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ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 132
8 January 1954

THE INFLUENCE OF MAGNESIUM ON THE OXIDATIVE ACTIVITY OF AZOTOBACTER*

*Subtask under Biological and Medical Aspects of Ionizing Radiation,
AMRL Project No. 6-59-08-014, Subtask, Effect of Irradiation on
Single Cell Organisms.



RESEARCH AND DEVELOPMENT DIVISION
OFFICE OF THE SURGEON GENERAL
DEPARTMENT OF THE ARMY

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Army Medical Research Lab. Project No. 6-59-08-014 Report No. 132

THE INFLUENCE OF MAGNESIUM ON THE OXIDATIVE ACTIVITY OF AZOTOBACTER

C. R. Goucher and V. F. Kocholaty

15 pp & ii

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The oxidative ability of Azotobacter is greatly diminished by washing the cells with phosphate buffer. The cause of this diminution appears to be the removal of magnesium from the cell by washing. This ion was shown to be important for the oxidation of glucose, acetate, succinate, malate and α -ketoglutarate. Experiments with washed cells of Azotobacter indicated that addition of magnesium restored oxidative ability to levels encountered with unwashed organisms.

1. Microbiology 2. Magnesium 3. Respiration 4. Trace metals.

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THE INFLUENCE OF MAGNESIUM ON THE OXIDATIVE
ACTIVITY OF AZOTOBACTER*

by

C. R. Goucher, Bacteriologist and W. F. Kocholaty, Biochemist

from

ARMY MEDICAL RESEARCH LABORATORY
FORT KNOX, KENTUCKY
8 January 1954

*Subtask under Biological and Medical Aspects of Ionizing Radiation,
AMRL Project No. 6-59-08-014, Subtask, Effect of Irradiation on
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Report No. 132
Project No. 6-59-08-014
Subtask AMRL S-2
MEDEA

8 January 1954

ABSTRACT

THE INFLUENCE OF MAGNESIUM ON THE OXIDATIVE ACTIVITY OF AZOTOBACTER

OBJECT

An exploratory study was undertaken with the purpose of finding micro-organisms especially suited for a study of the mechanism of destruction of anabolic processes caused by irradiation. The first objective was to determine the influence of various ions on the oxidative activity of micro-organisms.

RESULTS AND CONCLUSIONS

While investigating the effect of ultraviolet light on the induced oxidative activities of Azotobacter, wide variations in the oxidative activities of this organism were encountered. Investigation of these discrepancies revealed that the oxidative ability of Azotobacter was greatly diminished by washing with a potassium-phosphate buffer. The cause of this diminution in activity appeared to be the removal of magnesium from the bacterial cell by washing. This ion was found to be important for the oxidation of glucose, acetate, succinate, malate and α -ketoglutarate by Azotobacter cells. Experiments with washed Azotobacter cells indicated that addition of magnesium restored oxidative ability to levels encountered with unwashed organisms.

RECOMMENDATIONS

Adaptive oxidative manifestations in Azotobacter have been found to be drastically affected by ultraviolet light. The nature of the changes in oxidative activity has not been determined. Mechanisms responsible for adaptations, or for changes in oxidative capabilities, may represent alterations in permeability, or the functioning of induced biosynthetic mechanisms. It is suggested, therefore, that a study be made of the cause of the change in the adaptive oxidative activities of this organism with the object of discovering the metabolic systems that may be involved in irradiation injury.

Submitted by:

C. R. Goucher, Bacteriologist
W. F. Kocholaty, Biochemist

Approved: Ray G. Dagg
RAY G. DAGG
Director of Research

Approved: Carl F. Tessmer
CARL F. TESSMER
Lt. Colonel, M. C.
Commanding

THE INFLUENCE OF MAGNESIUM ON THE OXIDATIVE ACTIVITY OF AZOTOBACTER

I. INTRODUCTION

The oxidative capacities of a micro-organism are governed to a large extent by the composition of the medium in which the bacterium is grown. Studies of the oxidative abilities of a micro-organism are often performed with washed cells, suspended in salt solutions containing an oxidizable organic compound. In a study of the genus Azotobacter, using the resting cell technique, inconsistent fluctuations in oxidative capacities were traced to the mode of washing the cells, more specifically to the removal of magnesium from the cells during the washing process. The extent to, and the conditions under, which magnesium will influence certain oxidative properties of Azotobacter are the subjects of this communication.

II. EXPERIMENTAL

A. Organisms

Two strains of Azotobacter were used - Azotobacter agile A 44* and Azotobacter Q**.

B. Media

The synthetic medium described by Karlsson and Barker was used (1). The medium without the carbon source will be referred to as the "complete salt medium". The pH was adjusted to 7.0 with H₂SO₄. The carbon sources for growth were sterilized by autoclaving and were added to the sterile salt medium in such quantities as to give final concentrations of 1.0%.

C. Cultivation

The organisms were grown on a rotary type shaker at 30°C for 24 hours. At harvesting time, glucose grown cultures usually had attained a pH of 6.5 and an optical density of approximately 0.30, when measured with a Coleman Jr. Spectrophotometer at 6500 Å. Cells

* Obtained by courtesy of Dr. H. A. Barker, Biochemistry and Virus Laboratory, University of California, Berkeley, California.

