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The Effects of Arsenical Compounds on Growth and Enzymes of Klebsiella Pneumoniae

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THE EFFECTS OF ARSENICAL COMPOUNDS ON GROWTH AND ENZYMES OF KLEBSIELLA PNEUMONIAE

BY

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1967

THE EFFECTS OF ARSENICAL COMPOUNDS ON GROWTH AND ENZYMES OF KLEBSIELLA PNEUMONIAE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Head, Bacteriology Department

TABLE OF CONTENTS

	Page
INTRODUCTION.	1
LITERATURE REVIEW	2
Arsenical Chemistry.	2
Reactions of Arsenicals with Thiols.	4
Effect of Arsenicals on Proteins	7
Metabolism of Arsenicals	9
Inhibition of Growth and Enzymes by Arsenicals	10
MATERIALS AND METHODS	13
Organism	13
Growth Study	13
Enzyme Studies	14
Chromatography	17
RESULTS	20
Growth Response to the Presence of the Arsenicals. . .	20
Effect of the Arsenicals on the Enzymes.	20
Chromatographic Evidence for Partial Tricarboxylic Acid Cycle	22
DISCUSSION.	34
Inhibition of Growth	34
Inhibition of Enzyme Activity.	35
Products of Enzymatic Activity	37
SUMMARY	39

Page

CONCLUSIONS 41

LITERATURE CITED. 42

LIST OF TABLES

Table		Page
1.	Aerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide Measured in Klett-Summerson Units.	23
2.	Anaerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid Measured in Klett-Summerson Units	24
3.	Anaerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsine Oxide Measured in Klett-Summerson Units	24
4.	Relationship of Klett-Summerson Units to Number of Cells per ml	25
5.	Microliters of Oxygen Uptake in the Oxidation of Pyruvate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.	27
6.	Microliters of Oxygen Uptake in Oxidation of Succinate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide	27
7.	Microliters of Oxygen Uptake in Oxidation of Fumarate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide	28
8.	Microliters of Oxygen Uptake in Oxidation of Malic per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide	28
9.	Microliters of Oxygen Uptake in Oxidation of Citrate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide	29
10.	Microliters of Oxygen Uptake in Oxidation of α-ketoglutarate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.	29

Table | Page

11. Products of the Oxidation of the Tricarboxylic Acid Cycle Intermediates by the Cell Free Enzymes . . . 33

LIST OF FIGURES

Figure		Page
1.	Structures of Organic Arsenicals.	3
2.	Standard Curve for Protein Determination in Milligrams Protein per ml in Relation to Absorbance.	26
3.	Oxygen Uptake in the Oxidation of Pyruvate, Succinate, Fumarate, and Malate	30
4.	Oxygen Uptake in the Oxidation of Citrate and α-Ketoglutarate in the Presence of 3-NO_2-4-OH Phenylarsine Oxide	31
5.	Oxygen Uptake in the Oxidation of Citrate in the Presence of Increasing Concentrations of 3-NO_2-4-OH Phenylarsine Oxide	32
6.	Oxygen Uptake in the Oxidation of α-Ketoglutarate in the Presence of Increasing Concentrations of 3-NO_2-4-OH Phenylarsine Oxide	32

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INTRODUCTION

For many years people have equated arsenic or compounds containing arsenic with poison. Inorganic arsenic compounds are used as chemotherapeutic agents as well as poisons. Organic arsenicals were introduced at the start of this century launching modern chemotherapy. The introduction of antibiotics in the 1940's virtually replaced organic arsenicals in chemotherapy. Solutions of inorganic arsenic compounds are used as tonics for animals and to finish animals for show.

Because arsenicals are excreted by animals in much the same structure as they are consumed and arsenite prevents both putrefaction and growth, it is possible the decomposition of animal excreta will be inhibited. The decomposition of animal wastes is of interest to this laboratory and to the nation. Thus, this study was undertaken to determine if growth could be inhibited by either the feed additive 3-nitro-4-hydroxyphenylarsonic acid or 3-nitro-4-hydroxyphenylarsine oxide. Enzyme investigations were made to determine whether these two arsenicals inhibited enzymes of the tricarboxylic acid cycle.

LITERATURE REVIEW

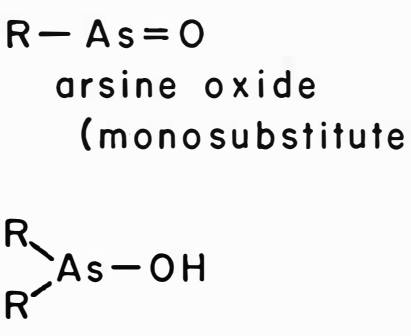
Arsenical Chemistry

In the biological field many different arsenicals have been used. There are many classes of arsenicals but this discussion will be limited to the trivalent and pentavalent organic arsenicals (Figure 1). In these trivalent compounds, the arsenic is bound to either a substituted or unsubstituted benzoid group and oxygen. In the pentavalent compounds, the arsenic is bound to either a substituted or unsubstituted benzoid group and four atoms of oxygen.

Arsenoxides may or may not be hydrated when in aqueous solution (Webb, 1966). R-As=O is the form that is generally accepted for arsenoxides in solution, but the form $R\text{-}AsO(OH)_2$ may be the dominant form. Phenylarsenoxides as well as phenylarsenolates are quite stable in solution. The form $R\text{-}AsO(OH)_2$ is the structure that is accepted for the phenylarsonic acids in solution.

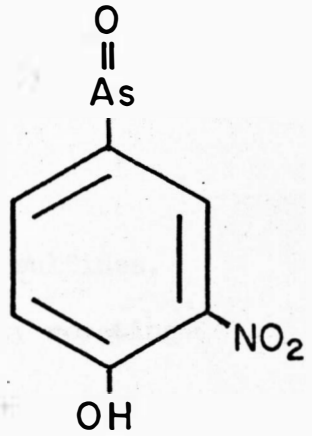
The structures of the arsenicals can be related to their activity against protozoans and to toxicity for man. Inorganic arsenite, which is considered to be a potent poison, is almost "inert" when compared to the relative toxicity of the trivalent organic arsenical (Johnstone, 1963). Parasiticidal activity and human toxicity of the arsenical is generally reduced with the substitution of the aromatic ring of phenylarsenoxide. Eagle and Doak (1951) classified the substitutent groups in relation to their relative parasiticidal activity. One of these groups was the inert group, which includes both nitro and hydroxy groups. These substitutent

Trivalent Organic Arsenicals



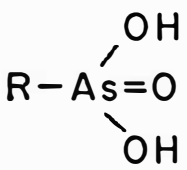
arsine oxide
(monosubstituted)

-arsinous acid
(disubstituted)

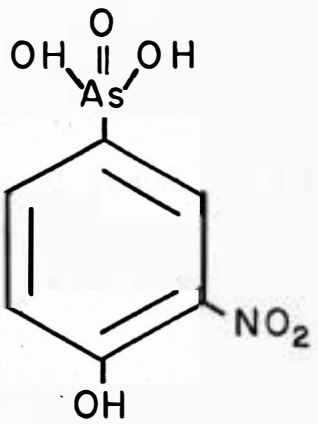


3-Nitro-4-hydroxy-
phenylarsine oxide

Pentavalent Organic Arsenicals



arsonic acid



R = benzoid group may or
may not be substituted

3-Nitro-4-hydroxy-
phenylarsonic acid

Figure I. Structures of Organic Arsenicals

groups will usually only slightly reduce the activity and toxicity of phenylarsenoxide.

