PHYSIOLOGICAL DIFFERENCES BETWEEN AZIDE RESISTANT AND AZIDE SUSCEPTIBLE VARIANTS OF *AZOTOBACTER AGILE*

By

CHARLES R. GOUCHER

Bachelor of Science

University of Oklahoma

Norman; Oklahoma

1951

Submitted to the Faculty of the Graduate School of

the Oklahoma Agricultural and Mechanical College

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

1951

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THESIS AND ABSTRACT APPROVED:

A. Eisenstark

Thesis Adviser

J. L. Johnson

Faculty Representative

W. L. Stolz

Dean of the Graduate School

ACKNOWLEDGMENT

I should like to thank Dr. A. Eisenstark for his indefatigable assistance throughout the course of this work. Thanks are also extended to the Chemistry Department for the use of their semi-micro-kjeldahl apparatus and for their many helpful criticisms.

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I. INTRODUCTION

The purpose of this investigation was first, to determine metabolic differences between two variants of Azotobacter agile, one of which is susceptible to poisoning by sodium azide and the other is resistant to such poisoning, and secondly, it was hoped that a knowledge of such differences might assist in elucidating the mechanism of azide inhibition of physiological processes. Also, the method used might yield information pertaining to the pathway of carbohydrate oxidation in Azotobacter.

Recent studies have placed emphasis upon biochemical mutants as tools for investigation of metabolic characteristics. The biochemical implications of genetic constitution are not entirely new, however, since they were realized quite early in the history of genetics. The relationship between the single gene and the simple biochemical character was demonstrated by Onslow (1909) by chemogenetic studies of flower color. She understood the genetic determinants which were responsible for differences in pigmentation, but during this period of her investigation knowledge of the chemical structure of the pigments with which she worked, apigenin and magenta anthocyanin, did not exist.

In 1931, Scott-Moncrieff, using chemical techniques of his day, was able to demonstrate the conversion of one anthocyanin into another by the action of a single dominant gene. During the same period Garrod (1923) made detailed studies of albinism, cystinuria, pentosuria, and alkaptonuria. He found that all of these conditions were inherited recessively, and he considered them to be inborn metabolic errors. He recognized the important biological implications that chemical differences considered inborn metabolic errors in one species may be normal in another species. Such a recognition was indeed a foundation for the biochemical orientation of genetics.

Since the time of these early observations, the biochemical aspect of

genetic differences has been investigated in many plants and animals, and especially in microorganisms, using the method of Beadle (1948). Assuming a one-to-one correspondence existing between gene and enzyme, Srb and Horowitz (1945) were able to show a pathway for the catalytic liberation of urea, using several mutants of the mold, Neurospora.

Thus, some knowledge has been gained of the nature of the aberration of cellular integrity, which is the locus of mutation and which represents the first difference between the physiology of the parent organism and the mutant organism.

As may be noted in the review of the literature which follows, investigators have considered azide inhibition to result from its reaction with one or more of the following metabolic entities:

1.) It may react with cytochrome oxidase and thus prevent the reduction of molecular oxygen and the formation of water.

2.) It may react with the iron of catalase and peroxidase preventing the destruction of the often toxic hydrogen peroxide.

3.) It may interfere with the synthesis of enzymes.

4.) It may inhibit the uptake of inorganic phosphorus.

5.) It may prevent the formation of adenosin-triphosphate.

In the present investigation, each of these categories was considered. However, the physiological machinery of Azotobacter is at the present time an unexplored subject, apart from a rather extensive investigation of the means by which this organism fixes atmospheric nitrogen. Therefore, in the hope of making the data which might evolve from such an investigation more intelligible, the glycolysis scheme and the tricarboxylic acid cycle of Kreb was assumed to exist in this organism. It was assumed also that the Azotobacter possess a cytochrome system or a system comparable to the Keilin-Warburg system of other

cells. In other words, the investigation was begun by assuming that the oxidative mechanism of Azotobacter was like that of muscle or yeast cells, although the data obtained at the beginning of the study places some question upon the tenability of such a hypothesis.

By consideration of the above possible modes of actions of azide using Azotobacter variants susceptible and resistant to the drug, it is hoped that some contribution is made to an understanding of

1.) The difference between azide sensitive and resistant organisms,

2.) The inhibitory mechanism of azide, and

3.) The mechanism of carbohydrate oxidation in Azotobacter.

II. REVIEW OF LITERATURE

This review is concerned with the effect of azide on living systems. It is presented with the view of describing the physiological areas in which azide has been shown to elicit certain well defined phenomena, as well as some that are rather poorly understood. At the present time the following biological effects are associated with azide:

a. Inhibition of catalase activity.

b. Inhibition of cytochrome oxidase.

c. Inhibition of synthesis of cellular units.

d. Inhibition of uptake of inorganic phosphorus.

e. Inhibition of the uptake of ammonia and biotin.

f. Inhibition of ATP synthesis.

g. An enzyme stabilizer.

h. Inhibition of hydrogenase.

a. Inhibition of catalase. In a study of the mechanism of the decomposition of hydrogen peroxide (Keilin, 1938), it was observed that azide inhibited catalase activity in concentrations as small as 10^{-3} molar. An investigation of this phenomenon revealed that azide reacted with catalase iron, preventing its reoxidation and hence its further reduction.

Even before this observation, Keilin (1936) had used azide as an inhibitor of yeast respiration. He found it especially effective at reactions between pH 5 and 7 and rather weakly inhibitory at more alkaline reactions, thus implying that the active element of azide inhibition is hydroazoic acid.

Keilin has also pointed out that yeast peroxidase is inhibited at low concentrations by this substance (Lardy, 1949). On the other hand, Stannard (1939) found no correlation between inhibition of respiration by azide and inhibition of catalase in frog muscle.

Lichstein and Soule (1944a), however, were able to design a medium bacteriostatic to gram negative organisms by the use of azide. This medium permitted the growth of most gram positive organisms.

These workers (1944b) found that most gram positive resistant organisms showed a very great catalase activity, as the gram positive Streptococci, or they showed no catalase activity, as the Clostridia, while the organisms most susceptible to the effect of azide were the gram negative bacteria, which were weak catalase producers.

Thus, it may be seen that the reports of effect of azide on catalase activity are encouraging enough to warrant further investigation.

b. Inhibition of cytochrome oxidase. This is another area affected by azide, as indicated by the following reports.

While working with systems capable of oxidizing and reducing cytochrome c, Keilin (1939) found that azide could be substituted for hydrogen cyanide as an inhibitor of cytochrome oxidase. Winzler (1943) asserted that azide was a more specific inhibitor of yeast respiration than was cyanide. He showed that azide reacted with and inhibited the function of the Atmungsferment in these organisms. It was later discovered (Stanley, et al., 1946) that while azide, like cyanide, did react with the oxidase of the cytochrome system of rat liver, the extent of the inhibition of these two poisons varied with the hydrogen donor, with the substance being oxidized.

Giese (1945) found that azide was capable at low concentrations of stimulating the respiration of luminescent bacteria and at high concentrations of inhibiting respiration, while at every concentration tested growth was inhibited.

c. Inhibition of synthesis of cellular components. Clifton (1937) studied the influence of azide on the oxidation of acetic and butyric acids by Pseudomonas calco-acetica. He found that two-thirds of the oxygen required for the

complete oxidation of a given quantity of these substrates was taken up by cells of this organism. It was assumed that the remaining unoxidized material was used in the synthesis of cellular material. But in the presence of sodium azide, the acetate and butyrate oxidation approached completion. It was demonstrated by Clifton and Logan (1938) that azide produced a similar effect in the metabolism of E. coli. In the present of azide, lactate, acetate, succinate and fumarate were oxidized more completely. Yeast oxidized acetate to completion in the presence of azide, and azide completely prevented assimilation from glucose (Winzler, 1944). Doudoroff (1940) reported that a number of sugars as well as lactate were utilized by Pseudomonas saccharophila. These substances were generally only two-thirds oxidized. However, in the presence of azide the oxygen consumed by the cells accounted for almost complete oxidation of these substances. Pickett and Clifton (1941) observed that azide affected an aerobic fermentation with the production of alcohol in such quantity as to account for thirty to forty percent of the substrate added, while under the same aerobic conditions in the absence of azide, only traces of alcohol could be found. It has also been noticed (Bernstein, 1944) that poisoning with azide does not affect assimilation and synthesis of four carbon di-carboxylic acids to the extent that it does sugars such as glucose.