**Kindly furnished by Dr. A. Eisenstark, Department of Bacteriology, Kansas State College, Manhattan, Kansas.

grown on an acetic acid medium attained a pH value of 8.4 and an optical density of about 0.20. Regardless of the growth medium used, in terms of the carbon source, the inocula in all cases were glucose grown cells washed two times in the manner described below.

D. Harvesting

At the end of a 24 hour growth period, the cells were centrifuged from the growth medium and resuspended in M/15 potassium-phosphate* buffer at pH 7.0, to an optical density of approximately 0.25.

E. Washing

Cells contained in 10 ml of this optically adjusted suspension were washed in the centrifuge five times with 10 ml of M/15 potassium-phosphate buffer. At the end of the fifth washing the suspensions were adjusted to an optical density of 0.25 with the same buffer, and 0.5 ml of such a suspension was used for respiration studies.

F. Respiration Studies

Each Warburg flask had the following fluid contents added in the order indicated: Center well - 0.15 ml 20% KOH; reaction chamber - 1.0 ml of $MgSO_4$ or other salt solution as indicated, 1.0 ml of buffer, and 0.5 ml of cells at the optical density indicated; side arm - 0.5 ml of substrate. The amount of substrate added to each flask would theoretically require 448 microliters of O_2 for its complete oxidation to carbon dioxide and water. The gas phase was air and the reaction was maintained at a temperature of 35°C. Cells were left in contact with magnesium for about 45 minutes at room temperature after washing and before the introduction of substrate to the reaction chamber, unless otherwise indicated. All salt and substrate solutions were prepared in water previously distilled in a metal still and then passed through an ion exchange column (Deeminizer**). The distilled water thus obtained had a conductivity corresponding to 0.25 p. p. m. NaCl.

III. RESULTS

The term "adapted cells" as used in this report refers to cells which can oxidize a substrate at a constant or diminishing rate during

*M/15 potassium-phosphate buffer consisted of 9.07 gm KH_2PO_4 adjusted to pH 7.0 with KOH and made up to 1000 ml with distilled water.

**Crystal Research Laboratories, Inc., Hartford, Conn.

the interval of exposure to the substrate. "Unadapted cells" designates cells which can oxidize substrate at a rate which increases as the interval of exposure to substrate is lengthened. These terms are intended to be superficially descriptive of phenomena without specifically indicating the nature of the mechanism by which modifications of the oxidative ability are accomplished.

A. The Influence of Washing Procedure and Composition of Washing Solution upon the Oxidative Capacity of Azotobacter.

Figure 1 illustrates the changes in oxidative capacity of acetate grown *Azotobacter agile* A 44 which occurred with repeated washings of the cells. Cells washed 5 times with the complete salt medium had approximately identical oxidative activities as the unwashed cells. When cells were washed with M/15 potassium-phosphate buffer, the first washing halved, and subsequent washings further diminished the oxidative capacity of the organisms. However, when magnesium was added to cells washed with phosphate buffer, practically full oxidative capacities were restored, whether the cells were washed once, or five times with phosphate buffer.

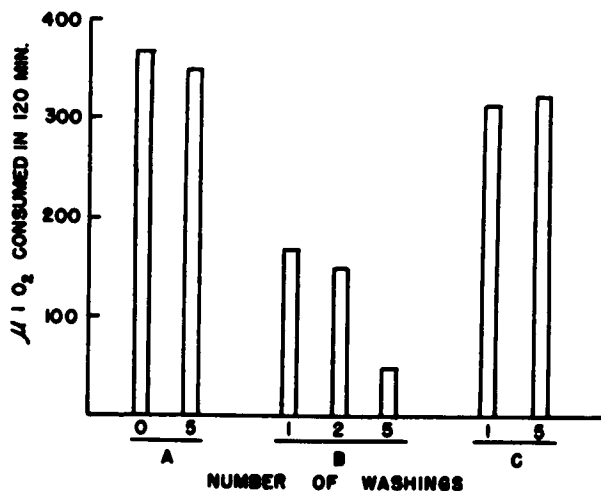


FIG.1- INFLUENCE OF WASHING PROCEDURES ON OXIDATIVE ACTIVITY.

