1954

The effect of organometallic and quaternary ammonium compounds on the growth of microorganisms

Lowell Lawrence Wallen
Iowa State College

THE EFFECT OF ORGANOMETALLIC AND QUATERNARY AMMONIUM COMPOUNDS ON THE GROWTH OF MICROORGANISMS

by

Lowell L. Wallen

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1954

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

I. INTRODUCTION

The presence of microorganisms upon the earth is universal, and they can be found almost anywhere man may search for them. The fact of their existence has not always been known since they are too small to be observed by the naked eye. However, this does not presume that they are of little importance, for some are endowed with tremendous potentialities for good and some for evil. They can survive under a great variety of conditions, and in addition, they multiply so rapidly that the destruction of millions of them is often of no consequence to their survival.

The man chiefly responsible for enabling microorganisms to be seen was a Dutch linen merchant turned investigator, Antonj van Leeuwenhoek (1632-1723), who was skilled in the art of making lenses to the extent that he fashioned a type of crude microscope. With such an instrument, van Leeuwenhoek was able to observe microorganisms, which he called "animalcules" (45).

Since then, the study of microorganisms has increased in scope, and as a result, the entire overall concept of a bacterial cell is so vast that no single person is able to master it in its entirety. The scope included in the genesis, lifespan and death of a cell involves all branches of chemistry, as well as physics, genetics, bacteriology, cytology, physiology, and many other branches of science, making it

necessary for one who endeavors to work with microorganisms to specialize in a limited area of research and thus add his findings to those of past years to help complete the ever-growing picture.

The activity of harmful and undesirable microorganisms has constantly challenged mankind in his attempt to gain mastery over them. Among the primary reasons for this effort is the control of diseases, many of which are usually attributed to microorganisms. Thus there have been many attempts to destroy these causative agents and therefore rid the world of diseases. This search continues unabated, and has resulted in a tremendous collection of published material dealing with methods for controlling or eliminating troublesome and pathogenic microorganisms from the body or wherever they may be found.

Chemotherapy is the science of therapeutics which employs chemical reagents toxic to the organisms, but not toxic (or only slightly so) to the host. Some compounds have involved a metal atom in their structure and have been very beneficial to man. Metals or their salts have been used for many years since the second century when mercury was employed by the ancients of the medical trade (149), but of more recent note is Salvarsan, an organometallic compound prepared by Paul Ehrlich, which has been used for the treatment of syphilis for many years.

Organometallic compounds have been defined very

generally as organic compounds which incorporate a metal in their structure. This could include the metal salts of organic acids, so this generalization usually excludes salts of this kind. A true organometallic compound, in the strictest sense, is one in which all normal valences of the metal atom are employed in a carbon-to-metal bond for each valence. The chemical literature has many references to organometallic compounds of the type where one carbon-to-metal bond is present, but the other bond(s) of the metal is usually partially or totally ionic.

This investigation entails the use of organometallic compounds in the strictest sense of the meaning only; i.e., where all valence bonds of the metal are to carbon, with the exception of triphenylsilanol. These compounds have received little or no attention as to their effect on microorganisms, so it was felt that an investigation of their action was justified. By observing the influence of varying types and concentrations of organometallic compounds upon different phases of a fermentation, one can draw helpful conclusions which would be useful in comparing these compounds with others of a similar type. Because many new organometallic compounds have been synthesized, and because their history has shown promise for chemotherapeutic usage, the growth of different organisms in their presence was observed and studied.

One of these organometallic compounds; i.e., triphenyl-γ- (diethylmethylammonium)-propyllead methosulfate, is a

true organometallic compound as well as a quaternary ammonium compound. In order to test the compound for the effect of the presence of the lead atom, the lead-free, carbon atom analog was synthesized and characterized since it is a new compound. The quaternary ammonium compounds are members of another class of bactericidal agents which has been exploited by man. A study of their effect in the case of the organometallic type mentioned was also undertaken because of the dual nature of this compound.

It is hoped that the conclusions reached on the basis of research accomplished with these compounds may be useful to persons interested in extending this investigation. These two classes of compounds may possibly possess some points in common in regard to their modus operandi in attaining their ultimate action on the microorganisms. Such a viewpoint has been proposed by Valko and DuBois (142), and will be discussed later.

II. REVIEW OF LITERATURE

A. Introduction

The scope of the investigations to date on bactericidal and bacteriostatic methods has grown to overwhelming proportions. Specialization within rather narrowly defined fields has been followed of necessity, and therefore, an all inclusive review of bacteriostasis is not possible here. The background of history relative to this problem includes research associated with the chemotherapeutic approach in which metal-containing organic compounds (i.e., organometallic compounds in general) are considered; also that approach which involves a broad generalization of the development of cationic surface-active compounds of the substituted "ammonium" types.

A representative group of microorganisms was studied in the course of the investigations reported in this thesis, including a yeast, a mold, and different bacteria which are aerobic, microaerophilic, and anaerobic in behavior. The effects of the different compounds used here were evaluated in terms of the growth observed and the sugar utilized; also measured was the respiration of the cells at different concentration levels of compounds added. Many workers (31, 76, 89, 95) have undertaken investigations of the action of organometallic and quaternary ammonium compounds on the

respiratory activities of cells, so it was felt that the effect of the compounds used here should also be studied. In addition, the chemotherapy of diseases involves either the cessation or inhibition of growth of the toxic organism in the host, so a review of this aspect of research is included. Some literature describing the organisms in a generally descriptive sense is also included as review material for orientation purposes, and additional reference is made to the historical background of organometallic compounds.

B. Microorganisms

1. Saccharomyces cerevisiae

The yeasts are classified as a part of the fungi, and are further subdivided into two groupings of Ascomycetes, i.e., "yeast-like" and "mold-like". The organism, Saccharomyces cerevisiae, is a "yeast-like" Ascomycete, or a true yeast. Its cells are large compared to bacterial cells. It reproduces by forming buds on the surface of the parent cell which enlarge and break away to be new cells. The yeast cells are non-motile, and obtain their nutrient requirements by absorption of soluble metabolites through their cell walls. They give a Gram positive stain, and can be cultivated on glucose and yeast extract medium, as the latter material contains the necessary growth factors.

This organism is fermentative in its activity, producing ethanol and carbon dioxide from sugar anaerobically via the Meyerhof-Embden-Parnas scheme. Physiologically, the cells of Saccharomyces cerevisiae are very similar to bacterial cells. Schmitthenner (123) has observed that carbon dioxide, a normal product of this fermentation, will halt multiplication of Saccharomyces cerevisiae and other strains if it is present in a concentration of 1.5 per cent at 7.7 atm. at 15° C. The cells were killed only if a pressure of 30 atm. of carbon dioxide was maintained for a long period. A similar effect was observed with acetic acid and lactic acid organisms.

An analysis of the cell wall of Saccharomyces cerevisiae by Northcote and Horne (106) showed it to be made of two membranes, one of which was composed of glucan polyglucides, and the second one made up of protein or mannan, or mannan associated with the protein. Their examination of the structure showed glycogen apparently is not a part of the structure of the cell wall. Bud scars were found, however, and their existence was regarded as important to the behavior of the cell wall as an osmotic boundary. This information may be of importance in this study of Saccharomyces cerevisiae in regard to the entrance of nutrilites and organometallic compounds into the yeast cell.

There are a multitude of compounds in yeast so a complete discussion of Saccharomyces cerevisiae cannot be made here.

A listing of the compounds in yeast has been compiled by Smith (129).

2. Aspergillus niger

This organism is classified as a mold, and has the necessary enzyme systems to cause the breakdown of starch to soluble sugars, which are then converted to the products of fermentation. Thus, by means of the glucose oxidase of this organism, which was first described by Müller (102), this mold oxidizes glucose to gluconic acid, the product resulting from oxidation of the number one carbon atom of glucose to a carboxyl group.

As the production of gluconic acid will cause a drop in the pH, the addition of calcium carbonate to the medium serves to produce the salt, calcium gluconate, and thus prevent development of too acid conditions.

The conversion of glucose to gluconic acid requires oxygen so this mold is grown as a "shake culture", in a suitable shaking device, allowing the admixture of oxygen and medium. Other strains of Aspergillus niger have been developed which produce citric acid instead of gluconic acid.

3. Lactobacillus delbrueckii

The production of lactic acid is an old process in which fermentation has played a major role. One of the

organisms producing lactic acid is Lactobacillus delbrueckii, which utilizes sugars such as glucose for its production. During the fermentation, the consequent increase in acid content will lower the pH of the medium to a level where growth will soon cease. Therefore, it is necessary to add calcium carbonate to a medium in which this organism is growing; this removes the lactic acid by conversion to calcium lactate.

This organism has no cytochrome or cytochrome oxidase, and no catalase, but because of the flavoprotein enzyme present, it is able to utilize oxygen of the air for its metabolic processes. However, because hydrogen peroxide is formed and the organism has no catalase present, it ceases to grow because of the accumulation of this peroxide.

This organism is classified as a homofermentative lactic acid bacterium; i.e., it produces only lactic acid. It is grown best at 45° C., so is one of the thermophilic bacteria. Riboflavin is a required metabolite which must be added to the medium for growth.

4. Acetobacter suboxydans

Another of the aerobic organisms requiring oxygen is Acetobacter suboxydans. The Acetobacter species includes the acetic acid organisms and are of great importance industrially. Acetobacter suboxydans is able to selectively

dehydrogenate the fifth carbon atom of the sugar alcohol sorbitol, giving rise to the keto sugar sorbose. The reaction is aerobic, so shake cultures are used. If the organism is allowed to grow in a flask which is left undisturbed a pellicle or scummy growth will result, possessing a characteristic stale odor. Japanese workers (7), by use of the Thunberg technique, have shown strong glucose oxidase and gluconic acid oxidase activity in Acetobacter suboxydans. King and Cheldelin (74), however, have investigated the oxidative metabolism of this organism and say that the citric acid (Krebs) cycle does not play a major role, if any, in Acetobacter suboxydans.

Because Acetobacter suboxydans lacks catalase, the hydrogen peroxide which is formed during intensive oxidation is not removed by decomposition, thereby causing a retardation of growth. Russian workers (99) have added catalase to the media of two Acetobacter organisms, Acetobacter melanogenum and Acetobacter suboxydans and noted an increase in the rate of oxidation of sorbitol to sorbose. Blood was also found to replace catalase in regard to this increase in rate.

It was shown in the discussion of Saccharomyces cerevisiae that positive pressures of carbon dioxide would halt cell multiplication and even cause death (123). This effect was also noted with Acetobacter organisms.

5. Lactobacillus casei

Another bacterium of the Lactobacillus species was used in this study; like Lactobacillus delbrueckii it has no catalase or hematin pigments (133), but in addition, it does not seem to have any biological system for carrying out the oxidation of substrate. Oxygen is not toxic to this organism, and no hydrogen peroxide is formed.

Biotin and folic acid are required for growth (146) and improved growth will result from alanine, isoleucine, threonine, methionine, lysine, and histidine (133), although many other amino acids must be present as requirements as well as riboflavin. The use of yeast extract and peptonized milk powder in the medium will supply these acids, however, so these materials are used, along with glucose and calcium carbonate to control the pH. Because of these amino acid requirements, Lactobacillus casei is often used in the microbiological assay for certain amino acids. The optimum temperature for cultivation and growth is 30° C.

6. Clostridium acetobutylicum

All bacteria classified as Clostridia are anaerobic Gram positive rods, and differ from the other organisms used in this study in the respect that they are spore-formers. Clostridium acetobutylicum is a non-pathogenic member of the Clostridium species, and produces butanol, acetone, and

ethanol in a ratio of 6:3:1 using grain starches as a substrate. Use of a cooked whole grain mash suffices to provide the necessary nutrients and starch for the fermentation.

Clostridium acetobutylicum can degrade starch because of the presence of the two enzymes, amylase and maltase, which act together to produce glucose. This can then be metabolized by the organism. Stephenson (133) has stated that both amylase and maltase are formed if the organism is grown on corn meal, but when yeast autolysate and maltose are used, only maltase is formed. If sucrose or glucose replaces maltose, however, neither amylase nor maltase will be produced, so the organism apparently produces amylase or maltase only if either of them is needed. The maltase of Clostridium acetobutylicum is a "true" maltase; i.e., it acts specifically on maltose and not on α- glucosides (such as sucrose). French and Knapp (44) found, however, that although glucose was the only product resulting from the action of maltase on maltose, the maltase of Clostridium acetobutylicum will remove glucose units individually from the non-reducing terminus of a starch chain resulting in extensive hydrolysis of starch.

Clostridium acetobutylicum produces butyric and acetic acids as does Clostridium butyricum, but goes a step further, reducing these acids to produce the corresponding four and two-carbon alcohols, respectively, as well as acetone. The latter compound arises from acetoacetic acid through

decarboxylation as shown by Johnson, Peterson and Fred (72). A very interesting study of the origin of the butanol, ethanol and acetone by means of added acetic and butyric acids and acetone which were labeled with C^{13} has been made by Wood, Brown and Werkman (148). It shows that added acetate is not quantitatively converted to acetone, but much of it appears as butanol (55 per cent). This organism has a hydrogenase and also dehydrogenases for glucose and pyruvic acid. The decarboxylation of acetoacetic acid is possible because of acetoacetic acid decarboxylase which is present and which has been isolated. The variety of products produced by Clostridium acetobutylicum is the reason why this organism has been studied extensively and is being used in industrial processes.

C. Organometallic Chemotherapy

1. History of organometallic compounds

When Frankland attempted the preparation of free ethyl radicals in 1849 by reacting ethyl iodide with zinc metal, he obtained the first organometallic compound prepared by man; diethylzinc (43). He extended his work to include compounds of arsenic, antimony and phosphorus, thereby laying a firm foundation upon which the entire field of organometallic chemistry has grown. The observation of the use of the maximum possible valences of the metals producing

organometallic compounds was hailed by some early workers who supported the then current views held by Kekule in the fixity of valence (50). An example of this behavior on the part of organometallic compounds is tetraphenyllead. Normal inorganic salts of lead have divalent lead present, whereas the organolead compound has lead with a valence of four. Even hydrolysis of tetraphenyllead by acids to give, for example, diphenyllead dichloride will yield a product in which the valence of lead is still four.

Ehrlich's research in the field of chemotherapy resulted in other early contributions to the organometallic field when he prepared organoarsenic compounds and applied them to the treatment of syphilis and trypanosome infections such as sleeping sickness. Ehrlich was interested in the curative properties of dyes and attributed these properties to the nitrogen atoms of the azo linkages. Thus he began research on a lower member of the Group V elements, arsenic (40), and from this interest came arsphenamine (Salvarsan), atoxyl, neoarsphenamine and others which advanced the science of organometallic chemistry in the chemotherapeutic field.

Before Ehrlich's discoveries, Victor Grignard had expanded and developed the earlier discoveries of his teacher, Barbier, and had presented a method of preparing organomagnesium halides which possessed such a fairly high degree of reactivity as to make them extremely valuable as intermediates in the synthesis of alcohols, hydrocarbons, acids, etc. This work has had a very large influence on the methods used today

for the preparation of organometallic compounds which employ other elements as the metal atom.

