Sodium, an Obligate Growth Requirement for Predominant Rumen Bacteria

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Received for publication 31 December 1973

Sodium is an obligate growth requirement for most currently recognized predominant species of rumen bacteria. The isosmotic deletion of Na⁺ from a nutritionally adequate defined medium completely eliminated growth of most species. Growth yields and rates were both a function of Na⁺ concentration for Na⁺-requiring species, and Na⁺ could not be replaced by Rb⁺, Li⁺, or Cs⁺ when these ions were substituted for Na⁺ at a concentration equivalent to an Na⁺ concentration that supported abundant growth. Li⁺, Cs⁺, or Rb⁺ was toxic at an Na⁺-replacing concentration (15 mM) but not at a K⁺-replacing concentration (0.65 mM). K⁺ was also an obligate growth requirement for rumen bacteria in media containing Na⁺ and K⁺ as major monovalent cations, but K⁺ could be replaced, for most species, by Rb⁺. The quantities of Na⁺ that support rapid and abundant growth of Na⁺-requiring rumen bacteria show that these organisms are slight halophiles. A growth requirement for Na⁺ appears more frequent among nonmarine bacteria than has been previously believed.

Sodium is a frequent growth requirement for halophilic and marine bacteria (10, 11, 13), but reports of Na⁺ growth requirements among terrestrial bacteria are rare (8, 15) and have previously been restricted to a few species (2, 8, 16, 20) and special growth conditions (5, 8, 18). The work of Bryant et al. has shown that Na⁺ is required for the growth of Bacteroides succinogenes (2), and recent work has shown that Na⁺ is an obligate growth requirement for B. amylophilus (4) as well as both subspecies of B. ruminicola (D. R. Caldwell and C. M. Arcand, unpublished data). These results suggest that Na⁺ may be a frequent growth requirement for other species of predominant rumen bacteria. The present study shows that Na⁺ is an obligate growth requirement for most of the currently recognized predominant rumen bacteria, and that Na⁺ influences the growth yields and rates of these organisms independently of K⁺. The latter ion is also obligately required in media containing Na⁺ and K⁺ as major monovalent cations but can frequently be extensively replaced by Rb⁺.

MATERIALS AND METHODS

Organisms studied. The organisms studied included Bacteroides sp. strain B107, Fusobacterium necrophorum strain B85, Selenomonas ruminantium strains GA31 and PC18, Succinivibrio dextrinosolvens strains 22B and 24, Butyribrio fibrisolvens strains D1 and 49, Bacteroides clostridiformis (Clostadium sp.) strains B58 and T90 (6), Lactobacillus vitulinis (19) strains GA1, GA19, B62, and T185, B-385-1-like organism strain 2-33, Megashaera (Peptostreptococcus) elsdenii strains B159, C8, and T81, Eubacterium ruminantium strains B4, GA195, and B,C23, Lachnospira multiformis strains D32 and 40, Streptococcus bovis strain FD10, and Ruminococcus albus strain 7.

The organisms were obtained from the stock culture collection maintained by the Division of Microbiology and Veterinary Medicine, University of Wyoming, Laramie, but were originally isolated by M. P. Bryant and associates.

Media and culture conditions. The organisms were maintained by loop transfer in the rumen fluid, glucose, cellobiase agar medium described by Bryant and Robinson (1) by use of the Hungate anaerobic technique (7) and incubation at 37 C. The medium used for the preparation of inocula and from which experimental media were prepared was that described by Caldwell et al. (4), except for the equimolar replacement of PO₄³⁻ by CO₃²⁻-equilibrated KHCO₃ as the buffering system during Na⁺ requirement studies, the replacement of maltose with glucose, and the addition of an amino acid mixture containing glycine, valine, alanine, leucine, isoleucine, serine, threonine, asparagine, aspartic acid, glutamic acid, lysine, arginine, cystine, phenylalanine, histidine, tyrosine, tryptophan, and proline, each at a final medium concentration of 65 μM. Except for glycine, the amino acids were supplied in either the L configuration or as a D L mixture. Abundant growth of all of the organisms was
obtained in mineral-sufficient media. All of the inorganic media components were supplied as reagent grade salts.

