Utilization of Inorganic Sulfate by Rumen Microorganisms

II. The Ability of Single Strains of Rumen Bacteria to Utilize Inorganic Sulfate[1,2]

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The ability of the rumen microbiota to collectively utilize inorganic sulfate in the formation of protein is well established (Block *et al.*, 1951; Emery *et al.*, 1957). However, there are some indications that a relatively small number of the many organisms present in this mixed population are responsible for sulfate incorporation (Emery *et al.*, 1957). For this reason, pure cultures of 10 widely differing types of rumen microorganisms have been tested for their ability to incorporate inorganic sulfate.

Materials and Methods

The cultures were grown according to the method of Bryant and Burkey (1953). The initial tests for ability to incorporate sulfate were performed on broth A, which contained inorganic salts, glucose, cellobiose, and 40 per cent centrifuged sterile rumen liquid. This broth differs from the one described by Bryant and Burkey (1953) only in that the sulfate salts were replaced by equivalent weights of their chlorides. Sufficient magnesium sulfate was included to give a final sulfate-sulfur concentration of about 0.03 mg per ml and an additional 0.03 mg sulfur per ml was present as cysteine. The nitrogen content was 33 mg per 100 ml which was supplied in nearly equal amounts by ammonium chloride and rumen liquid.

Heavy inocula from the slants were transferred to broth A and incubated 24 hr at 39 C. Sixteen hr subcultures from these tubes were used as the inocula for the sulfate incorporation tests. Four tubes containing 9 ml of broth A and 1 ml of inocula were prepared for each test culture. Three-tenths ml of inorganic radioactive sulfate solution was then added to two tubes for each culture. These tubes then contained 138 μc of sulfur^{35} with less than 0.01 mg of added carrier sulfur. One set of control and one set of radioactive cultures were removed at 12 hr and the other set at 24 hr. The isotope-containing cultures were poured into 40 ml of 0.05 M ethanolic H_2SO_4 and fractionation procedures continued as previously described (Emery *et al.*, 1957).

Radioactivity was assayed by transferring 0.02 ml of the clear liquid to Whatman no. 1 filter paper with a capillary pipette. The sample was allowed to cover a circle 2 cm in diameter and air dried before counting. The measured activity was found to be directly proportional to the μc applied within the range of 10 to 110 counts per min. Duplicates agreed within 5 per cent when more than 1000 counts were accumulated and corrected for background. All activities are reported as per cent of the total recovered radioactivity.

The incorporation of nitrogen was determined by adding 5 ml of the thoroughly mixed broth culture in the nonisotope containing tubes to 20 ml of absolute ethanol to precipitate the protein. The ethanol-soluble nitrogen was determined by a micro-Kjeldahl method. The difference between the ethanol-soluble nitrogen before and after incubation was assumed to be incorporated by the bacteria.

The culture, *Lachnospira multiparus* (Bryant and Small, 1956b), was chosen for detailed studies because it was found that it could be grown on a chemically defined medium. This medium, broth B, differed from broth A in that the rumen liquid was replaced by distilled water and the following amounts of other chemicals were added: 0.05 per cent agar, 0.0062 per cent valeric acid, 0.0062 per cent isovaleric acid, 0.25×10^{-6} per cent biotin, 0.001 per cent *p*-aminobenzoic acid, 0.008 per cent *dl*-tryptophan, 0.04 per cent *dl*-threonine, and 0.025 per cent *l* (−)-leucine.

Two batches of broth B were prepared simultaneously one of which contained 0.053 per cent *dl*-methionine. Eleven hundred microcuries of S^{35} labeled inorganic sulfate in 1 ml of distilled water were added to 100 ml of each batch and 10 tubes containing 10 ml each were dispensed. The radioactive sulfate used in this trial was essentially carrier-free. Nine tubes in each series (methionine and no-methionine) were inoculated with a straight needle from a 3-day old culture of *L. multiparus* on rumen fluid-glucose-cellobiose agar (Bryant and Burkey, 1953) and incubated at 39 C. One tube was removed from each series after incubation for 18 hr. Four tubes were removed after 25 hr and the remaining four tubes in each series were

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removed after 42 hr incubation. The tenth tube in each series was a sterile control used for determining nitrogen incorporation as described above.