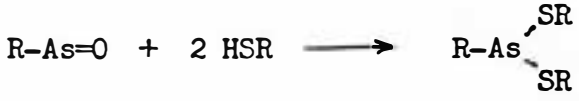
The "active" form of organic arsenicals is considered to be the trivalent form. Thus, the pentavalent form of the arsenical must first be reduced before it will be active.

Reactions of Arsenicals with Thiols

It is known that inorganic arsenic reacts with sulfides. Johnson and Voegtlin (1930) obtained a thioarsenite by reacting arsenious chloride and cysteine.

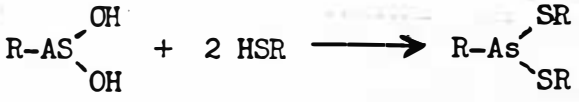
Little is known about the mechanisms of the reaction of arsenicals with thiols. The general overall reaction for the combination of sulphydryl groups with arsenicals is given in Reaction I (Johnstone, 1963).

Reaction I



Webb (1966) showed the reaction of sulphydryl groups and the hydrated form of the arsenical oxide (Reaction II).

Reaction II



He has also suggested that perhaps it is a progressive addition and substitution reaction as shown in Reaction III. However, he questioned whether the monothioarsenite actually exists or if the simultaneous replacement of the oxygen atom occurs as shown in Reaction I.

Reaction III

$$R\text{-}As{=}O + HSR \longrightarrow R\text{-}As\begin{matrix}OH\\OH\end{matrix} + HSR \longrightarrow R\text{-}As\begin{matrix}SR\\SR\end{matrix}$$

Webb (1966) discussed whether or not monosubstituted or disubstituted trivalent arsenicals react with different thiol groupings. The monosubstituted arsenical probably reacts with vicinal adjacent sulphydryl groups so that a five membered ring is formed, while disubstituted trivalent arsenicals bind with the monothiols.

The compound 2-3-dimercaptopropanol, more commonly known as British-Anti-Lewisite (BAL), has vicinal sulphydryl groups. Organic trivalent compounds will react with these groups. The combining of the arsenical to BAL will cause it to be inactive as a sulphydryl reagent. BAL can thus suppress the inhibition of enzymes by arsenicals.

Although there has been little work on the binding of arsenicals to proteins, Rosenthal (1932) determined indirectly that arsenicals probably react with proteins. He added oxophenarsine to serum proteins and found, by dialyzing the protein and arsenical mixture, that only the denatured proteins had bound appreciable amounts of the arsenical. This result was interpreted to mean that the denaturation of proteins released sulphydryl groups. Webb (1966) stated that the reaction of an arsenical with a sulphydryl group of a protein could be sterically impossible or prevented due to the electric fields about the arsenical as well as the sulphydryl groups. It has not yet been determined if there must be vicinal sulphydryl groups to insure the binding of primary trivalent arsenicals.

Lotspeich and Peters (1951) established the first criterion for characterizing enzymes as having either single or vicinal sulphydryl groups. Enzymes with vicinal sulphydryl groups are more sensitive to phenylarsenoxides, while those with single groups are more sensitive to diphenylcholorarsine. The reactions would be similar to the Reactions I, II, III.

As Webb (1966) pointed out this criterion of sensitivity could give an interpretation of the results that would be misleading. The inhibition may be due to the electrostatic forces or to hydrogen bonding caused by the arsenical and not by the binding of a sulphydryl groups in the active center, but for other reasons inhibition would not be observed. Possibly there is steric hindrance. It should also be possible for two disubstituted arsenicals to react with vicinal sulphydryl groups.

Lotspeich and Peters (1951) found that α-ketoglutarate oxidase of the rat liver was eleven times more sensitive to phenylarsenoxide than was isocitrate dehydrogenase and pyruvate oxidase was forty times more sensitive to phenylarsenoxide. Aldridge and Cremer (1955) found isocitrate dehydrogenase was more sensitive to diphenylchloroarsine than phenylarsenoxide. It was suggested that isocitrate dehydrogenase has a single sulphydryl groups near or at the active center, while the α-keto oxidases are dependent on vicinal sulphydryl groups.

The active form of the arsenicals is the trivalent form. Pentavalent arsenicals do not react with sulphydryl groups and therefore

have little effect on enzymatic activity (Gordon and Quastel, 1948; Mamelak and Quastel, 1953). It is likely if any inhibition is observed it may be attributed to trivalent impurities or to reduction by enzymes. Likewise, to be effective as chemotherapeutic agents, pentavalent forms must be reduced to the trivalent form in the body. The arsenoxides are enzyme inhibitors because of their ability to combine with sulphydryl groups.

Effect of Arsenicals on Proteins

Because early investigators studied enzymes that were not sensitive to the arsenicals which were used, the mechanism of their inhibition was not known for many years (Webb, 1966). The first evidence of changes in metabolism by the trivalent arsenical arsenite was shown by Krebs (1933). Using a liver homogenate, Krebs found arsenite caused the accumulation of the α-keto acids in the breakdown of amino acids. Peters, Sinclair, and Thompson (1946), after a detailed investigation, found these oxidases to be very sensitive to arsenite.

The mechanism of inhibition does not necessarily have to be the reaction of the arsenical with the sulphydryl groups of the apoenzyme, but could also be the reaction with the sulphydryl groups of a cofactor, substrate, or some intermediate. Also, arsenicals may inhibit enzymes if their structure is similar to the substrate.

Much of the inhibition observed in enzyme studies may be the result of the reaction of the arsenical with lipoic acid. It is now established that α-keto acid oxidation reactions are lipoic acid dependent (Reiss, 1958). It was reported by Reiss and Hellerman

(1958) and Reiss (1958), that all lipoic acid dependent reactions were inhibited by trivalent organic arsenicals. At one time it was suggested that the site of inhibition may have been related to the coenzyme A because it possesses a sulphydryl group (Sanadi, Littlefield, and Bock, 1952). It is now accepted that the arsenical acts on the lipoate. This means that the lipoate is not reduced, and no oxygen is used in the oxidation of the lipoate.

Oxidation of the pyruvate and α-ketoglutarate would be expected to be inhibited by arsenoxides because these are lipoic dependent reactions (Reiss, 1958). It is possible that these two α-keto acids are oxidized by another pathway involving enzymes that are not sensitive to arsenicals. Hellerman, Reiss, and Gey (1962) found the utilization of pyruvate but did not detect the formation of citrate.

Wessels (1959) and Avron and Biale (1957), found arsenite to inhibit oxidation of citrate. The enzyme aconitase converts citrate to cis-aconitate. Two cofactors, Fe^{2+} and a sulphydryl group were listed as essential for aconitase activity (Dixon and Webb, 1964). Morrison (1954), found that the prosthetic group was lost when aconitase was partially purified. However, the enzyme could be reactivated by the addition of Fe^{2+} and cysteine. Peters (1955) found that aconitase was not inhibited by monosubstituted arsenicals.

Webb (1966) stated that the arsenical inhibition may be recorded as either competitive, noncompetitive, or mixed depending on two things: the method used to determine the type of inhibition, and the tightness of the bond between the enzyme and the arsenical. The

site of inhibition is thought to be at or near the active site. When the arsenical is tightly bound to the enzyme, the final equilibrium is not dependent on the substrate concentration. Thus, the time that the inhibition is determined will dictate if competitive, non-competitive, or mixed inhibition is observed.

Glycolysis is not inhibited to a great extent by arsenite (Webb, 1966). However, little work has been done with organic arsenicals in glycolytic studies, thus it is not known what the effect is.