An early preparation of di-sec-butylmercury occurred in 1906 when Tafel (137) isolated this compound as a result of its formation by methyl ethyl ketone and sulfuric acid in an electrolysis which employed a mercury cathode.

Other historical examples could be given for the early preparation of organometallic compounds, such as dicyclohexylmercury (59), tetraphenyllead, in 1914, tetracyclohexyllead and tetracyclohexyltin (60), or diphenyldi-o-tolyllead (84) in 1916, but these should be sufficient to show that widespread efforts on the part of many organic chemists helped to bring about the rapid expansion of the ever-growing list of organometallic compounds which led to the great variety of today. New methods of preparation are constantly being found, and industrial uses exploited in this rather "young" branch of chemistry.

2. Nature of organometallic compounds

The physical properties of organometallic compounds are similar to those of any compound one might use for comparison. One is known which is a gas and the others are liquids or solids. Those of low molecular weight are liquids at room temperature, as there is only one gaseous organometallic compound, trimethylboron (50). A well known organolead

compound in wide use today, tetraethyllead, for example, is a heavy, colorless liquid, insoluble in water, having a deceptive, pleasant fruity odor in spite of its highly toxic nature. The corresponding tetraaryllead derivatives such as tetraphenyllead, are colorless solids with definite melting points. Like the tetraalkyllead derivatives, they are insoluble in water, but soluble in benzene, chloroform, and carbon disulfide (23).

The thermal stability of organometallic compounds covers a large range from the extremely unstable compounds to those which can be distilled at atmospheric pressures. Some compounds will decompose spontaneously yet are not chemically reactive, such as organosilver and organogold compounds; methylsodium decomposes with spontaneous flammability and also shows high reactivity, while tetramethyllead can be distilled at atmospheric pressure, and distills without decomposition at 110° C. (23). Ultraviolet irradiation was shown to lead to the breakdown of diphenylmercury (113), depending on the solvent used for these studies. In ethyl bromide, isopropyl bromide or bis-(β-chloroethyl)-ether, diphenylmercury gave 74, 59, and 71 per cent yields of benzene and phenylmercuric bromide or chloride in 12 to 15 hours, respectively. Chlorobenzene as a solvent gave calomel and some tars in 120 hours, whereas bromobenzene gave phenylmercuric bromide (40 per cent) and unreacted diphenylmercury (50 per cent) in 18 hours. No decomposition occurred in carbon

disulfide or carbon polysulfide solutions. Because of these observations it is best to keep organometallic compounds and solutions stored in a dark place.

A classification into three groups based on chemical reactivity can be made for all organometallic compounds. These three types are: (1) highly reactive, (2) moderately reactive, and (3) relatively unreactive (50). As organometallic compounds are best employed, in a synthetic sense, by addition to a double bond of another reactant, they have been classified on this basis, placing all those of type 1 in the class of compounds which add to the olefinic and carbonyl double bonds. Type 2 organometallic compounds add only to the carbonyl double bonds, and type 3 add to neither the olefinic nor the carbonyl type of bond. Thus one finds the organoalkali compounds in type 1, the Grignard reagents, organocalcium, organomagnesium and other types in type 2, and organolead, organomercury and organotin compounds in type 3 (50).

Various studies of the nature of the carbon-to-metal bonds of organometallic compounds have been made. Anantakrishnan (5) has examined alkali alkyl compounds (such as ethylsodium, methylpotassium) and found them to be essentially salt-like as the carbon-metal bond was ionic in character. He showed that the melting and boiling points of such compounds are also in agreement with this postulated ionic character, and in addition, he cited the spectroscopic

studies of Frank and Herzberg, the electronegativity values obtained by Gordy (58), as well as potential energy curves of Baughan et al. (15). Smyth (130), in a study of the dipole moments of organic molecules containing mercury, germanium, tin, lead, antimony, and chlorine, bromine and iodine, found the bonds between carbon and metal to be essentially covalent. Those joining metal with the halogens in the same compound were as ionic as bonds in typical salts where the bond consists of oppositely-charged ions.

These observations show that metals of the periodic table differ in their properties when present as inorganic compounds as contrasted to organometallic compounds. It can be seen that tetraphenyllead would have four covalent bonds from the lead atom, rather than the usual ionic valence value of two usually ascribed to this metal. These findings have aided greatly in explaining a part of the chemical behavior and reactivity of organometallic compounds and in understanding the reasons why one can classify these compounds into three groups based on their reactions with unsaturated double bonds.

These compounds can also be differentiated on the basis of their case of cleavage by acids, halogens, water, etc. It has been found that unsymmetrical organometallic compounds, such as RMR', are cleaved more readily than symmetrical molecules. Diphenylmercury is sufficiently stable that it will not be decomposed in the presence of water, and

tetraphenyllead will resist acid cleavage to a greater degree than will triphenyl-p-tolyllead, which is an unsymmetrical molecule.

A more complete discussion of the chemical reactivity of organometallic compounds has been given by Gilman in his Treatise on Organic Chemistry (50).

3. Chemotherapeutic properties of organometallic compounds

The chemotherapeutic approach to treatment of diseases involves the inhibition or death of the invading organism, so that chemotherapeutic agents must be able to accomplish this action by any means possible. There are multitudinous compounds which will and can accomplish this, and many have been tried on a number of organisms. Kinsey and Grant (75) tested the toxicity of mustard gas, a deadly war gas, and other poisons on yeast cells; Higuti (65) studied the inhibition of triphenylmethane dyes on different strains of Saccharomyces, while Fels and Cheldelin (38, 39) have used selenium (as hydrogen selenate) in their studies of its toxicity and its reversibility by sulfate or methionine. These three examples differ widely in their structure and mode of action, in all probability, yet they all accomplish the same thing; i.e., they inhibit the growth of yeast cells.

Thus it is of interest that organometallic compounds are yet another class apart from the three mentioned above which

can show toxic effects to microorganisms and therefore qualify as potential chemotherapeutic agents. Metals have played an important part in chemotherapy for centuries; mercury was used in the second century as the metal and as salts, while antimony seemed to enjoy popularity in the sixteenth century. For example, wine was allowed to stand in antimony goblets until it acquired the properties of an emetic, and solid antimony pellets were swallowed by patients and recovered from the feces for reuse after they had fulfilled their function (149).

It was not until Ehrlich's work on triphenylmethane dyes as chemotherapeutic agents interested him in the properties of the nitrogen that he considered the neighbor of nitrogen in Group V of the Periodic Table, arsenic, and thus began to synthesize organoarsenic compounds, giving organometallic chemotherapy its beginning. Other organometallic compounds had been synthesized prior to this date (59, 60, 84, 137), but Ehrlich was the pioneer in their application to human diseases. The organoarsenic compound, cacodyl, was discovered in the eighteenth century by Cadet (21,100) and the structure elucidated in 1840 by Bunsen, but it was very odoriferous and extremely toxic, so was of little use other than as a laboratory curiosity.

An interesting variation of the poisonous cacodyl molecule is that of cacodylic acid in which oxidation has produced a compound whose sodium salt has been used in medicine as a

tonic, as well as for syphilis and malaria (21). The two structures are given below:

$(CH_3)_2$ As - As$(CH_3)_2$ $(CH_3)_2$ As-OO-Na • 3 H_2O

CACODYL CACODYLIC ACID TRIHYDRATE (Sodium Salt)

Carlson (24) has confirmed the passage of cacodylic acid through the organism (human beings, here) in an unchanged form. It is not changed into a toxic inorganic form of arsenic as most of the acid was found to be excreted unchanged in the urine, although some is converted to a volatile cacodylic oxide and is largely eliminated in the expired air.

Ehrlich's first contribution, atoxyl, was so named because of a preliminary belief that it was not toxic. Clinical use established that it was, but the name remained. Atoxyl is p-aminophenylarsonic acid, although it was originally given the wrong structure. Further research by Ehrlich, however, corrected this mistaken belief and also led to synthesis of other organoarsenic compounds which have been used for chemotherapeutic treatment of syphilis; sleeping sickness, and other trypanosome diseases. Disadvantages of atoxyl, which was renamed arsanilic acid, were the possibility of blindness and the generally toxic nature of the arsenic atom throughout the body. The mercury salt of atoxyl was also used for syphilis treatment (98).

The action of the arsenic compounds was found to be due

to reduction products in which trivalent arsenic is present. The reduction products such as R-As = AsR and R-As = O were said to be very fatal to trypanosomes (36); a solution of p-hydroxyphenylarsenic oxide (p-HOC_6H_4AsO) with a dilution of 1:10,000,000 was fatal. The action of the substituted arsenic oxides is believed to be due to the reaction of the oxide with two adjacent mercapto groups to form a stable five-membered ring, which is dependent in part, upon the pH of the medium for its stability (22):

$$R\text{-}AsO + 2\ R\text{-}SH \underset{\text{alkaline}}{\overset{\text{neutral or acid}}{\rightleftharpoons}} R\text{-}As\begin{matrix}S\text{-}R\\S\text{-}R\end{matrix} + H_2O$$

The presence of -SH-containing compounds, glutathione, cysteine, or thioglycolic acid, can cause a lack of toxicity of the arsenic compound to the microorganisms, supposedly because these -SH compounds will compete with the enzymes of the microorganisms for the arsenic compounds. This has been shown to occur with compounds of arsenic, bismuth and mercury in the lack of inhibition against Treponema pallidum when glutathione, cysteine or thioglycolic acid was present in sufficient excess (35). No effect was obtained with methionine or thiamine chloride when a sulfide type of sulfur link is present.

4. Recent studies of organometallic chemotherapy

In the last 25 years, much work has been done on the

effect of metallic compounds in chemotherapy, and it may be generally stated that when an inorganic compound of a metal is used and found toxic, the aromatic organometallic compound of the same metal is far less toxic. Bischoff (19) conducted a study on the toxicity of lead compounds and classified them on the basis of their destructive effect on erythrocytes. The lead derivatives of glycerophosphate, oleic and palmitic acids were most destructive, followed by the basic carbonate, oxychloride and carbonate. Tetraethyllead and triethyllead chloride were reported as very slightly toxic, while lead sulfide and lead acid phosphate had no destructive effect. This report would give some promise for masking the normally toxic reaction of lead by attachment of alkyl or aryl groups to the metal.

Levaditi and Lepine (85) made a study of 45 elements against spirilloses, trypanosomes and syphilis, and found that gallium, indium and platinum have slight effects, while vanadium, arsenic, antimony, tellurium, gold, mercury and bismuth have a more or less pronounced effect. A later study by Levaditi and co-workers (86) showed that a tellurium suspension had no effect against herpes virus. Sollman et al. (132) reported on 12 different mercurials and found that 31 to 77 per cent of the mercury of the diffusable mercurials (those excreted in the urine) was fixed indefinitely in the tissues where it had no therapeutic value and was potentially harmful.

A report of magnesium chloride in rabbits (4) is not one showing organometallic properties in regard to the compound used, but serves only to give the effect of magnesium metal ions. The author reported immediate azotemia in a rabbit which was injected with magnesium chloride, and which had been given a previous experimental uremia. Mudd (101) examined bacterial cells treated with salts of silver, lead, mercury and nickel, and found structural changes due to penetration of these heavy metal ions into the cells.

Gilman (49) reported on biological applications of organometallic compounds and stated that the biological action of metals and inorganic salts may differ widely from organometallic compounds. He placed metal hydrides as the lowest members of the organometallic series and exemplified them as the "link" between organic and inorganic chemistry because a hydride can be thought of as the first member of the homologous series in such compounds as these: NaH (sodium hydride); $NaCH_3$ (methyl sodium); $NaCH_2CH_3$ (ethyl sodium) and so on.

A series of alkyl- and aryl- mercuric acetates has been synthesized by Coleman *et al.* (29) to determine the relative toxicities of the attached organic radicals *versus* the toxicity of the compound to bacteria. In aliphatic radicals, the toxicity of $RHgOOCCH_3$ increased uniformly from methyl to butyl. The toxicity of the aromatic series was found to be greater than the aliphatic, although no significant difference among the aromatic acetates was noticed. The toxic

effect of organomercury compounds is often enhanced for the purposes of disinfection rather than purposely suppressed, or masked, as in medicinal applications. Bonrath and Klös (20) reported a series of organomercury acetylides as seed-grain disinfectants, and Gassner (46) confirmed the use of mercury alkyls, especially methyl mercury compounds, as good fungicides suitable for seed-grain use, having a chemotherapeutic index of one-tenth (0.1). A number of organic hydroxymercuri-type compounds were rated on the basis of their toxicity to Staphylococcus, Escherichia coli or Eberthella typhi; the antibacterial activity was found to be lowered by the introduction of a nitro group (90). This effect is similar to that noted by Ehrlich with the arsenicals. Levine found alkylmercuric chlorides and dialkylmercury compounds active against horse strongyle larvae at concentrations of these organometallic compounds of 0.0005M, whereas triphenylarsenic, triphenylbismuth and tributylborate were all inactive at 0.01 M (87).

Other metals have been tested for their activity in many ways. Archdeacon et al. (6) tested radioactive gallium, gold and hafnium in rats, using intravenous injection, checking their physiological action. Much metal was found in the liver, and gallium was found in the bile, but no impaired liver function resulted. Becker et al. (18) used 3-nitro-4-hydroxyphenylarsonic acid in swine feeding, and noted that although it has some ability to improve performance, it did

not show any significant complementary effect with aureomycin for growth stimulation in swine. An interesting recent report (1954) by Rosenfeld (119) using albino rats showed that germanium, a member of the Group IV-B elements which also includes tin and lead, is relatively inert metabolically, has low toxicity, and is not bound by tissue proteins as are arsenic, mercury and many other metals.

In the industrial field, the use of phenylmercuricdinaphthylmethane disulfonate in paper manufacturing is being tested as a fungicide against C. globosum, Aspergillus niger and a Penicillium species (115). In a concentration of 0.25 per cent, growth of these organisms is prevented. This organic mercury compound is also used as a wound dressing.

This review is not complete but is intended to point out the type of research now being conducted in the field of organometallic chemistry aside from the purely synthetic approach. For a more thorough treatment of organometallic chemotherapy, the reader is referred to other sources (22, 70).

D. Quaternary Ammonium Compounds

1. History of quaternary ammonium compounds

The background of quaternary ammonium compounds as of 1954 is short but extensive because of the rapid progress in

this field. It was only recently that Domagk (34) described the compound, Zephirol, which he revealed to be an aqueous 10 per cent solution of mixed alkyldimethylbenzylammonium chlorides. Prior to this, however, Hartmann and Kägi (63) reported on the antibacterial action of surface-active cations. Zephirol was reported to be colorless, odorless, non-irritating, not affected by proteins and easily wet. Domagk found it to be effective against Staphylococci, Streptococci, Pneumococci, diphtheria organism and others at dilutions of 1:10,000 to 1:50,000.