Fermentation was terminated by transferring the cultures to sufficient ethanol to yield a final concentration of 80 per cent. The precipitate was removed by centrifugation using 0.2 g celite as a co-precipitant and washed with additional 80 per cent ethanol containing 0.01 M H_2SO_4 per L. The ethanol soluble and insoluble fractions were then fractionated as described above (Emery *et al.*, 1957) with the exception that all drying operations were carried out *in vacuo*. The concentrated desulfated ethanol filtrates and the protein hydrolysates were submitted to paper chromatography by the descending method using the 70 per cent isopropanol, 10 per cent formic acid, 20 per cent water solvent of Roberts *et al.* (1955). The ethanol filtrates were further developed in the second direction using 80 per cent phenol in 1.5 per cent aqueous ammonia solvent (Roberts *et al.*, 1955). The chromatograms were cut into 2 cm squares for quantitative assay of radioactivity.

An additional incorporation trial with *L. multiparus* was being carried out using broth B modified in the following manner: the reducing agent, 0.015 per cent cysteine hydrochloride was replaced by an equal concentration of *l*-ascorbic acid; the sulfur content was maintained at 0.03 mg per ml by including 0.003 per cent sulfuric acid in the media; valeric and isovaleric acids were omitted. Two batches of this modified broth B were prepared simultaneously, one of which contained 0.053 per cent *dl*-methionine. Sixty microcuries of S^{35} labeled inorganic sulfate were added to each ml batch and five tubes of 10 ml each dispensed as described above. One tube from each series served as a sterile control while the other four were inoculated with a 24 hr broth B culture of *L. multiparus*. These tubes were incubated for 9 days at 39 C before carrying out the chemical and chromatographic separations described above.

Results and Discussion

The activities recovered in organic sulfur compounds in four of the ten rumen microorganisms surveyed are presented in table 1. The nitrogen incorporation data are also reported here. Radioactivity could not be detected in protein hydrolyzates from cultures of *Succinivibrio dextrinosolvens* (Bryant and Small, 1956b), *Selenomonas ruminantium* (Bryant, 1956), *Bacteroides* strain GA-20 (Bryant and Burkey, 1953), and strain 7. Strain 7 is a cellulolytic coccus, often occurring in chains. *Bacteroides succinogenes* (Bryant and Doetsch, 1954) and strain B 17 contained 0.6 and 0.1 per cent of the total activity in their protein hydrolysates. Strain B 17 belonged to the +SR-gGXC group of Bryant and Burkey (1953). Growth was easily visible in all tubes even though it could not always be chemically demonstrated by nitrogen incorporation. The results reported are the higher of the 2 values obtained after 12 and 24 hr incubation.

TABLE 1

Recovery of the radio activity of S^{35} labeled inorganic sulfate as organic sulfur following incubation with some rumen microorganisms

Microorganisms	Per Cent of the Total Activity*		Microbial-N Formed Per 10 ml Media
	Ethanol filtrate	Protein hydrolyzate	
			mg
Butyrivibrio fibrisolvens†	2.3	10.4	0.3
Lachnospira multiparus‡	0.2	2.4	0.5
(Strain C94)§	0.3	0.6	0.9
Bacteroides (strain B_1-4)¶	0.1	1.7	0.9

* Activity in organic sulfur compounds expressed as per cent of the total activity.

† Bryant and Small, 1956a.

‡ Bryant and Small, 1956b.

§ Cellulolytic coccus, often in chains; description received from Dr. M. P. Bryant in a personal communication; classification incomplete.

¶ Bryant and Burkey, 1953.

The formation of microbial nitrogen for all tubes averaged 0.4 mg and these tubes each contained 0.25 mg of sulfate sulfur. Roberts *et al.* (1955) found that *Escherichia coli* contain 11.2 mg of sulfur per g of dry cells. If we assume that the microbial cells in this study had the same sulfur content and a nitrogen content of 80 mg per g of dry cells, the activity recovered as organic sulfur would equal about 22 per cent of the total activity provided all microbial sulfur incorporation came from sulfate. Therefore, table 1 demonstrates that all of these cultures utilized the organic sulfur from cysteine or rumen liquid in preference to inorganic sulfate.