REACTION MIXTURE:

0.5 ml of acetate grown *Azotobacter agile* A 44 cells in M/15 potassium-phosphate buffer, pH 7.0

0.5 ml of substrate (succinate) in M/15 potassium-phosphate buffer, pH 7.0

2.0 ml of M/15 potassium-phosphate buffer, pH 7.0

A - 0 = unwashed cells

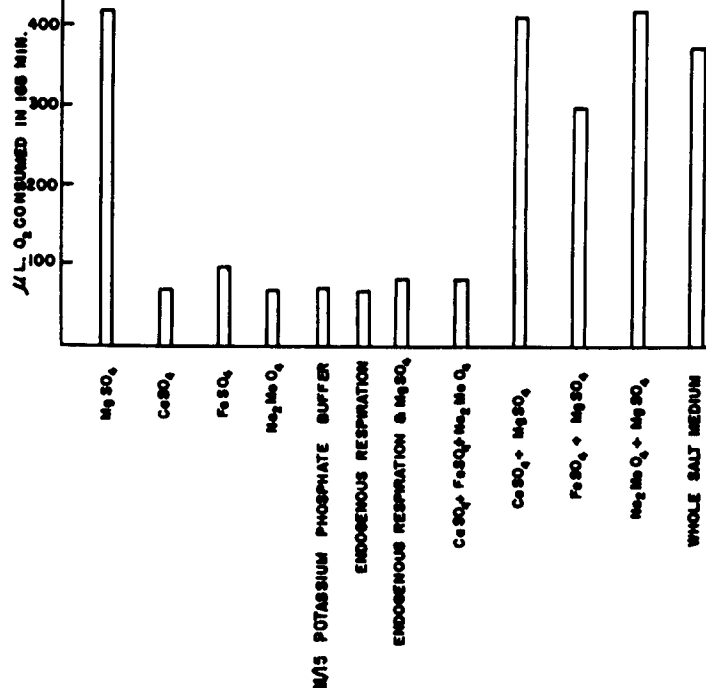
5 = cells washed 5 x in whole salt medium

B - Cells washed 1, 2 and 5 x in M/15 potassium-phosphate buffer at pH 7.0 no magnesium added

C - Cells washed 1 and 5 x in M/15 potassium-phosphate buffer at pH 7.0 with magnesium added

B. Effect of Various Ions on the Restoration of Oxidative Activity of Washed Azotobacter Cells.

The abilities of the cations present in the growth medium to influence, singly and in various combinations, the oxidation of succinate were investigated. Strain A 44 of Azotobacter agilis was grown on an acetate medium and washed in potassium-phosphate buffer as described. The various cations studied were in the same concentrations as found in the growth medium. Figure 2 shows the effects of CaSO_4 , FeSO_4 , Na_2MgO_4 , and MgSO_4 and of various combinations of these ions in M/15 potassium-phosphate buffer on succinate oxidation by washed cells. None of these ions, or ion combinations, except MgSO_4 , were capable of enhancing the rate of succinate oxidation. The activity imparted by magnesium could not be enhanced by the addition of calcium, iron or molybdenum. The effect of magnesium could not be duplicated by manganese, cobalt or zinc using concentrations of 0.02%. Ammonium sulfate is known to stimulate adaptive enzyme formation in yeast (2) and possibly could be implicated in the phenomenon of magnesium stimulation; however, it was found to be without effect on the oxidation of succinate by washed cells, in concentrations of 0.02%.



REACTION MIXTURE: FIG. 2—INFLUENCE OF VARIOUS IONS ON OXIDATIVE ACTIVITY.

0.5 ml of acetate grown Azotobacter agilis A 44 cells in M/15 potassium-phosphate buffer.

Cells were washed 5 x with M/15 potassium-phosphate buffer at pH 7.0

0.5 ml of substrate (succinate) in M/15 potassium-phosphate buffer

2.0 ml of M/15 potassium-phosphate buffer, pH 7.0; temperature 35°C

Cations were added in the same concentrations as found in the whole salt medium

When cells were washed with M/20 tris-hydroxy-methyl-aminomethane (THAM)-buffer, pH 7.0, instead of the phosphate buffer, the importance of magnesium to succinate oxidation was still evident, although revealed in a different pattern (Figure 3). No single cation of the growth medium restored the original oxidative capacity of THAM-washed Azotobacter cells; Fe, Mo and Ca, showed almost no effect and Mg alone showed only about 50% of the original oxidative capacity. However, the combination of Fe, Ca or Mo ions as present in the whole salt medium with Mg resulted in a restoration of activity and the combination of potassium phosphate and magnesium appeared to enhance the oxidative ability.

