SOME MINERAL REQUIREMENTS OF THE LACTIC ACID BACTERIA*

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Although the requirements of several lactic acid bacteria for organic nutrients are known in considerable detail (1, 2), knowledge of the mineral nutrition of this same group of bacteria is scanty. In chemically defined media, the mineral mixture most commonly used has been that of Speakman (3), which contains K^+, Na^+, Mg^{++}, Mn^{++}, Fe^{++}, $PO_4^{=}$, $SO_4^{=}$ and Cl^- ions. Of these, previous investigations have shown that potassium is required for growth of *Streptococcus faecalis* (4) and *Lactobacillus casei* (5), while manganese is essential for growth of *Lactobacillus plantarum* (6), and is stimulatory, in crude media, to *Lactobacillus casei* (7) and various other lactic acid bacteria (8).

The extensive use of lactic acid bacteria and purified media for the assay of vitamins and amino acids (1) makes knowledge of their mineral requirements of special importance. Such a study is made difficult, however, by the complexity of the nutritive requirements of these organisms. Complex media suitable for their growth usually contain, as contaminants, sufficient essential mineral elements to permit limited or extensive growth even though none of the mineral is added, and the presence of large amounts of organic materials renders ineffective many of the procedures used in other investigations for removal of traces of inorganic ions. Recently, it was shown (9) that contaminating traces of manganese could be removed from a medium by permitting a manganese-requiring organism, *Lactobacillus arabinosus*, to grow in the medium for 24 hours. After subsequent removal of the organism and reinoculation, growth occurred only if manganese were added to the medium. This biological method of removing trace impurities, which has been employed occasionally in the past (*cf.* (10)), has been applied below to a study of the Mn^{++}, Mg^{++}, Fe^{++}, K^+, and $PO_4^{=}$ requirements of several species of lactic acid bacteria. Previous work (11) has indicated that moderate amounts of citrate are toxic for these organisms; data below indicate that this toxicity is due to the complex-forming action of citrate with bivalent metallic ions, since it can be prevented by addition of adequate amounts of manganese and magnesium ions.

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EXPERIMENTAL

Cultures and Inocula—Cultures used were *Lactobacillus arabinosus* 17-5, *Lactobacillus casei*, *Lactobacillus delbrueckii* LD-3, *Lactobacillus fermenti* 36, *Lactobacillus pentosus* 124-2, *Leuconostoc mesenteroides* P-60 and 9135, and *Streptococcus faecalis* R. Cultures were carried as stabs in yeast-dextrose agar. Inoculum cultures were incubated 16 to 18 hours in yeast extract-glucose broth (1 per cent Difco yeast extract and 1 per cent glucose). On some occasions, the inoculum may be grown in the mineral-deficient

TABLE I

*Composition of Basal Medium**

	Amounts per 10 cc. final medium		Amounts per 10 cc. final medium
1. Casein (enzymatic hydrolysate)	100 mg.	13. Riboflavin	2 γ
2. Asparagine	1 "	14. Niacin	2 "
3. DL-Tryptophan	0.5 "	15. *p*-Aminobenzoic acid	1 "
4. Cystine	1 "	16. Folic acid	0.05 "
5. Adenine HCl	0.1 "	17. Biotin	0.01 "
6. Guanine "	0.1 "	18. KH_2PO_4	10 mg.
7. Uracil	0.1 "	19. K_2HPO_4	10 "
8. Glucose	100 "	20. $MgSO_4 \cdot 7H_2O$	2 "
9. Sodium acetate	60 "	21. NaCl	0.1 "
10. Pyridoxal HCl	1 γ	22. $FeSO_4 \cdot 7H_2O$	0.1 "
11. Thiamine "	1 "	23. $MnSO_4 \cdot H_2O$	0.1 "
12. Calcium pantothenate	2 "		

* For convenience separate solutions were prepared of (*a*) purines and pyrimidines (Constituents 5 to 7), (*b*) the vitamins (Constituents 10 to 17), (*c*) Salts A (Constituents 18 and 19), and (*d*) Salts B (Constituents 20 to 23). Salts A and Salts B constitute Speakman's salt mixture. Constituents 1 to 7 plus Salts A were combined to form a single stock solution, to which glucose, acetate, the vitamin solution, and the appropriate salts were added to form the final medium.

medium. Cultures of *Leuconostoc mesenteroides* and *Lactobacillus pentosus* were incubated at 30°; other cultures were incubated at 37°.

