobus 264
Temperature	39 C	Not taken
Sex	Male	Male
Weight (kg)	9.5	12.3
Estimated age	3–4 years	
Composition of stomach gas sample	27% H_2	26% H_2
	50% CO_2	29% CO_2
	No CH_4	No CH_4
Wet weight of whole stomach and contents (kg)	1.0	1.36
Dry matter in contents of saccular portion (%)	18.7	31.7
pH	≤5.5	≤5.5
Culture count	5.0×10^9/ml	2.6×10^9/ml
Direct microscopic count	3.8×10^{10}/ml	2.6×10^{10}/ml
N content, based on dry wt (%)	4.73	1.37
Starch content, dry wt (%)	3.75	5.64
Total carbohydrate, dry wt (%)	29.6	32.8

TABLE 2. *Results of the zero time rate analysis*

Fermentation products	Amt (μmol/ml) in colobus 263 after incubation for:			Amt (μmol/ml) in colobus 264 after incubation for:		
	0 min	15 min	30 min	0 min	24 min	41 min
Acetic acid	61	64	66	338	166	162
Propionic acid	19	15	19	48	17	20
n-Butyric acid	18	18	26	32	25	24
Isovaleric acid	2.2	2.6	4.5	0	0	0
Valeric acid	7	10	10	16	14	6
Caproic acid	Trace	Trace	Trace	Trace	Trace	Trace
L(+)-Lactic acid[a]	7.4	3.8	1.97	1.10	3.04	3.23
D(−)-Lactic acid[a]	2.22			0.455		

[a] Forestomach contents of colobus 263 contained 1.97 μmol of L(+)-lactate per ml and 1.20 μmol of D(−)-lactate per ml. For colobus 264, the values were 1.26 and 2.64, respectively.

incubated at 39 C, and the gas production was measured to determine whether the rates in the three vessels were comparable. After 15 min of incubation, excess acid was added to one vessel, releasing 4.3 ml (corrected) of gas from bicarbonate. The other vessels, incubated an additional 10 min before acid was added, also showed only 4.3 ml of gas liberated by the acid. The amount of bicarbonate remaining after equilibration was apparently insufficient, and the experiment did not measure the fermentation acids formed. The rates of gas production in the three vessels were 31, 27, and 25 μmol per h per g of fresh stomach contents, respectively.

In the run the next morning with colobus 264, only 32 g of stomach contents was used, and the bicarbonate solution was increased to 50 ml. Figure 1 shows the gas evolution in the three vessels and also the amount of residual bicarbonate in each when acid was added. The rates of gas production in bottles 1, 2, and 3 were 148, 194, and 195 μmol per g per h, respectively.

Comparison of bottles 2 and 3 between 17 and 23 min (Fig. 1) shows a difference of 7.1 ml (corrected) of CO_2 released from the residual bicarbonate, indicating that 316 μmol of the gas evolved during this part of the fermentation was CO_2 released from bicarbonate by the fermentation acids. The total gas produced and released by acid in bottle 2 during this period was 408 μmol, giving 92 μmol as the amount of CO_2 and H_2 produced in the fermentation and 316 μmol as the fermentation acids.

In calibration experiments done previously in California with bovine rumen contents plus mineral solution AB containing 0.5% $NaHCO_3$, incubated in an atmosphere of CO_2 (giving a pH of about 6.7), the amount of CO_2 released by added lactic acid was equivalent to only about 70% of the amount expected if eq of acid liberates 1 mol of CO_2. Correction for a similar
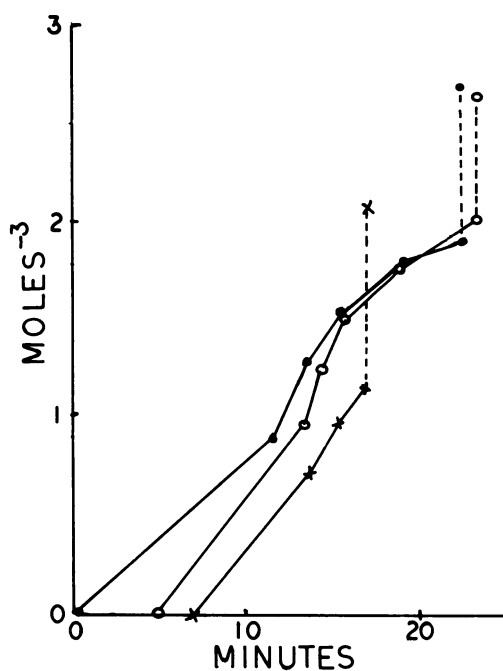