Spiegelman (1947) could not show the formation of adaptive anzymes in yeast in anaerobic conditions in the presence of azide. Azide did not interfere with the fermentation of the substance for which adaptation was complete. From these considerations he asserted that the site of action of azide is obviously not the cytochrome system.

Tests were carried out for the production of yeast carbohydrate (Pickett and Clifton, 1943) from glucose in the presence of azide, but the production of carbohydrate was not observed.

Burris and Wilson (1942) experimented with the effect of azide on the assimilatory processes of the Rhizobia. They discovered that in this organism the effect of azide need not be attributed to an interference with assimilation. In their experiments azide was added to a suspension of Rhizobium trifolii after oxygen uptake had ceased and substrate was entirely assimilated from the medium, but with the addition of azide, oxygen was consumed in such a quantity as to account for almost the complete oxidation of the substrate added.

Thus, not only does azide exert an effect on a number of rather distinct systems, but its effects on cell syntheses are themselves rather diverse, differing with difference organisms and various substrates oxidized.

d. Inhibition of phosphorus uptake. In the hope of gaining some information on the metabolic site of azide action, Spiegelman, et al. (1948) undertook an investigation of azide inhibition of synthesis in yeast cells. They considered the yeast cells to be carrying on a glycolysis of glucose in the manner of muscle cells, and from this hypothesis it was inferred that each time a molecule of glucose was glycolized, two molecules of phosphate were used in the production of phosphate esters, and that this phosphate was eventually used in the synthesis of adenosintriphosphate. Using radioactive phosphorus, these workers found that azide inhibited the uptake of inorganic phosphorus while not affecting the rate of glucose fermentation, but as previously observed, inhibiting synthesis of cell materials from glucose. It was concluded that azide inhibited the ability of the cell to esterify inorganic phosphate, and that this inhibition occurred at concentrations which did not interfere with glucose oxidation or cellular respiration.

e. Inhibition of ammonia and biotin uptake. The rate of fermentation of yeast has been observed to increase in the presence of ammonia and biotin. However, the low concentrations of azide and cyanide (Winzler, et al., 1944), while not affecting the initial fermentation rate, prevent the increase in rate caused

by these substances.

f. Inhibition of ATP synthesis. Winston (1948) in a study of bacteriophage formation without bacterial cell division observed that azide prevented the division of bacterial cells, the formation of phage, and the synthesis of adenosin-triphosphate.

g. Inhibition of enzyme destruction. If glucose grown cells of Saccharomyces cerevisiae are exposed to galactose, this substance is oxidized after a short period of time in which a galactozymase system is synthesized. In the presence of azide and galactose this synthesis does not occur. If galactose adapted cells are exposed to maltose only, their galactose system is slowly lost with the development of a maltozymase system. If, however, the galactose adapted cells are exposed to maltose in the presence of azide there is no loss of galactozymase activity (Sussman et al., 1950) and no gain in maltozymase activity. The destruction of the galactozymase system is inhibited.

h. Inhibition of hydrogenase. It was observed (Wilson et al., 1943) that azide is one of the poisons affecting the hydrogenase system of Azotobacter vinelandi. However, a concentration range of azide which is definitely inhibitory to cell respiration has little effect on the hydrogenase activity of these cells.

Thus, it may be seen that sodium azide is not a specific inhibitor in that it affects one enzyme only, or one class of enzymes only. An inquiry into the effect of azide and the physiological conditions requisite to azide resistance must deal with aspects of organismic integrity ranging from the relatively simple inhibition of catalase to its more enigmatic effect on protein synthesis.

III. MATERIALS AND METHODS

The organisms used in this experiment were Azotobacter agile M.B.4.4 obtained from Professor C. B. van Neil, and a variant of this organism capable of growth in the presence of 10^{-3} molar azide. The mutant organism was isolated from a medium containing azide by Dr. Nancy Ziebur, and it was kindly donated by her for this study.

Respiration studies were done with the Warburg constant volume respirometer. Unless otherwise specified, the reaction flask contained 3.2 ml. of fluid with the following composition:

1. 1 ml. of substrate at such a concentration as to require 448 micro liters of oxygen for complete oxidation to carbon dioxide and water.
2. 1 ml. of M/15 phosphate buffer at pH 7.2 or 1 ml. of inhibitor brought to neutrality and dissolved in phosphate buffer.
3. 1 ml. of a standard cell suspension representing approximately 0.05 mgs. of nitrogen per flask.
4. 0.2 ml. of 10% KOH with filter paper adsorption wick.

Oxygen uptake was measured at 34-35 C. (Lineweaver, 1933). Cells were harvested at 24 hours of growth on a rotary type shaker, and were then washed three times in 100 ml. phosphate buffer, and re-suspended in the complete medium without carbon source. This suspension was then aerated for approximately 12 hours on the shaker until endogenous respiration was negligible.

The basal medium used in this investigation had the following composition:

1. K_2HPO_4 - - - - - - - - 0.1000 %
2. $MgSO_4$ - - - - - - - - 0.0200 %
3. $CaSO_4$ - - - - - - - - 0.0100 %

4. $FeSO_4$ - - - - - - - 0.0030 %

5. $NaMoO_4$ - - - - - - - 0.0002 %

6. Carbon Source - - - - 1.0000 %

The Thunberg technique for estimating dehydrogenase activity was used after the method described by Burris (1951). Reduction was followed by photometric means, and one hundred percent reduction was accomplished by the addition of sodium hydrosulfite.

Catalase activity was measured by the method of von Euler and Josephson (1927), at room temperature.

Growth was measured by turbidimetric means using a photon reflectometer.

Cell nitrogen was determined by the usual semi-micro Kjeldahl method.

Counts of radioactivity were determined by a thin-walled Geiger tube and scaler and a Berkeley decimal scaler. Cells were grown in media containing carbohydrate source and radioactive phosphorus for three hours, at room temperature, on a rotary shaker.

Each experiment in this investigation was repeated several times until it was felt that the data obtained represented values easily repeatable and precise determinations. Imperical data used in all graphs and tables which follow represent average experiments.

The azide resistant organism will be designated az-r, or mutant, and the azide susceptible az-s, or parent, in order to facilitate the reading of the material. However, this designation should not imply that one organism has arisen from the other. The genetic relationship of these organisms has not been determined.

IV. RESULTS

Experiment 1: The effect of azide on the oxidation, assimilation, and growth of parent and mutant cells when utilizing glucose. Azide, like cyanide, has been described as an inhibitor of cytochrome oxidase (Keilin, 1939). If such an enzyme exist in Azotobacter and were the site of the inhibition of cell multiplication, it may be supposed that a resistant variant possesses an alternate pathway for the reduction of molecular oxygen, or that the resistant form possesses an azide insensitive cytochrome oxidase. If azide were the inhibitor of a terminal oxidase, increasing concentrations of this poison should produce an increasing inhibition of substrate oxidation, regardless of the substrate being oxidized, and this inhibition should be reflected in a corresponding decrease in cell growth.

A comparison of figures 1 and 2 will reveal that such relationships do not hold true for azide inhibition of Azotobacter. Azide concentrations from 1×10^{-3} to 3×10^{-3}M affect the rate of glucose oxidation only slightly, but they drastically affect the growth of both az-s and az-r organisms. It is observed that the effect of azide on the rate of oxidation and on growth is not great for the az-r as it is for the az-s. At 1×10^{-3} M azide, growth of the az-s on glucose is prevented and growth of the az-r is reduced to approximately one half normal.

Clifton (1937) reported that one effect of azide was the uncoupling of cell synthesis from the oxidative energy-yielding metabolic pathway of the cell. Under the influence of azide, cells which might normally oxidize only half of the carbon of the glucose molecule, using the other half for cell synthesis, would oxidize the entire molecule to carbon dioxide and water. In the Warburg apparatus, such an oxidation to completion is represented by an increase in total oxygen consumption per molecule of substrate assimilated, with or without a change in the rate at which oxidation is accomplished.