Basal Medium—The medium used (Table I) was a slight modification of that reported by Roberts and Snell (12). The enzymatic digest of casein was prepared as described previously (12) with the exception that hydrochloric acid was used to neutralize the digest and all charcoal treatments were omitted. The single mineral element to be studied was omitted from the medium in each case.

Preparation of Mineral-Deficient Medium—Despite omission of the inorganic ion under study, sufficient is usually present as a contaminant to

promote considerable growth. If the amount present is not too great, it is often possible, merely by growing in the medium an organism requiring the ion for growth, to reduce the concentration of the ion in the medium to a level which will not support growth of the organism. This procedure for removing traces of inorganic ions is referred to throughout this paper as "pretreatment" of the medium. It is carried out as follows:

1 liter of the double strength medium, complete except for its complement of the inorganic ion under investigation, is autoclaved 5 to 10 minutes at 15 pounds pressure. The time of autoclaving is reduced to a minimum to prevent excessive caramelization, should repeated pretreatment be necessary. After cooling, the sterilized medium is inoculated with 10 cc. of the inoculum culture, then incubated for 24 hours at the appropriate temperature. The cells are removed by centrifugation. For this purpose, plastic (Lustron) centrifuge tubes and a centrifuge equipped with a conical head are very satisfactory. After centrifugation, the clear medium is decanted from the cells and pooled. The pH is readjusted to 6.5 with a solution of ammonia prepared by boiling commercial ammonium hydroxide and redissolving the evolved ammonia in distilled water. The medium thus pretreated may be stored under toluene in the cold room until use. Since the concentrations of certain essential ingredients of the medium (other than the essential mineral element) may be reduced to levels below those required for optimal growth, the medium is "fortified" just before use by adding amounts of glucose, vitamins, and purine bases equal to those used in the original medium.

Test Procedure—The customary techniques of microbiological assay were used (*cf.* (12)). 5 cc. of the pretreated double strength medium were added to each of a series of 18 × 150 mm. Pyrex test-tubes. Appropriate quantities of solutions of the inorganic ion to be investigated were then added, and the total volume of each tube adjusted to 10 cc. with distilled water. In all cases, Mn^{++} was added as $MnSO_4 \cdot H_2O$, Mg^{++} as $MgSO_4 \cdot 7H_2O$, Fe^{++} as $FeSO_4 \cdot 7H_2O$, K^+ as KCl, and $PO_4^{\equiv}$ as KH_2PO_4. All salts were of reagent quality. Additions were made to supply the amounts of the *ions* indicated, not the salts. All tubes were covered with aluminum caps, autoclaved 10 minutes at 15 pounds pressure, cooled, and inoculated. For inoculum, the inoculum culture (10 cc.) was centrifuged, the supernatant medium removed, and the cells resuspended in sterile 0.9 per cent NaCl solution. This suspension was again centrifuged, the supernatant liquid discarded, and the cells resuspended as before in 10 cc. of sterile saline. When a "heavy" inoculum was desired, 1 drop of this suspension was added to each culture tube; if a "light" inoculum was needed, 1 cc. of this suspension was added to 100 cc. of sterile saline and 1 drop of this suspension added to each culture tube. After the growth period (usually 24 hours) the turbidity of each

culture was determined in the Evelyn colorimeter, with the 660 mμ filter and the 10 cc. aperture.