FIG. 1. *Gas production in incubated samples of forestomach contents of colobus 264. Solid lines show gas production during the fermentation; broken lines show the gas evolved from added acid. Stomach contents (32 ml) were incubated in 125-ml rubber-stoppered bottles with 50 ml of balanced salt solution containing 3,168 μmol of $NaHCO_3$. Fermentation was stopped with 4 ml of 5 N H_2SO_4. Symbols: ●, bottle 1; ○, bottle 2, ×, bottle 3.*

lack of stoichiometry in the colobus 264 experiments gave a ratio of fermentation acid to gas of about 5.

For bottles 1 and 3 between 17 and 22 min, 132 μmol of CO_2 was released by fermentation acids, and 118 μmol of CO_2 and H_2 was produced in metabolism. With the 0.7 correction,

the ratio of acid to gas was 1.6. The low ratio can be explained in part by the longer incubation of sample 1, with consequent greater depletion of bicarbonate. The experimental error was fairly large in these determinations because of the short incubation time.

Nature of the bacteria. The Formalin-fixed stomach contents were examined at Muguga with an oil immersion phase microscope. The microscopic appearances of the two animals were remarkably similar. Large cocci arranged in diplo and tetrad forms were morphologically the most distinctive bacteria observed. Some of them were very refringent, bright in appearance under dark-phase illumination, and regularly arranged in twos or tetrads. A few spirochetes were seen. Photomicrographs of the Formalin-fixed material gave the appearance shown in Fig. 2. In colobus 264, the large refringent cocci were relatively more abundant in the contents from the glandular region of the stomach as compared with the forestomach material. The former was not collected from colobus 263.

Culture counts for colobus 263 on the CF series and on the sheep rumen fluid series showed 2.2×10^9 and 7.7×10^9 colony-forming units per ml of fresh forestomach contents, respectively. For colobus 264, the counts were the same for both of these media, namely 2.6×10^9. Direct counts on the Formalin-fixed material were 3.8×10^{10} and 2.6×10^{10}/ml for colobus 263 and 264, respectively.

None of the culture series containing cellulose showed any cellulose digestion even after 7 weeks of incubation. Two large, non-cellulolytic colonies in tubes 5 and 6 of the CC medium were subcultured as strains 1-6cc-1 and 1-7cc-1. In this strain designation, the first number indicates the first, 263, or second, 264, monkey. The second number is the dilution tube from which the colony was picked, with the letters indicating the medium (cf for colobus fluid agar, cc for colobus fluid cellulose agar, and sf for sheep rumen fluid agar). The third number is that of the colony picked, the letter following being used to designate the strain when more than one was isolated from that colony.

Ten colonies were subcultured from the eighth dilution tube of the CF agar series inoculated from animal 263, and five were subcultured from the ninth dilution. Ten colonies were subcultured from the eighth dilution tube of the CF series inoculated from 264, five were subcultured from the eighth dilution of the sheep rumen fluid agar series, and nine were subcultured from the ninth dilution. Three first subcultures and some subsequent subcultures
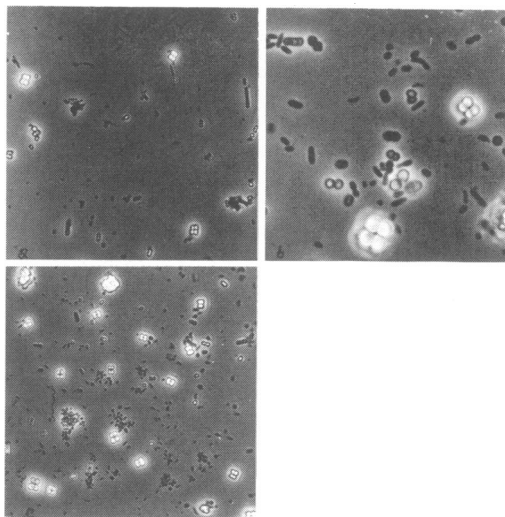

FIG. 2. (a) Forestomach contents of colobus 263. Dark-phase illumination; ×330. (b) Forestomach contents of colobus 264. Dark-phase illumination; ×825. (c) Glandular stomach contents of colobus 264. Dark-phase illumination; ×330.

failed to grow even though some colobus fluid was included in the medium. Of the remaining picked colonies, 27 survived further vicissitudes of shipment and various mishaps. From one of them two different bacteria were isolated, giving a total of 28 strains that were studied.