Zephirol, sometimes called Zephiran, was found to possess very good antibacterial activity if the alkyl substituent was of the order of 8 to 18 carbon atoms (143). This newly-discovered type of bactericide gave great impetus to the study of other compounds which would accomplish similar results. The main line of thought and research has centered about compounds which will markedly reduce the surface tension of aqueous solutions, and from this type of thinking has evolved cationic, nonionic and anionic surface-active agents. This brief survey will consider only those which are known as cationic compounds, in which the largest and most important part of the molecule carries a positive charge. The most commonly known type, and the one that will be discussed here, is the substituted ammonium cation in which all hydrogen atoms are replaced by alkyl, aryl, or aralkyl groups, and with which is associated an inorganic

anion to give an electrically neutral molecule. The resultant molecule therefore has a quaternary ammonium ion carrying a single positive charge and a monovalent negative anion. It may be expressed as:

$$R_4 N^+ X^-$$

quaternary ammonium compound

An historical accounting of these compounds is so intimately associated with the chemical, physical and physiological nature of these quaternary ammonium types, that a record of their introduction to science will, of necessity, overlap with other phases of the discussion.

2. Nature of quaternary ammonium compounds

The physical characteristics of quaternary ammonium compounds underline their most outstanding features, because the reduction of the surface tension of aqueous solutions is the chief reason for the interest in this kind of molecule. Quaternary compounds are usually colorless solids, and possess definite melting points. Decomposition occurs upon heating beyond the melting point, however. They are soluble in water as a rule, although some form colloidal gels which liquefy on warming, indicating that they do not form true solutions. Solubility and surface tension characteristics can change by the choice of the R groups about the nitrogen, or by choice

of the anionic part of the molecule.

The chemical behavior of quaternary ammonium compounds is relatively simple. By suitable reaction, the anionic part of the molecule can be changed, thus altering its physical properties. The manner of decomposition of quaternary ammonium hydroxides upon excessive heating to yield a tertiary amine is a well known reaction. The Hofmann Rule says: "In the decomposition of quaternary ammonium hydroxides, that olefin will be formed which will have the smallest number of alkyl groups attached to it." (3) Use of this behavior has been made in organic syntheses where a desired olefinic-type of compound is sought. The proper quaternary ammonium molecule is synthesized and the desired compound obtained by what is known as a Hofmann reaction.

Alkylations with quaternary ammonium salts have been described by Snyder et al. (131) in which reaction with a carbanion results in loss of the desired R group from the quaternary salt to the carbanion, and production of a tertiary amine as a by-product.

Chemically, quaternary ammonium hydroxides are very strong bases, equaling sodium or potassium hydroxide in their caustic attack on glass containers. They are prepared by reacting a quaternary ammonium halide with silver oxide and filtering the aqueous solution of the hydroxide. Removal of water usually gives crystalline material which will possess water of hydration. These bases find use as catalysts in

organic synthesis.

Of greatest interest here is the physiological nature of quaternary ammonium compounds. Their first mention by Hartmann and Kägi (63) and Domagk (34) included a report on their potential antibacterial activities, and many papers and reviews have reported new findings in this line of thought (11, 16, 67, 78, 83, 116, 142).

Among the earlier workers were Kuhn and Bielig (81) who demonstrated the precipitation of proteins by surface-active agents in 1940. Enzymes such as pepsin, trypsin, insulin and catalase were affected when they were on the alkaline side of their isoelectric points. An excellent study of the action of cationic, nonionic and anionic detergents on microorganisms was undertaken in 1941 by Baker et al. (11), who tested many such compounds under different conditions. The pH of the medium was found to be instrumental in enhancing the action of detergents against microorganisms. For example, cationic-type compounds are more bacteriostatic in alkaline solutions than in acid, while the reverse is true for the anionic types. This has been explained by some as due to the existence, in alkaline media, of reactive groups on protein molecules in a negatively-charged state which will thereby produce an increased attraction for the large, hydrophobic, positively-charged cation in the cationic-type of detergent. In acid media, more positively charged groups would be present, offering less attraction for the positive

cation.

Baker and his co-workers also remarked that the "wetting power" of a compound; i.e., its ability to reduce the surface tension, is not the sole reason for its germicidal activity. The cationic compounds were less effective as detergents than were the anionic types, but the former were superior germicides; they inhibited Gram positive and Gram negative organisms, whereas the anionic type selectively inhibited Gram positive organisms. Another interesting observation was concerned with the lack of inhibition from lecithin. Lecithin has a quaternary ammonium hydroxide structure, yet it did not inhibit the metabolism of three different Gram positive and three Gram negative organisms; instead, metabolism was stimulated in most cases.

An interesting variation of quaternary ammonium compounds has been the use of other Group V elements and a Group VI element in place of nitrogen. Thus Kuhn and co-workers have prepared organic sulfonium iodides (82), ammonium chlorides (83), phosphonium and arsonium chlorides (71) by replacing the nitrogen of a quaternary ammonium compound by sulfur, phosphorus and arsenic, respectively. These compounds were tested against Escherichia coli, Staphylococci, and other microorganisms and the effectiveness of different chain lengths was studied. The chain lengths varied from eight to sixteen carbon atoms; all compounds were solids, melting at a definite temperature for each individual entity, in a range

of 144-146° C. for C_8 to 181-183° C. for C_{16}. Their preparation was identical in type of reaction to that used for quaternary ammonium types.

A review of antibacterial surface-active substances by Valko and Du Bois in 1944 (142) related interesting information about the reversal of the bactericidal effect of Zephirol, cetylpyridinium bromide, and acriflavine (a mixture of 2,8-diaminoacridine hydrohloride and 2,8-diamino-10-methylacridine hydrochloride) by Duponol PC (sodium dodecyl sulfate), an anionic detergent. In a number of experiments, the authors have postulated from their results that the antibacterial behavior of surface-active cations is in agreement with the action of toxic metallic ions and dye cations because all of them can be classed as phenomena resulting from ionic exchanges by bacteria. Therefore, they do not regard surface-activity as an important part of antibacterial activity, and go on to say that adsorption is not a surface phenomenon associated with the bacterial cell surface because mercuric ions and dye cations are strongly adsorbable, yet have low surface activity. In a study of highly toxic surface-active cations the presence of relatively harmless surface-active cations was found to be subtractive, due to a shift in the "adsorption equilibrium" between the two molecules via their competition for the same sites on the bacterial cell. These sites were presumed to be the -COOH (carboxyl) groups of proteins, and to read of them reminds one of

Ehrlich's "chemo-receptor" theories in the action of arsenicals in chemotherapy.

A study of the inhibitory action of Zephiran by Sevag and Ross in 1944 showed that the inhibitions on yeast were counteracted by horse serum, phospholipids and high molecular weight anions (125). The inhibition by horse serum is perhaps contradictory to Domagk's earlier reference to Zephiran (Zephirol) when he said that it was not affected by proteins.

Many other studies of the mode of action of surface-active compounds against microorganisms have served to extend the knowledge of their bactericidal effect. Hotchkiss (67) observed a leakage of nitrogen and phosphorus compounds from Staphylococci, Streptococci, Escherichia coli and Saccharomyces cerevisiae due to cytolytic damage caused by these types of compounds. Russian workers using yeast noted the liberation of lipoids and destruction of lipo- and nucleo- protein cell components (97) with cetyl-zephirol. The cytoplasm and chondriosomes were quickly affected and the proteolytic activity of the cells was increased. Hotchkiss thus attributed the lethal effect of the quaternary ammonium compounds to establishment of equilibrium between the internal and external media of the cells, causing a drastic dilution of metabolically active components. He did not attribute a depression of the metabolic activity to any effect of the detergents on the enzyme systems of the cells as the concentrations of the former were below those which would depress the enzyme

activity in suspensions of broken cells. Werkman and Wilson (146) confirm these findings in their text, attributing the denaturation of proteins or breaking of the osmotic barrier to "mechanical strain imposed by attachment of detergent."

Knox and co-workers, however, attributed the metabolic inhibition, cell death and increased permeability in bacteria to the specific action of detergents on sensitive enzymes such as lactic acid oxidase. Massart and his associates (32, 92) have examined the acridines which form quaternary compounds in which the nitrogen is part of the aromatic polycyclic nucleus. These compounds inhibit respiration and yeast cell multiplication, forming adsorption complexes with nucleoproteins, thus displacing catalytically active ions.

Beck's studies (16, 17) on adsorption of detergents show a similarity between the adsorption on erythrocytes and on lecithin, and also between adsorptive carbon (Norit) and yeast cells. He therefore attributes the activity of quaternary ammonium compounds to (a) reaction specifically with the lipids of red blood cells or with lecithin, whose structure resembles fats, and to (b) adsorption on Norit or on yeast cells. Cationic detergents were adsorbed more strongly than anionic types, although no correlation between adsorption on yeast and the disinfectant action towards yeast was found.

Voets (143) related the reduction of surface tension to the antibacterial efficiency of detergents, as well as pointing out the dependence of the efficiency upon the molecular

structure and the positive and negative charges. Sexton, on page 253 (126), reviewed quaternary compounds and pointed out that the nature of the anion of cationic surface-active compounds has little effect other than on the solubility of the entire molecule. Another interesting mode of action was given, however, when it was stated that cationic surface-active substances can sever the loose union between coenzymes and proteins as well as that between carotene and chlorophyll and their naturally-associated macromolecular carriers.

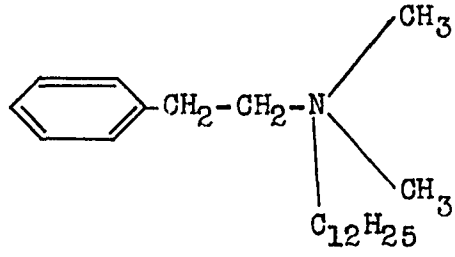
3. Recent studies of quaternary ammonium compounds

Older studies related in the preceding section have been concerned with theories of action and the effects of well known, generally similar compounds upon pure, unadulterated strains of various microorganisms. More recent literature (since 1950) has centered about the effect of altering the molecular structure of these detergents in experiments designed to determine the effect, on the bactericidal power, of altering the chain length in one of the R groups. This has been carried out by Reck and Harwood (114) who synthesized quaternary ammonium chlorides with 16 to 18 carbon atoms in one of the R groups, and methyl groups in the other three positions on the nitrogen. The long chains possessed varying degrees of unsaturation, but this alteration in the molecule did not alter the bactericidal properties to any significant

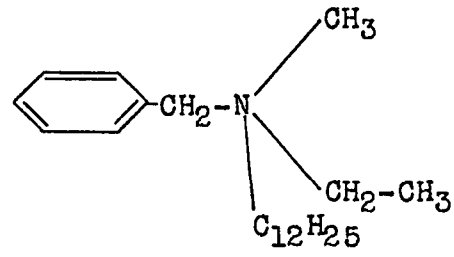
degree. The C_{16} chain in combination with three methyl groups around the nitrogen proved to be the most satisfactory of those tested. A series of syntheses were undertaken of quaternary bromides involving a benzyl group, two β-hydroxyethyl groups and a β-alkoxyethyl group on nitrogen. These compounds were prepared by Sturm (135) and had alkyl groups of 12, 16, and 18 carbon atoms in length. The antibacterial tests were inconclusive except that they indicated a low toxicity for these compounds.

The relationship between structure and bactericidal action was also investigated by Cella (25). The length of the higher aliphatic chain, and substitution on the phenyl ring of phenyl- or benzyl- type compounds are factors affecting bactericidal activity. Cella gave examples to show that the positive (+) character of the cationic compounds was to be associated with high activity, stating that the higher the charge density on the nitrogen atom, the greater was the bactericidal effect of the detergent. His examples were two compounds listed in the order of decreasing bactericidal action: meta-trifluoromethylphenyl compound phenyl because the highly electronegative trifluoromethyl ($-CF_3$) group competes with the nitrogen for the ring electrons, thereby increasing the (+) charge "density" on the nitrogen. Cella attributes the effect of differences in bactericidal activity resulting from added methylene groups between a phenyl group and the nitrogen to be caused by an electrical rather than a

bulk effect of added carbon atoms. Isomer A (see below) killed Micrococcus aureus in 0.73 minute while isomer B required 10 minutes.



A



B

Cella (25) related the bactericidal activity of cationic colloidal electrolytes to the tendency of these quaternary ammonium compounds to leave solution as manifested by their ability to form colloidal micelles. He also stated that lower molecular weight compounds do not exhibit these surface-active properties.

In Sexton's review (see page 91) (126) was noted the effect which the conversion of a tertiary nitrogen atom to a quaternary nitrogen would have upon well known compounds. The change from trimethylamine to a tetramethyl ammonium ion will produce a compound which exerts marked activity on the nervous system, while compounds of high antibacterial activity are produced if one of the methyl groups is replaced by dodecyl, for example. Nicotine, when converted to an N-alkylnicotinium ion, loses its former insecticidal activity, and N-alkylquinine ion loses its antimalarial power, giving instead a curare-like activity which is not shown by quinine hydrochloride.

The adaptation of Serratia marcescens cultures to dimethylbenzylammonium chloride has been achieved by Chaplin (26) who succeeded in growing this organism in detergent concentrations as high as 100,000 p.p.m. whereas the cultures were suppressed previously in concentrations of only 63 p.p.m. Evidence of cell changes were seen in an intense staining of the resistant cells by Sudan black B, an increase in the ether-soluble fraction, a decrease in electrophoretic mobility and the removal of the cell's resistance by the action of lipase. These observations led Chaplin to assume that cellular excretion of a lipid which is retained on the cell surface is responsible for the increased resistance of these cells. This lipid is capable of withstanding the disruptive forces exerted on the cell by the detergent. The possibility was also advanced that this lipid may be a lipid-protein complex which occurs in Gram negative bacteria only.

E. Respiration and Metabolism of Microorganisms

Although the exact nature of the mechanisms involved in respiration and metabolism is not known, the understanding of the enzymatic reactions which occur has expanded greatly in the last score of years.

Microorganisms have been grouped conveniently into two classes; aerobic and anaerobic, with subdivisions under each

heading implying partial adherence to either of the two types of activity. The discovery of the action of the dehydrogenases, oxidases, flavoproteins and cytochrome systems have elucidated the means by which enzymes aid in the utilization of oxygen by bacterial cells. Similarly, the advent of the Meyerhof-Embden-Parnas scheme for anaerobic metabolism and the Krebs cycle for aerobic oxidation of acetate have given a logical evaluation of the fate of carbohydrates in bacterial metabolism.

A large number of studies on the respiration and metabolism of microorganisms has been carried out, and it is the purpose in this section to refer to representative literature references which tell of the many different modes of study of these problems.

Cyanide ion is a well known inhibitor of oxidases and has been studied widely. Winzler (147) has proposed three ways in which this occurs: First, the usual inhibition may occur; second, an increase in the apparent Ko_2 of the reduced respiratory enzymes in a non-competitive manner may occur, and third, a combination of cyanide with the enzyme system to thus limit the maximum rate of respiration in the absence of inhibitor. Azide was found to be more specific as an inhibitor of the respiratory enzymes than cyanide.

