FURTHER MINERAL REQUIREMENTS OF STREPTOCOCCUS FAECALIS[1]

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The chief obstacle in the path of any study of the mineral nutrition of microorganisms is the difficulty encountered in obtaining media sufficiently free of inorganic ion contaminants to permit mineral requirements of the organisms to be demonstrated. The complexity of the nutritive requirements of the lactic acid bacteria makes the purification of suitable media by the usual methods even more difficult. With the aid of a biological purification procedure, however, it has been possible to demonstrate with relative ease that a number of lactic acid bacteria require K^+, Mn^{++}, and $PO_4^{=}$ for growth (MacLeod and Snell, 1947). In the purification procedure applied, use was made of the ability of an organism requiring an inorganic ion to remove contaminating traces of that ion from the medium during growth. After growth of the organism in the medium and removal of the cells, no further growth of the organism would take place in the medium on reinoculation unless the essential ion was added back. This purification procedure, however, will remove a metal ion successfully only if the amount of the ion present as an impurity in the medium is equal to or less than the amount that the organism used for purification can remove during growth. In the previous study, a medium containing an enzymatic casein hydrolysate was used. One might expect this medium, due to the presence of the casein, to be contaminated with relatively high concentrations of at least some inorganic ions. A study, therefore, was made to determine whether further inorganic requirements of the eight lactic acid bacteria previously studied would be revealed if the biological purification procedure previously used was applied to a medium containing crystalline amino acids in place of the casein hydrolysate. For only one of the organisms studied, *Streptococcus faecalis*, strain R, could further mineral requirements be demonstrated. In the case of this organism, a requirement for both Mn^{++} and Mg^{++} was revealed. In addition, the rate of autolysis of *S. faecalis* was found to be greatly increased in a medium containing insufficient amounts of Mn^{++} and Mg^{++} for maximum growth. The results of studies with *S. faecalis* are presented in the following pages.

METHODS

Culture and inoculum. A stab culture of *Streptococcus faecalis*, strain R ATCC 8043, was carried in yeast glucose agar. Unless otherwise stated, inoculum cultures were grown 16 to 18 hours at 37 C in a medium prepared by replacing the amino acid mixture of the basal medium described later with an enzymatic casein hydrolysate.

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Basal medium. The medium used (table 1) was a slight modification of one described elsewhere (MacLeod and Snell, 1950). The phosphate level was increased to raise the buffer capacity of the medium and the cystine level tripled to ensure an adequate supply of this amino acid after autoclaving (see Rabinowitz and Snell, 1947).

Preparation of mineral-deficient medium. The procedure used to remove contaminating traces of inorganic ions from the medium was essentially the same as that described previously (MacLeod and Snell, 1947). In this procedure, which

TABLE 1

Composition of the basal medium

COMPONENT	AMOUNT PER 100 ML*
Glucose	2 g
Potassium acetate	0.7 g
KH_2PO_4	0.2 g
Adenine sulfate	1 mg
Guanine hydrochloride	1 mg
Uracil	1 mg
Thiamine-HCl	100 μg
Riboflavin	100 μg
Pyridoxal	20 μg
Calcium pantothenate	100 μg
Niacin	100 μg
Para-aminobenzoic acid	20 μg
Biotin	1 μg
Folic acid	2 μg
L-Cystine	30 mg
DL-Alanine	100 mg
DL-Aspartic acid	50 mg
L-Glutamic acid	50 mg
L-Arginine HCl	20 mg
L-Lysine-HCl-H_2O	20 mg
Other amino acids†	
L-forms	10 mg each
DL-forms	20 mg each

* The quantities indicated are for 100 ml final volume (ten 10-ml tubes).

† Histidine, iso-leucine, leucine, methionine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, serine, and glycine.

will be referred to as "pretreatment" throughout the text, a suitable volume of double strength basal medium containing an excess of those ions known from the previous study to be required for growth was inoculated with *S. faecalis*. After a 24-hour incubation period at 37 C the cells were removed by centrifugation and the pH of the medium adjusted to 7 with a solution of NH_3 in glass-distilled water. In the previous study the medium was supplemented with glucose, vitamins, purines, and pyrimidines after pretreatment to ensure that sufficient of these substances would be present to permit further growth of the organism in the medium on reinoculation. No attempt was made then to determine whether

each of the organisms studied required the addition of all of these supplements to the medium after pretreatment. Since such supplements might contaminate the purified medium with traces of essential metal ions, additions to the medium after pretreatment in the present study were reduced to the minimum necessary to permit growth of the organism. Preliminary experiments revealed that both a folic acid and a tyrosine deficiency developed in the medium during pretreatment with *S. faecalis*. Folic acid and recrystallized tyrosine were therefore added to the pretreated medium in amounts equal to those added to the original medium. Certain of the factors responsible for the development of a tyrosine deficiency during pretreatment have been considered elsewhere (MacLeod, 1951).