Manganese—The manganese requirements of eight lactic acid bacteria were determined in a medium which had been pretreated once with *Lactobacillus arabinosus*. The results are given in Table II. With the exception of *Lactobacillus casei and Streptococcus faecalis*, none of the organisms grew appreciably unless Mn^{++} was added. Maximum growth in 24 hours was achieved with considerably less than 100 γ of Mn^{++} per 10 cc. of medium.

TABLE II

*Response of Lactic Acid Bacteria to Added Manganese on Manganese-Deficient Medium**

	Mn^{++} per 10 cc.				
	0 γ	0.1 γ	0.3 γ	1.0 γ	100 γ
	Galvanometer readings†				
Lactobacillus arabinosus	98	80	61	37	28
" *delbrueckii*	85	58	47	33	27
" *casei*	73	43	30	25	25
Streptococcus faecalis	66	62	59	58	54
Leuconostoc mesenteroides (P-60)	98	84	71	49	42
" " (9135)	95	81	72	63	63
Lactobacillus pentosus	97	71	51	38	38
" *fermenti*	95	87	80	63	34
" *casei*‡	93	69	54	40	30

* The basal medium, with manganese omitted, was pretreated once with *Lactobacillus arabinosus*.

† The galvanometer readings (Evelyn colorimeter) represent per cent of the incident light transmitted. The uninoculated medium was set to read 100.

‡ In this instance, the basal medium, with manganese omitted, was pretreated twice with *Lactobacillus casei*.

L. casei and *Streptococcus faecalis* grew appreciably without added manganese; growth was greatly enhanced, however, by its addition. It is possible either that the organisms do not require manganese and are stimulated by it, or their manganese requirements are low and sufficient ion remains in the medium after pretreatment with *L. arabinosus* to permit their limited growth.

To decide between these possibilities, a portion of the basal medium was pretreated twice in succession with *Lactobacillus casei*. Inoculum for the second pretreatment was washed with saline to avoid introduction of manganese from this source. The response of *L. casei* to added manganese in the twice pretreated medium is also shown in Table II. Growth in the absence of manganese was slight; good growth occurred when manganese

was added. It is concluded, therefore, that *L. casei* also requires manganese for growth. Even on media similar to this, however, it was not possible to demonstrate that *Streptococcus faecalis* required manganese for growth.

The specificity of the requirement for manganese was investigated to a limited extent. Reagent grade salts of Mg^{++}, Co^{++}, Ni^{++}, and Ca^{++}, when tested at a concentration of 1000 γ of the metallic ion per 10 cc., showed slight growth-promoting effects. After recrystallization from distilled water, however, they were inactive. Their initial activity was thus due to contamination with manganese. Ferrous salts showed low activity (about 0.08 per cent that of manganese) which was not altered by reprecipitation from dilute sulfuric acid solution with ethanol. Ferrous ion prepared by dissolving analytical grade iron wire in dilute hydrochloric acid showed similar activity. Although the activity of ferrous iron is believed to result from contamination, no positive data are available to prove this point.

Magnesium—Preliminary growth trials on a medium to which no magnesium was added showed that, of the eight cultures tested, only *Lactobacillus arabinosus* and *L. casei* were significantly stimulated by addition of magnesium. Three successive pretreatments of the deficient medium with *L. casei* failed to reduce growth in the unsupplemented medium. It was decided, therefore, to pretreat the medium with yeast, which is known to require magnesium for growth. For this purpose, the magnesium-deficient basal medium was supplemented with $CaCl_2$ (1 mg. per cc.) and inositol (50 γ per cc.), adjusted to pH 5.5, dispensed in 100 cc. quantities into 500 cc. Erlenmeyer flasks, and autoclaved for 5 minutes. After cooling, it was inoculated with a suspension of *Saccharomyces carlsbergensis* 4228, and incubated with shaking for 24 hours at 30°. The heavy growth which resulted was removed by centrifugation and the pretreatment repeated twice. The ability of this pretreated medium to support growth of *S. carlsbergensis* and of *L. casei* is shown in Table III. It is evident that the medium was deficient in magnesium for yeast, since it supported only slight growth of this organism unless magnesium was added. The medium supported good growth of *L. casei* in the absence of added magnesium, although magnesium does stimulate growth considerably.