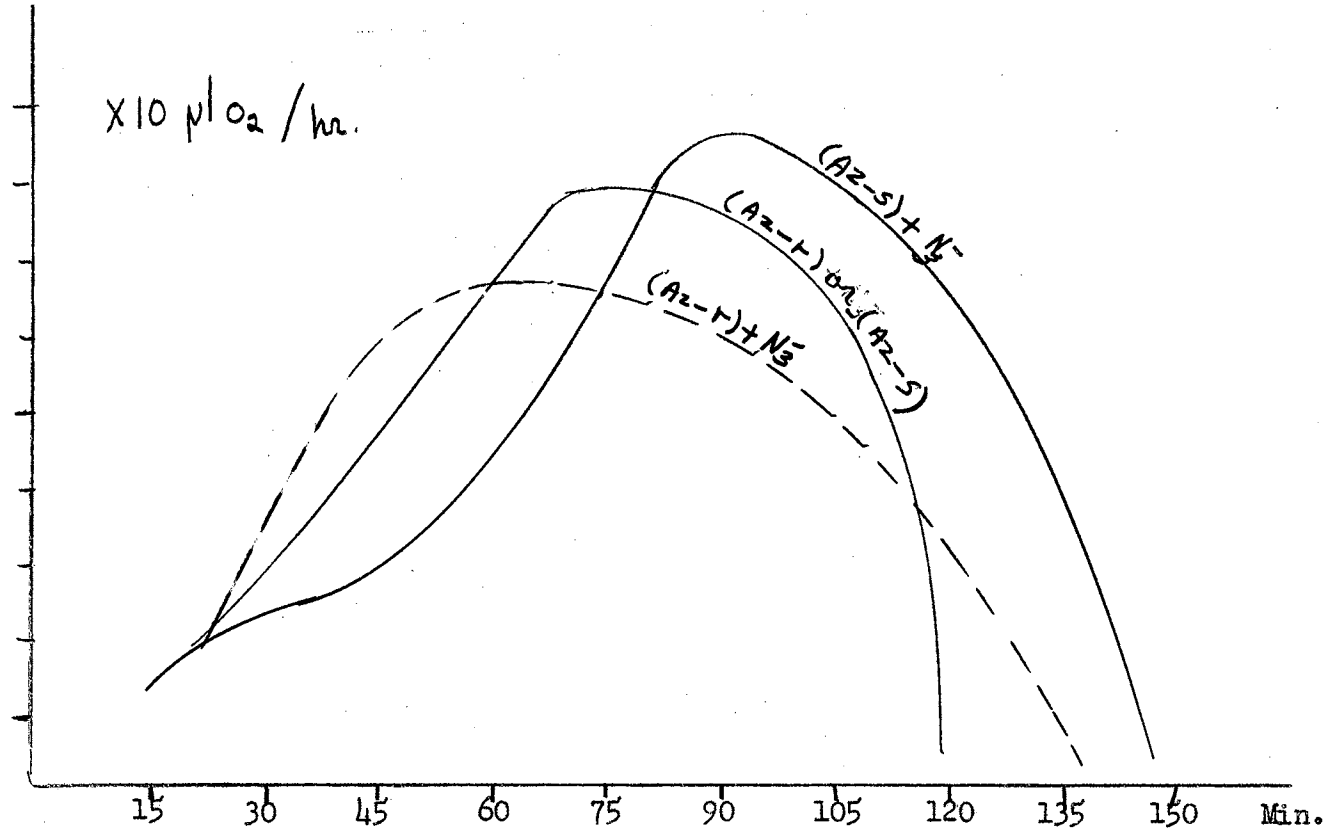

Fig. 1. Respiration curve for glucose oxidation conc. NaN_3 is 3×10^{-3} molar.

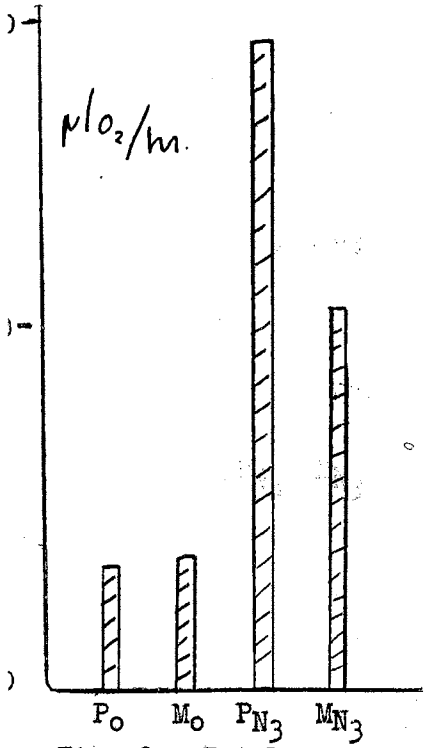

Fig. 3. Total oxygen used in glucose oxidation.

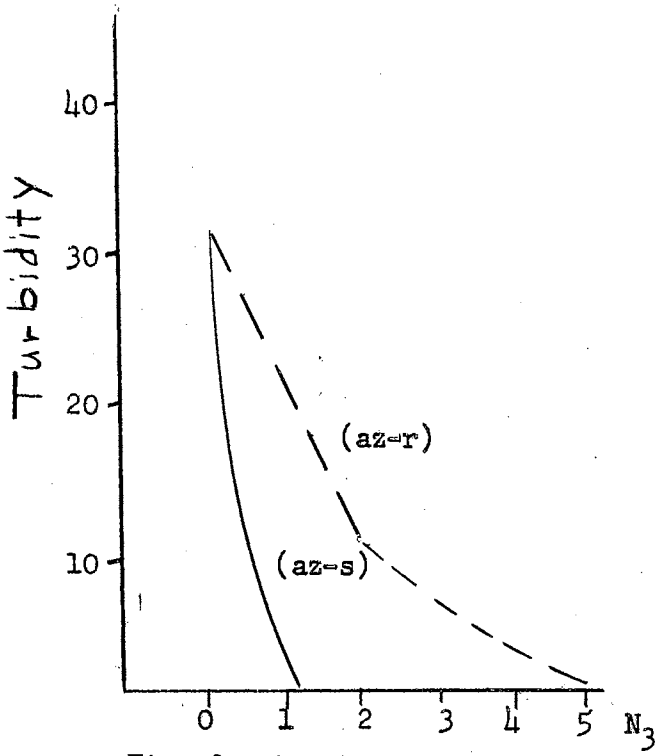

Fig. 2. Growth determination on glucose conc. of NaN_3 is 0, 1, 2, 3, 4, 5, $\times 10^{-3}$ molar.

Reference to figure 3 will reveal the same process operative in Azotobacter oxidation of glucose as it is affected by 2×10^{-3} and 3×10^{-3} molar azide. However, the total uptake of oxygen in the presence of azide is less with the az-r than with the az-s, indicating that the synthetic processes of the az-r are less affected by this poison than are those of the az-s organism.

A more complete oxidation of substrate by az-s and az-r occurs in the presence of azide, but the fraction of substrate oxidized by az-s is larger than that oxidized by the az-r in the presence of the same azide concentration.

This fact, that the az-s strain oxidizes a larger fraction of substrate, may partly account for the sensitivity of az-s and the resistance of az-r.

Experiment 2: The effect of azide on the oxidation, assimilation, and growth of parent and mutant cells when utilizing sodium acetate and sodium pyruvate. In yeast and in muscle cells, as well as in some bacteria, the formation of water as a neutral end product accounts for a majority of the hydrogen liberated by the oxidative processes of the cell, and most of this water results from the reduction of molecular oxygen by the terminal oxidase of the cytochrome system. Regardless of the compound from which the oxygen originated, it is eventually transferred by some co-enzyme, such as triphosphopyridine nucleotide (TPN), or some flavin carrier to the cytochromes, and at that instant the enzymatic stage is set for the formation of water.

Inhibition of cytochrome oxidase results in the inhibition of the many dehydrogenases which feed into the carriers supplying the cytochrome system, and the metabolism of the cell is retarded, regardless of the nature of the substance being oxidized, fatty acid or carbohydrate.

It was of some interest, therefore, to discover whether or not the respiration and synthesis of the az-r organism and the az-s organism suffered in-

hibition in the presence of azide when oxidizing various classes of compounds, and to ascertain the effects of various concentrations of azide on the oxidative and synthetic processes of the cell when pyruvate and acetate were utilized. The former, a product of glycolysis, occurs after the last known phosphorylation step, and the latter lies outside the glycolysis scheme.

Data expressed in figures 4, 5, and 6 are derived from experiments in which acetate was used as the oxidizable substrate.