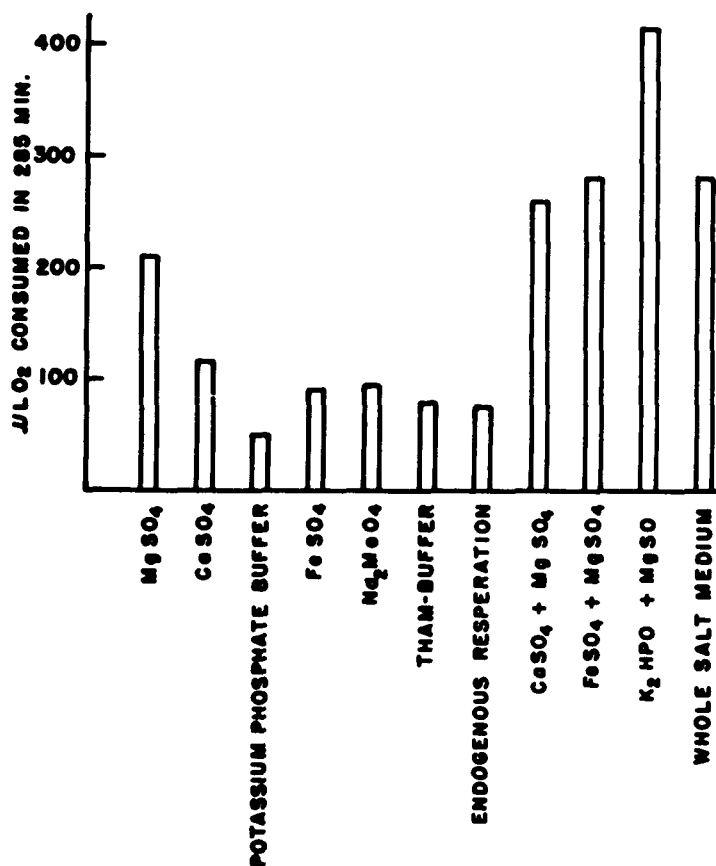


FIG. 3-INFLUENCE OF VARIOUS IONS ON OXIDATIVE ACTIVITY WITH THAM BUFFER.

Reaction mixture and conditions same as for Fig. 2., except M/20 THAM buffer was used in place of M/15 potassium-phosphate buffer.

Only under conditions indicated in Figure 3 was the stimulation of succinate oxidation associated with calcium. Stimulation of respiration in *Azotobacter* by calcium at low pH values has been reported by Harris and Gainey (3). Using cells washed and suspended in M/15 potassium-phosphate buffer, succinate oxidation was determined at pH 5.8 in the absence of calcium and magnesium, as well as in the presence of these ions, singly and combined. At this pH, oxidation of succinate was not observed to occur, regardless of the cation content of the reaction mixture.

C. Rate of Substrate Oxidation as Influenced by the Sequence of Addition of Magnesium and Substrate.

The rate at which the Mg ion was able to impart maximal oxidative activity to washed cells of *Azotobacter* was tested with succinate as substrate. Using acetate grown cells, washed 5 times with potassium-phosphate buffer, it was observed that succinate oxidation occurred as rapidly when succinate and magnesium were added simultaneously as when magnesium was added 90 minutes before the substrate.

When similarly treated cells were brought in contact with succinate in the initial absence of magnesium, a longer period of adjustment and a lower rate of oxidation ensued, than when magnesium was added at the same time as succinate (Figure 4).

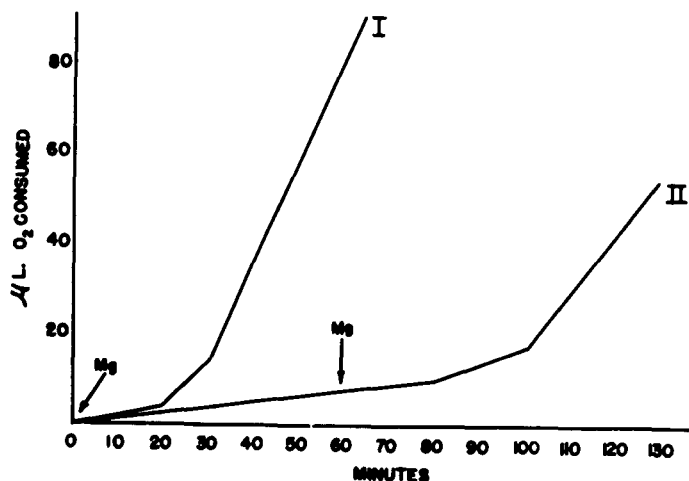


FIG. 4- RATE OF OXIDATION IN RELATION TO TIME OF ADDITION OF MAGNESIUM.

Reaction mixture and conditions same as for Fig. 2., except that in I magnesium was added immediately and in II magnesium was added after 60 min.

D. The Effect of Magnesium on the Oxidation of Glucose, and Acetate by Adapted Cells.

Azotobacter agile A 44, when grown on a glucose medium, is adapted to the oxidation of both acetate and glucose. Data presented in Figure 5 indicate the effect of various $MgSO_4$ concentrations on the oxidation of glucose by glucose grown cells. At suboptimal concentrations of magnesium, the rate of oxidation of glucose decreased slightly with time, but at the highest concentration of magnesium used, glucose oxidation continued for 120 minutes at a constant rate. Data presented in Figure 6, in addition to showing the effects of various magnesium concentrations on acetate oxidation also show the inability of glucose grown cells of Azotobacter agile A 44 to oxidize acetate in the absence of magnesium.