No definite conclusions concerning the magnesium requirements of *Lactobacillus casei* can be drawn from these data, except that this requirement, if it exists, is of a lower order of magnitude than that of yeast. If the amount required is sufficiently small compared to that initially present as a contaminant, even repeated pretreatments will fail to show up the requirement. Similarly, contamination from glassware, etc., may be sufficient to supply the amounts required, and this source of essential ions could not be removed by the procedures used here. Further evidence bearing on the magnesium requirements of these organisms will be discussed later.

Potassium—To prepare a potassium-deficient medium, the potassium phosphates of the basal medium were replaced by equimolar amounts of sodium phosphates, and the medium was pretreated once with *Lactobacillus arabinosus*. It is evident from Table IV that all of the organisms tested require potassium for growth.

TABLE III

*Response of Lactobacillus casei and Saccharomyces carlsbergensis to Magnesium in Magnesium-Deficient Medium**

	Mg^{++} per 10 cc.				
	0 γ	0.1 γ	1 γ	100 γ	1000 γ
	Galvanometer readings				
S. carlsbergensis	86				19
L. casei	41	35	34	28	27

* The basal medium with magnesium omitted was pretreated three successive times with *Saccharomyces carlsbergensis*.

TABLE IV

*Response of Lactic Acid Bacteria to Added Potassium in Potassium-Deficient Medium**

	K^{+} per 10 cc.			
	0 γ	100 γ	1000 γ	10,000 γ
	Galvanometer readings			
Lactobacillus arabinosus	100	94	35	33
" *delbrueckii*	84	67	35	32
" *casei*	81	62	28	25
Streptococcus faecalis	88	80	68	65
Leuconostoc mesenteroides P-60	94	73	69	69
" " 9135	96	88	59	53
Lactobacillus pentosus	93	67	35	35
" *fermenti*	89	70	57	57

* The basal medium, with potassium salts omitted, was pretreated once with *Lactobacillus arabinosus*.

The requirements of *Lactobacillus casei* and *L. delbrueckii* appear somewhat less than for *L. arabinosus*, and growth of these organisms occurred to a slight extent in the tubes without added potassium. This is undoubtedly due to residual traces of potassium in the medium, since on longer incubation organisms such as *L. arabinosus* also show some growth in the "blank" tubes (Table V). Without added potassium, however, growth does not reach a maximum, regardless of the period of incubation.

The amount of potassium required for maximum growth of these organisms agrees well with that found by others (4, 5) with different media. However, this amount is somewhat higher than one would expect to be required for direct metabolic use. Preliminary experiments indicate that factors other than the amount thus utilized contribute to the high requirement.

Iron—In no case did omission of iron from the medium affect growth deleteriously. Pretreatment of the medium with *Saccharomyces carlsbergensis* reduced the amount of iron to a level slightly below that required for optimal growth of this organism, but all of the lactic acid bacteria grew optimally on this medium without addition of iron. From the known facts that lactic acid bacteria grow anaerobically, do not contain cytochrome, and are catalase-negative, it would be expected that if iron is required at all, it would be required in extremely small amounts.

TABLE V

Relation of Incubation Time to Potassium Requirement of Lactobacillus arabinosus

K^+ per 10 cc.	Galvanometer readings		
	24 hrs.	47 hrs.	95 hrs.
γ			
0	90	76	72
10	82	72	69
50	74	64	61
100	69	58	54
500	41	31	28

Production of Mineral Deficiencies by Use of Citrate—Although sodium or potassium citrate has been used as a buffer in media for *Streptococcus faecalis* (13), its addition to media for certain other lactic acid bacteria is known to inhibit growth (11). The ability of citrate to form non-ionic complexes with certain metallic ions has long been recognized (*e.g.* (14)), and a closer investigation of its inhibitory action on microorganisms seemed warranted.