Many of the metabolic pathways are closely associated with the formation and utilization of acetyl-Co A and other compounds of the tricarboxylic acid cycle. It is thus expected that arsenicals will effect the metabolism of compounds directly as well as indirectly.

Metabolism of Arsenicals

It is possible for pentavalent arsenicals to be reduced to the trivalent form without an enzymatic reaction taking place. Gordon and Quastel (1948), attribute this reduction to reductants in the body such as glutathione. Crawford (1947) points out that it is only an assumption that the pentavalent arsenicals must be reduced to be active, but there is no direct evidence or demonstration of the reduced product in the tissue. Also, it had not been shown that arsenite can be formed from organic arsenicals in the body. Subsequently, Crawford and Levvy (1947), armed with better analytical techniques found that when trivalent arsenicals were injected into rabbits some of the arsenicals were excreted in the pentavalent form. Likewise, a

small amount of the trivalent form could be detected in the excreta when only the pentavalent form had been included in the diet.

Crawford and Levvy (1947) found that the rate of oxidation of phenylarsenoxide to phenylarsonate determined the toxicity of the compound and the rate of excretion. They found neither arsenite nor arsenate to be formed.

Inhibition of Growth and Enzymes by Arsenicals

Some bacteria are more sensitive than others to arsenite and organic arsenicals. Albert, Folk, and Rubbo (1944) determined the concentrations of oxophenarsine, acetarsone and $HgCl_2$ which would inhibit growth. Their data showed Escherichia coli and Proteus vulgaris to be fairly resistant to the arsenicals, but not to $HgCl_2$. Clostridium welchii, Streptococcus hemolyticus A, and Staphylococcus aureus were sensitive to the trivalent arsenicals and $HgCl_2$. The arsenicals are as potent as $HgCl_2$ against certain bacteria. They found pentavalent arsenicals to be inactive. Larson and Carpenter (1952) found no reduction in the fecal clostridia population when pigs were fed 3-nitro-4-hydroxyphenylarsonic acid.

The growth of Aerobacter aerogenes was not inhibited under aerobic or anaerobic conditions in the presence of 3 μM arsenite, but pyruvate and certain amino acids accumulated (Fowler and Werkman, 1955).

An acquired tolerance or resistance of organisms to arsenicals has been observed in protozoa, fungi, and bacteria (Albert, Folk, Rubbo, 1944). Webb (1966) suggested classifying the mechanisms

possibly responsible for the resistance of microorganisms to high concentrations of arsenicals as follows:

(1) Decreased penetration of the arsenicals into the cells.

(2) Decreased affinity of the cells or their receptors for the arsenicals.

(3) Increased destruction of the arsenicals.

(4) Alteration of the metabolic pathways so that vulnerable components are no longer necessary.

Savchuck, Loy and Schiaffino (1960) found a concentration of 0.0017 μM arsenite inhibited growth by 50% in HeLa cell cultures. The concentration needed for inhibition by 3-nitro-4-hydroxyphenylarsonate was 1.22 μM and for phenylarsenoxide 0.00059 μM. This pattern of concentrations needed for inhibition was also observed in studies using human liver cells, skin 2198, and Earle's L-929 cells.

Two studies have been reported on the inhibition of the tricarboxylic acid cycle by arsenite. Avron and Biale (1957) used avocado particles and found inhibition of oxygen uptake in the oxidation of tricarboxylic acid cycle intermediates. Alpha-ketoglutarate oxidation was completely inhibited by 0.001 M arsenite, while pyruvate was 94%, malate 84%, and fumarate 81% inhibited. About 50% inhibition was observed with citrate and cis-aconitate. Inhibition was 25% with the substrate succinate. Wessels (1959) working with the fungus Schizophyllum commune found 9% inhibition with succinate, 20% with citrate, 46% with malate, 96% pyruvate, and 99% with α-ketoglutarate.

A careful review of the literature failed to indicate any enzyme inhibitor studies employing either 3-nitro-4-hydroxyphenylarsonic acid or 3-nitro-4-hydroxyphenylarsine oxide.

MATERIALS AND METHODS

Organism

The organism used in this study was strain M5a1 of *Klebsiella pneumoniae* obtained from the Bacteriology Department of South Dakota State University. The organism was originally obtained from the University of Wisconsin as *Aerobacter aerogenes* strain M5a1 (Hamilton and Wilson, 1955) and later the organism was found by serotyping to be a *K. pneumoniae* (Mahl, Wilson, Fife, and Ewing, 1965). The culture was streaked on a nutrient agar plate and a typical colony was picked and inoculated into a tube of nutrient broth. After incubating the nutrient broth tube at 37 C overnight the culture was used to inoculate five tubes of plate count agar for a stock culture. One of the tubes of the stock culture was used to inoculate tubes of plate count agar for a working culture. After the working culture tubes had been incubated at 37 C for 24 hours and abundant growth was observed, the tubes were stored at 4 C in a refrigerator. One tube was used for each week.

Growth Study

K. pneumoniae was inoculated into 100 ml of sterilized medium of modified nutrient broth. The medium consisted of nutrient broth to which had been added 1.0% glucose, 0.8% NaCl, and 0.3% yeast extract. The culture was incubated at 32 C overnight on a Cutler & Hammer rotary shaker. This culture was used to inoculate the series of 500 ml side arm flasks containing 75 ml of the modified nutrient broth used in the growth studies.

The inhibitor arsenical compounds used in these growth studies were 3-nitro-4-hydroxyphenylarsonic acid and 3-nitro-4-hydroxyphenylarsine oxide. The arsonic acid which was donated by Salsbury Laboratories, Charles City, Iowa, was used at a level of 0.0025%, the concentration suggested for this arsenical in feeds. The oxide was prepared by reducing the arsonic acid using the method of Kano and Tayshima (1954) and was used at a concentration of 0.0022% which was equivalent in arsenic concentration to the arsonic acid. The flasks were incubated at 32 C on a Cutler & Hammer rotary shaker. Klett-Summerson scale readings were taken using the side arm tubes. The red filter was used in the Klett-Summerson Photoelectric Colorimeter so the approximate spectral range was 640-700 mμ. Growth of the organism was measured turbidimetrically. Nitrogen gas was used to flush the flasks to produce anaerobic conditions. A slight negative pressure was left as anaerobic growth produced gas.

K. pneumoniae was inoculated into 500 ml side arm flasks and incubated at 32 C on a Cutler & Hammer rotary shaker. The flasks were removed when the Klett-Summerson scale readings were 3, 50, 100, 150, 200, 250, and 300. The number of viable cells per ml was determined using plate count agar in a poured plate procedure.

Enzyme Studies

K. pneumoniae was inoculated into 100 ml of Koser Citrate medium to which had been added 0.3% yeast extract, 0.3% glucose, and 0.1% nutrient broth. The inoculum was prepared by washing the surface of a working stock culture tube with sterile medium. A sterile pipet

was used to transfer the organism to 250 ml flasks. The cultures were incubated for 10 to 12 hours at 32 C on a Cutler & Hammer rotary shaker. The cells were harvested at 2,000 g for 10 min using a Sorvall Type II-A centrifuge head and washed twice with Sorenson's buffer, pH 7.0. The cells were resuspended in buffer at pH 7.0 and disrupted using a cooled (4 C) French pressure cell. The force exerted on the cells was 20,000 psi. The broken cells were then centrifuged twice in a refrigerated (1 C) Beckman Model L Ultracentrifuge Rotor Type 50 for 10 min at 20,000 rpm. Microscopic examination of the resultant supernatant revealed no intact cells.