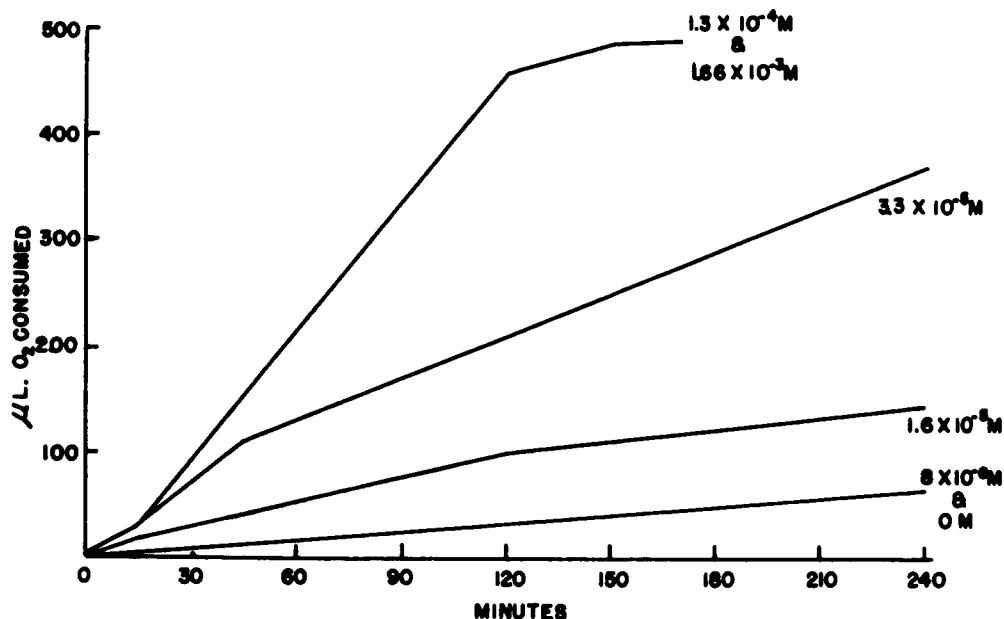


FIG.5- MAGNESIUM CONCENTRATION AND THE RATE OF GLUCOSE OXIDATION.

REACTION MIXTURE:

0.5 ml of glucose grown Azotobacter agile A 44 cells in M/15 potassium-phosphate buffer pH 7.0. Cells were washed 5 x with M/15 potassium-phosphate buffer at pH 7.0.

0.5 ml of substrate (glucose) in M/15 potassium-phosphate buffer

2.0 ml of M/15 potassium-phosphate buffer, pH 7.0, temp. 35° C

Varying molar concentrations of $MgSO_4$ were used

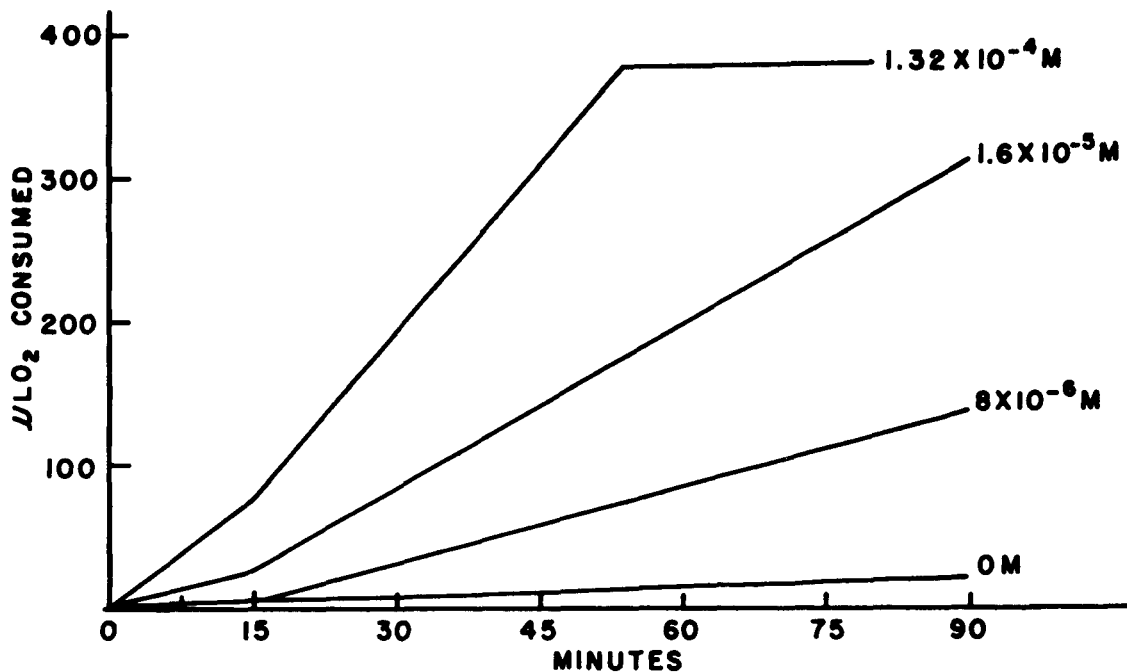


FIG. 6-MAGNESIUM CONCENTRATION AND THE RATE OF ACETATE OXIDATION.

Reaction mixture and conditions same as for Fig. 5, except acetate was used as the substrate in place of glucose.

E. The Effect of Magnesium on the Oxidation and/or the Adaptation to α -Ketoglutarate, Succinate and Malate by Unadapted Cells.