Such investigation soon showed that inhibition could be prevented by addition of extra mineral salts to the medium, and that the effective ions were Mn^{++} and Mg^{++}. Illustrative data are given in Tables VI to IX. These data were obtained on the basal medium described earlier, but with both Mn^{++} and Mg^{++} omitted, and pretreated once with *Lactobacillus arabinosus* to remove traces of Mn^{++}. The growth response of *L. arabinosus* and *L. casei* to Mn^{++} in the presence and absence of added Mg^{++} is shown in Table VI. The response of *L. arabinosus* to Mn^{++} is essentially unchanged by addition of amounts of Mg^{++} up to 3.0 mg. per 10 cc. As noted earlier

(Table III), growth of *L. casei* is enhanced by Mg^{++}, but the amount of Mn^{++} necessary to achieve maximum growth is nearly the same whether or not Mg^{++} is added (Table VI). In the presence of citrate, these relationships are altered. With *L. arabinosus* (Table VII), much larger amounts of Mn^{++} are required to permit growth when citrate is present; when the

TABLE VI

*Response of Lactobacillus arabinosus and Lactobacillus casei to Additions of Manganese and Magnesium in Medium Deficient in These Ions**

		Mn^{++} per 10 cc.			
		0 γ	0.1 γ	1 γ	10 γ
		Galvanometer readings			
L. arabinosus	No added Mg^{++}	95	86	54	31
	3 mg. Mg^{++} added per tube	94	83	52	27
" *casei*	No added Mg^{++}	86	69	43	33
	3 mg. Mg^{++} added	80	61	34	26

* The basal medium, with both manganese and magnesium salts omitted, was pretreated once with *Lactobacillus arabinosus*.

TABLE VII

*Effect of Varying Citrate and Magnesium Ion Concentration on Manganese Requirement of Lactobacillus arabinosus**

	Mn^{++} per 10 cc.							
	0 γ	10 γ	50 γ	100 γ	150 γ	200 γ	250 γ	500 γ
	Galvanometer readings							
No citrate	99	29		24				
1% "	100		95	58	28	22	21	20
2% "	100			98	92	91	39	22
2% " + 100 γ Mg^{++}	100			80	44	34	27	
2% " + 200 " "	100	75	43	36		24		
2% " + 300 " "	100	35	29			23		

* Medium prepared as described in foot-note to Table VI. Citrate was added after pretreatment.

amount of citrate present is doubled, the amount of Mn^{++} necessary is doubled. With the addition of Mg^{++}, however, the amount of Mn^{++} required is greatly decreased, and with excess Mg^{++} present, the amount of Mn^{++} required for growth is only slightly larger when citrate is present than in its absence.

With *Lactobacillus casei* (Table VIII), a similar reciprocal relationship exists. In this case, however, manganese alone does not prevent inhibition

of growth by citrate; magnesium is also essential for growth. Magnesium is undoubtedly essential also in the absence of citrate; failure to observe more than a stimulatory effect probably reflects its incomplete removal from the medium.

A comparison of the relationship of other lactic acid bacteria to manganese, magnesium, and citrate is given in Table IX. All of the organisms grow well in the presence of citrate when both Mn^{++} and Mg^{++} are present; only *Streptococcus faecalis* grows in the absence of these added ions. The other organisms resemble either *Lactobacillus arabinosus* or *L. casei* in their behavior, or are intermediate between them.

TABLE VIII

*Effect of Concentration of Manganese and Magnesium Ions on Inhibition of Lactobacillus casei by Citrate**

Mg^{++} per 10 cc.	Mn^{++} per 10 cc.						
	0 γ	1 γ	10 γ	100 γ	500 γ	1000 γ	10,000 γ
	Galvanometer readings						
γ							
0	100				98	98	98
500	95				35	27	
1000	84	85	54	24	22	23	
1500	81	39	35	26	23		

Mn^{++} per 10 cc.	Mg^{++} per 10 cc.				
	0 γ	300 γ	350 γ	400 γ	500 γ
1000	100	82	49	29	24
1500	98	65	43	26	24

* The medium was pretreated as described in the foot-note to Table VI. 2 per cent sodium citrate was added after pretreatment.