Protein was determined by using the Biuret method (Clark, 1964). A standard curve for protein determinations was made using crystalline bovine albumin as a standard. Absorbance was determined with a "Spectronic 20", Bausch & Lomb Spectrophotometer, at 550 mμ. The absorbance figures were read in mg of protein from the standard curve (Figure 2). To each Warburg constant volume respirometer 10 to 10.5 mg protein were added.

The Warburg flasks contained 18 μmoles of $MgSO_4$ and 84 μmoles of phosphate buffer pH 7.0 (Storck and Wachsman, 1957). The cofactors, purchased from Calbiochem, Los Angeles, were added in the following quantities:

Cofactors	Quantity
Adenosinetriphosphate	0.12 mg
Nicotinamide-Adenine Dinucleotide	0.12 mg
Nicotinamide-Adenine Dinucleotide Phosphate	0.24 mg
Thiamine Pyrophosphate Chloride	0.06 mg
Coenzyme A	0.06 mg
Cytochrome C	0.03 mg

The volume of the contents added to Warburg flasks was 3.2 ml with a final pH of 7.0.

Three flasks were designated as endogenous controls. One contained no substrate and the arsonic acid. The second flask contained the arsine oxide and no substrate. The third flask did not contain either substrate or inhibitors. The endogenous flasks were always duplicated for each cell free enzyme preparation.

The tricarboxylic acid cycle intermediates were used for substrates in enzyme studies and standards in the chromatography studies. The following chromatographically homogenous compounds were purchased from Calbiochem: α-ketoglutaric acid, pyruvic acid (sodium salt), succinic acid (disodium salt hexahydrate), l-malic (NaOH neutralized), and oxaloacetic acid. Cis-aconitic acid and citric acid (Mallinckrodt) as well as the other acids were neutralized with Na_2CO_3. The quantity of tricarboxylic acid cycle intermediates added to the Warburg flasks was varied: 175 μmoles of citrate, pyruvate, or α-ketoglutarate; 250 μmoles of malate, succinate, or fumarate. The cell free preparation was placed in the side arm and after an equilibration time of 10 min in a 32 C water bath, the cell free extract was added to the main chamber of the flasks. The Warburg flasks were incubated using atmospheric oxygen. The manometer was closed before the addition of the enzyme. Immediately after the addition of the enzyme a zero reading was taken. Manometer readings were recorded every 10 min for an hour. To absorb any carbon dioxide evolved 0.2 ml of 10% KOH and 2 sq. cm. of folded filter paper were placed in the center well.

The oxidation of the tricarboxylic acid cycle intermediates was first tested for inhibition in the presence of the arsonic acid (0.0025%) and the arsine oxide (0.0022%). The oxidation of the intermediates found to be inhibited were placed in the presence of decreasing amounts of the inhibitor.

BAL was placed in Warburg flasks at 0.10, 0.25, 0.5, 1.0 and 2.0 times molar concentrations in relation to the molar concentration of the arsine oxide in the presence of the substrates citrate and α-ketoglutarate.

Chromatography

Thin-layer chromatography was used to observe the products of enzymatic activity. Acids of the tricarboxylic acid cycle were separated using the method of Goebell as described by Marini-Bettólo (1964).

Cellulose plates of 250 micron thickness were prepared using 12 g Brinkman Cellulosepulver MM300, and 72 ml of distilled water which had been adjusted to a pH of 11.5 with NaOH (Marini-Bettólo, 1964). The water and cellulose were mixed in a Waring Blendor for 1 min and poured into a Brinkman thin-layer chromatographic applicator Model S-11 which had been set for 250 micron (0.25 mm). Plates of 500 micron thickness were prepared using 20 g Cellulosepulver MM300 and 120 ml distilled water (pH 11.5). The 200 mm x 200 mm plates were coated immediately and allowed to air dry for 10 min. The plates were placed into a rack and dried in a Dispatch oven for 10 min at 105 C.

The plates were then stored in a desiccator over calcium chloride until used.

The thin-layer plates were developed in a battery jar with the organic phase of a mixture of 90 ml 5 M formic acid and 60 ml of isobutanol (Marini-Bettòlo, 1964). The 7 cm x 24 cm x 24 cm chromatographic tank chamber was lined with filter paper. The bottom edge of the paper was immersed in the solvent system. The chamber was covered and allowed to equilibrate for 20 min before the plates were placed in.

Contents of the flasks were chromatographed before the enzyme preparation was added to the flasks by removing 10 µl from the flasks with a 10 µl Hamilton syringe. The Warburg flasks contents were chromatographed after the incubation of the flasks. The contents of the flasks were poured into test tubes and placed in boiling water for 2 min (Wessels, 1959). Two drops of about 0.5 N HCl was added to each tube. The tubes were then centrifuged and the clear supernatant was chromotographed. A 10 µl sample was spotted on the plates. When streaking occurred in the separation of the acids, 500 µ plates were used. If no separation could be detected using 10 µl samples on 250 µ plates, then 20 µl samples were used and 500 µ plates. About 3 mm of the cellulose at the side of the plates was removed before the plate was placed in the tank for 100 mm or 90 min developing. Sometimes the plates were developed longer when separation was not distinct with the 100 mm developing length. Standard of unincubated tricarboxylic acid cycle intermediates were used.

To examine the plates they were sprayed with a solution of 95% ethyl alcohol and 0.04% Bromocresol Green (Block, 1952). The spray was adjusted to a green color using dilute NaOH. The acids appeared as yellow spots against a blue background. The compounds were identified by comparing the distances traveled with standards. The plates were documented by the tracing of the chromatograph on onion-skin paper (Stahl, 1965).

RESULTS

Growth Response to the Presence of the Arsenicals

Comparison of the average of Klett-Summerson scale readings between the control culture and the flasks containing 3-nitro-4-hydroxyphenylarsonic acid showed no difference after 9 hours of incubation (Table 1). Thus, when K. pneumoniae was grown under aerobic conditions in the presence of the arsonic acid, growth was not inhibited. However, when this organism was grown anaerobically in the presence of the arsonic acid, no inhibition was observed during the first 4 hours of incubation. After 4 hours the first changes in Klett-Summerson readings appeared so by the end of a 9 hour incubation period, the Klett-Summerson readings were at least 100 units less for the flasks containing the arsonic acid than the control cultures.

Growth of K. pneumoniae was inhibited by 3-nitro-4-hydroxyphenylarsine oxide under aerobic and anaerobic conditions. The Klett-Summerson scale reading never differed by over one unit from the initial reading taken at the beginning of the incubation period (Tables 2, 3).

The number of viable cells per ml increased with an increase in Klett-Summerson scale readings. While at a reading of 3 there were 3×10^8 cells per ml, there were 53×10^8 cells per ml at a reading of 300 (Table 4).

Effect of the Arsenicals on the Enzymes

No inhibition of oxygen uptake by the cell free extract in the oxidation of pyruvate, succinate, fumarate, and malate was observed

in the presence of 3-nitro-4-hydroxyphenylarsonic acid or 3-nitro-4-hydroxyphenylarsine oxide. Fumarate and malate were oxidized rapidly. The rate of oxygen uptake was 70 µl oxygen per mg protein per hour. About 23 µl of oxygen was taken up per mg protein per hour in the oxidation of the pyruvate and malate (Tables 5, 6, 7, and 8, and Figure 3).