Azotobacter agile A 44 cells grown on glucose were unadapted to the oxidation of α -ketoglutarate, succinate and malate. Data presented in Figures 7 and 8 show the effect of various magnesium concentrations on the rate of oxidation of α -ketoglutaric acid by washed cells of Azotobacter agile A 44 and Azotobacter Q, respectively. In the absence of magnesium this acid was not oxidized by either strain of Azotobacter.

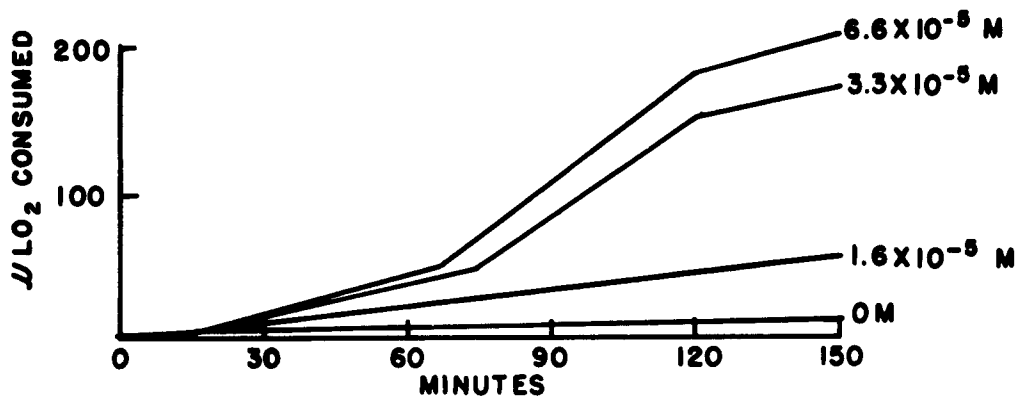


FIG. 7-MAGNESIUM CONCENTRATION AND THE RATE OF α -KETOGLUTARATE OXIDATION BY AZOTOBACTER AGILE A44.

REACTION MIXTURE:

0.5 ml of glucose grown Azotobacter agile A 44 cells in M/15 potassium-phosphate buffer. Cells were washed 5 x with M/15 potassium-phosphate buffer at pH 7.0
 0.5 ml of substrate (α -ketoglutarate) in M/15 potassium-phosphate buffer
 2.0 ml of M/15 potassium-phosphate buffer, pH 7.0, temp., 35°C
 Varying molar concentrations of $MgSO_4$ were used.

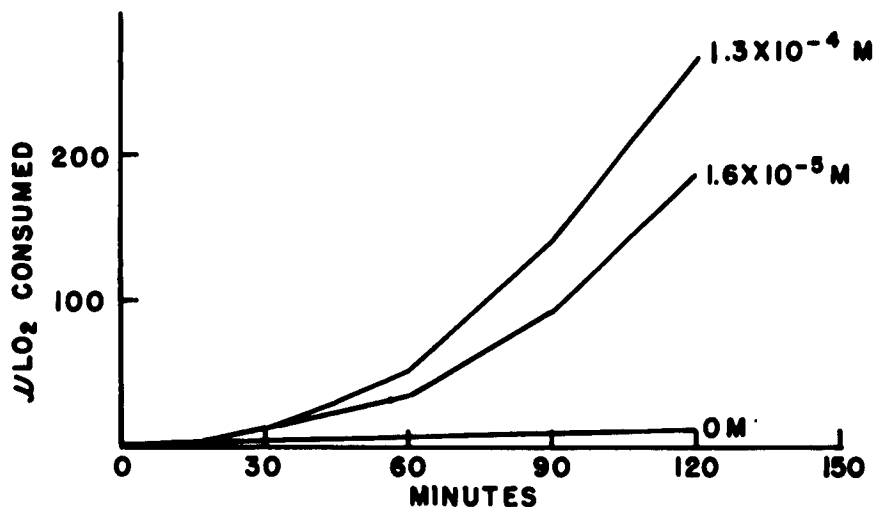


FIG. 8-MAGNESIUM CONCENTRATION AND THE RATE OF α -KETOGLUTARATE OXIDATION BY AZOTOBACTER Q.

Reaction mixture and conditions same as for Fig. 7, except Azotobacter Q cells were used in place of Azotobacter agile A 44

Data presented in Figure 9 show the influence of various concentrations of $MgSO_4$ on the ability of glucose grown and washed cells of Azotobacter agile A 44 to oxidize succinate. Succinate oxidation was not observed in the absence of magnesium. At low $MgSO_4$ concentrations (1.6×10^{-5} molar), succinate was oxidized without any appreciable increase in rate of oxidation after the first 60 minutes of incubation. Increasing increments of magnesium lead to increasing and eventually maximal rates of substrate oxidation. Similar conditions prevailed with Azotobacter Q, when grown on a glucose medium and tested with succinate as substrate. The effect of $MgSO_4$ concentration on the oxidation of malic acid by glucose grown Azotobacter agile A 44 cells is indicated in Figure 10. It is evident that 1.6×10^{-5} molar $MgSO_4$ stimulated malate oxidation but did not permit an increase in rate of oxidation comparable to the rates observed with higher $MgSO_4$ concentrations. With diminishing concentrations of magnesium the rate of oxidation of malate approached zero.