Lachrymators were investigated by Macworth (89) in 1948 and a progressive and irreversible inhibition was found with thiol (-SH) enzymes; yeast respiration was not irreversibly

inhibited by chloropicrin, this being the only such case found with lachrymator. The importance of concentration was emphasized when it was found that the respiration of yeast could be either stimulated or depressed by altering the amount of fluoride used (105).

Investigations of organic compounds have been made; benzoic acid inhibits the D-amino acid oxidase for D (+)-methionine, but will not interfere with transmethylation reactions of L (-)-methionine (62). A series of 3-alkyl-2-hydroxynaphthoquinones showed apparent activity against respiratory enzymes which acted below cytochrome c and above cytochrome b by a possible combination with the unknown enzymes (12). Also investigated and found inhibitory to yeast were a series of organic compounds such as trypaflavin, methylene blue, crystal violet, protamine sulfate and quinine. The effects of these agents were counteracted by streptomycin and other organic compounds (93).

Selzer and Baumberger (124) stirred a yeast cell suspension with minutely-divided metallic mercury with glucose as a substrate, obtaining complete inhibition of oxygen consumption. Use of lactate or pyruvate as a substrate gave no inhibition, but the possibility of reaction of the mercury with a thiol group of the enzyme inhibited was considered. Four years later, in 1946, Cook and co-workers (30) investigated the effect of the organometallic compounds, phenylmercuric nitrate, on enzymes and demonstrated complete or

partial inhibition depending upon the enzyme and/or the molar concentration used; succinoxidase was completely inhibited while catalase suffered only 36 per cent depression of activity. Later work by these workers (31) demonstrated a depression of cytochrome oxidase and of yeast respiration by basic phenylmercuric nitrate. This depression could be prevented but not reversed by addition of -SH-containing compounds. Mercuric chloride inhibited the action of ox liver catalase in the decomposition of hydrogen peroxide, according to Okamoto and Nagayama (107).

Synthetic detergents, both of the anionic and the cationic types, were shown to inhibit bacterial metabolism as was stated previously (11), while other workers found that non-toxic concentrations of trimethylhexadecylammonium bromide would protect yeast against respiratory inhibition caused by uranyl, thoric and silver ions if the former was added first. However, in the presence of either cupric or mercuric ions, this detergent increased the inhibition. Possible competition for active sites on the bacterial surface was suggested, possibly the formation of complexes with amino groups (95). Other cases can be cited for protection of the respiration of yeast by salts (94), or the increase of respiration by concentrations of less than one per cent where higher concentrations are inhibitory (76). Examples of the latter include mercuric chloride and sodium fluoride.

Metabolism inhibition studies, like those on respiration,

have followed similar lines. The effect of sodium fluoride (111), metabolic inhibition by dyes (65), mustard gas and derivatives (75), para-aminobenzoic acid (PABA) (138) and benzoic acid (118) have been evaluated for yeast. The action of metal ions on phosphopyruvate reactions in Escherichia coli by Utter and Werkman (141), and of organic metal salts on yeast by Kreke and Nadeau (80) were set forth for evaluation. The results demonstrate a variety of effects from that of no action to stimulation or to depression of enzyme and/or metabolic activity. Lupolone and humulone, found in hops, will inhibit growth of yeasts and bacteria as well as some of the fungi which are found to infect beer (10).

Intimately associated with these problems of metabolism and respiration are the enzymes which mediate these reactions in living cells. Of interest is the report that some molds will methylate inorganic compounds of arsenic and tellurium to the corresponding organometallic compounds (49). This was correlated with the synthesis of compounds such as chlorophyll or hemoglobin.

The enzymes of microorganisms are indispensable to their survival; thus they are endowed with extracellular and intracellular enzymes (exo- and endo-enzymes) which are correlated in their activity so as to allow full growth of the cells. The maltase of Clostridium acetobutylicum (44) and other organisms helps to utilize starch as substrate, while other organisms have other enzymes (such as invertase of yeast (33)

cells) for making various types of substrate available. Various persons have studied the cell surface of microorganisms as a possible site of enzyme inhibition, using toxic reagents. These will be discussed later, but the inhibition of enzymes in general must also take into account the cell membrane as inhibitors must be able to penetrate it in order to act upon the intracellular enzymes. Northcote and Horne (106) have examined the yeast cell wall by electron microscopy and have detected at least two membranes. One is composed in part of glucan polyglucides; the second was found to remain intact after the lipids were removed, and to be made of either protein or mannan, or both together. An analysis of the cell wall showed 29 per cent glucan, 31 per cent mannan, 13 per cent protein, 8.5 per cent lipid and 3 per cent ash. Mechanical breakdown of yeast cells did not give any glycogen present. The presence of bud scars was felt to be important to the behavior of the cell wall in its function as an osmotic boundary and the relationship of the scars to the process of budding.

A function of the cell membrane is described by Werkman and Wilson on page 357 (146): "it regulates not only the rate but also the kind of penetration." Thus the type of inhibitory agent used would conceivably vary with the organism and the type of inhibition produced should differ in degree, at least. Quagliozzi and Rescigno (112) found the permeability of Saccharomyces cerevisiae to Cu^{64} to be 7.2×10^{-6} g.

metal per g. of yeast per hour.

Enzyme inhibition may be due to many factors, among them, the inactivation of specific groups. In 1907 the existence of labile thiol groups in tissues was shown, and it was predicted that they would be found to play an important part in respiration. Two years later, Hata (64) demonstrated inhibition of pepsin, trypsin, and several other enzymes with mercuric chloride, and also showed their reactivation by precipitation of the mercury with potassium sulfide. After many years, it has become increasingly apparent that the -SH groups of enzymes are very necessary to respiration and to metabolism. Hopkins and Morgan (66) treated succinic dehydrogenase with GSSG (oxidized glutathione) and found that the enzyme lost its activity. The enzyme was reactivated by GSH (reduced glutathione), thus showing that the GSSG oxidized the essential -SH groups of the enzyme to give a disulfide structure, while GSH reversed this reaction to restore the mercapto (-SH) groups of the dehydrogenase, and thus restore its activity.

Barron and Singer (14) demonstrated the prevention of inhibition of succinoxidase by addition of glutathione (GSH) and divided -SH groups into two types; freely active and sluggish. Those reagents which react with -SH groups were classified into three groups according to their mode of action (oxidation of, or combination with -SH groups) and the degree of reactivity. Heavy metal salts and compounds are toxic in

lieu of their reaction with essential mercapto groups. Ing (69) listed four enzymes; yeast alcohol dehydrogenase, urease, hexokinase and phosphoglyceraldehyde dehydrogenase as extremely sensitive to arsenicals while King (126) listed several methods by which arsenic compounds may act in a cell, saying that their final mode of action is likely to be by combination with an essential sulfhydryl group.

Stoppani has discussed yeast carboxylase in the light of the role played by these sulfhydryl (-SH) groups, and goes on to say that -SH reagents will also inactivate some enzymes such as cytochrome oxidase or catalase which, in all probability, are not thiols (134).

The use of BAL (British antilewisite) as an -SH reagent was introduced in 1945 by Waters and Stock (145) who found it to compete with the sulfhydryl groups of protein for the halogen of the vesicant war gas, Lewisite. Lewisite is β-chloroethylarsenic dichloride, and reacts with -SH groups by reason of the reactive chlorine atoms present. The BAL molecule supplies these -SH groups so that the reaction of Lewisite and protein -SH groups is prevented. Thus BAL has come into wide usage in inhibiting the toxic action of antibacterials, especially where heavy metal compounds are concerned, although BAL may be toxic in its own right for it is a strong reducing agent and will interfere with cytochrome oxidase activity by keeping cytochrome c in the reduced state.

Because the cell surface is more readily susceptible to

inhibitory agents than the cell interior, Rothstein and various associates have investigated the relationship of the cell surface and its enzymes to metabolism. Rothstein and Larrabee (121) have indicated that uranium uptake by yeast cells is associated with complex formation on the cell surface, and that no uranium penetrates into the cell. These results were interpreted by Rothstein et al. (120) in 1948 by examination of the formation of these complexes. Later, Rothstein and Meier (122) studied cell surface phosphatases of yeast and concluded that they appear to play no part in carbohydrate metabolism or in transfer of phosphate into the cell, but may serve to cleave organic phosphates which the cell cannot utilize, into products the cell can use.

Of recent interest is the report of work by Demis, Rothstein and Meier (33) which gives evidence that the enzyme invertase, responsible for hydrolytic cleavage of sucrose, is located on the surface of Saccharomyces cerevisiae. Uranyl ion reversibly inhibited invertase by a non-competitive mechanism which the authors assume to involve reaction with carboxyl groups.

These examples of activity involving enzymes and their inhibitors as involved in respiration and metabolism have been "spotty" as far as a complete review is concerned. It was intended to create a very generalized picture of the background work which led to the experimental work in this thesis, thus laying the foundation for the conclusions drawn from the results obtained.

III. MATERIALS

A. Cultures

All microorganisms used in this study were obtained from Dr. L. A. Underkofler, as designated below. They were obtained as needed as slant cultures grown on the proper agar media except for Clostridium acetobutylicum which was obtained as a spore culture.

	Microorganism	Code designation
1.	Saccharomyces cerevisiae	NRRL Y567
2.	Lactobacillus delbrueckii	NRRL B445
3.	Aspergillus niger 67	NRRL 67
4.	Clostridium acetobutylicum	POS from Dr. L. A. Underkofler
5.	Lactobacillus casei	ATCC 7469
6.	Acetobacter suboxydans	ATCC 621

B. Ingredients of Media

1. Glucose

The glucose used in this investigation was the C.P.

anhydrous sugar obtained from the Pfanstiehl Chemical Co., Waukegan, Illinois.

2. Yeast extract

Dehydrated powdered yeast extract was obtained as Difco Bacto yeast extract from Difco Laboratories, Detroit 1, Michigan.

3. Peptonized milk

This material is an enzymatic digest of fresh milk containing proteins, albumins and globulins. It was obtained from the Difco Laboratories, Detroit 1, Michigan.

4. Corn

The corn used in these experiments was purchased on the open market.

5. Sorbitol

Anhydrous sorbitol was obtained from the Atlas Powder Co., Wilmington, Delaware.

C. Special Solutions and Chemicals

1. Solutions of test compounds

See Table 1 on page 51.

2. BAL

This chemical, British antilewisite, is 2,3-dimercapto-propanol and was purchased from Hynson, Westcott and Dunning, Inc., Baltimore 1, Maryland.

3. γ-Diethylaminopropanol

This amino alcohol was obtained from Distillation Products Industries division of Eastman Kodak Co., Rochester 3, New York.

4. Diphenylmercury

The organomercury compound was obtained from the Eastman Kodak Co., Rochester 3, New York.

5. Other organic compounds

a. Tetrakis (p-dimethylaminophenyl) lead tetramethiodide. This organometallic compound was obtained from Dr. Henry Gilman, Dept. of Chemistry, Iowa State College, Ames, Iowa.

Table 1. Stock solutions of compounds used.

	Compounds	Mol. wt.	Solvent	Conc'n.[a] (moles/ml.)
1.	Tetrakis (p-dimethyl-aminophenyl)-lead tetramethiodide	1255.73	water	3×10^{-3}
2.	Triphenyl-γ-(diethyl-methylammonium)-propyl-lead methosulfate	678.85	water	1×10^{-4}
3.	4,4,4-Triphenyl-n-butyldiethylmethylammonium methosulfate	483.65	water	1×10^{-4}
4.	n-Propyldiethylmethyl-ammonium methosulfate	241.34	water	1×10^{-4}
5.	Diphenylmercury	354.81	ethyl lactate	1×10^{-4}
6.	Triphenyl-p-tolyllead	529.64	dioxane	4×10^{-5}
7.	Triphenylbenzyllead	529.64	Butyl Cellosolve[b]	1×10^{-4}
8.	Triphenylsilanol	276.40	Butyl Cellosolve	1×10^{-4}
9.	Sodium benzoate	144.11	water	1×10^{-4}
10.	BAL	124.13	ethanol (35 per cent)	1×10^{-3}

[a]Dilutions of these stock solutions supplied the various concentrations of compounds required.

[b]Butyl Cellosolve is the trade name for ethylene glycol mono-butyl ether, manufactured by Carbide and Carbon Chemicals Corp., New York, New York.

b. Triphenylsilanol. The silanol was donated by Mr. Jay Curtice of the Dept. of Chemistry, Iowa State College, Ames, Iowa.

c. Organometallic compounds. Those organometallic and non-metallic compounds not mentioned previously were prepared according to procedures given in the Experimental section of this thesis.

6. Reagents

All chemicals used during this investigation were obtained from ordinary commercial sources and were of C.P. quality.

IV. METHODS

A. Warburg Apparatus

The study of the respiration of microorganisms was given a tremendous advance with the introduction of the well known Warburg apparatus for following manometrically the up-take or evolution of gases. The mechanism involved imparts a reciprocating motion to small flasks held at constant temperature by immersion in a thermostatically-controlled water bath. Each flask contains the respiring organisms, tissue, or enzyme system plus substrate and is connected to a previously calibrated manometer filled properly with manometric fluid of known density. Volume changes occurring within each flask can then be measured accurately and the volume of change of the manometer plus flask interior calculated in terms of microliters (µl).

Umbreit et al. (139) have described the procedures by which the Warburg apparatus may be best utilized, and their procedures were followed in the calibration of the manometers and flasks. The volume of each flask was calculated by weighing it when empty and when filled with pure mercury, employing a balance which was accurate to 0.0001 g. with loads of 300 to 400 g. A similar procedure was used for each manometer and the connecting glass tubing between the flask and its manometer, so that each flask was calibrated to its own

manometer.

B. Fermentation Procedures

The determination of glucose remaining after a fermentation made it necessary to insure that known quantities of medium were present in each flask or tube used. A common volume used was 10 ml. per flask, although larger fermentations of the order of 100 to 300 ml. were also employed when this was warranted.

A 10 ml. pipet fitted with a 3-way stopcock connected to a funnel for automatic refilling was used to dispense equal volumes of medium to each flask in a series. The media were usually sterilized at 15 p.s.i. for a minimum of 30 minutes; the grain mashes were allowed to remain in the autoclave an additional 15 to 30 minutes.

1. Aerobes

The use of aerobic microorganisms made aeration necessary for the proper growth of these cultures. Aeration may be accomplished by bubbling air or air/oxygen mixtures through a suitable sparger immersed in the medium, or by shaking the cultures in a reciprocating fashion so as to mix air with the medium. Such cultures are known as "shake cultures."

The growth of Aspergillus niger was carried out by bubbling sterile air through a series of tubes leading from a

water flask (for humidification of the air) to each flask of medium. No sparger was used; a glass tube placed within one-half inch of the bottom of each flask gave sufficient agitation and provided aeration for the cultures.

The cultures of Acetobacter suboxydans were grown as "shake cultures" by placing each 50 ml. flask in a reciprocating shaker in a 30° C. incubator, and shaking at 100 strokes per minute. This rate was sufficient for good aeration of this microorganism.