No inhibition of oxygen uptake was observed by the cell free extract in the presence of 3-nitro-4-hydroxyphenylarsonic acid in the oxidation of citrate and α-ketoglutarate. Inhibition of oxygen was observed to be 60% at a 0.0022% concentration of the arsine oxide. The cell free extract utilized about 13 µl of oxygen per mg protein per hour in the oxidation of the substrates citrate and α-ketoglutarate, while with the arsine oxide present only 5 µl of oxygen were taken up (Tables 9 and 10). Inhibition increased with the amount of the inhibitor present (Figures 5 and 6).

The preliminary experiments using BAL gave irreproducible results. The BAL could not be readily dissolved, and at the conclusion of the Warburg incubation a thick white curd was present in the flasks. Gas was taken up in flasks containing BAL when the enzyme preparation was left in the side arm. Endogenous uptake of oxygen was high with as much as 125 µl oxygen per flask. Inhibition appeared to be overcome at 0.5, 1.0, and 2.0 times arsine oxide molar concentrations of BAL when compared to controls with no BAL added but the arsine oxide present. The amount of oxygen uptake was greater than the substrate control. The amount of oxygen taken up varied from

experiment to experiment and within an experiment replicate flasks varied. Differences were as much as 6 µl oxygen per mg protein per hour or 60 µl per flask.

Chromoatographic Evidence for Partial Tricarboxylic Acid Cycle

A partial tricarboxylic acid cycle was constructed using chromatography. The products of the oxidation of the cycle intermediates are shown in Table 11. Citrate was partially converted to cis-aconitate. Oxaloacetic acid was found as a product in the oxidation of malate. Malate was partially converted to fumarate.

TABLE 1. Aerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide Measured in Klett-Summerson Units.

Time	Klett-Summerson Scale Readings					
Hours	Control Flask 1	Control Flask 2	3-Nitro-4-hydroxyphenylarsonic Acid Flask 1	3-Nitro-4-hydroxyphenylarsonic Acid Flask 2	3-Nitro-4-hydroxyphenylarsine Oxide Flask 1	3-Nitro-4-hydroxyphenylarsine Oxide Flask 2
0.0	4	3	2	2	11	4
0.75	6	5	3	4	10	4
2.0	41	42	38	45	10	4
2.5	98	98	95	99	10	4
3.0	136	140	144	137	11	4
3.5	155	154	150	154	10	4
4.0	190	195	188	195	11	4
4.5	218	222	218	226	11	4
5.5	266	276	282	290	11	4
6.5	302	312	318	325	11	4
7.5	315	348	350	360	11	4
8.25	350	360	375	380	10	4
10.0	395	405	400	400	10	4

TABLE 2. Anaerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid Measured in Klett-Summerson Units.

Time	Klett-Summerson Scale Readings				
Hours	Control		Arsonic Acid		
	1	2	1	2	3
0.0	3	4	5	4	5
0.33	4	3	5	5	5
1.6	11	11	13	11	12
2.5	35	31	33	31	30
3.0	55	57	52	50	47
3.5	77	63	64	62	60
5.0	135	110	100	95	97
6.0	180	145	123	117	118
7.0	226	188	147	142	140
7.5	250	210	150	150	150
8.5	270	272	172	165	-
9.0	278	278	173	166	-

TABLE 3. Anaerobic Growth of Klebsiella pneumoniae in the Presence of 3-Nitro-4-hydroxyphenylarsine Oxide Measured in Klett-Summerson Units.

Time	Klett-Summerson Scale Readings			
Hours	Control		Arsine Oxide	
	1	2	1	2
0	3.5	3	3	3
1	3	3	3	3
2	16	17	3	3
3	47	50	3	3
4	70	74	3	3
5	105	110	3	3
6	140	148	3	3
7	175	167	3	3
8	-	228	3	3
9	-	274	3	3

TABLE 4. Relationship of Klett-Summerson Units to Number of Cells per ml.

Klett-Summerson Readings	10^8 Cells per ml
3	3
50	12
100	17
150	23
200	-
250	40
300	53

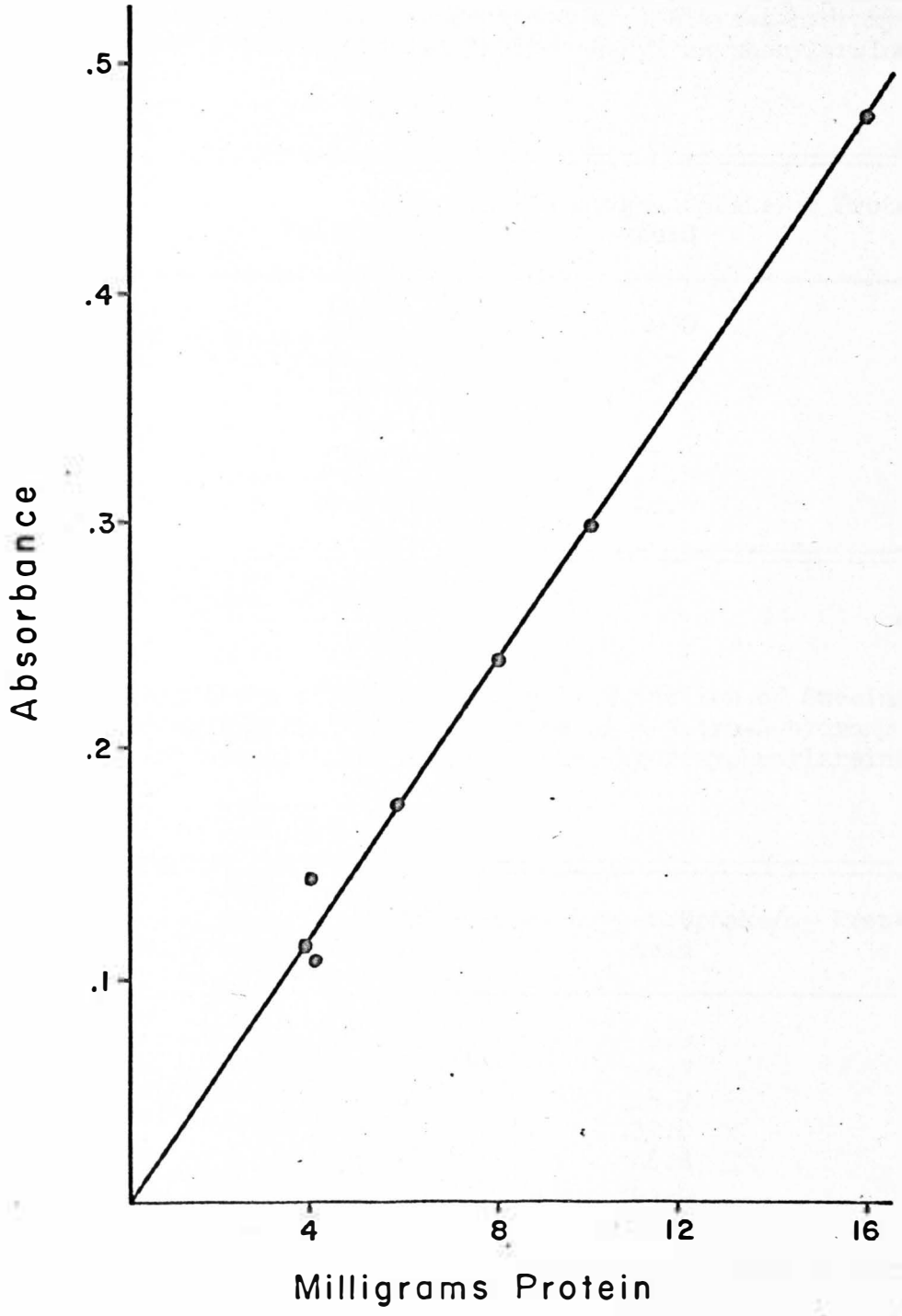


Figure 2. Standard Curve for Protein Determination in Milligrams Protein per ml. in Relation to Absorbance.