It is observed that while azide inhibits respiration considerably for a short time (figure 4), it does not cause the oxidation of acetate to approach completion, (figure 6). Both az-s and az-r, after exposure to azide and acetate for several hours, are able to affect an adjustment of such a nature that the cells oxidize acetate in the presence of azide at a rate very close to that of the control, which is without azide. This may explain the apparent inhibition of growth of az-r organism on acetate in the presence of azide.

Figure 5 pictures the relative growth rate of these organisms on acetate and acetate-azide media. It is seen that resistance is characteristic of the az-r even with the oxidation of acetate.

Figures 7, 8, and 9 show the effect of azide on the oxidation, growth, and assimilation of sodium pyruvate. In contrast to acetate (figure 7), the rate of oxidation is only very slightly affected by concentrations of azide as high as 0.01 molar, and in no instance was the extent of pyruvate oxidation (figure 9) observed to exceed that of the control which was without azide.

No significant difference could be observed between az-s and az-r organisms when acetate or pyruvate were tested in the respirometer. However, the az-r organism proved to grow more luxuriently in the presence of azide than was possible for the parent (figures 5 and 8).

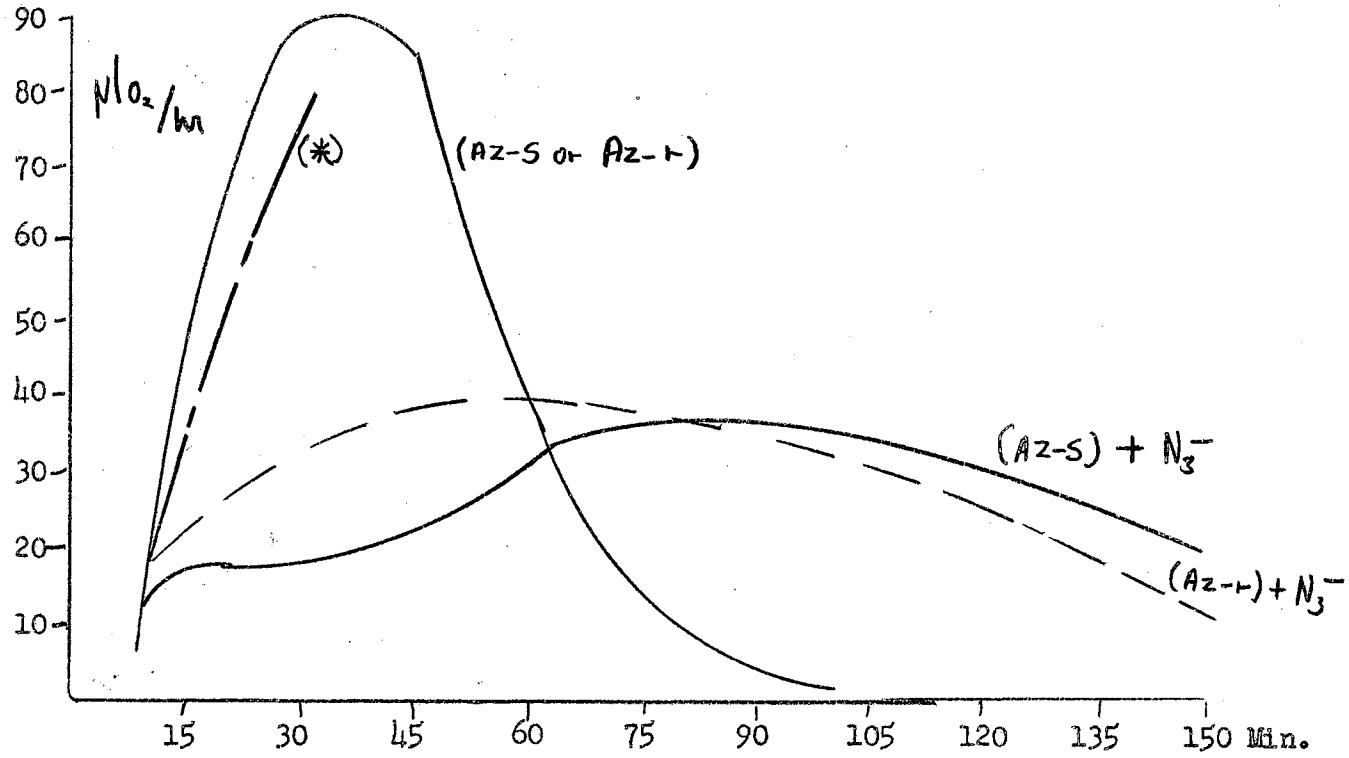

Fig. 4. Respiration for acetate oxidation. NaN_3 conc. is 6×10^{-3} molar.
(*)Acetate grown cells on 6×10^{-3} molar NaN_3

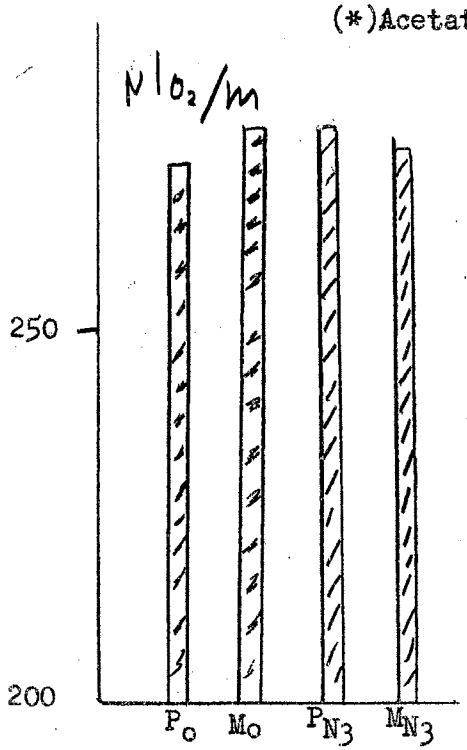

Fig. 6. Total oxygen consumed in acetate oxidation.

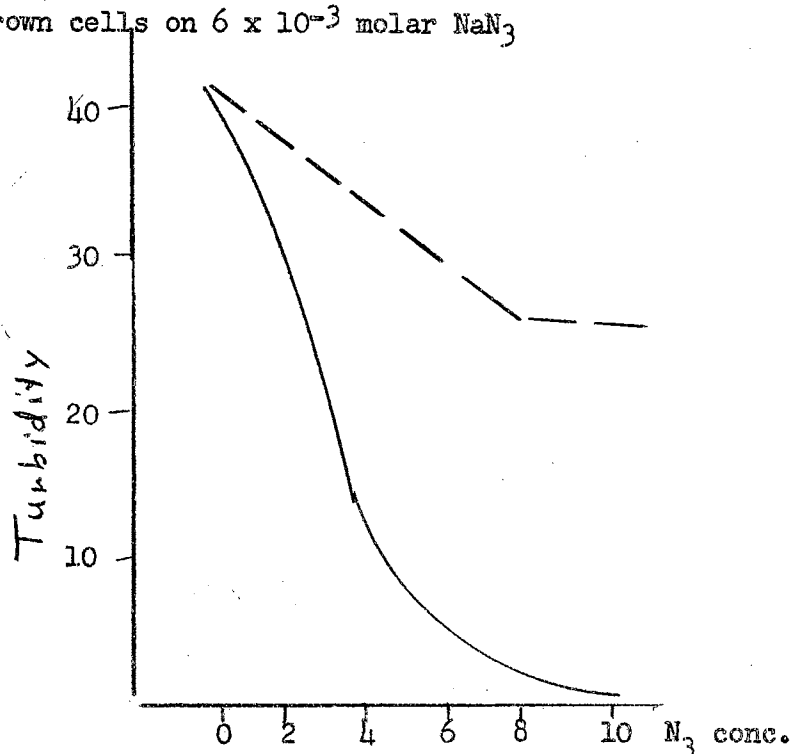

Fig. 5. Growth determination on acetate NaN_3 conc. 0, 2, 4, 6, 8, 10, $\times 10^{-3}$ molar.