Test procedure. The usual procedures of microbiological assay were used and have already been described (MacLeod and Snell, 1950). The inoculum was prepared by first centrifuging an inoculum culture, removing the supernatant, and suspending the cells in 10 ml of sterile glass-distilled water. This procedure was repeated three times. The final suspension was diluted until it transmitted 70 per cent of the light transmitted by a water blank in an Evelyn colorimeter with a 660 mμ filter. A 1:100 dilution of this suspension was made then with sterile glass-distilled water and one drop of the diluted suspension added to each assay tube.

Metal ion solutions. Metal ions were added to the medium as solutions of their respective reagent grade salts. Mn^{++} was added as $MnSO_4 \cdot H_2O$, Mg^{++} as $MgCl_2 \cdot 7H_2O$, Be^{++} as $BeSO_4 \cdot 4H_2O$, Ca^{++} as $CaCl_2 \cdot 6H_2O$, Sr^{++} as $SrCl_2 \cdot 6H_2O$, Ba^{++} as $BaCl_2 \cdot 2H_2O$, Cd^{++} as $CdCl_2 \cdot 2H_2O$, Fe^{++} as $FeSO_4(NH_4)_2SO_4$, Ni^{++} as $NiSO_4 \cdot 6H_2O$, Co^{++} as $CoSO_4 \cdot 7H_2O$, and Zn^{++} as $ZnSO_4 \cdot 7H_2O$. The Mn^{++} and Mg^{++} salts were recrystallized three times from glass-distilled water before use.

All solutions were prepared using distilled water redistilled in an all-glass still. Glassware was cleaned in a hot HNO_3:H_2SO_4 mixture and rinsed thoroughly first with tap water and finally with glass-distilled water.

RESULTS

The response of S. faecalis to Mg^{++}. *S. faecalis* has been shown previously to require K^+ and $PO_4^{=}$ for growth. Some evidence for a Mn^{++} requirement was also obtained (MacLeod and Snell, 1947).

In the present study, the double strength medium, which contained adequate amounts of K^+ and $PO_4^{=}$ for growth, was supplemented with 20 μg of Mn^{++} per 5 ml and pretreated with *S. faecalis*. The resulting medium, after supplementation with folic acid and tyrosine, supported little or no growth of *S. faecalis* unless Mg^{++} was added to the medium. The response of the organism to Mg^{++} in this medium is shown in figure 1, curve 1. Under the conditions used, the organism required 4 to 5 μg of Mg^{++} per 10 ml of medium for maximum growth.

The effect of citrate on the response to Mg^{++}. In contrast to other lactic acid bacteria studied, the growth of *S. faecalis* is not inhibited by the presence of 2 per cent sodium citrate in a medium containing the usual levels of inorganic ions. Since the inhibition produced by citrate has been shown to be due to the

ability of citrate to bind divalent inorganic ions essential for the growth of these organisms, it was concluded that the requirement of *S. faecalis* for such ions, if such a requirement existed, must be exceedingly small (MacLeod and Snell, 1947). Since *S. faecalis* required a readily measurable amount of Mg^{++} in the medium used in the present investigations, it was of interest to know whether the presence of citrate would inhibit the growth of the organism in this medium. To ascertain this point, 2 per cent citrate was added to the pretreated medium and the response to Mg^{++} determined. The results are shown in figure 1, curve 2

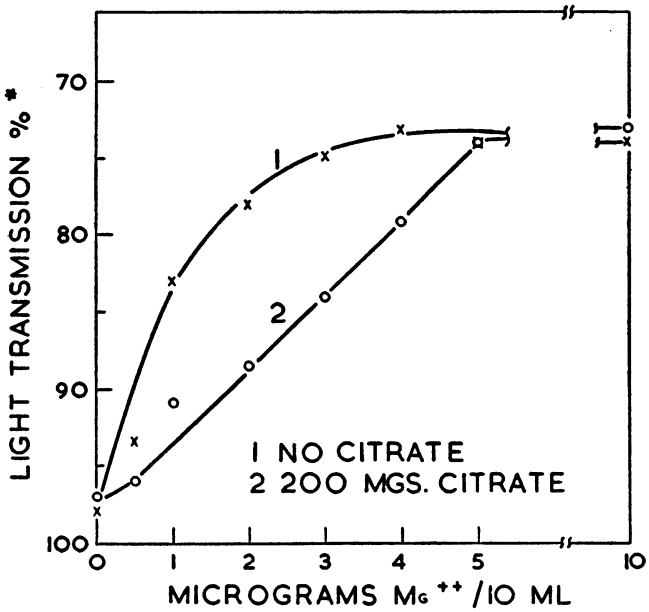

Figure 1. The response of *Streptococcus faecalis* to Mg^{++} in the presence and absence of citrate.