Inoculation of these cultures was done under aseptic conditions using sterile 1 ml. pipets or a sterile wire loop for transfer of the inocula. The flasks were plugged with sterile cotton plugs during growth of the organisms except for aeration of the Aspergillus niger, cultures, where gas inlet tubes were used.

2. Anaerobes

Only one anaerobic microorganism, Clostridium acetobutylicum, was used; it was started from a soil spore culture from Dr. L. A. Underkofler's private collection. Anaerobes are not aerated as oxygen is inhibitory to their proper development and growth. Therefore, these cultures were allowed to stand and the Clostridium acetobutylicum provided its own oxygen-free atmosphere by the production of carbon dioxide. The use of long narrow culture tubes (19 x 254 mm.) was

preferable here so as to limit the total surface area exposed to air.

Cultures of Clostridium acetobutylicum are initiated by inoculation of freshly-prepared grain mashes with spores under sterile conditions. The organism is then heat-shocked by immersion of the newly-inoculated medium in boiling water for two minutes, followed by cooling. Such treatment serves two purposes: (a) it aids in killing vegetative organisms which may be present, and (b) it activates the spores to help them in adjusting to the new medium. When vigorous growth has begun, the organisms are transferred to fresh sterile medium, allowed to attain good growth (usually in 24 hours) and again transferred. The fifth transfer should be made into the medium which is to be used for the fermentation proper, as the organisms are at their peak of efficiency at this point. Additional transfers will materially reduce the yield of solvents produced by Clostridium acetobutylicum.

Culture tubes were also used for carrying the yeast, Saccharomyces cerevisiae. It was grown in 50 ml. Erlenmeyer flasks for the tests with compounds, but no shaking or agitation was allowed so conditions became anaerobic because of the production of carbon dioxide, as in the case cited previously.

C. Analytical Procedures

1. Detection of sorbose by chromatography

The method used for detection of sorbose was that employed by Suhadolnik (136). The silver-reducing properties of sorbose are utilized to cause reduction of silver ions to free silver which appears as a black spot on the chromatogram (68).

Sheets of Whatman No. 1 paper were cut so as to be 8 5/8 in. long and 9 in. wide in order to fit into the wide mouthed jars used. Each jar was provided with a screw cap, and each had a flat bottom.

Along a line drawn 1 in. from the bottom of the paper were placed samples of the sugar solutions to be tested. Samples were spaced at intervals not less than 2 cm., depending upon the number of samples desired on one chromatogram, and were applied with a small wire loop of approximately 1/8 in. diameter.

The "carrier" solvent consisted of a mixture of three parts of distilled water, four parts of pyridine and six parts of 1-butanol, to give a miscible homogeneous solution, called 3:4:6, and which was good for four or five ascents before it was discarded. Fifty ml. of solvent was placed in the bottom of each jar, and the paper cylinder, formed by stapling the right edge of the paper next to, but not touching, the left

edge, was placed in the jar so the bottom was soaked in the solvent.

The paper chromatogram was allowed to remain in the jar until the solvent had traversed the entire length of the cylinder. This required from 6 to 8 hours. The chromatogram was then removed from the jar, dried very briefly at 110° C. and developed.

For development of the chromatogram, an aqueous 8.5 per cent silver nitrate-ammonium hydroxide solution was sprayed on the paper by means of a No. 31 De Vilbiss atomizer with a continuous air stream until the paper was uniformly moistened. Drying at 110° C. for five minutes caused dark brown or black spots to form at that place where the reducing sugar was positioned on the paper. The chromatogram was then washed with dilute ammonium hydroxide and distilled water to remove excess silver which would subsequently darken the entire chromatogram if not removed. Drying at 110° C. gave a series of dark brown spots on a tan background.

The silver nitrate developing solution was prepared by dissolving 8.5 g. of silver nitrate in 100 ml. of distilled water. Concentrated ammonium hydroxide was then added, with stirring, until the silver oxide dissolved and a clear solution resulted. This reagent was prepared fresh for each development of any one series.

2. Determination of acetone

The acetone was determined by the method of Messinger as given by Goodwin (57).

3. Determination of total solvents

The total solvents present in cultures of Clostridium acetobutylicum were determined by values of the Specific Gravity of a 100 ml. sample of distillate as explained by Christensen and Fulmer (27).

4. Determination of reducing sugars

The Shaffer-Somogyi method for the analytical determination of reducing sugars as modified by Underkofler et al. (140) was used for the determination of glucose remaining in media, or for the presence of sorbose.

The media of these experiments varied in sugar concentration according to the type of microorganism for which it was used, so samples were withdrawn and diluted to a standard reference in volumetric flasks, and portions from these dilutions then analyzed for reducing sugar concentration. Use of 2 per cent glucose solutions was frequent, so a 1 ml. aliquot was removed, diluted to exactly 10 ml., and a 1 ml. portion of the diluted sample withdrawn to give a sample containing no more than 2.50 mg. glucose. A similar procedure was under-

taken for sorbose except that only 0.2 ml. aliquots were removed from the original media as their concentration was 10 per cent.

The total reducing sugars in the fermentation media was computed on the basis of the total volume of mash from which the aliquot was withdrawn. Blank determinations on sterile media as well as controls in which no inhibitors were present, were carried out.

5. Determination of volatile acids (total)

The volatile acids were determined by the procedure given by Neish (103) and were titrated with standardized sodium hydroxide using phenol red as an indicator. The results were reported as total milliequivalents of acids.

6. Determination of concentrations of organometallic intermediates

For purposes of calculating the molar concentration of a reactive alkali or alkaline-earth organometallic intermediate which is to be used in a reaction involving stoichiometric equivalents of other reactants, it is necessary to determine the concentration of active metal present in solution as RM compound. An aliquot (usually 2 ml.) is withdrawn by pipet and the organometallic compound present is hydrolyzed by running it rapidly into excess distilled water,

thus forming an amount of alkali hydroxide equivalent to the milliequivalents of alkali metal in the organometallic compound added. Titration with standardized 0.2 N sulfuric acid using phenolphthalein as an indicator will allow the exact calculation of organometallic compound in the aliquot, and therefore, in the total volume to be used in the reaction.

7. Determination of the presence of organometallic intermediates

The determination of the presence of reactive alkali or alkaline earth organometallic compounds in a reaction mixture is accomplished by means of Color Test I as developed by Gilman and Schulze (53). This test depends upon the addition of a reactive organometallic compound to the double bond in the carbonyl group of Michler's ketone to produce a colored dye upon hydrolysis and oxidation. Michler's ketone is bis (p-dimethylaminophenyl) ketone; reaction with phenyllithium, for example, produces an intense, green color.

The procedure has been described by Gilman (50) as follows:

> One-half to one cubic centimeter of the organometallic solution is added to an equal volume of a 1 per cent solution of Michler's ketone in dry benzene; the reaction product is then hydrolyzed by the slow addition of 1 cc. of water; and, finally, the addition of several drops of a 0.2 per cent solution of iodine in glacial acetic acid develops a characteristic greenish-blue color.

If phenyllithium reacts with Michler's ketone, one will obtain malachite green as a product, but if the phenyllithium has already reacted with another ketone by an addition, the resultant lithium-containing intermediate will not give a positive test as only carbon-to-metal linkages will do so.

8. Qualitative determination of elements

The qualitative analytical procedures given by Shriner and Fuson (127) were used for determining the presence of sulfur, halogens and nitrogen in organic compounds. The nitrogen was determined as ferrocyanide and the sulfur as lead sulfide precipitate and by a positive nitroprusside reaction.

9. Quantitative determination of elements

The analyses for carbon, hydrogen, nitrogen and sulfur were performed by:

Huffman Microanalytical Laboratories
3830 High Court, P.O. Box 125
Wheatridge, Colorado

D. Synthetic Procedures

1. Nitrogen purification

Attainment of an inert atmosphere free of highly reactive

gases and moisture was accomplished by flushing all apparatus used for organometallic compound preparations with dry, purified nitrogen gas as a preliminary step. This was followed by maintenance of a slight, positive pressure of nitrogen in a sealed system throughout the reaction period.

Purification of the nitrogen as it was obtained from the tank demanded complete removal of oxygen and water vapor as well as any acidic gases or carry-over from the purification train. The purification train is diagrammed in Figure 1. The first two bottles each contain 50 g. zinc amalgam, 2 g. vanadyl sulfate dihydrate in 100 ml. water, and 1.5 ml. concentrated sulfuric acid. These bottles are fitted with dropping funnels for adding additional sulfuric acid to regenerate the vanadous state of the vanadium. The nitrogen is freed of any oxygen by the reducing action of the vanadous ions.

The following bottle of water removes volatile vanadium vapors carried over from the first two bottles. Empty bottles serve as safety features in the event of any back-up or carry-over of other reagents. The sodium hydroxide removes acid vapors plus some water, while the sulfuric acid removes ammonia, other basic constituents, and water vapor. The calcium chloride is anhydrous and removes any other water vapor. The nitrogen then is distributed through glass tubing to various points along the laboratory bench.

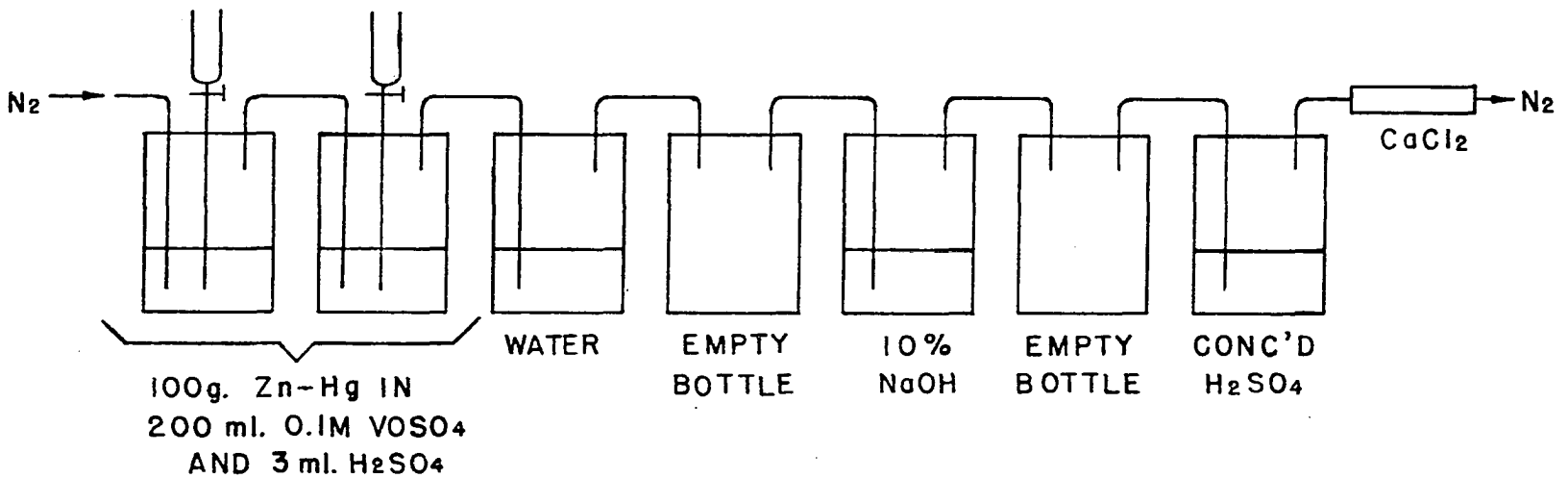


FIGURE I. NITROGEN PURIFICATION TRAIN

2. Melting points

All melting points were taken by immersing a 1 mm. tube containing the sample and attached to a 360° C. thermometer in a bath of DC 550 Silicone fluid which was stirred constantly and heated by a gas burner. A melting range of 0.5 to 1 degree indicates pure material in use of a bath of this type. The temperature was raised approximately two degrees per minute near the melting point.

V. EXPERIMENTAL RESULTS

A. Synthesis of Organic Compounds

1. Preparation of phenyllithium (56)

In a 300 ml. round bottom flask previously flushed with dry, oxygen-free nitrogen and fitted with stirrer, condenser and dropping funnel were placed 2 g. (0.29 gram atom) lithium metal in 80 ml. dry ethyl ether. Then 20.9 g. (0.133 mole) of bromobenzene in 35 ml. dry ethyl ether was added slowly to the stirred suspension at such a rate as to maintain a constant reflux. Stirring was continued for one hour after the cessation of refluxing, and the system maintained in a nitrogen atmosphere.

A two ml. aliquot of the phenyllithium solution was withdrawn, hydrolyzed with water and analyzed as described previously. A two ml. sample required 11.2 ml. of 0.205 N sulfuric acid, giving a yield of 0.127 mole of phenyllithium in 110 ml. of ether, or 95.0 per cent.

2. Preparation of triphenyllead lithium (55)

Using 105 ml. (0.120 mole) of freshly-prepared phenyllithium, triphenyllead lithium was prepared by adding the filtered, clear solution of phenyllithium to a vigorously-

stirred suspension of 11.1 g. (0.04 mole) anhydrous lead chloride in thirty ml. of dry ethyl ether cooled to -10° C. by an ice-salt bath. The flask was flushed and constantly filled with pure dry nitrogen gas. The initial canary yellow color suddenly became light gray which is typical behavior for this preparation. A quantitative yield is obtained when the reaction follows this behavior.

3. Preparation of γ-diethylaminopropyl chloride (18)

This compound was prepared according to the procedure obtained from Nelson (104). A solution of 197 g. (1.5 moles) of redistilled -diethylaminopropanol in 200 ml. of chloroform was added slowly to a solution of 357 g. (3.0 moles) of thionyl chloride in 1200 ml. of chloroform cooled in a salt-ice bath. The resultant mixture was refluxed for twelve hours, the excess solvent and thionyl chloride removed by distillation, and the remaining oil distilled. There was obtained 169.5 g. of clear, colorless liquid which boiled at 70-71° C. at 15 mm. Glass wool was placed in the distilling flask to reduce excessive frothing during the distillation. Yield: 76 per cent.

The material was stored in the refrigerator to reduce dimerization.

4. Preparation of triphenyl-γ-diethylaminopropyllead (54)

A 0.04 mole preparation of triphenyllead lithium was undertaken as described, and to the stirred gray suspension was added 6.6 g. (0.04 mole plus 10 per cent excess) of γ-diethylaminopropyl chloride in 25 ml. of dry ethyl ether. An atmosphere of dry purified nitrogen gas was present at all times. The temperature was maintained at -10° C. for 10 minutes; then the solution was refluxed for 1.5 hours and after cooling, the mixture was hydrolyzed by the cautious addition of 1.5 per cent ammonium chloride when Color Test I was negative (53). The ether layer was dried over anhydrous sodium sulfate and the dry ether solution subsequently distilled to remove the ether. The product was an oil which was left in the flask.