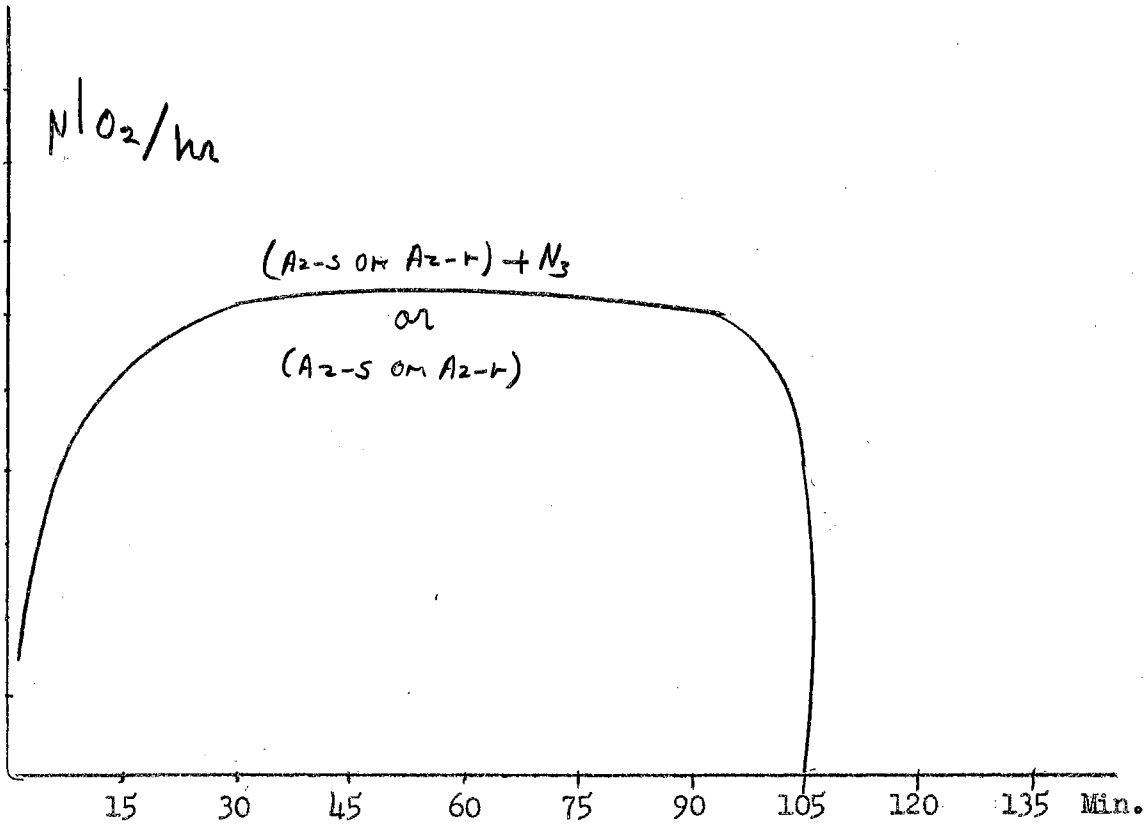

Fig. 7. Respiration curve for pyruvate oxidation. NaN_3 conc. is 1 x 10^{-2} molar.

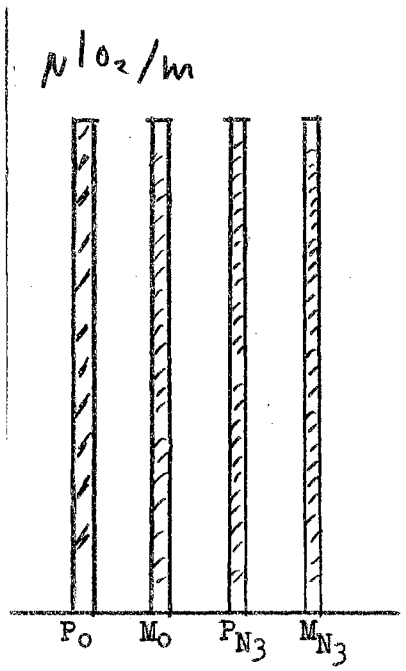

Fig. 9. Total oxygen consumed in pyruvate oxidation.

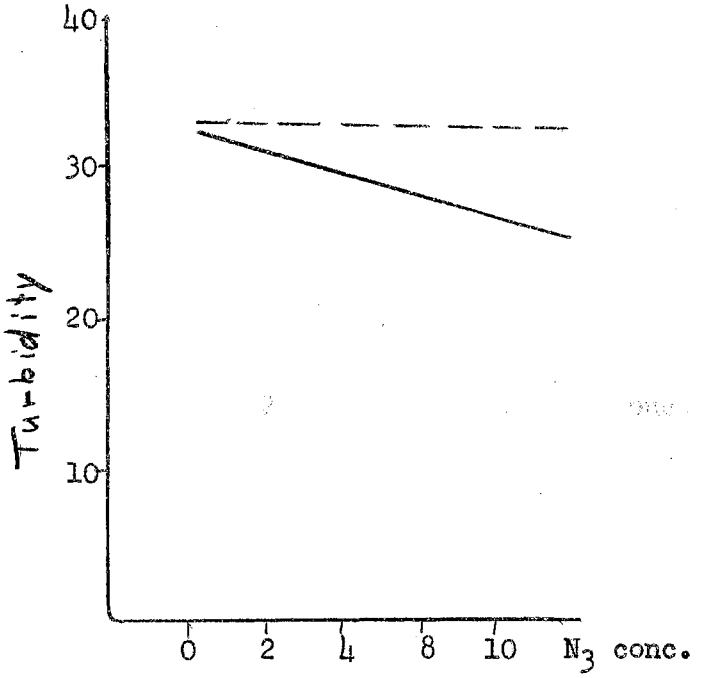

Fig. 8. Growth determination on pyruvate. NaN_3 conc. = 0, 2, 4, 6, 8, 10

From a consideration of the growth curves of these organisms, it is apparent that the locus of mutation in the az-r organism is responsible for an inhanced ability to grow in the presence of azide not only when oxidizing glucose but also with the oxidation of acetate and pyruvate. This suggests a common pathway for the assimilation of carbon fragments or production of energy used in synthesis, regardless of their origin, from glucose, pyruvate, or acetate. If such a common pathway did not exist, a distinct mutation would be implied for every compound utilized more readily in the presence of azide by the mutant than by the parent which is a rather unlikely circumstance.

Experiment 3: The effect of azide on the oxidation, assimilation, and growth of parent and mutant organisms when utilizing alphaketoglutarate, succinate, fumarate, and malate. As an extension of the endeavor to discover a compound or a group of compounds whose oxidation by the az-s and az-r cells followed a similar patter, some of the members of the tricarboxylic acid cycle were used.

Of the four compounds studied the oxidation of alphaketoglutarate and fumarate were most profoundly affected by the presence of azide, while the oxidation of malate was least affected by this inhibitor. In the studies of the effect of azide on the oxidation of these compounds the poison acted neither as an inhibitor of the rate of oxidation nor did it tend to affect an oxidation to completion (figure 12).

The inhibitor caused a considerable increase in the time required for adaptation to the new substrate. Such periods of adaptation in Azotobacter have been thought to occur during the formation of specific enzymes capable of acting on the substrate which elicited their production (Karlsson, 1948).

Reference to figure 10 will reveal the facility with which the az-r organism is able to make this adjustment as compared with the az-s, both in the

absence and in the presence of azide. If the time required for adaptation were a measure of the synthetic capacities of az-s and az-r for the production of enzymes, then the superior ability of the az-r in this regard is implied both from manometric and growth considerations.

The same pattern of adjustment and inhibition were observed with fumerate and alphaketoglutarate, but the delaying effect of azide was much greater in the case of these two compounds than for any others studied. A similar pattern of inhibition with a much abbreviated period of adjustment was encountered during the oxidation of malate.

From the data obtained in the study of the effect of azide on the assimilation of intermediate compounds, it is concluded that azide affects cell synthesis as manifested in the time required to produce an optional adjustment to substrates for which adaptation has not occurred.

However, the effect of azide on the oxidation of these intermediate compounds is not as great as its effect on glucose or fructose oxidation and assimilation. (Fig. 10, 11 and 12)

Experiment 4: The effect of sodium azide on the catalytic destruction of hydrogen peroxide by az-s and az-r organisms. Keilin (1938) reported that 10^{-3} molar azide completely inhibited the action of catalase. Lichstein and Soule (1944a) distinguished between three classes of bacteria differing in resistance to sodium azide, and differing also in catalase activity. Organisms with a strong catalase activity, as the gram positive streptococci showed a relatively great resistance to azide. Clostridia, with no catalase, are greatly resistant to azide. However, the gram negative bacteria, which are for the most part poor catalase producers, were the most susceptible to azide poisoning.