Butyrivibrio fibrisolvens, *L. multiparus*, and *Bacteroides* strain B_1-4 were the only organisms of the 10 tested which showed an appreciable ability to utilize inorganic sulfate under the conditions of this trial. This is in contrast to the intense incorporation of inorganic sulfate by the entire rumen microbiota previously reported (Emery *et al.*, 1957). The low sulfate utilization found here could mean that bacteria other than those which were studied are responsible for sulfate utilization in the rumen, or it could mean that synergistic effects between the different strains occurs in the mixed population. The ability of rumen bacteria to incorporate sulfate in the presence of organic sulfur varies with the strain while the pattern of incorporation with the individual strains studied is not the same as with the mixed culture fermentation in rumen fluid.

Preliminary studies with *L. multiparus* revealed that it could be grown on the chemical medium, broth B. The 0.05 per cent agar was required for reasonable growth although minimal growth could be obtained

without it. Growth promotion studies with biotin and *p*-aminobenzoic acid were conflicting. Shiota and Clark (1955) demonstrated that *Lactobacillus arabinosus* requires *p*-aminobenzoic acid for methionine synthesis. Either methionine or a combination of tryptophan, threonine, and leucine greatly stimulated growth, but were not essential. The effects of these aminoacids upon growth were largely nonadditive. Growth promoting effects were not noted with the addition of the following compounds: pyridoxine, nicotinic acid, glycine, or lysine; or with a combination of glutamic acid, aspartic acid, and alanine; or a combination of guanine, adenine, uracil, and xanthine. Valeric and *iso*-valeric acids were also without growth promoting effect.

The utilization of S^{35} labeled inorganic sulfate by *L. multiparus* when grown in broth B reduced with either 0.015 per cent cysteine hydrochloride or 0.015 per cent ascorbic acid is shown in table 2. The sulfate and nitrogen incorporations at 18 hr were too small to be accurately determined. When the reducing agent was cysteine hydrochloride, methionine slightly increased the utilization of inorganic sulfate per mg microbial nitrogen formed for the 25 and 42 hr period. However, if we again assume a nitrogen to sulfur ratio of 8 to 1 in the microbial cells, only 1.04 mg of microbial nitrogen would need to be formed in each tube to give a 100 per cent incorporation of the inorganic sulfate if it was used preferentially. Therefore, the actual utilization of inorganic sulfate was very small and the only other source of sulfur, cysteine, was apparently utilized preferentially.

Microbial growth, or nitrogen incorporation, was about 3 times larger with cysteine than when it was replaced with ascorbic acid. However, omission of all but a trace of cysteine increased the incorporation of sulfate by more than 100 times. It should be noted also in table 2 that the ethanol filtrates were virtually devoid of activity with the ascorbic acid medium while the majority of the activity was usually ethanol extractable with the cysteine medium. Two dimensional paper chromatography of the concentrated sulfate-free ethanol filtrates from the cysteine medium revealed that 81 per cent of their organic sulfur activity was present as glutathione with only 19 per cent appearing as cysteic acid, methionine, and unidentified compounds. Moreover, ninhydrin reactive spots corresponding to the locations of oxidized and reduced glutathione were easily visible on these chromatograms suggesting a glutathione synthesis of much greater magnitude than the radioactivities would indicate. This can be explained by a preferential utilization of the nonradioactive cysteine as opposed to the radioactive sulfate. Methionine seemed to have little effect on this pattern.

The activity of cysteine and its decomposition products in the protein hydrolyzates was more than 25-fold that of methionine and its decomposition products

TABLE 2

Recovery of the radioactivity of S^{35} labeled sulfate as organic sulfur following incubation with Lachnospira multiparus

	Incubation Time	Per Cent of the Total Activity*		Microbial-N Formed Per 10 ml Media
		Ethanol filtrate	Protein hydrolysate	
				mg
0.015% Cysteine hydrochloride	25 hr	0.118	0.033	0.76
	42 hr	0.091	0.203	1.11
0.015% Cysteine hydrochloride and 0.053% *dl*-Methionine in medium	25 hr	0.173	0.031	0.13
	42 hr	0.144	0.099	0.60
0.015% Ascorbic acid†	9 days	trace	25.42	0.021
0.015% Ascorbic acid and 0.053% *dl*-methionine in medium†	9 days	trace	29.66	0.238

* Activity in organic sulfur compounds expressed as per cent of the total activity.

