EFFECT OF MANGANESE AND OTHER FACTORS ON EARLY AUTOLYSIS OF CULTURES OF STREPTOCOCCUS FAECALIS[1]

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This study grew out of an unsuccessful attempt to employ *Streptococcus faecalis r*, strain ATCC 8043, for the differential assay of pyridoxamine and pyridoxal, according to Rabinowitz and Snell (1947). It was found that the organism had lost its requirement for pyridoxal and pyridoxamine, and that other factors, such as length of time of autoclaving the medium and selection of buffers, influenced its growth. Both these factors affect growth in a somewhat unpredictable manner. For example, autoclaving is essential for the growth of *Lactobacillus bulgaricus* (Snell *et al.*, 1948) but may inhibit that of *Lactobacillus bifidus* (Rose *et al.*, 1952). Analogous differences exist for buffers: citrate and phosphate are superior for *S. faecalis r* (Teply and Elvehjem, 1945), but citrate, because of its chelating power, is toxic for *Lactobacillus casei* (MacLeod and Snell, 1947).

In the present study, cultures of *S. faecalis r* grown in the presence of a moderately high level of citrate exhibited an enhanced autolysis. This autolysis has been shown to be due to a relative deficiency of manganese. Even though the cultures of *S. faecalis r*, strain ATCC 8043, in our possession could not be used for the assay of pyridoxal and pyridoxamine, it was considered important to investigate the growth behavior of this particular strain, on the medium employed, since it is used widely for the assay of folic acid and methionine.

EXPERIMENTAL METHODS

One of the cultures of *S. faecalis r*, strain ATCC 8043, used was in this laboratory before this work was started, and three were obtained later from the American Type Culture Collection.[2] None of these showed an absolute requirement for any form of vitamin B_6. All experiments reported were performed with the culture originally in possession of this laboratory. This stock culture was carried, with monthly transfers, on a medium consisting of yeast extract (Difco), 1 per cent; proteose peptone, 0.5 per cent; glucose, 1 per cent; and agar, 1.5 per cent.

The composition of the synthetic basal liquid medium is listed in table 1. Redistilled water was used throughout. This basal medium was dispensed in 5 ml quantities into Evelyn colorimeter tubes, supplements were added, and the volume adjusted with redistilled water to 10 ml. Whenever manganese response was under investigation, the manganese salt was omitted from the basal medium. When manganese was added as supplement, $MnSO_4 \cdot 4H_2O$ was employed. All tubes were set up at least in duplicate and sterilized either by autoclaving for 5 minutes at 126.5 C, or by filtration through a Seitz or an ultrafine sintered glass filter. The inoculum consisted of one drop of a dilute washed cell suspension, made by growing the organism 24 hours prior to the experiment in 10 ml of a broth, consisting of yeast extract, 2.0 per cent; proteose peptone, 0.5 per cent; glucose, 1.0 per cent; and KH_2PO_4, 0.2 per cent.

The tubes were incubated at 37 C. At specified intervals, growth was estimated in an Evelyn colorimeter, employing a 660 filter.

Experiments designed to verify the autolysis provoking effect of suboptimal concentrations of

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[2] The original culture was obtained from Dr. Ilda McVeigh, from the Vanderbilt University Biology Department. We are grateful to the American Type Culture Collection for sending us the additional three cultures. Dr. E. E. Snell (personal communication) reported that a culture of this organism, lyophilized in 1941, retained its vitamin B_6 requirement, but substantiated the loss of requirement in our cultures.

TABLE 1

*Composition of the basal synthetic medium**

"Vitamin-free" casein hydrolyzate (10%)†	100 ml
L-Asparagine	200 mg
L-Cystine	800 mg
DL-Tryptophan	800 mg
Xanthine	2 mg
Guanine	2 mg
Adenine	2 mg
Uracil	2 mg
Glucose	20 g
KH_2PO_4	1 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	800 mg
$FeSO_4 \cdot 4H_2O$	40 mg
$MnSO_4 \cdot 4H_2O$	40 mg
Thiamin Cl·HCl	0.5 mg
Riboflavin	0.5 mg
Pyridoxamine·2HCl	0.5 mg
p-Aminobenzoic acid	1.0 mg
Nicotinamide	1.0 mg
Folic acid	20 μg
Leucovorin‡	20 μg
α-Lipoic acid, sodium salt‡	44 μg
Biotin	1 μg
Polyoxyethylene sorbitan monooleate (tween 80)	25 μg

* All amounts are for one liter double strength medium.

† Nutritional Biochemical Corporation.

‡ Courtesy of Lederle Laboratories Division, American Cyanamid Company.

manganese were performed on the inoculum broth. This medium was rendered free of the metal by the biological purification procedure of MacLeod and Snell (1947), employing as the pregrowing organism the same strain of *S. faecalis r* as used in the rest of this study. It was noted that, on pregrown broth, reasonably good growth was not obtained unless citrate was added to the medium at a level of 2.5 per cent.