Experimental media were prepared by the isosmotic deletion of Na+ or K+, by the isosmotic alteration of Na+ or K+ concentrations, or by the equimolar replacement of Na+ or K+ with related monovalent cations as previously described (4). The methods of media preparation, sterilization, dispensation into rubber-stopped test tubes (13 by 100 mm), inoculation, and incubation were as previously described (4).

**Growth measurements.** The effects of Na+ or K+ deletion, alteration of Na+ or K+ concentrations, or replacement of Na+ or K+ with related ions on growth were assessed by measurement of turbidity (optical density [OD]) changes at 600 nm (11-mm light path) with a Bausch and Lomb Spectronic 20 spectrophotometer by using uninoculated media tubes as standards. Yield differences were assessed from the maximum OD values obtained in media of differing ionic composition. Growth rates were calculated from OD changes observed at frequent (0.25 to 2 h) intervals and the associated measurement times. The fastest growth rate observed was used to calculate the time required for an OD change from 0.2 to 0.4.

**RESULTS AND DISCUSSION**

**Effects of sodium concentration on growth.** Preliminary studies indicated that the isosmotic deletion of either Na+ or K+ from a nutritionally adequate medium completely eliminated growth of all the organisms studied, with the exception of strains of *M. elsdenii* and *S. bovis*. Growth of the latter organisms was completely eliminated by K+ deletion but was unaffected, as measured by turbidity, by isosmotic Na+ deletion. The failure to detect an effect on growth of *M. elsdenii* and *S. bovis* as a function of Na+ deletion does not preclude an Na+ requirement for these organisms, but such a requirement, if present, is small, since the inoculated Na+-depleted defined medium contained a residual Na+ concentration of only 43 μM. The effect of Na+ concentration on the growth yields and rates of strains of Na+-requiring rumen bacteria is shown in Table 1. In media containing excess K+, increasing Na+ concentration increased both growth yields and rates of most currently recognized predominant rumen bacterial species. The maximal doubling times (OD) observed in Na+-sufficient media were between 0.5 and 4 h, depending on the species. The concentration range that increased yield was similar to that which affected rate, within a species, but differences were found among species in the minimal Na+ concentrations that supported rapid and abundant growth. Strains representing *L. vitulinis, B. fibrisolvens, E. ruminantium,* and *S. dextrinosolvens* grew abundantly at Na+ concentrations similar to those required for abundant growth of *B. succinogenes* (2), *B. amylophilus* (4), and *B. ruminicola* (D. R. Caldwell and C. M. Arcand, unpublished data).

Optimal yields and rates of these organisms were achieved at Na+ concentrations similar to those found in rumen fluid (≥60 to 120 mM). Strains representing *L. multiparus, Bacteroides sp., B. clostridiiformis, F. necrophorum,* and *S. ruminantium* grew abundantly at lower Na+ concentrations. The requirement of *S. ruminantium* was especially low, since substantial growth was obtained at the lowest concentration added (1.5 mM). The demonstration of an Na+ requirement for *F. necrophorum* and *B. clostridiiformis* is interesting. The strains studied were isolated from a young calf rumen (3). *F. necrophorum* and *B. clostridiiformis* are not normally considered predominant rumen species, and they are usually isolated from other sources (6). It would be interesting to determine whether nonruminating isolates of these species require Na+. The quantitative Na+ requirements of rumen strains of *B. clostridiiformis* are lower than those for most predominant rumen *Bacteroides* species and are similar to the requirements of many nonruminating *Bacteroides* species (D. R. Caldwell and C. M. Arcand, unpublished data).