TABLE 5. Microliters of Oxygen Uptake in Oxidation of Pyruvate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	-	-	-
20	5.7	8.0	8.0
30	17.1	16.5	17.3
40	22.3	21.1	22.4
50	26.2	24.3	24.5
60	27.2	26.6	26.7

TABLE 6. Microliters of Oxygen Uptake in Oxidation of Succinate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	4.7	4.7	4.1
20	15.8	15.9	16.8
30	29.6	32.8	34.1
40	42.0	46.8	46.7
50	55.1	63.8	61.3
60	67.8	73.0	71.0

TABLE 7. Microliters of Oxygen Uptake in Oxidation of Fumarate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	5.0	5.9	4.5
20	17.2	18.5	16.7
30	30.7	32.6	31.8
40	48.9	35.0	44.1
50	57.3	49.3	57.8
60	70.4	70.2	68.1

TABLE 8. Microliters of Oxygen Uptake in Oxidation of Malic per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	4.1	6.5	11.2
20	9.4	13.5	13.6
30	11.5	15.3	17.5
40	16.1	18.3	19.9
50	16.7	21.6	23.6
60	24.8	25.3	26.3

TABLE 9. Microliters of Oxygen Uptake in Oxidation of Citrate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	0.8	1.4	1.5
20	4.3	5.2	3.5
30	9.3	7.7	4.3
40	10.3	9.8	4.7
50	11.9	12.7	5.0
60	12.7	12.8	4.7

TABLE 10. Microliters of Oxygen Uptake in Oxidation of α-Ketoglutarate per mg Protein in the Presence of 3-Nitro-4-hydroxyphenylarsonic Acid and 3-Nitro-4-hydroxyphenylarsine Oxide.

Time Minutes	Microliters Oxygen Uptake/mg Protein		
	Substrate	Acid	Oxide
0	0.0	0.0	0.0
10	3.8	4.0	2.3
20	6.6	6.8	4.3
30	8.5	5.2	4.6
40	9.8	9.8	4.7
50	11.1	11.6	4.8
60	12.5	12.5	4.8

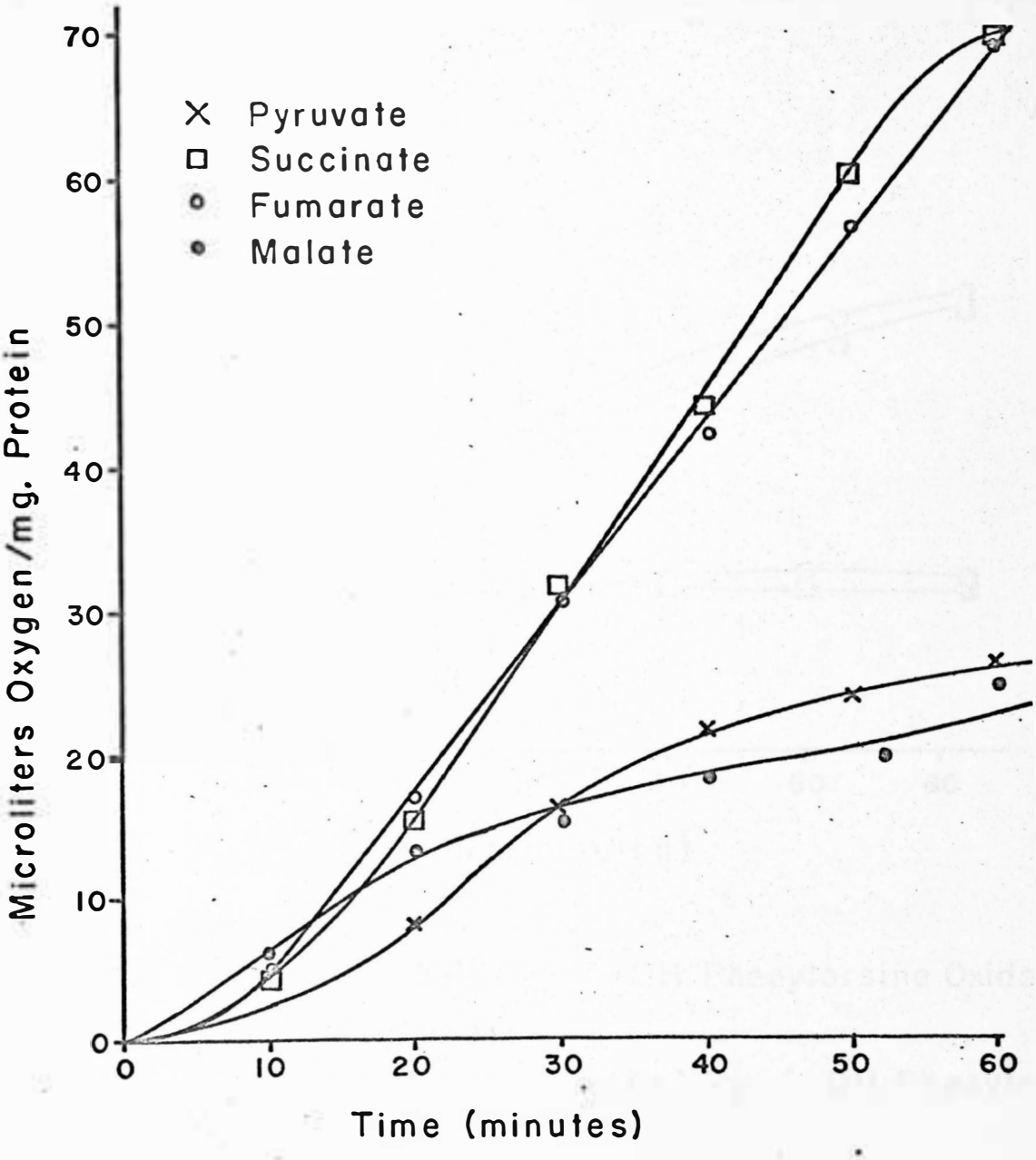
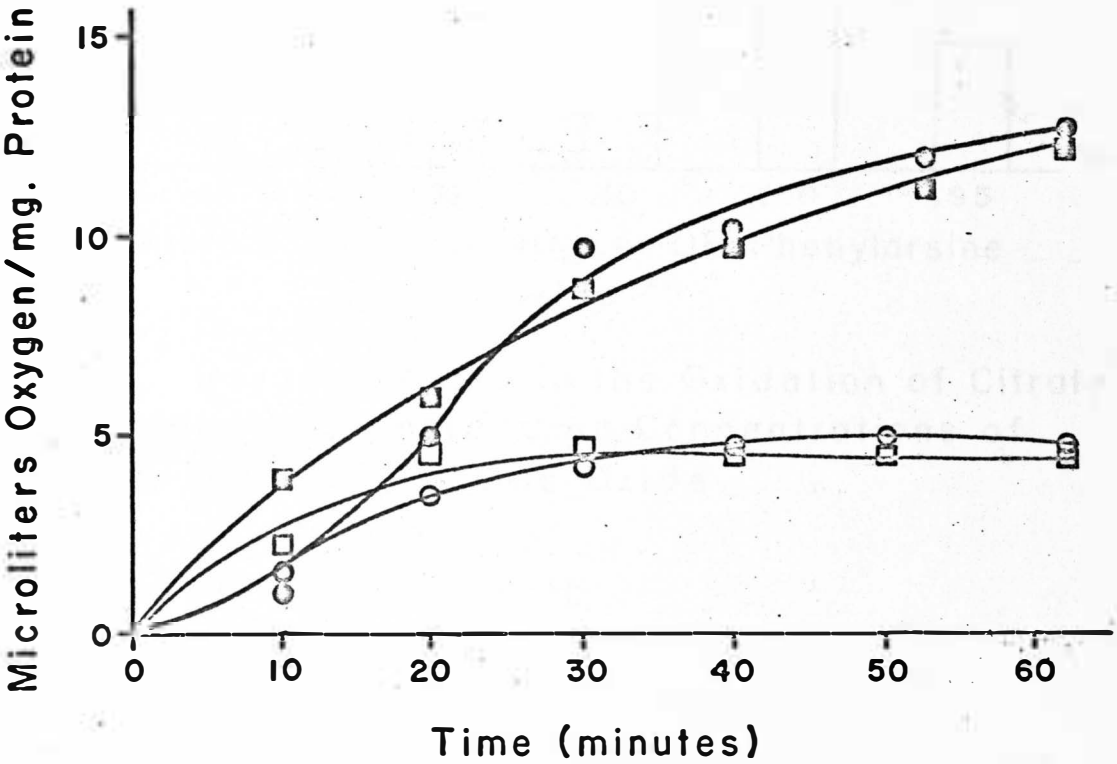