In this experiment, the quantity of hydrogen peroxide remaining after an hour exposure to the intra-cellular catalase of az-s and az-r organisms was

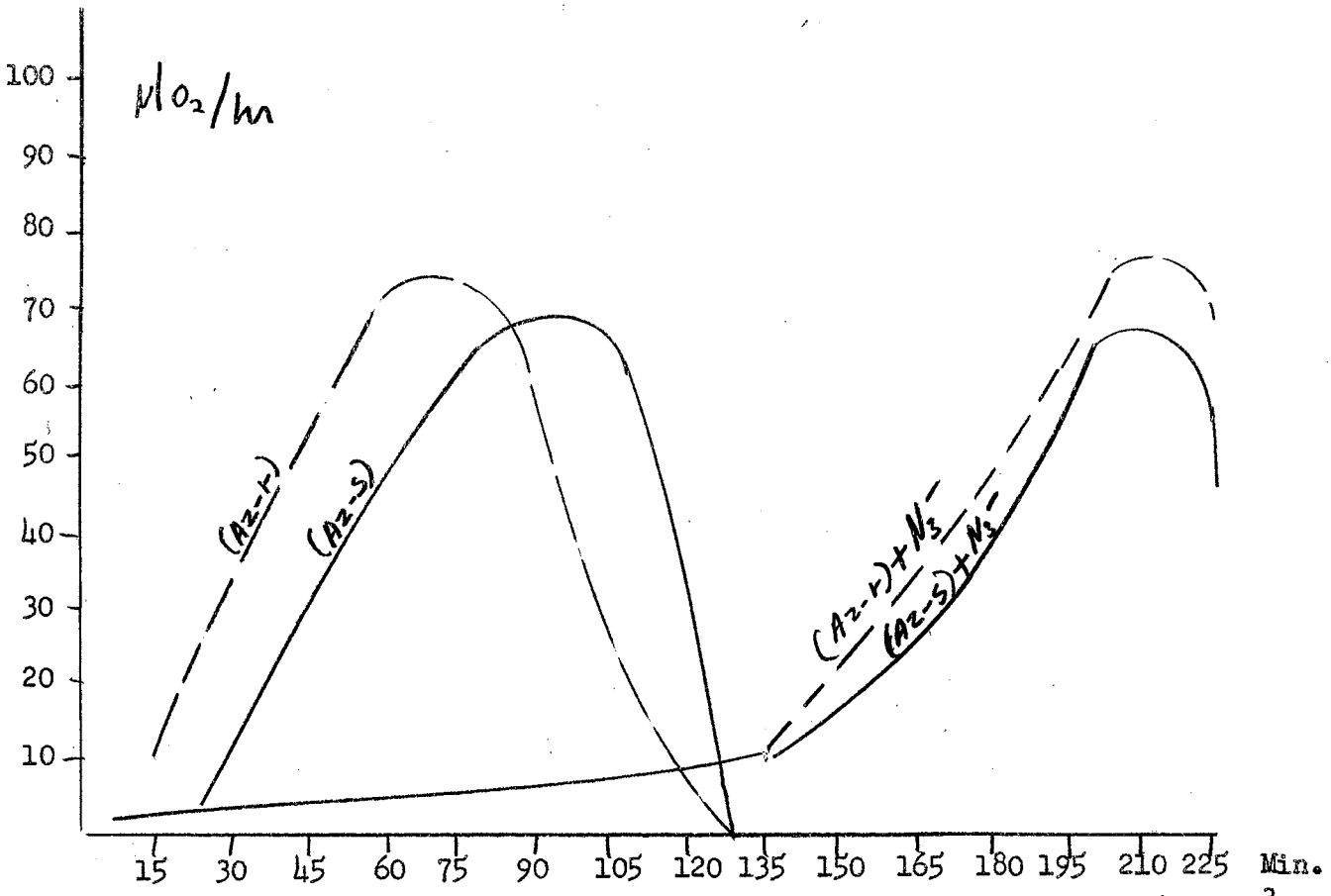

Fig. 10. Respiration curve for succinate oxidation. NaN_3 conc. = 6×10^{-3} molar.

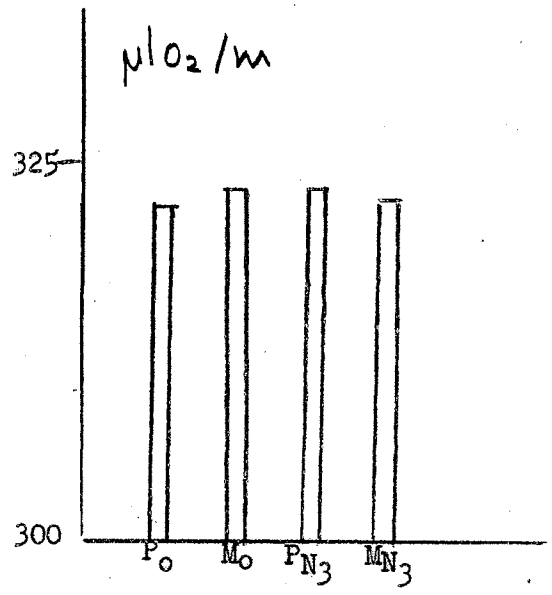

Fig. 12. Total oxygen consumed in succinate oxidation.

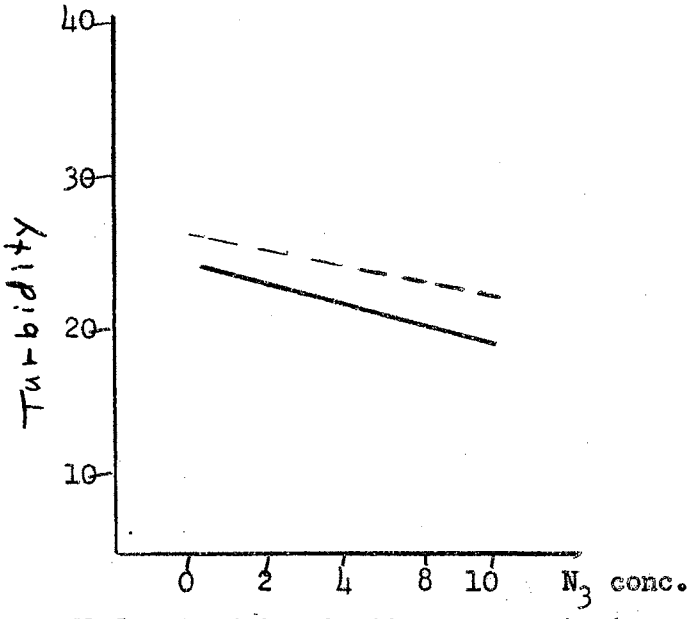

Fig. 11 Growth determination on succinate. NaN_3 conc. = 0, 2, 4, 6, 8, 10

determined by titration with 0.1 M potassium permanganate at room temperature, The results of this titration in the absence and in the presence of azide, with az-s and az-r organisms is summarized in figure 13.

It is apparent from this summary that azide at a concentration of only 10^{-3} molar entirely prevents the destruction of hydrogen peroxide. The az-s cells used in this experiment were glucose-grown while the az-r cells were both glucose-grown and glucose-azide-grown. While azide did not prevent the production of catalase in the az-r organism, its effect is indicated by the inability of az-r cells, glucose-azide-grown, to decompose hydrogen peroxide at the same rate as az-r cells or az-s cells grown on glucose only.

It has been observed repeatedly that both az-s and az-r organisms are capable of abundant growth in media which contains as high as 0.01 M sodium azide. The question naturally arises whether or not these media which sustain prolific cell growth maintain an azide residual after growth has commenced capable of inhibiting catalase activity.

To test some aspects of this problem, cells were grown on an acetate medium containing 5×10^{-3} molar azide for 36 hours. At the end of this time heavy suspensions of both organisms were obtained. These organisms were centrifuged out of the media and the media were passed through ultra-fine glass filters in order to remove any cells which may have remained after centrifugation. Using these filtrates, it was determined that the filtrates contained sufficient azide to inhibit completely the catalytic destruction of hydrogen peroxide; yet these filtrates permitted and maintained abundant cell formation (figure 14).

Controls were maintained in order to determine (1) the activity of the az-s and az-r cells, (2) determine the catalytic properties of the filtrates of az-s and az-r, (3) determine the non-catalytic destruction of hydrogen peroxide. These are summarized also in figure 14.