**Effects of potassium concentration on growth.** Since the deletion studies showed that K+ was required for growth of all the strains studied independently of Na+, studies were conducted to determine the effects of K+ concentration on growth. In media containing Na+ and K+ as major monovalent cations, the addition of K+ to media containing excess Na+ increased growth yields. The doubling times of all the organisms studied were also increased with increasing K+ concentration. The K+ concentration range that supported rapid and abundant growth of most rumen bacteria was substantially lower than the corresponding range for Na+, although the quantitative Na+ and K+ requirements of *S. ruminantium* were similar.

**Effects of related monovalent cations on growth.** Since obligate Na+ growth requirements are rare for nonhalophilic and nonmarine bacteria (8, 13, 15), and since the Na+ requirement of some bacteria can be replaced by Li+ (8, 15), whereas the K+ requirement of many bacterial species can be replaced by Rb+ (8, 12, 14, 15), it was interesting to study the effects of related monovalent cations on the growth of Na+- and K+-requiring rumen bacteria. The addition of Li+, Cs+, or Rb+ to Na+- and
TABLE 1. Effects of sodium concentration on the growth yields (Y) and rates (R) of strains of representative species of predominant rumen bacteria as measured by turbidity (OD)

| mM Na⁺ | Organism | GA1* | B4 | D32 | B107 | B85 | T90 | 49 | 22B | PC18 | 2-33 |
|--------|----------|------|----|-----|------|-----|-----|----|-----|------|-------|
| 1.5    | Y        | R    | Y  | R   | Y    | R   | Y   | R  | Y   | R    | Y     |
| 0.5    | 12.0     | 0.22 | 18.0| 0.23| 15.0 | 0.18| 12.0| 0  | 0   | 1.05 | 0.95  |
| 3.1    | 0        | 0.68 | 7.0 | 0.38| 12.0 | 0.42| 10.0| 0.31| 10.0| 0.25 | 7.0   |
| 9.3    | 0.91*    | 5.0  | 0.52| 3.0 | 1.10 | 3.5 | 0.51| 4.0 | 0.45| 4.0   | 0.56  |
| 31.0   | 1.40     | 2.0  | 0.75| 2.0 | 1.10 | 3.5 | 0.55| 3.0 | 0.48| 3.5   | 0.60  |
| 91.0   | 1.52     | 1.0  | 0.98| 2.0 | 1.10 | 3.5 | 0.55| 3.0 | 0.50| 3.5   | 0.60  |

*The numbers designate L. vulininis GA1, E. ruminantium B4, L. multvarus D32, Bacteroides sp. B107, B. clostridiiformis T90, F. necrophorum B85, B. fibrisolvens 49, S. dextrinosolvens 22B, S. ruminantium PC18, and B-385-1-like organism strain 2-23. Similar results were obtained with L. vulininis GA19, B62, and T185, E. ruminantium GA195 and B,C23, L. multivarus 40, B. clostridiiformis B58, B. fibrisolvens D1, S. dextrinosolvens 24, S. ruminantium GA31, and R. albus 7.

Although OD measurements were used throughout to measure growth yields and rates, the OD values obtained were indicative of substantial cell concentrations. An OD of 0.5 was equivalent to the following cell concentrations per milliliter in millions for the strains indicated: GA1, 86; B4, 1,540; B107, 300; T90, 150; 49, 370; 24, 960; PC18, 230; 2-33, 380. The relationship between turbidity and cell numbers for strain B85 was not determined. Extensive clumping of cells precluded an accurate determination of the relationship between cells per milliliter and turbidity for L. multivarus strains.