Figure 3. Oxygen Uptake in the Oxidation of Pyruvate, Succinate, Fumarate and Malate.



- Citrate
- Citrate and 3-NO_2-4-OH Phenylarsine Oxide
- ∝-Ketoglutarate
- ∝-Ketoglutarate and 3-NO_2-4-OH Phenylarsine Oxide

Figure 4. Oxygen Uptake in the Oxidation of Citrate and ∝-Ketoglutarate in the Presence of 3-NO_2-4-OH Phenylarsine Oxide

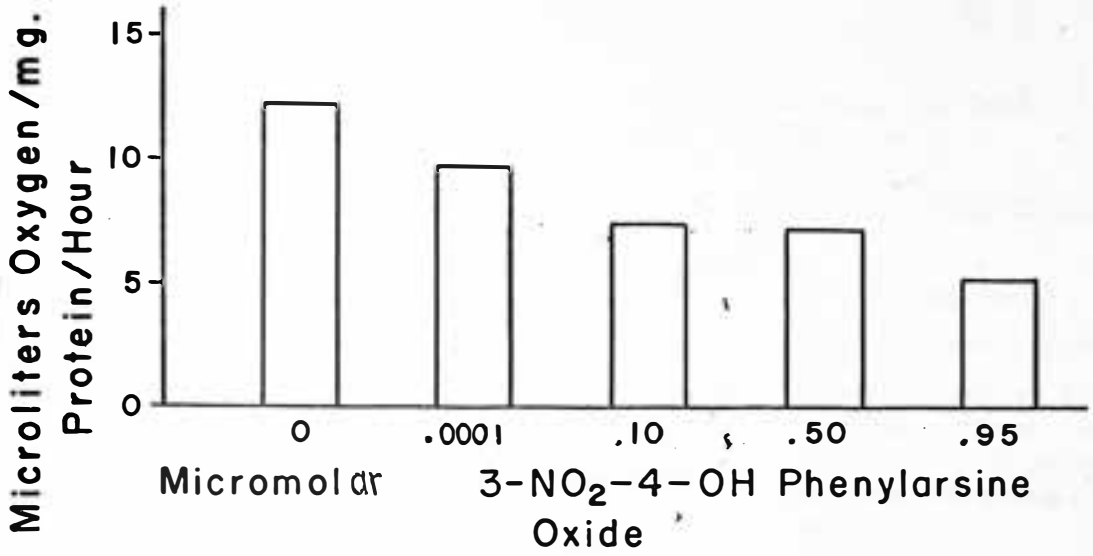


Figure 5. Oxygen Uptake in the Oxidation of Citrate in the Presence of Increasing Concentrations of 3-NO_2-4-OH Phenylarsine Oxide

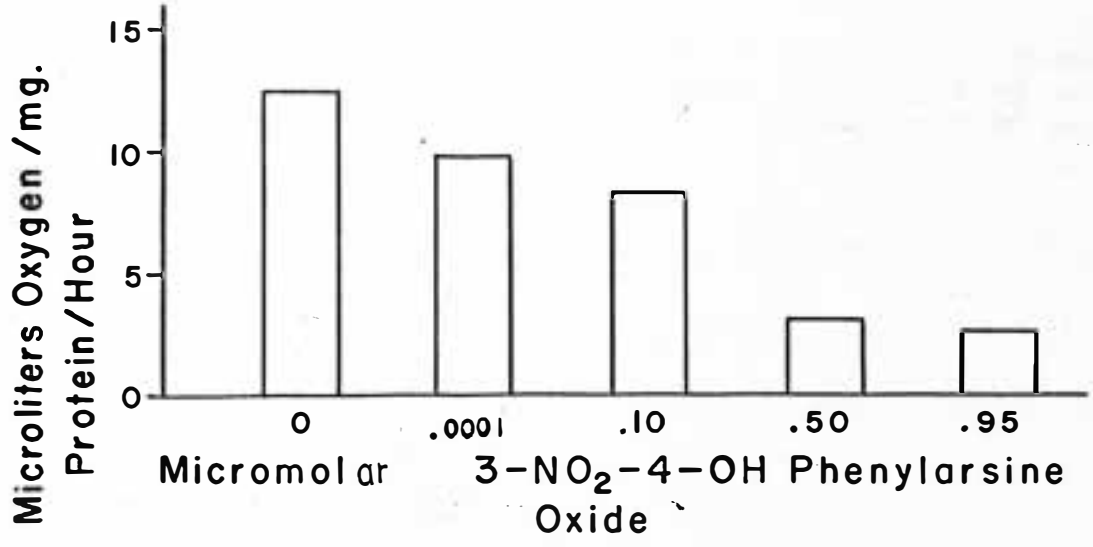


Figure 6. Oxygen Uptake in the Oxidation of α-Keto-glutarate in the Presence of Increasing Concentrations of 3-NO_2-4-OH Phenylarsine Oxide

TABLE 11. Products of the Oxidation of the Tricarboxylic Acid Cycle Intermediates by the Cell Free Enzymes

Intermediate	Chromatogram Before	Chromatogram After	Comments
Pyruvate	Pyruvic Acid	Pyruvic Acid	May be oxidized by another pathway, concentration of product may not be enough to detect
Citrate	Citric Acid	Citric Acid cis-aconitic	
α-Ketoglutaric Acid	α-Ketoglutaric Acid	α-Ketoglutaric Acid	Little activity, concentration of product may not be enough to detect
Succinate	Succinic Acid	Succinic Acid Possibly Malic Acid	Separation between malic acid and succinic acid not good
Malate	Malic Acid	Malic Acid Oxaloacetic Acid	
Fumarate	Fumaric Acid	Fumaric Acid Malic Acid	

DISCUSSION

Inhibition of Growth

The growth of K. pneumoniae was not inhibited in the presence of 3-nitro-4-hydroxyphenylarsonic acid under aerobic conditions as determined by Klett-Summerson readings. The report that atoxyl is inactive in vitro (Webb, 1966) and the observation that the arsonic acid under aerobic conditions does not inhibit growth, inhibition under anaerobic conditions was not anticipated. However, under anaerobic conditions inhibition was observed. Inhibition was not observed in the first 4 hours of incubation, but appeared later. Further studies are required to determine if the time needed for inhibition to appear could be correlated to the amount of inoculum. The presence of oxygen may either have inhibited an enzyme or enzyme system responsible for the reduction of the arsonic acid. The pattern of the cells metabolism may have changed under anaerobic conditions such that the presence of the arsenical caused the production of another compound that was inhibitory.