Sixteen of the 28 strains characterized were euryoxic, of which 14 formed catalase. These 14 were gram-positive cocci, in single, diplo, tetrad, or irregular chain arrangement, and fermented glucose without producing visible gas bubbles. The diameter of the cells varied from 0.6 to 1.2 μm, and in some strains was quite variable. Nine of the strains formed white colonies, of which four strains liquefied gelatin; four of them were light yellow and did not liquefy gelatin. One strain was golden yellow and fermented mannitol, whereas the others did not. It reduced nitrate but did not liquefy gelatin.

The golden strain (2-8sf-2) was identified as *S. aureus*. It was identical to *S. aureus* ATCC 14458 in morphology and in production of coagulase, proteinase, lipase, nuclease, and phosphatase. ATCC 14458 gave only delta-hemolysis on sheep, cow, rabbit, and human blood, whereas strain 2-8sf-2 showed a hemolysin for all bloods tested and was beta-hemolytic on sheep and cow blood. Strain 2-8sf-2 also differed in being sensitive to arsenic and cadmium, streptomycin, tetracycline, and penicillin. It was negative for production of enterotoxins A, B, C, and D (Table 3). The phage type was not

typable at routine test dilutions, but at 1,000 × concentration the phage type was 42A/52/42D/29/55/79 (4).

The other 13 euryoxic catalase-positive strains (Table 4) also showed the characteristics of the genus *Staphylococcus* and were presumably *S. epidermidis* (1). For several representative strains the guanosine-cytidine percentages were determined (Table 5).

Lactic acid was the chief fermentation product of the three tested strains of staphylococcus grown anaerobically on glucose (Table 6). The recovered lactate accounted for 75 to 88% of the glucose provided. The lactate was chiefly the L-form for two of the strains. The third strain formed almost equal amounts of L- and D-lac-

tate. The acetate in the experimental culture was no greater than in the uninoculated control. The calculated yield of cells was low for a homolactic fermentation, only 11.2, 13.2, and 10.6 μg of cells per μmol of glucose for the three cultures, respectively. The yield of cells was estimated from the nitrogen content of the sediment of the culture, because it was necessary to add $CaCO_3$ to obtain good growth. In an experiment without $CaCO_3$, the nitrogen content of the cells of strain 2-9sf-4 was 12.6% of the dry weight.

The two euryoxic catalase-negative strains were gram-positive cocci to rods, 0.2 to 0.4 μm in diameter in the original culture, but later, on media containing yeast extract, the diameter

TABLE 3. *Detection of enterotoxins A, B and C, if produced by Staphylococcus aureus strain colobus*

Test	S. aureus enterotoxin				
	ATCC 13565 enterotoxin A producer[c]	ATCC 14458 enterotoxin B producer[c]	ATCC 19095 enterotoxin C producer[c]	315 F.R.I. enterotoxin D producer[c]	Staphylococcus strain colobus
Gel diffusion tubes[a]					
Anti-enterotoxin A (1:40)	+	−	−		−
Anti-enterotoxin B (1:100)	−	+	−		−
Anti-enterotoxin C (1:60)	−	−	+		−
Gel diffusion tubes[b]					
Anti-enterotoxin A (1:40)	+	−	−		−
Anti-enterotoxin B (1:100)	−	+	−		−
Anti-enterotoxin C (1:60)	−	−	+		−
Micro-Ouchterlony slides[a]					
Anti-enterotoxin A (1:25)	+	−	−	−	−
Anti-enterotoxin B (1:100)	−	+	−	−	−
Anti-enterotoxin C (1:100)	−	−	+	−	−
Anti-enterotoxin D (1:50)				+	−
Micro-Ouchterlony slides[b]					
Anti-enterotoxin A (1:25)	+	−	−	−	−
Anti-enterotoxin B (1:100)	−	+	−	−	−
Anti-enterotoxin C (1:100)	−	−	+	−	−
Anti-enterotoxin D (1:50)				+	−

[a] With supernatants.
[b] With supernatants of sac cultures; 7.4% brain heart infusion broth inside dialysis sacs and surrounded by 3% NZ Amine NAK and 3% protein hydrolysate with staphylococci.
[c] Supernatants of control cultures.