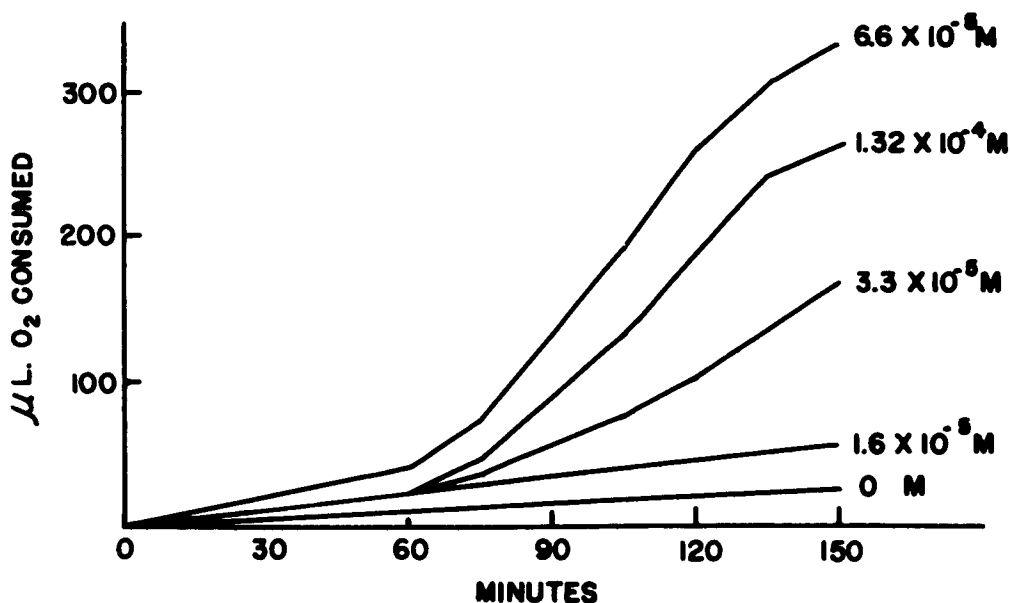


FIG.9- MAGNESIUM CONCENTRATION AND RATE OF SUCCINATE OXIDATION.

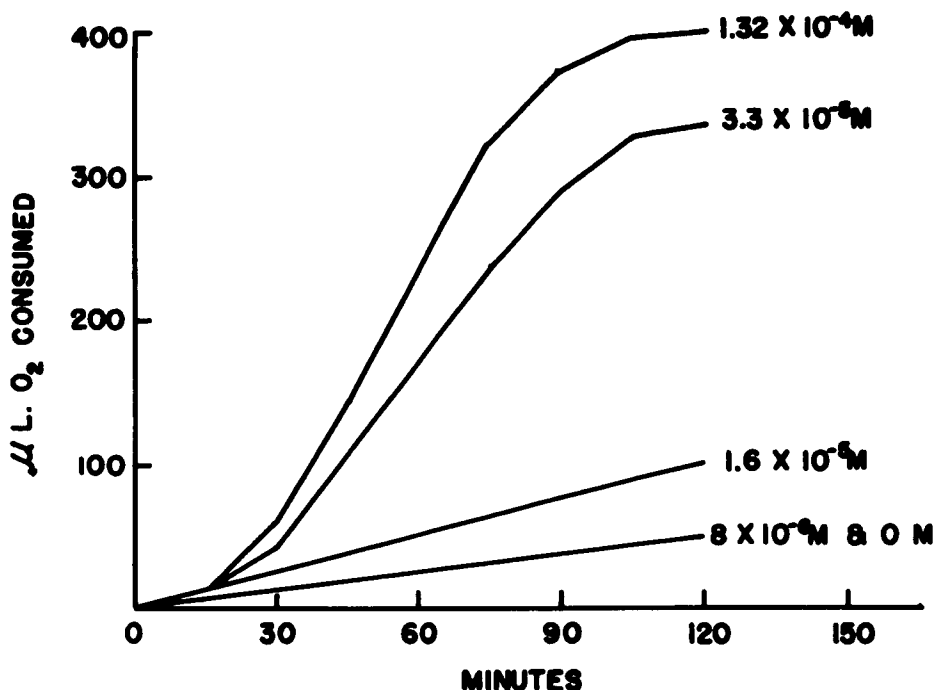


FIG.10- MAGNESIUM CONCENTRATIONS AND THE RATE OF MALIC ACID OXIDATION.

Reaction mixture and conditions same as for Fig. 7 except malic acid was used as substrate in place of α -ketoglutarate.

IV. DISCUSSION

The importance of magnesium to the chemical processes involved in intermediary metabolism has been reviewed by Ochoa (4). The essentiality and mode of participation of various ions in the growth of Azotobacter has been the subject of many investigations (see review by T. S. Kyle and A. Eisenstark (5)). In respiratory studies of the genus Azotobacter, Harris and Gainey (3) reported a stimulation of respiration by calcium at low pH values. Harris (6) and Karlsson and Barker (1) added calcium to the reaction mixture in studies of Azotobacter metabolism. However, Burris, et al., (7) demonstrated the oxidation of a large number of organic compounds in the absence of calcium, using washed cells of Azotobacter.