It was found that 3-nitro-4-hydroxyphenylarsine oxide inhibited growth under aerobic as well as anaerobic growth conditions. The reaction of an arsine oxide with sulphydryl groups is probably not influenced by aerobic or anaerobic conditions.

The relationship of Klett-Summerson readings to viable organisms per ml was determined by plate counts. It is not known whether the cells in the flasks containing the arsonic acid or arsine oxide were

viable or not. Klett-Summerson scale reading differences of a few units could possibly be no actual difference in the number of organisms per ml, because the side arms of the flasks used for the growth studies were matched to 1%.

Inhibition of Enzyme Activity

Cells grown in the modified Koser Citrate medium were found in preliminary experiments to show better tricarboxylic acid cycle activity than those grown in the growth medium or nutrient broth. The presence of the citrate and glucose could possibly have stimulated cycle formation.

No inhibition of oxygen uptake was observed by the cell free enzyme preparation in the presence of 3-nitro-4-hydroxyphenylarsonic acid with tricarboxylic acid cycle intermediates as substrates. Arsonates do not react with thiol groups, thus, if any inhibition was noted it would probably have been explained by the reduction of the arsenical by the enzyme preparation. This may be possible under anaerobic conditions.

The reaction of trivalent arsenicals with thiol groups should result in the inhibition of certain enzymes of the tricarboxylic acid cycle. The cycle contains two α-keto oxidases and an enzyme requiring a sulphydryl group as a cofactor.

No inhibition of oxygen uptake in the oxidation of succinate, malate, fumarate and pyruvate was observed in the presence of the arsine oxide. It is possible that inhibition would have been observed if higher concentrations had been used or more highly purified enzymes

had been used. The reaction of the arsenical with a sulphydryl group would then bind the arsenic to the group in a fairly stable complex or reduce the arsenic. It is possible no arsenical would then be left to combine with the sulphydryl groups of all the enzymes. The amount of inhibition was reduced by reducing the quantity of inhibitor. If an excess amount of inhibitor had been added it would be expected that there would be a level above which there would be the same or maximum amount of inhibition. As no plateau was found in this experiment, the level is probably greater than the 95 μM. It cannot be overlooked that the arsenical could cause other changes that would also cause an increase in inhibition with an increase in inhibitor concentration.

There appears to be a stimulation in the oxygen uptake with the substrates succinate and malate as shown in Tables 6 and 8, but the data for only one experiment are given. Several duplicate experiments were carried out with each substrate. These showed slight inhibition in some cases and stimulation in others. Similar results were observed with pyruvate and fumarate. In some cases there appeared slight stimulation and others slight inhibition. These substrates were considered not to be inhibited.

Reiss and Hellerman (1958) and Reiss (1958) reported all lipoic acid dependent reactions were inhibited by trivalent organic arsenicals. Inhibition of oxygen uptake would thus be expected in the oxidation of pyruvate as the enzyme complex contains lipoate (Reed and Cox, 1966). No inhibition was observed in the oxygen uptake in

the presence of the arsine oxide. It is possible that if the level of the arsenicals were increased that inhibition would have been observed.

The lipoate dependent enzyme system that oxidizes α-keto-glutarate was inhibited by the trivalent arsenical. It is possible that this system tied up all the inhibitor so that pyruvate was not inhibited or that other sulphydryl groups tied up the inhibitor. It may be that it is sterically impossible for the arsenical to react with the lipoate in the pyruvate system. Reed and Cox (1966) show different tentative models for the pyruvate and α-ketoglutarate oxidase systems.

The oxygen uptake was higher with citrate and α-ketoglutarate in the presence of BAL and the arsine oxide than it was for each of the substrates in the presence of the arsine oxide. The increase in oxygen uptake in the presence of BAL could be explained by either enzymes in the cell free extract using oxygen in metabolizing the BAL or the BAL suppressing some other inhibitor present. The irre-producibility of results could possibly be overcome by finding a way of dissolving the BAL into the flasks contents.

Products of Enzymatic Activity

Thin-layer chromatographs failed to detect pyruvic acid or oxaloacetic acid in quantities less than 80 µg. The other inter-mediates could be detected when present in quantities of less than 20 µg. Streaking occurred when the concentrations were high. In-creasing the thickness of the chromatograph enhanced its capacity to

separate compounds. When streaking occurred or concentrations were too high to separate, 500 μ thickness of absorbent was used. All the tricarboxylic acid cycle compounds were not present. This was not unexpected as pyruvic acid or acetyl Co A would have to be added so that acetyl Co A could condense with the oxaloacetate to form citrate to complete the cycle. If the incubation period of the Warburg respirometers had been longer more products might have been found. The longer incubation period would measure the amount of inhibition of a product of oxidation rather than the substrate if the substrate was not inhibited.

SUMMARY

The growth and enzymatic activity of K. pneumoniae strain M5al was observed in the presence of 3-nitro-4-hydroxyphenylarsonic acid and 3-nitro-4-hydroxyphenylarsine oxide.

Growth was not inhibited when the organism was grown aerobically in the presence of 3-nitro-4-hydroxyphenylarsonic acid, however, inhibition of growth was noted when organisms were grown anaerobically in the presence of this arsonic acid. The acid did not inhibit oxygen uptake in the oxidation of the tricarboxylic acid cycle intermediates.

Inhibition of growth was noted when the organisms were cultured in the presence of 3-nitro-4-hydroxyphenylarsine oxide. This inhibition was observed when the conditions were anaerobic or aerobic. The arsine oxide form of the arsenical showed inhibition of certain enzymes of the tricarboxylic acid cycle. No inhibition was noted in the oxidation of pyruvate, malate, succinate and fumarate. Inhibition was noted in the oxidation of α-ketoglutarate and citrate. An increase in arsine oxide concentrations resulted in the decrease in the amount of oxygen taken up for the substrates citrate and α-ketoglutarate. When BAL was added, inhibition by the arsine oxide was overcome and more oxygen was taken up than with the substrate alone. However, the results were not reproducible.

Thin-layer chromatography was used to determine products of the enzymatic oxidation of the substrates. Citrate was partially converted

to cis-aconitate. Oxaloacetic acid was found as a product in the oxidation of malate. Malate was partially converted to fumarate.

CONCLUSIONS

1. Inhibition of growth of K. pneumoniae in the presence of 3-nitro-4-hydroxyphenylarsonic is determined by whether conditions are aerobic or anaerobic. Aerobically growth was not inhibited, while anaerobically it was. If anaerobic conditions exist in lagoons where animal wastes contain 3-nitro-4-hydroxyphenylarsonic acid microbial growth could be inhibited.
2. At a concentration of 0.0022% 3-nitro-4-hydroxyphenylarsine oxide will inhibit growth under anaerobic and aerobic conditions.
3. Under the conditions and at the concentrations used the arsonic acid did not inhibit the enzymes of the tricarboxylic acid cycle.
4. The arsine oxide did not inhibit all the enzymes of the tricarboxylic acid cycle. The oxidation of citrate and α-ketoglutarate was shown to be inhibited by measuring oxygen uptake in a Warburg constant volume respirometer.
5. Inhibition by the arsine oxide appears from preliminary experiments to be suppressed by BAL.
6. Chromatographic evidence for the partial functioning of a tricarboxylic acid cycle was found.

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