Special techniques which were employed will be discussed in detail with the particular experiment involved.

RESULTS

Promotion of autolysis by citrate and its reversal by manganese. When the basal synthetic medium was supplemented with sodium citrate at a concentration of 2.5 per cent, a precipitous autolysis occurred, usually after 24 hours of

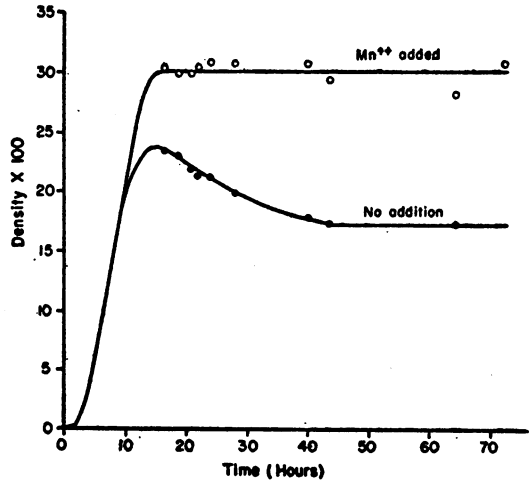

Figure 1. Autolysis of *Streptococcus faecalis* on pregrown medium and its prevention by manganous ion.

incubation (figure 1). This autolysis was estimated by the reduced density readings obtained after this time. It was not apparent when sodium acetate was employed as the buffer, instead of sodium citrate, at a concentration of 0.5 per cent. MacLeod and Snell (1947) demonstrated the the toxicity of citrate for several lactic acid bacteria is due to its chelating power. Furthermore, MacLeod (1951) noted that suboptimal concentrations of manganese give more rapid autolysis of *S. faecalis r*. We verified this observation by employing the same pregrowing technique as used by MacLeod. The typical experiment, shown in figure 2, confirms the observation that *S. faecalis* autolyzes in a pregrown medium containing citrate (presumably metal deficient), and that this autolysis can be prevented by manganous ion.

The autolysis preventing effect of manganese could be demonstrated also in the basal synthetic medium[3] provided this medium was autoclaved double strength. Manganese had much less effect when the single strength medium was autoclaved. This effect on autolysis of the autoclaved concentrated medium could be imitated wholly or partially in the diluted medium by increasing the glucose concentration

[3] Hereafter, manganese was omitted from the basal medium and, where needed, was added as a supplement.

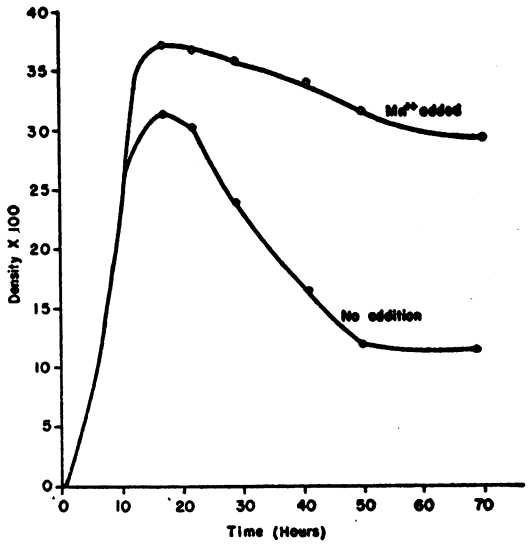

Figure 2. Autolysis of *Streptococcus faecalis* on synthetic medium containing 2.5 per cent citrate and its partial prevention by manganous ion.

or by supplementing the medium with inoculum broth or yeast extract (table 2). Hence, it was concluded that these supplements were not active *per se*. These results indicate that manganese deficiency provokes autolysis, but before manganese supplementation will prevent the autolysis, a second factor is required.

Enhancement of autolysis by manganese and its reversal by ascorbic acid. Since this second factor appeared only after autoclaving, growth on the synthetic basal medium sterilized by filtration was investigated. Again, autolysis could be clearly demonstrated. However, under these conditions, manganese actually enhanced the autolysis rather than prevented it (table 3, first set of figures). Since autoclaving induces a low redox potential (Raynaud and Viscontini, 1945) (which does not arise during filtration), the basal medium was supplemented with ascorbic acid to provoke a reducing state. From table 3, it is evident that increasing amounts of ascorbic acid reverse the action of manganese from autolysis enhancement to that of partial lysis prevention.