* Evelyn colorimeter, 660 mμ filter, uninoculated medium = 100. Incubation time, 20 hr.

** Citrate added as ammonium citrate (200 mg citrate ion per 10 ml tube).

It can be seen that citrate exerted an effect on the response to Mg^{++} only in the presence of suboptimal amounts of Mg^{++} and did not have any appreciable effect upon the amount of this ion required for maximum growth of the organism. This effect of citrate on the Mg^{++} level required for the growth of *S. faecalis* is in sharp contrast to its effect on the Mn^{++} level necessary to permit the growth of other organisms studied (MacLeod and Snell, 1947). For the latter organisms, the Mn^{++} concentration required for growth was increased 25 to 30 fold by the addition of 2 per cent citrate to the medium. The results in figure 1 were obtained using ammonium citrate, prepared by neutralizing reagent grade citric acid with a solution of ammonia gas in glass-distilled water, to ensure the maximum metal-binding capacity of the citrate ion. A similar response was obtained, however, with reagent-grade potassium citrate.

The sparing action of Mn^{++} on the Mg^{++} requirement. Mg^{++} has been shown to exert a marked sparing action on the Mn^{++} requirement of several lactic acid bacteria (MacLeod and Snell, 1950). It was therefore of interest to know whether, in the case of *S. faecalis*, the opposite relationship would hold, that is, that Mn^{++} would spare the requirement for Mg^{++}. To determine this point, a medium containing no added Mn^{++} was pretreated to remove traces of Mg^{++}. The response of *S. faecalis* to Mg^{++} was then determined in the presence and absence of added

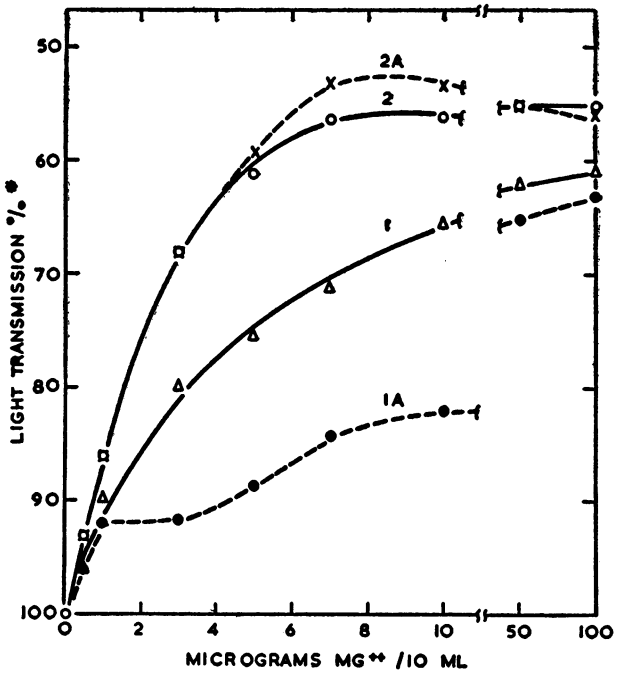

Figure 2. The sparing action of Mn^{++} on the Mg^{++} requirement and the effect of Mn^{++} and Mg^{++} on the rate of autolysis of *Streptococcus faecalis*. Curve 1—response to Mg^{++}, no added Mn^{++}, 16-hr incubation; curve 1A—response to Mg^{++}, no added Mn^{++}, 48-hr incubation; curve 2—response to Mg^{++} in the presence of 10 μg per 10 ml of added Mn^{++}, 16-hr incubation; curve 2A—response to Mg^{++}, 10 μg per 10 ml of added Mn^{++}, 48-hr incubation.
* Evelyn colorimeter, 660 mμ filter, uninoculated medium = 100.