5. Preparation of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate

The oil obtained from the preparation of the free amine was dissolved in 40 ml. of dry benzene and added to a stirred solution of 5.54 g. (0.04 mole plus 10 per cent excess) of freshly-distilled dimethyl sulfate in 15 ml. of dry benzene. After stirring for 1½ hours at room temperature, it was refluxed a short time and cooled. A white sticky solid material precipitated and was filtered. There was obtained 110 g. of white crystals by recrystallization from benzene. Yield: 40.6

per cent, based on the lead chloride.

6. Preparation of p-tolyllithium

The preparation of p-tolyllithium was identical to that of phenyllithium except that 22.74 g. (0.133 mole) of p-bromotoluene was used instead of the usual amount of bromobenzene. A quantitative yield was obtained.

7. Preparation of triphenyllead chloride (52)

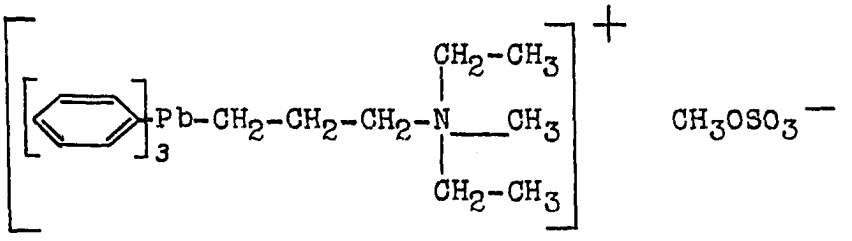
Following the directions given by Gilman and Robinson (52), an 0.08 mole preparation of triphenyllead chloride was undertaken. Hot chloroform (600 ml.) was used to dissolve 40.4 g. (0.08 mole) of tetraphenyllead after which hydrogen chloride gas was passed into the refluxing solution at a moderate rate. After 23 minutes, the gas flow was halted and the solution refluxed 5 minutes, then filtered. Removal of the chloroform and extraction of the resultant white solid mass of material with 800 ml. of hot ethanol gave a total of 22.8 g. of triphenyllead chloride upon cooling. The product melted at 205-206° C. Yield: 60.2 per cent.

8. Preparation of triphenyl-p-tolyllead

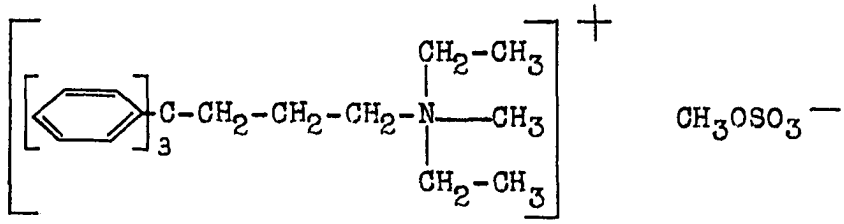
This compound was prepared using either triphenyllead chloride or triphenyllead iodide. For preparation of the

chloride, 19.0 g. (0.04 mole) was suspended in 200 ml. dry ethyl ether, and to the stirred suspension was added 32 ml. (0.04 mole) of freshly prepared p-tolyllithium in ether within 10 minutes. Color Test I (53) was negative at once, so the mixture was hydrolyzed and an insoluble portion removed by filtration. Following removal of the ether, the product was recrystallized from hot ethanol. A total of 19.8 g. of triphenyl-p-tolyllead, melting at 125.5 to 126.5° C., was obtained. Yield: 93.5 per cent. When triphenyllead iodide was used, the yield of triphenyl-p-tolyllead was 95.2 per cent. Triphenyllead iodide was prepared according to the method of Gilman and Bailie (51).

The use of the organolead detergent, triphenyl-γ-(diethylmethylammonium)-propyllead, led to the synthesis of the carbon atom analog in which the lead atom is replaced by a carbon atom. Such a compound would be known by the name, 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate. The formulas for the lead compound and its carbon analog are shown below.



Organolead compound
triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate



Carbon analog of organolead compound
4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate

The following syntheses were required for preparation of the carbon analog. The compound was called "444T" on the basis of the official name which would be given it by Chemical Abstracts. The compound found in the literature which most closely approximated the structure of "444T" was 4,4-diphenyl-n-butyldiethylamine which was prepared by Marxer (91) and by Clarke and Mooradian (28), and as no reference to any compound such as "444T" could be found, it was synthesized and characterized by analysis as befits a new compound.

It was noted that Bachmann and Wiselogle (9) prepared triphenylbenzylmethane by the reaction of triphenylmethyl sodium with benzyl chloride. Therefore, it was felt that the preparation of the desired amine; i.e., 4,4,4-triphenyl-n-butyldiethylamine, could be achieved by the reaction of triphenylmethyl sodium and the readily available γ-diethylaminopropyl chloride. This reasoning led to the preparation of triphenylmethyl chloride which was needed to obtain the necessary triphenylmethyl sodium.

9. Preparation of triphenylmethyl chloride (8)

This compound was prepared from recrystallized triphenylcarbinol by dissolving 85 g. of the latter in 30 ml. of benzene and cautiously adding 51 ml. of freshly-distilled acetyl chloride to the hot solution. Heating and shaking resulted in a clear solution which was refluxed for 30 minutes. Skelly B (68 ml.) was added and the solution cooled overnight in the refrigerator. There was obtained 34 g. of triphenylmethyl chloride as pure white crystals which were dried in a desiccator over paraffin and soda-lime. Yield: 37.3 per cent, (material in mother liquor not recovered).

10. Preparation of triphenylmethyl sodium

Following the procedure of Bachmann and Wiselogle (9), 30 g. (0.108 mole) of triphenylmethyl chloride was dissolved in a mixture of 240 ml. of driedethyl ether and 240 ml. of dried benzene; 12 g. (0.522 g. atom) of sodium sand (42) in 120 ml. of dried ether were added, and the bottle containing this mixture was tightly stoppered after preliminary flushing with dry nitrogen.

After several hours of shaking (7 hours were required in this instance), the appearance of a blood-red color indicated the presence of the triphenylmethyl carbanion. The shaking was continued for 10 minutes and the mixture allowed to stand for 2 hours in the refrigerator. Titration of a 5 ml. aliquot

(following hydrolysis) with 0.2050 N sulfuric acid gave a total of 0.096 equivalents of triphenylmethyl sodium, or a yield of 89 per cent.

11. Preparation of 4,4,4-triphenyl-n-butyldiethylamine

In a 1 liter round bottom flask flushed with dry nitrogen was placed 13.5 g. (0.0902 mole) of freshly-distilled γ-diethylaminopropyl chloride in 125 ml. of dry benzene. Then 550 ml. (0.0902 equivalents) of triphenylmethyl sodium in ether-benzene solution was added at a rapid rate of dropping to the stirred refluxing amine solution. The red color of the organometallic solution was discharged rapidly, giving an orange-yellow color. Addition of the triphenylmethyl sodium required 45 minutes, after which the mixture was refluxed 15 minutes more and then was allowed to stand overnight. Hydrolysis of any unreacted organometallic compound by addition of 1.5 per cent ammonium chloride was followed by washing the solvent layer with water and drying by standing over potassium hydroxide pellets. Most of the benzene was removed by distillation but a portion of it was left to act as a solvent for preparation of the methosulfate.

12. Preparation of 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate

The orange-colored benzene solution of the free amine

above was allowed to remain in the flask, and to it was added 11.4 g. (0.0902 mole) of freshly distilled dimethyl sulfate in 75 ml. of dry benzene. A cloudiness resulted and some heat was generated, although only enough to warm the solution. Upon cooling, crystals of product were obtained which were washed with Skelly B and recrystallized from acetone. This product weighed 21.5 g. and melted at 161-162° C. From the mother liquor was obtained 7.3 g. of another product which melted at 236-237° C.

As this compound was not reported in the literature, both products were analyzed qualitatively for nitrogen, halogen and sulfur and were tested for solubility. The low melting material (m.p. 161-162° C.) was called Compound A; the higher melting material was called Compound B. The data obtained for each is given in Table 2 on the following page.

On the basis of these preliminary results, it was decided to subject Compound A and the organolead compound analog to infrared analysis. The curves obtained are shown in Figure 2. They were sufficiently alike to believe that Compound A was the desired product. This evidence was therefore made the basis for subjecting Compound A to quantitative organic analyses for carbon, hydrogen, nitrogen and sulfur. The analyses were performed by the Huffman Microanalytical Laboratories, and are reported below.

Anal. Calculated for $C_{28}H_{37}O_4NS$: C, 69.53; H, 7.71; N, 2.89; S, 6.63. Found: C, 69.23; H, 7.67;

Table 2. Physical and chemical properties of Compound A and Compound B.

	Test	A	B
1.	M.P. determination	161-162° C. to a clear liquid[a]	M.P. 236-237° C. to a brown liquid
2.	Beilstein (halogen)	negative; compound burns	negative; compound burns
3.	Sulfur (Na fusion)	positive	positive
4.	Nitrogen(Na fusion)	positive (?)	positive
5.	Barium precipitate	addition of $BaCl_2$ to aq. solution gave no precipitate; gel formed[b]	precipitate formed which gelled on standing
6.	Grams obtained	21.5	7.3
7.	Solubility	soluble in water	partly soluble
8.	Surface tension reduction	very strong[c]	moderate

[a]Mixed M.P. with triphenylcarbinol: 153-154° C. with preliminary softening. Triphenylcarbinol melts at 161° C.

[b]Addition of $BaCl_2$ to the organolead analog gave identical behavior as A.

[c]Strong surface tension-reducing properties with organolead analog.

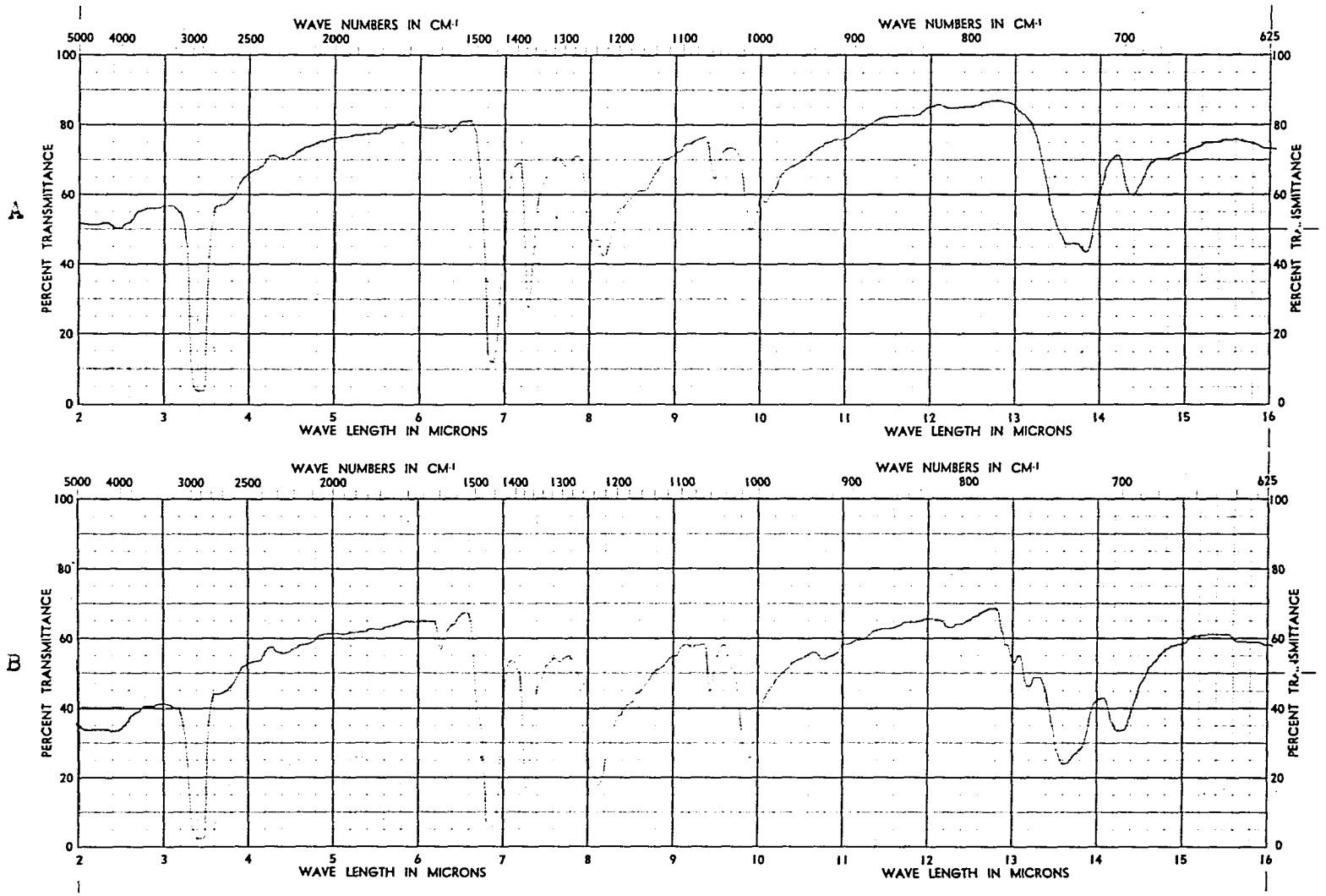


Figure 2. Infrared spectra

A. Spectrum for triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

B. Spectrum for 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate.

N, 2.98; S, 6.73.

These results have been taken as confirmation of the structure of Compound A as the carbon atom analog of the organolead compound (see structures on pages 70 and 71). This new compound has been called "444T", and will be referred to by this designated number in lieu of its entire name; i.e., 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate.

Compound B (m.p. 236-237° C.) is unknown as to its structure, although it has been felt that a halogen-metal interconversion between triphenylmethyl sodium and γ-diethylaminopropyl chloride may occur to give some γ-diethylaminopropyl sodium. This might then react with the chloride to give 1,6-bis-(diethylamino)-hexane. The bismethosulfate of such a compound would be expected to have a rather high melting point because of the two positive and negative centers in the molecule. Furthermore, the dimethiodide of 1,6-bis-(dimethylamino)-hexane prepared by von Braun (144) did not melt at a temperature of 270° C. so this adds support to the possibility of a rather high melting point for a bismethosulfate of the postulated diamine: The melting point of the methosulfate of n-propyldiethylamine (see below) is 196° C. No further work was done with Compound B, however.

13. Preparation of n-propyldiethylamine (37)

A 500 ml. round bottom flask was fitted with a water-

cooled condenser and a mixture of 100 g. (0.81 mole) of n-propyl bromide, 76 g. (1.04 moles) of freshly distilled diethylamine and 40 g. (0.435 mole) of glycerol was refluxed for 67 hours. The miscible solution formed two layers within two hours, and remained thus for the remainder of the reaction. When reaction was complete, the mixture was cooled, 40 ml. of water was added, followed by 160 ml. of 50 per cent potassium hydroxide. The mixture of amines was extracted with ethyl ether and the ether solution dried over pellets of potassium hydroxide.

Fractionation (following removal of the ether) of the solution of mixed amines gave 41 g. of product in the 104-108° C. range which was identified as n-propyldiethylamine by its index of refraction (n_D^{20} : 1.4064). A portion of this material was redistilled at 110-111°C. as a clear, colorless liquid. Yield: 44 per cent.