TABLE 4. *Classification of the 28 cultured bacterial strains*

Staphylococcus		Streptococcus	Propionibacterium	Peptostreptococcus
aureus	epidermidis			
2-8sf-2	1-6cc-1, 1-8cf-2a, 1-8cf-4, 1-8cf-6, 1-8cf-8, 1-9sf-3, 1-9sf-4, 2-8cf-1, 2-8cf-5, 2-9sf-2, 2-9sf-4, 2-9sf-6, 2-9sf-7.	1-7cc-1 1-8cf-7	1-8cf-2b, 1-8cf-5, 1-8cf-9, 1-8cf-10, 1-8cf-11, 2-8sf-1, 2-8sf-3, 2-8sf-4, 2-9sf-5, 2-8cf-4.	1-9sf-2 2-9sf-1

TABLE 5. *Base ratios in euryoxic catalase-positive strains*

Strain	Buoyant density	Guanosine-Cytidine (%)
1-8cf-8	1.6968	37.55
1-8cf-2a	1.6942	34.98
1-6cc-1	1.6946	35.31
2-9st-1	1.6946	35.31
2-9sf-2	1.6938	34.49
Escherichia coli	1.710	51.02
Micrococcus lysodeikticus[a]	1.730	72.45
Staphylococcus aureus (ref. 1)	1.694	

[a] Western Regional Research Laboratory strain 2039.

TABLE 6. *Fermentation products from three strains of Staphylococcus*

Strain	Glucose used (μmol/ml)	Fermentation products			Carbon recovery (%)
		L(+)-lactate (μmol/ml)	D(−)-lactate (μmol/ml)	Wt of cells[a] (μg/ml)	
1-6cc-1	35	44	8.9	3.9	82
1-8cf-8	35	47	4.1	4.6	80
2-9sf-4	35	38	27	3.7	99

[a] Estimated from the nitrogen content.

was about 1 μm. Chains were formed in typical streptococcal fashion, and the strains were assigned to the genus *Streptococcus*.

The obligately anaerobic strains included eight which were gram-positive, straight or variously bent, V-shaped or clustered long rods, 0.5 to 0.75 μm in diameter and 1 to 5 μm long. They were nonmotile and without capsules. They produced large, circular, entire, thin, lenticular, deep colonies and opaque, smooth, white colonies on the surface of the agar in the roll tubes. Three tested strains (1-8cf-2b, 1-8cf-10, and 2-8cf-4) were catalase positive and produced acetic and propionic acids in the ratios expected for *Propionibacterium*. The production of H_2 varied (Table 7). A slight amount of H_2 was produced by all three strains when the medium contained 0.5% peptone and 0.3% beef extract, and only in strain 1-8cf-10 was the H_2 production increased by glucose. One strain produced a slight amount of H_2 from rumen fluid. All three strains grew on the glucose-free peptone, and the amount of fermentation products was not greatly increased by addition of glucose (Table 8). The eight strains were assigned to the genus *Propionibacterium*.

The other four anaerobic strains were not as easy to classify. Cells of strain 1-8cf-9 ranged in shape from cocci to rods, 0.5 μm in diameter, single, in V's or close clusters. The Gram reaction was not clearly negative or positive. The strain produced 9 μmol of acetic acid per ml when grown on PB, and this amount was not increased if glucose was added. No propionic acid was formed on the PB medium, but if glucose was added, 1 μmol of propionic acid per ml was formed. The strain did not produce H_2 at any time and is probably closely related to *Propionibacterium*.

Strain 2-8sf-1 contained gram-positive rods to cocci, single, in pairs or chains, 0.75 to 1.25 μm in diameter and 1.25 to 2.0 μm long. They were nonmotile and without capsules, and formed acetic and propionic acids in the ratio expected for *Propionibacterium*, but they also produced a great deal of H_2 (Table 7). In its fermentation products, the strain appeared to be intermediate between *Propionibacterium* and *Veillonella*. A slight amount of H_2 was formed on PB or rumen fluid medium alone, but addition of glucose caused copious H_2 production. There was very little growth on rumen fluid medium alone but a fair amount with glucose added. Even with added glucose, the optical density on rumen fluid medium was not as great as that on PB, which was about half of that on PB plus glucose (Table 7). Traces of *n*-butyric and isovaleric acids were formed, the amounts being slightly increased by glucose.

Strain 1-9sf-2 was non-saccharoclastic and formed some acetate and H_2 from the PB medium without glucose. It grew very poorly on the few media tested. Morphologically it consisted of chains of cocci and is probably referable to the genus *Peptostreptococcus*. No propionic or butyric acid was formed. Strain 2-9sf-1 resembled 1-9sf-2 in morphology and culture characteristics.