The measurements in these experiments were complicated by the occurrence of "browning" during incubation. This was taken into account by carrying uninoculated blanks along with each inoculated tube. The densities of the uninoculated tubes were subtracted from the corresponding inoculated ones; the resulting value was considered the true density, due to bacterial growth. It is recognized that this correction is only an approximation because of the influence the growth has on "browning" in the inoculated tubes. Attempts to measure the "browning" in the supernatant after centrifugation of the cells

TABLE 2

The influence of manganese and various supplements on autolysis in a synthetic medium

SUPPLEMENTS TO BASAL MEDIUM* + 2.5% CITRATE	CHANGES IN TURBIDITY BETWEEN 24 AND 64 HR, EXPRESSED AS DENSITY × 100†
No additions	−21.4
Manganese	−17.1
Glucose	−22.3
Glucose + manganese	−10.1
Inoculum broth	−2.1
Inoculum broth + manganese	+4.6
Yeast extract	−6.6
Yeast extract + manganese	+5.7
Medium autoclaved double strength, no additions	−6.4
Medium autoclaved double strength, + manganese	+5.4

* Concentrations of the various supplements were: glucose, 10 per cent; inoculum broth, 10 per cent v/v; yeast extract, 10 per cent; and manganese, 4.5 μM/10 ml. The medium was autoclaved single strength, unless otherwise indicated.

† A negative value indicates autolysis.

TABLE 3

The influence of graded amounts of ascorbic acid on autolysis in a filtered synthetic medium

MG PER TUBE OF ASCORBIC ACID ADDED TO BASAL MEDIUM + 2.5% CITRATE	CHANGES IN TURBIDITY BETWEEN 24 AND 64 HR, EXPRESSED AS DENSITY × 100 (CORRECTED FOR "BROWNING"*)		DIFFERENCE†
	Tubes containing no manganese	Tubes containing manganese	
—	−10.9	−19.0	−8.1
2.5	−4.4	−12.2	−7.8
5.0	−3.3	−7.1	−3.8
10.0	−3.2	−4.1	−0.9
15.0	−6.0	−3.5	+2.5
20.0	−7.8	−6.3	+1.5

* See text.

† A negative value indicates autolysis enhancement.

were unsuccessful for two reasons. First, the supernatant possessed an inherent opalescence, and secondly, the few cells remaining in this layer grew at such a rapid rate that within an hour visible turbidity was again apparent.

Growth inhibition by manganese. The addition of manganese to the synthetic medium before or after autoclaving was equally effective in the prevention of autolysis. Hence, this property of manganese was not due to a catalytic effect during autoclaving. In the filtered basal medium it was observed that "browning" was constantly greater in tubes containing manganese than it was in their controls. However, when ascorbic acid was added, this "browning" difference was either not evident or in fact reversed.

To study this effect of manganese upon "browning" in a filtered medium, uninoculated tubes containing this medium and graded amounts of manganese were incubated at 37 C for 92 hours. Controls without manganese were included. At the end of this period one-half of the tubes were inoculated. Twenty hours after inoculation, i.e., before autolysis set in, growth was estimated as was the "browning" in the uninoculated blanks. The values so obtained were compared with identical tubes which had not been allowed to stand prior to inoculation.

TABLE 4

The influence of manganese on absolute growth of Streptococcus faecalis in a filtered synthetic medium

ADDITIONS PER TUBE TO BASAL MEDIUM + 2.5% CITRATE	"BROWNING" DENSITY (× 100) DUE TO MANGANESE AT TIME OF ESTIMATION OF GROWTH* (UNINOCULATED BLANKS)		GROWTH DENSITY (× 100) AT 20 HR AFTER INOCULATION (CORRECTED FOR "BROWNING")	
	Total incubation time 20 hr	Total incubation time 112 hr	Total incubation time 20 hr†	Total incubation time 112 hr‡
No additions	0.0	0.7	48.2	48.4
4.5 μM Manganese	1.1	4.1	42.6	29.5
18.0 μM Manganese	3.6	13.5	35.9	27.1
45.0 μM Manganese	7.1	25.0	33.5	18.5
135.0 μM Manganese	10.0	ppte	26.4	ppte
4.5 μM Manganese + 0.5 mg ascorbic acid	−0.4	—	48.8	—
4.5 μM Manganese + 2.5 mg ascorbic acid	−0.9	—	53.8	—

* The density of the tubes containing no manganese, at the onset of the experiment, is taken as 0.0.

† I.e., no incubation prior to inoculation, and 20 hr incubation after inoculation.

‡ I.e., 92 hours' incubation prior to inoculation, and twenty hours' incubation after inoculation.