Mn^{++}. The results are shown in figure 2, curves 1 and 2. In the absence of added Mn^{++}, *S. faecalis* requires about 15 times as much Mg^{++} for maximum growth as it does when grown in the presence of added Mn^{++}. Although the maximum amount of growth obtainable is somewhat greater in the presence of the Mn^{++}, the growth promoting effect of Mn^{++} is most pronounced at suboptimal levels of Mg^{++}, indicating a sparing action of Mn^{++} on the Mg^{++} requirement. Be^{++}, Ca^{++}, Sr^{++}, Zn^{++}, Ba^{++}, Cd^{++}, and Fe^{++} were each tested to determine their ability to spare or to replace Mg^{++} for the growth of *S. faecalis*. None of these ions showed any activity in either capacity. In addition, none of these ions proved toxic when added at levels up to 400 μg per 10 ml of medium, the highest

level which could be tested without causing appreciable amounts of precipitate to form in the medium.

The effect of Mn^{++} and Mg^{++} on the rate of autolysis of S. faecalis. Figure 2 also shows the effect of incubation time on the response of *S. faecalis* to Mg^{++} in the presence and absence of added Mn^{++}. In the absence of added Mn^{++} the maximum growth response to Mg^{++} was achieved in 16 hours. This response is shown in curve 1. With further incubation, a marked decrease in turbidity took place in all tubes except those containing the two highest levels of Mg^{++}. Turbidity readings after 48 hours are shown in curve 1A. Little further decrease in turbidity was observed after periods of incubation longer than 48 hours. In tubes containing 10 μg of added Mn^{++}, essentially maximum growth again was achieved in 16 hours (curve 2). No decrease in turbidity was observed in these tubes on longer incubation (curve 2A). None of the other ions tested for their sparing action could replace Mn^{++} in preventing this decrease in turbidity at suboptimal levels of Mg^{++}.

The response of S. faecalis to Mn^{++}. On the basis of results obtained previously, it was concluded that *S. faecalis* probably requires Mn^{++} for growth but in a very small amount (MacLeod and Snell, 1947). In view of the present demonstration of a Mg^{++} requirement, it was felt that the possibility of a Mn^{++} requirement for this organism should be reinvestigated.

A medium containing no added Mn^{++} or Mg^{++} when pretreated with *S. faecalis* supported adequate growth of the organism on reinoculation when supplemented with Mg^{++} (figure 2). Thus it was evident that if the organism required both Mn^{++} and Mg^{++} for growth, the Mg^{++} level became the limiting factor for growth during pretreatment before a Mn^{++} deficiency developed. One could therefore expect to produce a Mn^{++} deficiency in this medium by applying the pretreatment procedure only if the medium during pretreatment contained an excess of Mg^{++}. Thus a double strength medium containing no added Mn^{++} was supplemented with 200 μg of Mg^{++} per 5 ml and pretreated with *S. faecalis*. A dilute inoculum was used to inoculate the medium for pretreatment. To prepare Mn^{++}-deficient cells for the inoculum, inoculum cultures were grown in the basal medium supplemented with Mg^{++} (20 μg per 10 ml) but not with Mn^{++}. The results obtained under these conditions are presented in table 2, where it is clearly evident that even in the presence of Mg^{++}, *S. faecalis* requires Mn^{++}. The Mn^{++} requirement is small, however, and 1 μg of Mn^{++} per 10 ml of medium is sufficient for maximum growth of the organism. Neither Fe^{++}, Ni^{++}, nor Co^{++} was found to be capable of replacing Mn^{++} in the nutrition of the organism. Of the three ions tested, only Fe^{++} showed any evidence of an ability to spare the Mn^{++} requirement. In view of the smallness of the Mn^{++} requirement, it was not possible to decide whether the effect of Fe^{++} was due to a true sparing action or to the presence of traces of Mn^{++} in the iron salt used. Previous attempts to remove Mn^{++} activity from Fe^{++} salts by the application of purification procedures to the Fe^{++} salts have proven unsuccessful (MacLeod and Snell, 1947).

The effect of incubation time on the response to Mn^{++} is also shown in table 2. In the absence of added Mn^{++} and in the presence of 0.01 μg of added Mn^{++}, a

considerable decrease in turbidity took place between 17 and 48 hours. In the presence of 0.05 μg or more of added Mn^{++} no decrease in turbidity was observed during this same incubation period. It is notable that this increased rate of autolysis at low levels of Mn^{++} occurred in a medium containing a considerable excess of Mg^{++}.