It is logical to assume, by analogy with its effects on blood coagulation, that citrate enhances the requirements of these organisms for manganese and magnesium because it forms complexes with these ions which are unavailable for growth. Known complexes of citrate with metallic ions decrease in stability as the pH is lowered (14). This is consistent with the observation that progressively less manganese is required to counteract the inhibitory effect of citrate as the initial pH of the medium is lowered (Table X). The formation of similar complexes with citrate could also explain the observation that magnesium exerts a sparing effect on the requirement for manganese (and vice versa) in the presence of citrate, but not in its absence. Other bivalent metallic ions which form complexes with citrate also should have some effect in this direction. Most of these are toxic, and could not

be adequately tested. Ca^{++}, however, is about one-thirtieth as effective as Mg^{++} in this respect for *L. arabinosus*. An interesting incidental observation made in connection with these studies was that Zn^{++} and Ni^{++} showed

TABLE IX

*Effect of Citrate on Response of Several Lactic Acid Bacteria to Manganese and Magnesium**

	Micrograms ion added per 10 cc.						
Mn^{++}	0	0	0	1000	10,000	1000	1,000
Mg^{++}	0	1000	10,000	0	0	1000	10,000
	Galvanometer readings						
Lactobacillus casei	100	99	89	98	99	32	26
" *arabinosus*	100	100	100	25	27	25	50
" *delbrueckii*	100	100	96	82	55	37	28
Streptococcus faecalis	63	62		56		42	55
Leuconostoc mesenteroides P-60	100	100	97	100	63	59	57
" " 9135	99	100	99	50	45	50	47
Lactobacillus fermenti	100	99	100	93	69	75	61
" *pentosus*	100	99	99	23	20	20	22

* 2 per cent sodium citrate added to a medium prepared as described in the footnote to Table VI.

TABLE X

*Effect of pH on Availability of Manganese to Lactobacillus arabinosus in Presence of Citrate**

pH	Mn^{++} per 10 cc.					
	0 γ	100 γ	200 γ	300 γ	400 γ	600 γ
	Galvanometer readings					
7.0	99			48	34	27
6.5	100		97	55	33	25
6.0	100		85	41	26	23
5.5	100	91	48	29	24	22
5.0	100	48	29	28	27	27

* 2 per cent sodium citrate added to the medium prepared as described in Table VI.

much less toxicity in the presence of citrate than in its absence, whereas Cu^{++} was slightly more toxic when citrate was present.

Manganese Requirement of Streptococcus faecalis—Although *S. faecalis* was stimulated by addition of Mn^{++}, both on citrate-free and citrate-containing media, good growth without its addition occurred in both instances. To determine whether manganese was essential, the double strength basal

medium was pretreated with *Lactobacillus arabinosus* for 24 hours. After removal of the cells, sodium citrate (4 per cent of the double strength medium) was added, and the medium was sterilized and inoculated with a saline suspension of washed *S. faecalis* cells. After 24 hours, during which considerable growth occurred, the cells were centrifuged out and this doubly pretreated medium used to determine the response of *S. faecalis* to manganese. The results are given in Table XI. The growth-enhancing effect of manganese additions is clearly visible. When growth ceased at about 72 hours, the cultures with added manganese had grown much more than the unsupplemented tubes. It is tentatively concluded that *S. faecalis*, too, requires manganese for growth, but in far smaller quantities than do the

TABLE XI

*Response of Streptococcus faecalis to Manganese in Pretreated Medium Containing Sodium Citrate**

Incubation period	Mn^{++} per 10 cc.						
	0 γ	0.01 γ	0.03 γ	0.1 γ	0.3 γ	3 γ	100 γ
	Galvanometer readings						
hrs.							
12	88	88	84	83	82	81	77
72	82	77	70	70	66	60	57
116	83	77	70	70	65	63	56

* The basal medium, with manganese salts omitted, was pretreated once with *Lactobacillus arabinosus*, 4 per cent of sodium citrate was then added to the double strength medium, and the pretreatment repeated once with *Streptococcus faecalis*.

lactobacilli. This conclusion must remain tentative, however, since growth on this final medium was slow, and did not reach maximum levels even with added manganese.