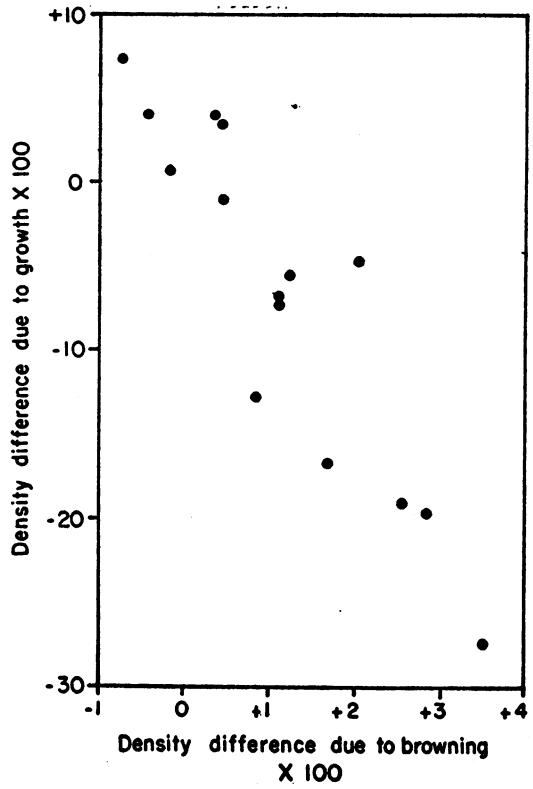

Figure 3. The relation between browning (due to manganese) and difference in maximal growth in tubes containing manganese and their controls. Incubation time 20 hours.

Table 4 shows that "browning" was proportional within limits to the level of manganese in the medium, and that growth was depressed by increasing manganese supplementation. Both the "browning" and the growth inhibition increase with the time of incubation. Thus, without implying a causal relationship between "browning" and the principle responsible for the growth inhibition, it can be stated that the growth inhibition is proportional to the "browning". This proportionality is expressed in figure 3 which presents the difference in growth density at time of maximal growth (20 hr after inoculation) between controls and tubes containing manganese plotted against the density difference induced by manganese "browning", measured at

540 mμ. The data of this figure are composed of several experiments, including those in which ascorbic acid was added, hence, the negative values in some instances for the "browning" difference. It is clear that an increase in "browning" was associated with a decrease in growth.

On this basis it may be inferred that the means by which ascorbic acid and autoclaving act in modifying the effect of manganese is by preventing the formation of an inhibitory product under the influence of manganese. Conversely, the autolysis enhancement may be explained by a growth inhibition since this growth inhibition increases with time. If we assume that the level of maximal density, which continues for several days in normal cultures of *S. faecalis r*, is a reflection of a balance between autolysis and growth, then continuously increasing inhibition will upset the balance in favor of autolysis.

DISCUSSION

It is evident that two components of the medium exert a dual effect, both beneficial and deleterious. The first component is the citrate ion. This acts as a superior buffer for *S. faecalis r*, but maximal growth is not obtained unless high levels are employed. At these high levels citric acid chelates sufficient manganese to provoke autolysis. The second component, manganese, is an essential element for the growth (MacLeod, 1951) of *S. faecalis r*, but under certain circumstances it will provoke the formation of a less suitable medium.

These observations are of practical importance, especially in the microbiological assay of folic acid. Urine samples assayed for folic acid[4] by the Association of Official Agricultural Chemists method (1950) gave different results depending upon the time at which the turbidimetric readings were taken. A representative assay of rat urine which was read at various intervals gave results that ranged from 38.9 to 29.1 mμg per ml of urine. The reason for this variation lies in the fact that the medium containing the urine samples and the medium containing the standard curve samples lysed differently both as to extent and time of onset of lysis. There was also a marked difference in the extent of autolysis at different levels of urine assay. This suggests strongly that titration is the better method of determining assay growth (as was originally proposed by Teply and Elvehjem, 1945) since in most cases the autolysis in both standards and samples is complete by 72 hours and the values obtained may be considered more comparable.

The exact mechanisms by which manganese acts are not known. From this study, however, emerges the warning that a bacterial medium is not defined by its initial composition, and that not only does bacterial growth change the medium during incubation, but that incubation modifies the medium and may thus influence growth.

[4] Since the culture of *Streptococcus faecalis*, strain ATCC 8043, showed a reduced sensitivity for folic acid, this vitamin is assayed currently in this laboratory with a strain designated *S. faecalis*, 29-21, isolated from turkey feces (Harrison and Hansen, 1950). This organism shows, however, the same phenomenon of lysis.

SUMMARY

The strains of *Streptococcus faecalis r*, strain ATCC 8043, used in this laboratory for vitamin assays, have lost their requirement for vitamin B_6. Citrate stimulates the growth of this organism, but large quantities produce autolysis. The induction of autolysis by citrate is due to a relative manganese deficiency. At high concentrations, manganese will depress growth in a filtered medium; however, this depression can be counteracted by the reducing agent, ascorbic acid. These findings suggest that turbidimetric assays, employing this organism, are subjected to error.

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