14. Preparation of methyldiethyl-n-propylammonium methosulfate

In a 250 ml. round bottom flask fitted with a stirrer and dropping funnel was placed a solution of 23 g. (0.2 mole) of freshly distilled n-propyldiethylamine in 25 ml. of dry ethyl ether. A solution of 25.2 g. (0.2 mole) of freshly distilled dimethyl sulfate in 50 ml. of dry ethyl ether was added dropwise, with cooling, to the stirred amine solution.

Stirring was continued for 15 minutes, followed by refluxing for 30 minutes which caused production of a heavy oily layer and an upper turbid ether layer.

The turbid ether contained a water-soluble substance in insignificant quantities; the bottom layer of slightly brown oil was saturated with ether but was water-soluble. This layer was dissolved in hot acetone, decolorized with Norit, and the acetone was removed in a nitrogen atmosphere at reduced pressure. A colorless oil remained which formed a glass when it was cooled in Dry Ice, but formed crystals when the glass was warmed. Recrystallization from acetone gave a white crystalline product which melted at 196° C.

B. Preliminary Experiments

1. Solvents and solubility

The use of water-soluble compounds for antibacterial studies is an ideal condition for the research worker as aqueous media are required for the successful growth of microorganisms. The soluble organolead compound used and the 444T presented no difficulties in regard to their addition to media except for gel formation of 444T at a concentration of 1×10^{-4} mole per ml. Heating the gel caused rapid liquefaction, however.

The introduction of water-insoluble organometallic

compounds into a medium presented difficulties. It was decided to find solvents which would satisfactorily dissolve a sufficient quantity of an organometallic compound to give a solution of at least 1 x 10^{-4} mole per ml., and which would be miscible with water. Furthermore, these solvents would have to possess little or no toxicity to the microorganism in the concentrations which would be used.

Such stipulations meant that a trial of any available solvents for their toxicity and solubility was necessary so some were tested in this regard. Some solvents were known to be toxic before trial (such as 100 per cent ethanol) so were not tested for toxicity after a preliminary solubility test showed them to be poor solvents, since too high a concentration of solvent would be needed in order to attain a suitable concentration of the organometallic compound in a medium.

The Butyl Cellosolve proved to be a good solvent but was shown to be inhibitory to the growth of Saccharomyces cerevisiae so it was not used except in limited instances where its presence was below the inhibitory level.

2. Effect of the presence of yeast extract on glucose analyses

It was recognized that the use of Difco yeast extract in a medium would probably cause an increase in the quantity of copper oxidizing reagent used for reducing sugar analyses.

Thus a sample of aqueous yeast extract corresponding to the concentration present in the normal media used for Saccharomyces cerevisiae was treated with Reagent G for the reducing sugar analyses, with glucose present in the normal concentration. Another sample containing no yeast extract but glucose

Table 3. Solubility of organometallic compounds in organic solvents.

Compound	Ethanol	Dioxane	Butyl Cellosolve
1. Triphenylbismuthine dichloride	insol.	insol.	mod. sol.
2. Triphenylsilanol	sol.	sol.	sol.
3. Diphenylmercury	sl. sol.	mod. sol.	mod. sol.
4. Triphenylbenzyllead	insol.	sol.	sol.
5. Triphenyl-p-tolyllead	insol.	sol.	sol.
6. Tetraphenyllead	insol.	insol.	----
7. Tetraphenyltin	insol.	insol.	----

only, was also treated with Reagent G.

Glucose solution: 2.065 mg. glucose present.
Glucose-yeast extract solution: 2.364 mg. "glucose" present.

This shows that 0.299 mg. or approximately 0.3 mg. of

"theoretical glucose" might be attributable to the presence of yeast extract. On the basis of this behavior, all reducing sugar analyses which were made included a blank run on a sample of sterile medium to nullify the adverse effect of the yeast extract on the analyses.

3. Exposure of media containing an organolead detergent to air-borne microorganisms

Three 10 ml. Erlenmeyer flasks, each containing a solution of 200 mg. of glucose and 2.5 mg. of yeast extract in 10 ml. of distilled water were sterilized and then allowed to stand opened to the atmosphere for five days. One flask was a control, while to another was added 10^{-4} mole of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate and to the third was added 10^{-6} mole of the same compound.

The control medium was covered with mold mycelia of several different varieties, among them Penicillium and what appeared to be Aspergillus niger, while the flask with 10^{-4} mole of lead compound was free of any contaminants. The flask with 10^{-6} mole of lead compound had a turbid medium indicating contamination although no mold mycelia were present. This would indicate the highest degree of toxicity toward molds and a moderate toxicity toward other microorganisms which are not, perhaps, molds.

4. Growth of an air-borne mold in the presence of an organolead compound

The results obtained by using the water-soluble organolead compound (triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate) led to the trial of this material against an air-borne mold which had been cultured from a contaminated glucose medium. The mold was grown in the presence of more dilute concentrations of the lead compound as the use of 10^{-5} mole of compound per ml. of medium was apparently toxic to all air-borne microorganisms. In Figure 3 the glucose utilized is plotted vs. the concentration of organolead compound (mole per ml. of medium), showing the large increase in glucose utilization by the mold when dilute solutions of the organolead compound are present. However, the lack of growth at a concentration of 10^{-7} mole of lead compound per ml. of medium is in accord with the exposure of the various media to the air for five days; no mold mycelia were to be found in the flask with this concentration (10^{-7} mole) of lead compound. The mold was allowed to grow for 36 hours only, so the glucose uptake for the normal culture containing no organolead compound is small. The glucose uptake was largest when 10^{-9} mole of the lead compound per ml. of medium was present. It is seen that at these concentrations the normally toxic effect of this organolead detergent has been suppressed and that the surface tension reducing properties are

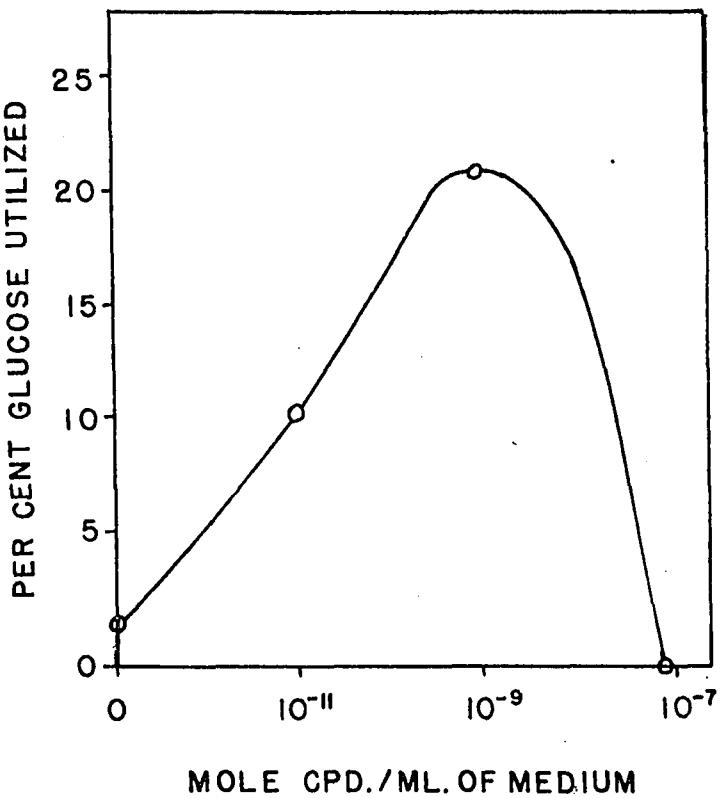


Figure 3. Glucose utilization of an air-borne mold in the presence of triphenyl-Y-(diethylmethylammonium)-propyllead methosulfate.

Mole of compound per ml. of medium	Per cent glucose utilized
None	1.77
10^{-11}	10.18
10^{-9}	19.45
10^{-7}	0.00

apparently aiding in the diffusion of nutrients into the cells to give the remarkable increase seen here.

5. Growth tests of microorganisms in the presence of an organotin compound

A small sample of an organotin dye acid was obtained from Mr. Sanders Rosenberg. It is a dark red compound called triphenyl-2-(p-carboxyphenylazo)-5-dimethylaminophenyltin sodium salt. This compound was impure so no extensive work involving its use was planned. A portion of it dissolved in water, so a saturated aqueous solution was prepared by agitating a warmed mixture of the tin compound and water at 60° C. The clear pink solution was used in a test culture of Saccharomyces cerevisiae. No inhibition was noted.

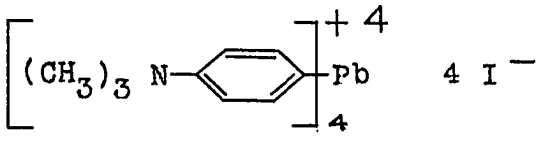
A more concentrated solution of the tin compound containing 0.0001 g. per ml. was obtained and used for growth of cultures containing Saccharomyces cerevisiae and Lactobacillus delbrueckii. Normal media were prepared with 2 per cent glucose and 0.25 per cent Difco yeast extract, using distilled water as the solvent. Identical media were made, using as a solvent the solution of the organotin compound prepared. Each organism was introduced as a 0.01 ml. inoculum into individual flasks containing 50 ml. of normal medium and tin-containing medium. These were incubated at 31° C. for 24 hours. No inhibition of growth of Lactobacillus delbrueckii

appeared in any case, as the normal medium and tin medium were both cloudy and had normal growth. Similar results for Saccharomyces cerevisiae were found. Heavy deposits of cells were found at the bottom of each flask.

These preliminary checks did not demonstrate a toxic effect of this tin compound on either Saccharomyces cerevisiae or Lactobacillus delbrueckii.

6. Growth of Saccharomyces cerevisiae in the presence of tetrakis-(p-dimethylaminophenyl)-lead tetramethiodide

This organolead compound has the following structure:



tetrakis-(p-dimethylaminophenyl)-lead tetramethiodide

A solution of 3×10^{-6} mole per ml. concentration was prepared by dissolving 0.0377 g. of this lead compound in distilled water to make a solution of 10 ml. total volume. Different concentrations within a range of 6×10^{-6} mole to 3×10^{-7} mole of compound were added to 2 per cent sucrose and 0.25 per cent yeast extract media which had been previously inoculated from an agar slant of Saccharomyces cerevisiae. After 16 hours, all flasks showed equally good growth of yeast cells.

C. Tests Conducted on Microorganisms Using Organic Compounds

1. Aspergillus niger

The preliminary observation made with the air-borne mold and with the water soluble organolead detergent, triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate led to an experiment with a known mold, Aspergillus niger.

Eight flasks containing 15 ml. of inoculum medium each were inoculated with a small wire loop of fermentation broth from a 36 hour old culture of A. niger 67. These inocula were grown as shake cultures for 24 hours at 30° C. and then each one was transferred to larger flasks containing 200 ml. of fermentation medium per flask. The large flasks were aerated by humidified air as described previously in the section entitled "Methods".

The fermentation was allowed to proceed for 3 days and the medium then removed. A carry-over of water vapor from the humidification flask caused an increase of total volume in several flasks (No. 3, 4 and 4A) which was accounted for in the calculation of the sugar remaining. The results of this determination were fairly satisfactory as they showed trends which were similar in the action of this same lead compound when it was used with the air-borne mold alluded to previously.

For properly comparing the activity of these compounds, the unit concentration of the organolead compound expressed as moles per ml. of medium must be calculated. A comparison of Figure 3 with Figure 4 will demonstrate some degree of similarity of the effect of adding 10^{-6} and 10^{-8} mole of the organolead compound.

The materials used in the media for the growth of A. niger for the inocula and the fermentation are given in Table 4.

Table 4. Ingredients of media for Aspergillus niger.

Grams/liter	Inoculum	Fermentation
Glucose	50.0	150.0
$MgSO_4 . 7\ H_2O$	0.12	0.156
KH_2PO_4	0.15	0.188
KCl	0.20	-----
$(NH_4)_2\ HPO_4$	0.60	0.388
Ferric tartrate	0.01	-----
Yeast extract	3.0	-----
Agar	1.5	-----
$CaCO_3$	-----	26.0[a]

[a]Sterilized separately by heating at 320° C. for 20 hours.

The results of the glucose determinations as plotted in Figure 4 are given below the plot. It may be said that

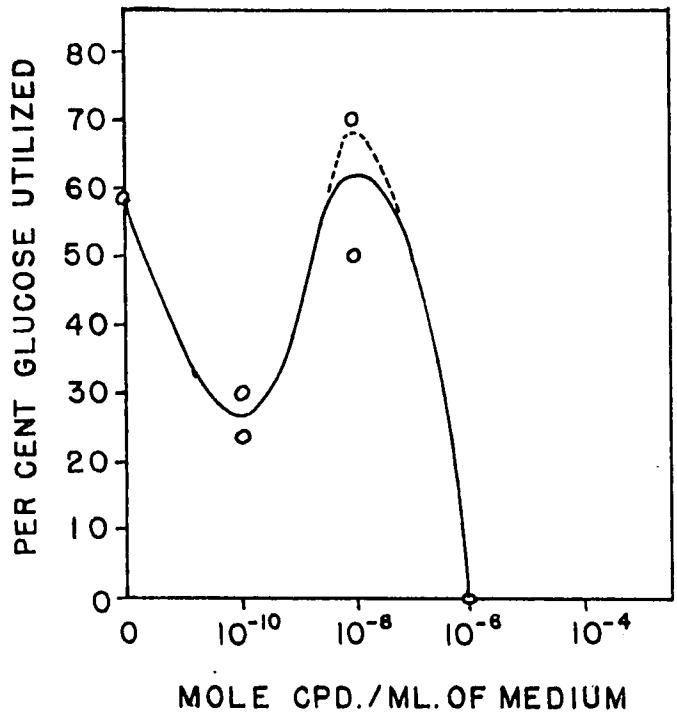


Figure 4. Glucose utilization of Aspergillus niger in the presence of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

Mole of compound added to medium	Mole of compound per ml. of medium	Per cent glucose utilized
None	None	58.4
2×10^{-4}	1×10^{-6}	0.0
4×10^{-4}	2×10^{-6}	0.0
2×10^{-6}	1×10^{-8}	50.0[a]
2×10^{-6}	1×10^{-8}	70.3
2×10^{-8}	1×10^{-8}	23.7
2×10^{-8}	1×10^{-10}	30.0

[a]Value may be too low; dotted line of graph may represent a truer presentation of the curve.

inhibition of A. niger by this organolead compound tends to parallel that shown against the air-borne molds; that is, a complete inhibition of glucose metabolism is observed at concentrations of 10^{-7} mole of compound per ml. of medium for the air-borne mold and of 10^{-6} mole of compound per ml. of medium in the case of A. niger. An enhancement of glucose utilization by A. niger occurred at a concentration of 10^{-8} mole of lead compound per ml. of the medium, with partial growth at the 10^{-10} mole per ml. level, perhaps due to an increased rate of passage of metabolites through the cell wall because of a reduction of surface tension of the aqueous phase.