† Ascorbic acid and inorganic sulfate used to replace the reducing action and sulfur content of the cysteine hydrochloride.

regardless of the presence or absence of cysteine in the media. Methionine could not be detected on the paper chromatograms of the protein hydrolyzates from the cultures incubated for 25 hr. This pattern is different from the 2 to 1 ratio of activities in cysteine and methionine found in the whole rumen microbiota by Emery *et al.* (1957) or the 1 to 1 ratio found in Block *et al.* (1951).

These experiments demonstrate that although *L. multiparus* can synthesize both cysteine and methionine, both of these amino acids are capable of stimulating growth. Cysteine appears to stimulate the synthesis of glutathione and is utilized in preference to inorganic sulfate. Roberts *et al.* (1955) found that *E. coli* utilizes cystine sulfur in preference to inorganic sulfate to such an extent that sulfate incorporation is blocked in the presence of cystine. Hift and Wallace (1949) found that several lactic acid bacteria which can grow with inorganic sulfate as their sole source of sulfur are stimulated by cystine, cysteine, or glutathione. Anderson (1956) reported that the whole rumen microbiota utilized sulfate in preference to amino acid sulfur.

L. multiparus does not produce H_2S from cystine which is a common reaction among rumen bacteria. It is present in considerable numbers in the rumen of animals on a variety of rations, but is never a predominant strain (Bryant and Small, 1956b.).

In this work, *L. multiparus* utilized inorganic sulfate in the presence of cysteine, but at a reduced rate.

Summary

A survey of ten cultures of rumen bacteria revealed that only five utilized a significant amount of inorganic sulfate in the synthesis of organic sulfur compounds

and only three incorporated inorganic sulfate into their microbial proteins when cysteine was present in the medium. One of these cultures, *Lachnospira multiparus*, incorporated about equal amounts of inorganic sulfate into glutathione and microbial protein when cysteine was present in the medium; but failed to form radioactive glutathione when S^{35} labeled inorganic sulfate was the only source of sulfur. *L. multiparus* utilized less than 0.2 per cent of the inorganic sulfate when cysteine was present as an alternate sulfur source as compared to 22 per cent when inorganic sulfur was the only sulfur source. This occurred even though much more growth took place in the presence of cysteine.

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The Effect of Catalase on the Lethality of Cobalt^{60} Gamma Radiation for Certain Anaerobic Bacterial Spores

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Two explanations are offered for the lethal action of ionizing radiations on living cells. The first postulates a direct action of the radiations on genetic material; the other assumes that an initial change takes place in the medium or at some nongenetic locus and that this change brings about lethal effects in the cell. The work of Hollaender *et al.* (1951) and Burnett *et al.* (1951) supports the latter theory. Similarly, the work presented in this paper further supports the indirect action theory by describing a protective effect of catalase for anaerobic bacterial spores when catalase is present in the suspending medium during irradiation. Obviously, such protection of anaerobic bacterial spores will probably increase the amount of ionizing radiations required to sterilize foods containing catalase.

MATERIALS AND METHODS

Spores used in this work were grown, harvested, and suspended in distilled water as described by Kempe *et al.* (1954).

Immediately prior to use in an experiment, the stock spore suspensions were shaken with glass beads for 5 min to disperse the spore clumps. The desired quantity of spores was next pipetted into a sterile test tube and heated at 85 C for 15 min to kill the vegetative cells. The spore suspension was then diluted into the final solutions to be irradiated.

The control for these experiments was sterile phosphate buffer to which only the spores of either *Clostridium botulinum* 213-B or Putrefactive anaerobe no. 3679 were added.

Purified crystalline catalase for this work was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. For use in an experiment, phosphate buffer (pH 7.02) was sterilized by autoclaving. Following this, 60 mg of catalase was added to 14.5 ml of the cooled experimental solution and then 0.5 ml of a spore suspension was added to both the control and the catalase solutions.

Four ml quantities of these preparations were pi-