Phosphate Requirements of Lactic Acid Bacteria—To establish the amount of phosphate necessary for growth, the basal medium was modified as follows: Sodium citrate was added at a level of 100 mg. per 10 cc. of final medium, phosphates were omitted, and 5 mg. of magnesium sulfate and 5 mg. of manganese sulfate were added per 10 cc. of medium. The enzymatic casein digest was replaced by a pancreatic fibrin digest[1] at a level of 75 mg. per 10 cc. The medium was pretreated once with *Lactobacillus arabinosus*.

[1] 120 gm. of fibrin were suspended in 2 liters of 0.8 per cent $NaHCO_3$. 1 gm. of pancreatin in 20 cc. of water was added, and the mixture incubated under toluene at 37° for 4 days. The hydrolysate was steamed at 100° for 30 minutes, cooled, and filtered. Precipitates were removed as they appeared when the pH was dropped to 3.5 with HCl. The hydrolysate was adjusted to pH 6.5. It contained about 17 mg. of solids per cc.

The response to added KH_2PO_4 was then determined for eight lactic acid bacteria (Table XII). A phosphate deficiency sufficient completely or nearly completely to inhibit the growth of all organisms except *Streptococcus faecalis*, *L. casei*, and *L. delbrueckii* was obtained. In general, these three organisms also showed a greater response to small additions of phosphate than did the other organisms. For maximum growth, most organisms require about 3 mg. of $PO_4^{\equiv}$ (equivalent to 4.39 mg. of KH_2PO_4) per tube. Separate experiments showed that prolonging the incubation period from

TABLE XII

*Response of Eight Lactic Acid Bacteria to Additions of Phosphate in Phosphate-Free Medium**

	$PO_4^{\equiv}$ per 10 cc.				
	0 γ	150 γ	600 γ	1500 γ	3000 γ
	Galvanometer readings				
Lactobacillus arabinosus	97	96	38	25	23
" *delbrueckii*	87	79	43	34	28
Streptococcus faecalis	86	82	67	63	62
Lactobacillus casei	78	51	27	21	19
" *fermenti*	90	79	55	53	52
" *pentosus*	98	80	38	30	28
Leuconostoc mesenteroides, P-60	97	75	45	40	38
" " 9135	99	94	62	50	52

* The basal medium, with fibrin digest substituted for the casein digest and with phosphate omitted, was pretreated once with *Lactobacillus arabinosus* (see the text).

24 hours to as long as 95 hours decreased the phosphate requirement only very slightly.

DISCUSSION

It is evident from the results cited above that in some cases a deficiency in essential inorganic ions can be obtained in a complex medium by preliminary growth in the medium of an organism requiring the ion for growth. The ability of an organism to reduce the concentration of an essential element to a level which will no longer support growth of that organism depends upon the concentration of the ion present as a contaminant in the medium and on the magnitude of the requirement of the organism for maximum growth. If the former concentration is high relative to the latter requirement, a single pretreatment of the medium, and perhaps several successive pretreatments, will not render the medium free of the ion. It is also evident that a medium sufficiently free of an element to demonstrate the requirement for it by one organism may contain sufficient of the mineral

to permit growth of other organisms. *Lactobacillus casei* and *Streptococcus faecalis*, for example, grow fairly well on a medium which contains insufficient manganese to support growth of *Lactobacillus arabinosus*, although both of the former organisms also appear to require manganese for growth.