To determine whether or not the lack of optimum amounts of any essential nutrient in the medium would increase the rate of autolysis of this organism, the effect of incubation time on the response of *S. faecalis* to folic acid was observed. For this purpose, folic acid was omitted and 20 μg each of Mn^{++} and Mg^{++} added in the preparation of the basal medium. No decrease, but rather a slight increase in turbidity, took place between the 17- and 120-hour incubation periods, when the organism was grown in the presence of suboptimal levels of folic acid. The

TABLE 2

The response of Streptococcus faecalis to Mn^{++} after two periods of incubation

	INCUBATION TIME (HOURS)	
μg Mn^{++} per 10 ml	17	48
	PER CENT OF INCIDENT LIGHT TRANSMITTED*	
0	83	95
0.01	79	89
0.05	74	75
1	67	66
10	67	65

* Evelyn colorimeter, 660 mμ filter, uninoculated medium = 100.

response of *Lactobacillus arabinosus*, strain 8014, to Mn^{++} was also determined in the basal medium. This organism showed no tendency to autolyze at suboptimal levels of Mn^{++} when incubated for periods up to 140 hours.

Test for further mineral requirements. The basal medium, containing an excess of both Mn^{++} and Mg^{++}, was pretreated with *S. faecalis*. No further mineral requirements of the organism could be detected on reinoculation of this medium.

DISCUSSION

The application of the biological purification procedure to an amino acid medium has permitted a clear demonstration of the requirement of *S. faecalis* for both Mg^{++} and Mn^{++}. This organism is the first of the lactic acid bacteria to be shown to require both of these ions for growth. The finding that Mn^{++} exerts a sparing action on the Mg^{++} requirement suggests that there are one or more functions of Mn^{++} and Mg^{++} within the organism for which the two ions are interchangeable. The possible mechanism of sparing actions of this type has been considered in some detail elsewhere (MacLeod and Snell, 1950).

Since citrate is known to be capable of binding Mg^{++} under physiological conditions (Hastings *et al.*, 1934), the inability of citrate to affect appreciably the response of *S. faecalis* to Mg^{++} is interesting. One possible explanation for the

phenomenon would be that this particular organism, acting through its Mg^{++} activated enzyme systems, has sufficient affinity for Mg^{++} to compete successfully with citrate for the Mg^{++} present in the medium. It has been stated, without the presentation of supporting chemical evidence, that Mg^{++} forms a complex with citrate in the neutral pH range whereas Mn^{++} and other divalent ions form complexes with citrate only under alkaline conditions (Smith, 1948). On the basis of this assumption it has been concluded that peptidases inactivated by citrate at a neutral pH are Mg^{++}-activated enzymes *in vivo*. The findings reported in this paper suggest that such a criterion for Mg^{++} activation of an enzyme may not always be valid.

The observation that small amounts of Mn^{++} prevent autolysis of *S. faecalis* indicates a role for Mn^{++} in reactions governing the rate of autolysis of this organism. The ability of Mg^{++} in much higher concentrations to exert a similar protective effect suggests that this metal ion may govern the same or similar reactions, though somewhat less efficiently. Autolysis of gram-positive staphylococci has been shown to involve first the removal of a ribonucleic acid complex from the cell surface followed by proteolysis of the residual cytoskeleton (Jones, Stacey, and Webb, 1949). The outer layer of the cell, which is responsible for the positive gram staining reaction, contains ribonucleic acid as a Mg^{++} salt in several organisms studied (Henry and Stacey, 1946). Instability or lack of this outer layer would be expected to increase the rate of autolysis of a gram-positive organism. The possibility that a lack of Mn^{++} or Mg^{++} would interfere with the formation of such an outer layer in cultures of *S. faecalis* due to an inability of the organism to form a Mn^{++} or Mg^{++} salt of ribonucleic acid was considered. Tests for the presence of the outer layer were made by applying the gram stain to the cells. Cells of *S. faecalis* grown in a medium deficient in both Mn^{++} and Mg^{++}, however, stained gram positively from the time of inoculation until the period when active autolysis was taking place.

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SUMMARY

By applying a biological purification procedure to a suitable medium, it has been possible to demonstrate that *Streptococcus faecalis*, strain R, requires both Mg^{++} and Mn^{++} for growth. A number of related ions tested were found to be unable to replace Mg^{++} and Mn^{++} in the nutrition of the organism.

Mn^{++} exerted a sparing action on the requirement of *S. faecalis* for Mg^{++}.

Citrate interfered with the response of *S. faecalis* to Mg^{++} only at levels of Mg^{++} insufficient for maximum growth in the absence of citrate.

Cultures of *S. faecalis* grown in the presence of suboptimal concentrations of Mg^{++} and Mn^{++} autolysed at a greatly increased rate. This increase in the rate of autolysis could be prevented completely by the addition of small amounts of Mn^{++} and partially by the addition of relatively large amounts of Mg^{++} to the medium.

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