Organism	az-s	az-r	az-s	az-r	az-s	az-r
Ml of $KMnO_4$	6.3	6.3	29	29	29	29
NaN_3 conc.	None		1×10^{-3}M		2×10^{-3}M	

Fig. 13
All cells grown in absence of NaN_3.
(Ml. of $KMnO_4$ is that required to eliminate H_2O_2 remaining after action by cell catalase.)

Explanation for Figure 14 (See next page.):

Example No. 1. Control of the non-catalytic decomposition of hydrogen peroxide.

Example No. 2. Control of the effect of azide on hydrogen peroxide decomposition.

Example No. 3. Control of the catalytic activity of the filtrate containing azide in which az-s has been grown.

Example No. 4. Control of the catalytic activity of the filtrate containing azide in which az-r has been grown.

Example No. 5. Azide filtrate in which az-s has been grown. Inhibition of the catalase activity of this organism may be noted.

Example No. 6. Azide filtrate in which az-s has been grown. Inhibition of the catalase activity of az-r variant may be noted.

Example No. 7. Azide filtrate in which az-r has been grown. Inhibition of the catalase activity of az-s variant may be noted.

Example No. 8. Azide filtrate in which az-r has been grown. Inhibits the catalase activity of this organism.

Example No. 9. Hydrogen peroxide solution containing no azide and az-s cells grown in the absence of azide. Lack of inhibition of the catalase of az-s may be noted.

Example No.10. Hydrogen peroxide solution containing no azide and az-r cells grown in the absence of azide. Lack of inhibition of the catalase of az-r may be noted.

Example No. 11. Az-s cells which have been grown in the presence of azide. Hydrogen peroxide is not decomposed as rapidly as those grown in an azide free medium.

Example No. 12. Az-r cells grown in the presence of azide. Hydrogen peroxide is not decomposed as readily as those grown in the absence of azide.

(In this figure, 1 M $KMnO_4$ refers to the quantity of potassium permanganate required to decompose the hydrogen peroxide remaining after catalase activity.)

Ex. No.	Ml. of $KMnO_4$	Type Solution with H_2O_2 added	organ-ism
1	29.7	H_2O_2 only	none
2	29.2	H_2O_2 with NaN_3	none
3	29.2	NaN_3 Filt. of az-s	none
4	29.2	NaN_3 Filt. of az-r	none
5	29.3	NaN_3 Filt. of az-s	az-s
6	29.2	NaN_3 Filt. of az-s	az-r
7	29.2	NaN_3 Filt. of az-r	az-s
8	29.2	NaN_3 Filt. of az-r	az-r
9	6.3	H_2O_2 only	az-s
10	6.3	H_2O_2 only	az-r
11	12.9	H_2O_2 only	az-s *
12	12.5	H_2O_2 only	az-r *

(*) Cells grown in acetate-azide medium. Conc. of NaN_3 = 5×10^{-3} M.

Fig. 14. NaN_3 inhibition of catalase.

No difference could be observed between az-r and az-s organisms when both were grown on glucose alone, but when az-r was glucose-azide grown, its catalase activity was impaired in the absence of azide. It remains a question whether or not this decreased activity of the glucose-azide-grown az-r cell is a result of inhibition of catalase production or the effect of inhibitor not being removed from the cells even after repeated washings.

It was concluded from these observations that az-s and az-r cells can grow well in media which inhibits catalase activity and that such media does not lose its inhibitory quality even after bacterial growth.

Experiment 5: Inhibition of phosphate assimilation by azide. Preliminary runs were made to determine what effect the presence of azide had on the assimilation of phosphate by the two variants. Radioactive phosphate was used in the medium. The results were as follows:

	First Determination			
	Acetate		Glucose	
	az-s	az-r	az-s	az-r
No Azide	37,074	33,993	31,316	14,347
0.003 M Azide	25,937	29,893	23,414	21,598
	Second Determination			
No Azide	14,599	13,650	14,064	7,852
0.003 M Azide	9,037	12,966	9,965	8,816
	Third Determination			
No Azide	15,889	8,377	8,103	4,455
0.003 M Azide	7,692	7,039	6,191	6,142

The numbers refer to counts per minute of radioactive phosphorus assimilated by the cells from the medium.

The following conclusions may be made from this data:

1. Inhibition of phosphorus uptake by azide is less apparent in the az-r organism than in the az-s variant.
2. Neither phosphorus uptake nor oxidation of substrate are related in a simple one-to-one manner with growth and re-

production (figures 5 and 11).

Further work will be undertaken using radioactive phosphorus to attempt to discover the nature of the quantitative relationship between substrate oxidation and phosphate uptake, as these are functions of growth.

V. DISCUSSION

Of the variety of cellular phenomena described as associated with azide action, the data obtained in this investigation are explicitly related to the following:

1. Azide as an inhibitor of a termical oxidase and catalase.
2. Azide as an inhibitor of cell synthesis.
3. Azide as an inhibitor of phosphorus uptake.
4. Azide as an inhibitor of growth.
5. Azide as an inhibitor of dehydrogenase.

The inhibition of respiration affected by azide was assumed to result from its reaction with the components of two distinct physiological processes (Keilin, 1936). First it was observed that azide could inhibit catalase activity, and the supposition followed that inhibition of growth resulted from the toxicity of hydrogen peroxide. Later, it was shown that azide could replace cyanide in the inhibition of cytochrome oxidase. This observation led to the conclusion that azide inhibition of respiration probably resulted more specifically from the latter reaction than from the former, and it was pointed out that an inhibition of cytochrome oxidase would account for the immediate inhibition which is produced by the presence of azide.

In the experiments it is noted that the inhibition of the rate of oxidation is significant in one instance only, acetate oxidation, and with this compound the inhibition was not appreciable (figure 4) when cells grown on acetate were tested for azide inhibition. The absence of inhibition in the rate of oxidation (figures 1, 4, 7, 10) suggests that the drastic effect of azide on growth (figures 2, 5, 8, 11) must be accounted for in some way other than by assuming that azide inhibits a terminal oxidase such as cytochrome oxidase. Other evidence against inhibition of a terminal oxidase as the cause of the inhibition of growth is accrued from a comparison of figures 2 and 5

with figures 8 and 11. Here it is seen that the magnitude of inhibition of growth is a function not only of azide concentration but also of the type substrate undergoing oxidation. The rate of oxidation of compounds as diverse structurally as alphaketoglutarate, and glucose was affected in much the same way by the presence of azide. This similarity of inhibition pattern might imply the existence of a common carrier system associated with the dehydrogenases involved in the oxidation of these compounds, and it may imply also the resistance of that system to the azide concentrations used.

In these experiments it has been shown that azide does indeed inhibit catalase activity in the concentrations which were used throughout the undertaking. However, it cannot be stated that inhibition of catalase is the sole cause of the inhibition of growth in the az-s, or the retardation of growth in the az-r organism, because both organisms have been shown to grow abundantly in media which are capable of entirely preventing the catalytic destruction of hydrogen peroxide.

Inhibition of growth must be partially accounted for by the effect of azide in causing the oxidation of glucose to approach completion. The work of Clifton (1937) has shown such a phenomenon occurs in many organisms. However, the effect of a substrate being oxidized to a greater extent than normal, and assimilation and synthesis from that substrate being inhibited, has not been quantitatively correlated with growth experiments. Also, this investigation with Azotobacter has shown that the azr organism does not oxidize glucose to the same extent that the az-s organism does when both are under the influence of identical concentrations of azide. In other words, the synthetic mechanism of the az-r is not impaired by azide to the same extent as that of the az-s organism (figure 3).

The resistant synthetic mechanism of the az-r has not been demonstrated manometrically with the intermediate compounds in the same manner as it has

been shown with glucose and fructose oxidation. With the intermediate compounds, there was no apparent approach to an oxidation to completion in the presence or absence of azide, with either organism (figures 6, 9, and 12).

It is observed, however, that the oxidation of every substrate other than glucose and fructose (figure 1) requires a period of adaptation or cellular adjustment before oxidation begins (Karlsson et al., 1948). The mutant organism was able to adjust in the presence of azide more readily than was possible for the parent under similar circumstances. In other words, the maximum rate of oxidation of pyruvate (figure 7), acetate (figure 4) and succinate (figure 10) was attained in a shorter time in the presence of azide by the az-r than by the az-s, although the extent of oxidation by both organisms was virtually the same.