DISCUSSION

The results from the zero time rate experiments demonstrate that within 30 min after death of the animal, the fermentation was inhibited by the acids formed, in agreement with the observations of Kuhn (14).

Rapid acid production was indicated by the in vitro fermentation rate experiments. With colobus 263, the bicarbonate added was insufficient to bring the pH into the range (about pH 7.0) at which the acids produced could be measured by release of CO_2 from bicarbonate. In this experiment, 2,520 μmol of $NaHCO_3$ (60 μmol/ml) were added. Addition of excess acid at 34, 44, and 45 min of incubation of the three

TABLE 7. *Optical density and total H_2 production for three colobus strains of Propionibacterium and three other anaerobic isolates*

Strain	Peptone beef extract				Rumen fluid			
	Glucose		No glucose		Glucose		No glucose	
	OD[1]	H_2 (nmol)	OD	H_2 (nmol)	OD	H_2 (nmol)	OD	H_2 (nmol)
1-8cf-2b	0.34	88	0.16	111	0.52	171	0.005	83
2-8cf-4	0.53	49	0.11	53	0.18	4	0.000	−2
1-8cf-10	0.42	446	0.26	86	0.09	−42	0.023	7
1-8cf-9	0.36	−12	0.36	−10	0.12	2	0.05	12
2-8sf-1	0.50	88,437	0.22	2,162	0.112	115,000	0.000	1,951
1-9sf-2	0.003	5,045	0.000	4,566	0.06	7,078	0.005	5,489

[a] Optical density (OD) was measured at a wave length of 600 nm in a cuvette with a 1-cm light path.

TABLE 8. *Fermentation acids formed by three colobus strains of Propionibacterium and three other strains[a]*

Strain	Peptone beef extract + glucose (μmol)				Peptone beef extract (μmol)			
	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid
1-8cf-2b	6.08	34.5	0.32	1.28	4.75	20.6	0.39	0.90
1-8cf-10	5.25	19.2	0	0.85	0.90	4.64	0	0.23
2-8cf-4	7.10	23.0	0.23	0.93	3.63	9.34	−0.03	0.16
1-8cf-9	77.6	9.43	0.32	0.25	77.7	16.0	0.13	0.30
2-8sf-1	26.2	50.9	1.38	1.78	8.76	41.44	0.54	1.21
1-9sf-2	0.45	−0.18	−0.06	−0.03	2.49	0.05	0.02	−0.03

[a] Each value is the total amount in the 10-ml culture minus the amount in the refrigerated control. Glucose provided was 55 μmol; residual glucose was not determined.

samples, respectively, released in each case only 192 μmol of gas from the residual bicarbonate.

The acid production in the live monkey stomachs must have been considerably greater than in ruminants collected in nature and similarly studied (12). In the ruminant studies, only 30 μmol of NaHCO₃ per ml of rumen contents was sufficient to maintain an excess of bicarbonate during the in vitro fermentation rate measurements made at about the same time after death of the animal.

With colobus 264, 99 μmol of NaHCO₃ per ml of stomach contents was added, and in this case 48, 714, and 536 μmol of gas were released by . acid added after 17, 23, and 24 min of incubation, respectively, as compared with the 3,168 μmol total of added bicarbonate. With colobus 264, the total gas released, both during the run and by acid added later, amounted to 2,076, 2,634, and 2,813 μmol for the three samples, respectively.

The more rapid development of acidity as compared with the rumen could be due to a greater availability of substrate in the colobus, to poorer buffering, or to poorer absorption. The stomach contents appeared to be chiefly finely comminuted, starch-like white material and

gave an intense black color when tested with iodine, though the percentage digestible by saliva was less than 5% (Table 1).

The fermentation gas production rate for the first animal, 28 μmol per h per g (wet weight), is of the same order of magnitude as the values of 38 and 12 reported by Kuhn (14) for *Presbytis cristatus* and *Procolobus badius*, respectively. Values of 63 to 79 μmol per h per g reported by Bauchop and Martucci (3) for *Presbytis cristatus* are considerably greater but are less than the average rate of 179 μmol per h per g for colobus 264. This high rate represents the potential for a very rapid fermentation when sufficient bicarbonate is added to maintain a favorable pH. It is doubtful that the high rate for colobus 264 obtained in the animal. More likely, after feeding, acid is produced more rapidly than it can be absorbed, and the resulting acidity slows the fermentation. The slower rate observed with colobus 263 is unexplained, but may be due to a longer interval between the last feeding and the time of sampling.