K⁺-sufficient media at a concentration equivalent to an Na⁺ concentration that supported abundant growth (15 mM) completely inhibited growth, whereas the addition of the same ions at an analogous K⁺ concentration (0.65 mM) permitted growth yields and rates equivalent to those obtained in media containing only Na⁺ and K⁺. The growth inhibition observed at 15 mM was not the result of osmotic changes, since the changes resulting from the addition of Cs⁺, Li⁺, or Rb⁺ to Na⁺- and K⁺-containing media were small, and additional experiments showed that all of the organisms grew abundantly over a wide osmotic range in nutritionally adequate media. The substitution of Li⁺, Cs⁺, or Rb⁺ for Na⁺ at 15 mM failed to permit growth, indicating that Li⁺, Cs⁺, or Rb⁺ cannot replace Na⁺ on an equimolar basis. The possibility of Na⁺ replacement by these ions supplied at lower nontoxic concentrations cannot be excluded but seems unlikely. The K⁺ requirement of most of the strains studied could be extensively replaced by Rb⁺, although S. dextrinosolvens 24 and L. vulininis B62 and T185 displayed an absolute requirement for K⁺. The K⁺ requirement of S. bovis was partially replaced by Cs⁺ as well as Rb⁺; but Cs⁺ or Li⁺, although noninhibitory at K⁺-replacing concentrations, failed to replace the K⁺ requirement of any of the remaining strains.

Significance of the sodium and potassium requirements of rumen bacteria. The K⁺ requirement of most rumen bacteria is similar to that of many other microbes and is apparently less stringent than that for Na⁺, since K⁺ cannot often be replaced by Rb⁺. The fact that differences in the specificity of the K⁺ requirement among strains within species were sometimes found is interesting. The differences among strains of S. dextrinosolvens are presumably strain differences. However, it is possible that differences in K⁺ requirement specificity, in conjunction with other demonstrated differences (19), may allow further species differentiation among isolates currently regarded as L. vulininis. It would be interesting to determine the extent to which K⁺ is concentrated in cells of rumen bacteria and to study the effects of Rb⁺ and Cs⁺ on the intracellular accumulation of K⁺ for those organisms for which Rb⁺ and Cs⁺ replace K⁺ as growth factors.

Many metabolic and physiological functions for K⁺ are well documented for bacteria (8, 15), and previous work has shown the presence of K⁺-activated enzymes in broken cell preparations of rumen bacteria (9). The function(s) of Na⁺ in the physiology and metabolism of these organisms is presently unknown. Since Na⁺ deletion in K⁺-sufficient media completely eliminated growth, Na⁺ appears to serve at
least one function that cannot be replaced by K⁺ and is also nonreplaceable by Li⁺, Cs⁺, or Rb⁺ unless these ions are active at molar concentrations substantially lower than those required for Na⁺.

Although differences were found among species in the quantities of Na⁺ that supported rapid and abundant growth, the quantities of Na⁺ required by rumen bacteria are substantially greater than those required by the relatively few terrestrial bacteria thus far shown to require Na⁺ (8). The quantities of Na⁺ required by rumen bacteria are similar to the carbon and nitrogen requirements of many of the organisms and suggest that most rumen bacteria are slight halophiles (10). Although the Na⁺ requirements of rumen bacteria are relatively large, preliminary studies suggest that Na⁺ is not accumulated against the concentration gradient (S. J. Hufsmith and D. R. Caldwell, unpublished data). The differences in the quantitative Na⁺ requirements among species should not substantially affect the growth of these organisms in the rumen, since the Na⁺ concentrations normally found in rumen fluid are more than adequate to support abundant growth of all of the Na⁺-requiring species, and the growth of species that require relatively small amounts of Na⁺ is apparently unaffected by Na⁺ concentrations much greater than the minimal concentrations that support rapid and abundant growth.

The rumen appears analogous to an inland sea, and appears to be the first terrestrial environment studied in which the majority of the predominant bacteria require Na⁺. The quantity and specificity of the Na⁺ requirements of rumen bacteria should be useful in distinguishing these organisms from many other anaerobes. An obligate Na⁺ growth requirement among terrestrial bacteria appears more frequent than has been previously believed.

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