The effect of magnesium on the respiration of Azotobacter was studied by Harris and Gainey (3) using a basal salt medium containing approximately 5×10^{-4} molar $MgSO_4$. The addition of magnesium to this salt solution, already high in magnesium content, did not result in an increase in respiration. It was concluded from these observations that the Mg ion is not essential for the respiration of Azotobacter cells. It should be noted, however, that a 5×10^{-4} molar $MgSO_4$ concentration is sufficient to saturate many times over every oxidase system examined in the present study. Data presented here show that acetate grown cells, washed thoroughly in potassium-phosphate buffer, required magnesium for the oxidation of, and/or for the adjustment to, the oxidation of various organic compounds. Combinations of Mg with other cations present in the growth medium, at the same concentrations as found in the medium, were not observed to enhance the stimulation effected by magnesium alone.

The effect of magnesium on the respiration of washed Azotobacter cells is in sharp contrast to the effect of magnesium on the oxidative capacities of B. cereus (8). The addition of Mg, Ca, and Fe ions to cells grown at optimal and sub-optimal magnesium concentrations, with reference to cell division, did not effect the rate of oxidation of glucose by B. cereus cells.

The importance of magnesium for the oxidative process was also evident when tris-hydroxy-methylaminomethane buffer was substituted for the phosphate buffer in the washing process. The addition of Ca, Fe, and Mo ions, singly or in combination, did not influence significantly the oxidation of the substrate. However, the combination of any of the aforementioned ions with magnesium effected a considerable increase in the rate of oxidation. A still greater increase was achieved by the combination of potassium phosphate with magnesium. This activation has also been observed by Wilson and Stone (9), who found the combination of phosphate and magnesium necessary for optimal oxidative activity of dialyzed cell-free extracts of Azotobacter vinelandii.

Chelating agents, such as citrate or Versene*, provide yet another means of demonstrating the need for magnesium in the oxidative processes involving Azotobacter. Indications are that loss of oxidative capacity effected by these complexing agents can be restored by the addition of magnesium. Preliminary experiments have indicated that drastic treatment with certain cation exchangers may cause damage to Azotobacter cells which may become irreparable with magnesium.

*Bersworth Chemical Co., Farmington, Mass.

Glucose grown cells of Azotobacter agile A 44, when suspended in a potassium-phosphate buffer which contained a certain magnesium concentration, were capable of a rapid and immediate oxidation of acetate and glucose at a rate which was relatively constant for a number of hours. Such a pattern of oxidation may be contrasted to the response of glucose grown cells to substances such as α -ketoglutarate, succinate, or malate. Before glucose grown organisms could oxidize these acids at a constant rate, a lag or adaptation period was observed, in which the rate of oxidation increased until a constant rate was obtained.

From the standpoint of oxidation, glucose-grown Azotobacter agile A 44 may be said to be adapted to the oxidation of glucose and acetate and unadapted to the oxidation of α -ketoglutarate, succinate and malate. The effect of magnesium on the oxidation of substrates to which this organism is adapted revealed that, in phosphate buffer, magnesium is required for the oxidation of glucose, acetate, α -ketoglutarate, succinate and malate.

The reported findings could be of importance in the interpretations of the various processes of intermediary metabolism in the whole bacterial cell. As has been shown, the optimal concentration of magnesium for the oxidation of various substrates tested differed considerably. Cell suspensions containing varying amounts of magnesium could be obtained, which would permit the oxidation of acetate by glucose grown cells, but not the oxidation of succinate. Such artifacts, which may lead to fallacious conclusions concerning the normal metabolism of the cell, may be abolished by increasing the concentration of magnesium to optimal levels.

V. SUMMARY

A. The oxidative activities of Azotobacter agile A 44 and Azotobacter Q were greatly influenced by the washing procedure employed in preparing the cells for metabolic studies.

B. Ca, Fe and Mo ions, singly or in combination, were found to be ineffectual in stimulating succinate oxidation by Azotobacter cells grown on acetate and suspended in potassium-phosphate buffer without added magnesium. Addition of magnesium to acetate grown cells suspended in a solution of potassium phosphate resulted in the oxidation of succinate.

C. Using washed glucose grown cells it was shown that magnesium was essential for the oxidation of acetate and glucose, to which the cells were adapted. Magnesium was also essential to the adaptation or oxidation of α -ketoglutarate, succinate and malate to which substrates the cells were not adapted.

VI. RECOMMENDATIONS

Adaptive oxidative manifestations in Azotobacter have been found to be drastically affected by ultraviolet light. The nature of the changes in oxidative activity has not been determined. Mechanisms responsible for adaptations, or for changes in oxidative capabilities, may represent alterations in permeability, or the functioning of induced biosynthetic mechanisms. It is suggested, therefore, that a study be made of the cause of the change in the adaptive oxidative activities of this organism with the object of discovering the metabolic systems that may be involved in irradiation injury.

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