A consistent difference in az-s and az-r is manifested in the ability of the az-r to grow more readily in the presence of high concentrations of azide than the az-s organism. This ability of the az-r emphasizes the possibility of a greater synthetic capacity in the az-r than in the az-s, regardless of the substrate used for growth and energy.

Unless the resistance of the az-r organism is to be explained on the basis of a distinct mutation for resistance on glucose, and a different but distinct mutation for resistance on all the intermediate compounds tested, it appears reasonable that the "key" reaction responsible for synthesis in this organism is the reaction which mutation somehow enforced and made more adequate in its functioning. Such a mutation could hardly be considered a gene deletion. The ability of the mutant to survive, in the chemical environments studied, has been inhanced, not mitigated.

Spiegelman et al. (1948) was able to demonstrate that inorganic phosphorus is not taken up in the oxidation of glucose by yeast cells in the presence of suitable concentrations of azide. This work was performed with radioactive

phosphorus. This finding was in accord with Winston's (1948) notation that the effect of azide was to prevent the synthesis of ATP in several species of bacteria.

In this investigation of Azotobacter it is shown that cells oxidizing pyruvate or acetate or the acids of the Kreb cycle are at least ten times more resistant to the effects of azide than are the same cells when oxidizing glucose or fructose.

Spiegelman's investigation was limited to considerations of phosphorus uptake with the oxidation of glucose. It was found here that Azotobacter cells take up phosphorus in the presence of azide in a manner similar to their growth in the presence of azide. The az-r cells and the az-s cells give a radiation count of 7,852 and 14,064 respectively when grown in a medium containing only glucose and radioactive phosphorus, but in a medium containing glucose and azide and radioactive phosphorus the radiation count of az-s dropped to 9,965 while that of the az-4 was 8,816. Thus, it appears that azide inhibits phosphorus uptake and possibly the esterification of that phosphorus in the az-s variant.

If phosphorus uptake predicates growth on glucose then the same must be true for growth on the lower compounds. As an extension of the earlier work of Spiegelman, acetate was selected as a substrate for oxidation in the presence of azide and an acetate medium was prepared containing P^{32}. In this medium the radiation count of the az-s cells was found to be 14,599, that of the az-r was 13,650, but when the cells were permitted to metabolize in this medium but in the presence of azide the radiation count of the az-s dropped to 9,037 while that of the az-r dropped only to 12,966.

Hence it is suggested that phosphorus uptake and growth are reciprocal functions while the oxidation of substrate may and may not occur with cell synthesis. It is concluded that the az-r organism is not inhibited by azide

to the extent of the az-s in the uptake of phosphorus.

The effect of azide on the dehydrogenase activity of the cells followed the same patters of inhibition encountered in the oxygen consumption experiments. However, because of the system of testing dehydrogenase activity, the results are difficult to interpretate. Even if methylene blue reduction were a measure per se of dehydrogenase activity the method would not be a rewarding one in determining time required to attain a certain percentage reduction which could be validly compared with reduction times for a number of compounds. This difficulty arises from the perculiar adaptive response of Azotobacter (Karlsson et al., 1948). When these cells have been glucose-grown or acetate-grown, a period of adaptation before oxidation occurs is required for almost every compound capable of being oxidized by these organisms, and further the length of time required for adaptation is more or less specific for the substrate employed and also varies with the concentration of substrate, especially is this latter variation significant if fumarate or alphaketoglutarate are being oxidized. Thus the time required for reduction represents more than time required for the activation, the dehydrogenation, of some particular substrate. However, it will also represent the time required for cellular adjustment which is itself a function of a number of variables.

a. time for the dehydrogenation of substrate

b. time required for adaptation to substrate
   (1) function of substrate concentration
   (2) function of nature of substrate

c. time required for dehydrogenation influenced by azide

d. time required for adaptation influenced by azide

These are a few of the variables which must be determined in evaluating Thunberg data with this organism. The only conclusion apparently justified by the data obtained from Thunberg experiments on succinate, fumarate, and glu-

cose is that the pattern of inhibition, and the pattern of adaptation to these substrates as judged from methylene blue reduction is the same as that observed in the manometric determination.

With regard to azide affecting growth, assimilation and extent of substrate oxidation, there are two classes of compounds: (a) Compounds such as glucose and fructose, 10^{-3} M azide inhibits assimilation by oxidizing the compound to completion, (b) Compounds such as pyruvate, acetate, succinate and fumurate, in which neither growth nor oxidation are materially affected by azide.

However, if adaptation time is considered, then three classes of compounds emerge: (a) Compounds such as glucose which are discussed above, (b) Compounds such as acetate and pyruvate with which oxidation is immediate in the presence of azide, and (c) Compounds such as succinate and fumurate in which the rate of oxidation and the extent of oxidation are not affected by azide when a period of adaptation to these compounds has ensued. Azide affects only the period of adaptation.

VI. SUMMARY

1. The rate and extent of glucose oxidation by az-s and az-r variants of Azotobacter agile in the absence of azide is essentially the same, but in the presence of azide glucose is more completely oxidized by az-s variant than by az-r variant, although the extent of oxidation is greater in the presence of azide for both organisms. This effect of azide on oxidation is thought to be partly responsible for its inhibitory effect on growth in both organisms. The greater resistance of the az-r to this effect of azide on glucose oxidation may be, in part, the reason that this variant can grow in the presence of the drug.

2. The oxidation of acetate and pyruvate is not more extensive in the presence than in the absence of azide. Both substrates were found to sustain growth of the variants at azide concentration which were entirely bacteriostatic to these organisms when glucose was used as the only carbon source. However, the az-r and az-s variants did suffer an inhibition of growth on all azide concentrations tested while metabolizing these acids, but the inhibition of growth of az-r was never found to be as great as the inhibition of growth of az-s. Because respiration and growth is less affected than the same processes when glucose is used as a substrate, it has been inferred that azide does not inhibit growth by inhibiting some terminal oxidase, which is the common pathway for the liberation of hydrogen from any substrate, such as cytochrome oxidase in yeast or muscle cells.

3. The rate of oxidation of alphaketoglutarate, succinate, fumarate, and malate are not appreciably affected by azide, nor are these compounds oxidized to a greater extent in the presence of azide. However, with azide, the adaptation time of glucose-grown cells was significantly lengthened by this poison. However, the period of adaptation in the mutant was not extended as long as that of the parent organism, nor was growth of the az-r inhibited to as

great an extent by azide as that of az-s. This implies that az-r is able to synthesize its adaptive enzyme system more readily in the presence of azide than is the az-s variant. Such a rapid synthesis would result in cell division beginning in the az-r before division began in az-s, hence accounting for the more abundant growth of az-r on these substrates in the presence of azide. If the effect of azide on the variants, when oxidizing these intermediates, were to lengthen the period of adaptation without affecting the extent of substrate oxidation, then the superior growth of az-r on azide-media could be reconciled with the lack of inhibition of assimilation of substrate.

4. It was pointed out that azide inhibits uptake of inorganic phosphorus when glucose and acetate are oxidized, and it was also shown that phosphorus uptake is less inhibited in az-r than in az-s.

5. Azide was shown to inhibit the action of catalase in these organisms and it was pointed out that such an inhibition of catalase did not account for the inhibition of growth on glucose because cells were found to grow, using other carbon sources, in media which inhibited catalase activity entirely.

6. The limitations of the Thunberg method in following dehydrogenation reactions was also discussed, and it was shown that azide inhibition of methylene blue reduction followed the same pattern as azide inhibition of oxygen uptake.

Therefore, it may be stated that the differences between az-s and az-r variants of Azotobacter agile may be associated with (1) quicker adaptation in az-r in the presence of azide; (2) in the presence of azide, az-r tends to remain normal, whereas az-s tends to use all of the substrate for oxidation, leaving little to be used for cell multiplication processes; (3) uptake of phosphorus is inhibited less by azide in az-r than in az-s. When azide poisons the az-s variant of Azotobacter agile, it does so by interfering with uptake of phosphorus and therefore, possibly, the synthesis of cellular com-

ponents. Azide does not prevent oxidation by inhibiting some terminal oxidase.

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TYPIST PAGE

THESIS TITLE: PHYSIOLOGICAL DIFFERENCES BETWEEN AZIDE RESISTANT AND AZIDE SUSCEPTIBLE VARIANTS OF AZOTOBACTER AGILE

NAME OF AUTHOR: CHARLES R. GOUCHER

THESIS ADVISER: DR. A. EISENSTARK

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