It seems unlikely after death that mixing of the acidic contents of the glandular stomach contents with the forestomach contents could account for the high acidity of the latter, since

the contents of both compartments were relatively dry. The percentage of dry matter in the saccular stomach contents of colobus 264 (Table 1) was much higher (31.7%) than in colobus 263 (18.7%), and the percentage nitrogen was much lower. Colobus 264, collected at 8:32 a.m., may have completed a morning feed on dried seeds and not yet consumed water.

The ratios of the concentrations of the various fermentation acids in the zero time rate samples and in the Formalin-preserved material (Table 2) are similar to those typical of rumen samples. For colobus 264, the total concentrations of acids is about as high as the highest values encountered in the rumen. There is more lactate in the colobus stomach than is usually found in forage-fed ruminants but comparable to those on a high concentrate ration (2).

The absence of methane from the gas in the colobus stomach contrasts with its occurrence in the langur (3) and in the ruminant. The stomach fermentation of the colobus differs also from that in a marsupial, the quokka (*Setonix brachyura*) (Moir and Hungate, unpublished data), which formed significant quantities of both hydrogen and methane.

Another difference in the colobus fermentation from that in ruminants is the absence of cellulolytic bacteria. No bacterial colonies surrounded by zones cleared of cellulose were observed in any of the cellulose agar cultures containing rumen fluid or colobus fluid, and none of the liquid cultures showed any disappearance of cellulose. These negative results might be explained in lower dilutions as due to too great an acidity introduced with the inoculum, but this could not explain the negative results in the higher dilutions. The stomach contents seemed to contain very little plant fibrous material, suggesting that "leaf eating" may not apply to *Colobus polykomos*.

Acidity may be a factor preventing the development of both methanogenic and cellulolytic bacteria in the colobus stomach. Acidity may also explain the absence of protozoa, though Kuhn (14) postulated that it was the consistency of the contents which prevented protozoal growth. Purser and Moir (15) found that the rumen protozoa could not survive continuous exposure to acidities much below pH 6.

Another difference from the forage-fed ruminant is the greater number of euryoxic cultures as compared with anaerobes. Inability to absorb traces of O_2 from the CO_2 used in the field experiments might be a factor in the increased proportion of aerobes and in the low ratio of culture count to direct count. But the absolute number of cultured aerobes per milliliter is much higher than for the rumen. The culture counts were 20 and 10% of the direct counts for the two monkeys, respectively. This is a slightly lower ratio than is obtained in careful culture experiments on rumen contents from forage-fed cattle, but is of the same order of magnitude. A higher ratio of culture count to direct count is usually obtained for ruminants on high concentrate rations.

Although propionibacteria are occasionally abundant in the rumen (9), they are not usually a prominent element, nor are staphylococci commonly found.

The morphology of the cultured bacteria is consistent with the morphology of some of those seen by direct microscope examination. The pure culture strain 1-8cf-4, a staphylococcus, showed tetrads of cocci very similar to the tetrads of smaller cocci in Fig. 2. None of the cultured strains resembled the very large refringent cocci. The appearance of these cells is similar to that of *Sarcina ventriculi*, and, in view of their greater abundance in the contents of the more acidic glandular stomach contents, it seems possible that this species is a normal inhabitant of the colobus stomach. The observed high refringence might be due to the cellulose produced by this species (5). It seemed to be more marked as the cells increased in size.

The relative abundance of staphylococci in the colobus might be the result of grooming with the teeth for fleas and other external parasites, a process which could conceivably provide a continuous inoculum, proliferating further under the conditions in the stomach. Thorough examination failed to demonstrate any enterotoxin production by the isolated *Staphylococcus aureus* (Table 3). This suggests that the staphylococcus strains in the colobus stomach may have been selected for host digestive compatibility and may be a normal component of the stomach microflora, maintaining themselves without ingested inocula.

The concentration of fermentation products affords some index to their rate of absorption, since absorption of volatile fatty acids is a function of the concentration gradient between stomach contents and blood. The concentration of volatile fatty acids in the stomach of colobus 263 ranged between 107 and 125 μmol per ml of stomach contents and between 212 and 434 for colobus 264 (Table 2). The values obtained from analyses of the Formalin-fixed material are roughly the same, if the dilution by the Formalin is taken into account.

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