For successful application of the pretreatment procedure used above for the preparation of deficient media, it is apparent that the inorganic ion (or other substance) to be removed must be the sole essential substance present in limiting amounts. Otherwise, a multiple deficiency may be encountered. Similarly, the organism used for the absorption must not produce, during growth, substances which will inhibit growth of organisms subsequently

TABLE XIII

Comparison of Amounts of Inorganic Salts Commonly Employed in Assay Media with Amounts Necessary for Maximum Growth

	Amounts commonly employed in assay media*	Amounts found necessary for maximum growth in present study	
		Acetate buffer	2 per cent citrate buffer
Mn^{++}, γ	32.5	10–100	10–1000†
Mg^{++}, "	197.4	0	0–1500†
Fe^{++}, "	20.1	0	0
K^{+}, *mg*	7.35	10	10
Na^{+}, γ	39.3		
$PO_4^{\equiv}$, *mg*	12.4	3	3

* Speakman's salts in the concentrations customarily employed in assay media.

† The amount of Mn^{++} required in the presence of citrate depends on the amount of Mg^{++} present. For *Lactobacillus casei*, at least 500 γ of Mg^{++} should be present per 10 cc. (see the text).

introduced into the medium. With the media and organisms used in this work, only one possible example of interference from one of these sources was encountered, *viz. Streptococcus faecalis* failed to grow optimally (after supplementation with manganese) on a medium which had been pretreated first with *Lactobacillus arabinosus*, then with *Streptococcus faecalis* itself. The reason for this is not yet known. In no other case was a depressing effect on growth of an organism by preliminary pretreatment of the medium with the same or another organism encountered.

It is highly probable that the lactic acid bacteria require inorganic ions in addition to those shown to be essential by this investigation. An absolute requirement for magnesium, for example, could not be demonstrated by the pretreatment procedure alone, and it was only through the complex-forming action of citrate that requirement for this cation could be demonstrated. No precautions have been taken in the present investigation to eliminate

the inorganic ions furnished by glassware, etc., to the organisms. The requirements demonstrated above, are, therefore, minimal requirements of the organisms tested, and probably represent those inorganic ions which are required in the largest amounts for growth.

It is interesting to compare the amounts of the inorganic ions commonly added to assay media with the amounts found necessary to promote optimal growth of the organism with the highest requirement in the present study. This comparison is made in Table XIII. For maximum growth in 24 hours, concentrations of manganese ion and of potassium ion previously used have been marginal for some organisms; the remainder appear to be present in adequate amounts. The figures in Table XIII were determined after a 24 hour incubation period; frequently longer incubation periods are used, which result in a slightly decreased requirement for the inorganic ions.

SUMMARY

Under favorable circumstances, growth of an organism in a complex medium can be used to remove completely from the medium traces of a substance essential for growth of that organism.

By this procedure it has been shown that Mn^{++} is essential for *Leuconostoc mesenteroides*, all lactobacilli, and probably for *Streptococcus faecalis*. Potassium ion is also required in comparatively large amounts by all lactic acid bacteria investigated. The level of phosphate required by lactic acid bacteria for growth in an adequately buffered medium has also been established.

With this procedure it was possible to show that magnesium stimulated growth, but not that it was essential. The amount of magnesium required for growth of the lactic acid bacteria is certainly less than that required by yeast. Addition of ferrous iron to an iron-low medium neither enhanced nor inhibited growth.

Citrate inhibits growth of lactic acid bacteria when added to the medium in large amounts. This inhibition of growth can be prevented by increasing the amounts of manganese and of magnesium present in the medium, and appears due to the action of citrate in forming complexes with essential metallic ions. For some organisms, manganese alone prevents the "toxic" action of citrate; for others, both manganese and magnesium are essential. The amount of manganese required is, however, always decreased by addition of magnesium. It can also be decreased by addition of other bivalent metallic ions, such as calcium, which form complex ions with citrate.

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