2. Saccharomyces cerevisiae

The use of this microorganism by other investigators for analyzing the inhibitory and toxic effects of organic compounds has been very extensive. In this thesis, this same yeast has been used also to a large extent. The cultures were allowed to grow at 30° C. without any agitation, so conditions were essentially anaerobic during the growth periods.

It was shown previously that the initial experiments with S. cerevisiae were conducted using the tin compound supplied by Mr. Sanders Rosenberg, and with tetrakis-(p-dimethylaminophenyl)-lead tetramethiodide supplied by Dr. Henry Gilman. These two compounds did not demonstrate any toxicity in the

concentrations which were used, and as their solutions were not stable over a period of time, the use of other compounds was undertaken.

The use of organic solvents was mentioned previously in their role as "carriers" for the water-insoluble organic compounds employed in this research. Some solvents were individually evaluated by introducing actively-growing cells of S. cerevisiae into media containing carying concentrations of them. Solvents which showed no toxicity resulted in a 100 per cent utilization of glucose.

Among the solvents tested, the results had to be correlated with their ability to dissolve the various compounds used.

a. Butyl cellosolve. Concentrations from 1 per cent to 50 per cent were used for a check of the toxicity of this solvent. It was seen to be quite toxic to S. cerevisiae as all concentrations above 1 per cent were inhibitory, whereas values of 2.3 per cent and 17 per cent glucose utilization were found for a medium where only 1 per cent of Butyl Cellosolve was present. This seems to indicate an inhibition which would be overcome were the yeast cells allowed to remain in the medium for a longer period of time. Thus it would perhaps be satisfactory if the concentration of Butyl Cellosolve were held to less than 1 per cent, although this level places severe limitations upon the suitability of this solvent in view of

its limited solvation of the organometallic compounds.

b. Ethyl lactate. It was thought that because lactic acid is a normal product of metabolic activity in many micro-organisms, that its ethyl ester, ethyl lactate, might prove to be a relatively non-toxic solvent. Therefore, a pure sample was obtained from the fractional distillation of impure material, and the liquid boiling at 63 to 66° C. at 20 mm. pressure was used for toxicity tests. Rodd (117) gave the boiling point as 69 to 70° C. at 36 mm. pressure. Smith and Claborn (128) gave the boiling point at atmospheric pressure, 154.5° C.

Media were prepared with different concentrations of ethyl lactate in glucose (2 per cent) and yeast extract (0.25 per cent) medium by adding the required amount of solvent to 5 ml. of medium containing 4 per cent glucose and 0.5 per cent yeast extract and adding sufficient distilled water to make a total volume of 10 ml. Each flask of medium was inoculated with a small loopful of an actively-growing 24 hour old culture of S. cerevisiae and incubated for 24 hours at 30° C. One ml. samples were removed and each diluted to 10 ml. One ml. aliquots of the dilutions were used for the glucose analyses. An individual flask of the corresponding concentration of ethyl lactate in sterile medium was also analyzed for glucose so that no error in the reducing sugar determination would be introduced by the presence of ethyl lactate. The data in Table

5 show the results of this experiment.

These data show better potentialities for ethyl lactate than for Butyl Cellosolve, as the uptake of glucose was complete for concentrations of 2 per cent, and high for 3 per cent, indicating that up to 2 per cent of ethyl lactate will be satisfactory for use in cultures of S. cerevisiae.

Table 5. Glucose utilization of Saccharomyces cerevisiae at different concentrations of ethyl lactate.

Per cent ethyl lactate	Per cent glucose utilized
0	99.3
1	100.0
2	100.0
3	73.2
4	0.0
5	0.0
10	0.0

A test of ethyl lactate and of acetal at a concentration of 4 per cent for each solvent in separate cultures was made. This experiment checked for any possible decrease of toxicity for ethyl lactate when the solvent was added after sterilization of the media. In the preceding experiment, the media

were sterilized in the autoclave with ethyl lactate present. This could conceivably give rise to partial hydrolysis of the ester to form free lactic acid and ethanol, thus altering the toxic qualities of a 4 per cent concentration of ethyl lactate. However, no growth occurred in the acetal or the ethyl lactate, indicating unsuitability of these compounds when present in concentrations of 4 per cent.

c. Dioxane. An interesting observation was that of the behavior of dioxane in media containing cells of S. cerevisiae. Ethyl ether has been considered to be toxic to this yeast, and as dioxane resembles this compound, it was felt that it would be equally or even more inhibitory. These two compounds are shown below:

ethyl ether

dioxane

However, a study of the toxicity of dioxane has shown that concentrations as high as 4 per cent do not appear to show any appreciable toxicity toward the metabolism of glucose by S. cerevisiae, so this result is an indication that dioxane would be a suitable solvent from the standpoint of non-inhibitory activity.

d. Triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate. Previous experimental work reported in this

thesis has indicated that this compound is inhibitory to microorganisms. Hence a study of its activity toward yeast was initiated, and results were based on the utilization of glucose as a measure of the growth of the cells, as glucose metabolism is the energy source for growing yeast cells.

A solution containing 10^{-4} mole of the compound per ml. was prepared by dissolving 1.6971 g. of the soluble lead compound in distilled water, and diluting the solution to 25 ml. total volume. A one ml. aliquot was removed and diluted to 10 ml. to give 10^{-5} mole per ml. of solution. A one ml. aliquot of the 10^{-5} mole per ml. solution was then used for preparing a 10^{-6} mole per ml. solution, and so on. This procedure is the one used in preparation of all organometallic solutions used in the experimental work reported in this thesis.

A preliminary trial experiment showed that addition of 10^{-4} mole of this lead compound to 10 ml. of glucose medium inoculated with S. cerevisiae would prevent all growth. Therefore this concentration was chosen as the starting point for a series of decreasing concentrations of the solutions of the lead compound added to cultures of S. cerevisiae. The results are given in Table 6.

A sudden increase in glucose utilization occurs at a concentration of 10^{-7} mole per ml. of medium; thus the toxic level is approximately 10^{-6} mole per ml. of medium for this compound with S. cerevisiae.

Sucrose, a disaccharide composed of one molecule of glucose and one molecule of fructose, is hydrolytically cleaved by the enzyme invertase. S. cerevisiae uses invertase to make glucose available when sucrose is present as the

Table 6. Growth of Saccharomyces cerevisiae in the presence of variable concentrations of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

Mole of compound per ml. of medium	Per cent glucose utilized
None	100.0
10^{-5}	0.0
10^{-6}	0.0
10^{-7}	100.0
10^{-8}	97.4
10^{-9}	99.1
10^{-10}	94.6

substrate, so it was decided that because sucrose is a nonreducing sugar, any glucose which is formed might be detectable in the medium. Demis et al. (33) have shown that their results point to invertase as an extracellular enzyme which is attached to the outer cell wall of S. cerevisiae, and which forms glucose and fructose at a rate far in excess of the rate of diffusion of these monosaccharides into the cell.

It was hoped that a reducing sugar analysis might demonstrate the presence of glucose and fructose if the invertase was not inhibited, because in flasks where the presence of the organolead compound prevented growth, the lack of invertase inhibition might cause a detectable amount of glucose and fructose to be present in the medium. Such a behavior would indicate that the invertase of yeast was not inhibited by the soluble organolead compound. Reducing sugar analyses, however, failed to demonstrate the presence of any significant amount of such compounds.

An interesting study of the addition of the soluble lead compound to growing cells showed the effect of this detergent on yeast cells of different ages. A series of flasks containing identical quantities (10 ml.) of the same glucose-yeast extract medium were inoculated at the same time with 0.5 ml. of an actively growing 24 hour old culture of S. cerevisiae. A 10^{-4} mole addition of the soluble organolead compound was made to the first flask, and to succeeding flasks at one hour intervals over an eleven hour period. The flasks were all allowed to remain in the incubator at 30° C. for 24 hours before being removed.

A cell count of the inoculum showed that 0.5 ml. contained 3.1×10^{7} cells. Thus there were 29.5×10^{5} cells per ml. of medium or a total of 3.1×10^{7} cells present when 10^{-4} mole of organolead detergent was added. A mole of any compound contains 6.02×10^{23} molecules; hence there was added

one ml. of solution containing $6.02 \times 10^{23} \times 10^{-4}$ or 6.02×10^{19} molecules of organolead compound. By division, one finds that there were 1.95×10^{12}, or 1,950,000,000,000 molecules present for each yeast cell at the initiation of the experiment. A cell count taken seven hours later indicated a concentration of approximately 3.4×10^{8} cells in the flask, giving 1.77×10^{11} molecules per cell. Such a ratio of molecules per cell was also inhibitory to the metabolism of glucose as only 27.2 per cent of the glucose was utilized showing that the uptake of it was halted.

In the data of Table 7 are presented the results of the addition of the lead compound at the various times shown.

Some of the glucose utilization values appear to be abnormally low, such as number 5, 7 and 8, but this may be due to poor initial growth following inoculation on the part of some of the cultures. In spite of these abnormal examples, it is interesting to observe the graded inhibition of glucose uptake with time, showing that this soluble organolead compound is toxic over a large variation in the yeast cell concentration of a medium.

It was desired to determine whether the prolonged exposure of living cells of S. cerevisiae to 10^{-4} mole of the water-soluble organolead compound in 12 ml. of medium for several days would result in a loss of cell viability. In a flask of 10 ml. of medium containing 2 per cent glucose and 0.25 per cent yeast extract was placed 10^{-4} mole of the lead compound

Table 7. Effect of 10^{-4} molar concentration of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate on yeast cultures of different ages.

Sample No.	Inoculum	Lead compound added at elapsed hours following inoculation	Per cent glucose utilized in 24 hours
1	-	0	0.0[a]
2	+	none added	94.8
3	+	0	6.6
4	+	1	11.0
5	+	2	5.7
6	+	3	11.4
7	+	4	9.7
8	+	5	11.4
9	+	6	21.5
10	+	7	27.2
11	+	8	37.3
12	+	9	53.6
13	+	10	64.1

[a]Control flask; not inoculated.

in one ml. of water, followed by aseptic inoculation with 1 ml. of a 24 hour culture of S. cerevisiae. After 9 days at 30° C., no growth had occurred, so the cells were resuspended by shaking and a loopful of this cell suspension used to inoculate 10 ml. of fresh medium. After two weeks, no growth had occurred, so a repeated inoculation of 0.2 ml. of the original cell suspension was placed in 21 ml. of fresh medium, but no growth took place.

Another addition of 10^{-4} mole of soluble organolead compound to 10 ml. of an active, 24 hour old culture of S. cerevisiae was made and allowed to stand at 30° C. for five weeks. The yeast cells were then centrifuged at ca. 10,000 r.p.m. for 10 minutes and the supernatant liquid removed by decantation. The cells were then washed by resuspending them in distilled water and centrifuging. This was repeated five times in an effort to remove the water-soluble lead compound completely. The cells were resuspended in fresh sterile glucose and yeast extract medium and incubated at 30° C. After two days, no growth had occurred.

The appearance of the yeast cells (S.cerevisiae) after exposure to the organolead detergent is of interest as they exhibit "coagulation", forming large clumps of cells. When the cells were washed with water, these clumps were reduced in size although there were still some evidences of abnormal behavior in their physical appearance, for a sufficient number

of cells were still in a coagulated or clumped state as to render them visible to the naked eye. This is possibly due to the adsorption of the organolead detergent compound onto the yeast cell surfaces, as described by Beck (16) and Beck and Meier (17) who have described the action of phenoxyethyldimethyldodecylammonium bromide on yeast. They cite the parallelism between the type of adsorption isotherm given by the detergent with Norit vs. that given by the detergent and yeast cells.

The potency of the lead atom in the molecule used here is less than that of the quaternary nitrogen and the five chemical groups bound to it. Later experimental work was done on the carbon atom analog of this lead compound and the alkyl chain and nitrogen atom portion of this molecule. This will be reported later.

It has been established that the viability of S. cerevisiae cells is destroyed by the presence of the water-soluble organolead compound, triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate. It was next decided to see how quickly the viability (as measured by the cell's ability to reproduce when transferred to fresh, sterile medium) of a cell was destroyed when a known toxic concentration of the organolead detergent was added to a large concentration of them. The following procedure was adopted:

To each of six tubes containing 12 hour old actively-growing cells of S. cerevisiae was added one ml. of an

organolead detergent solution whose concentration was 10^{-5} mole per ml. This gave a concentration of 10^{-6} mole of compound per ml. of medium. After predetermined intervals of time, 0.1 ml. aliquots from different tubes were transferred to fresh medium and incubated for 24 hours at 30° C. A check for viable (growing) cells was made at the end of this time, but no growth was observed in any sample.

A tube from this experiment in which no growth had occurred was inoculated with pure, resting cells of S. cerevisiae to see if growth would occur as a check for any toxic or inhibitory conditions existing in the tube of medium. After only 5 hours, growth had taken place sufficiently for visual detection. Observation of the other tubes in the order of their increasing time of exposure of the cells to the lead compound showed increasingly smaller amounts of white material at the bottom of each tube. These were agitated by shaking, and were incubated at 30° C. Results after 4 days are given below. It can be seen that these cells did not lose all their viability within 5 minutes as the 24 hour check reveals, but are only inhibited and retarded for an indefinite length of time. The resultant inhibition appears to affect the vigor of the ensuing fermentation.

These data may be summarized by saying that inhibition of S. cerevisiae cells in varying degree results from an exposure of the cells to a concentration of organolead detergent of 10^{-6} mole per ml. of medium when the time of the exposure

is less than 45 minutes. Exposure of greater than 45 minutes duration results in loss of cell viability. This indicates that the reaction or the process of adsorption is not an instantaneous one, but proceeds at a rate that is effectively complete in 45 minutes, and that the process is irreversible in the presence of fresh medium.

Table 8. Effect of short time exposure of S. cerevisiae to triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

Tube No.	Exposure time (min.)	Growth after 24 hours	Growth after 4 days
1	5	none	excellent
2	10	none	excellent
3	15	none	fair
4	30	none	fair
5	45	none	none
6	60	none	none

The cationic type of detergents has been shown to possess greater bacteriostatic properties in alkaline media than in acidic media. A study of cationic and of anionic detergents upon the respiration and glycolysis of both Gram positive and Gram negative organisms was undertaken by Baker et al. (11). They found the cationic types to be enhanced in their

activity in alkaline solution, while acid solutions enhanced the anionic varieties. The organolead detergent, triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate, is a cationic detergent, so its effectiveness was tested at different concentrations of the compound at different pH values within the pH range of 3 to 8.

Buffer solutions were prepared according to the method of MacIlvaine (88). Two solutions, designated as Solution A and Solution B, are added to each other in the proper proportions so as to produce a solution having the desired pH. These solutions are described here:

Solution A: Dissolve 107.3 g. of dibasic sodium phosphate heptahydrate in carbon dioxide-free distilled water and dilute this solution to exactly 2 liters total volume in a volumetric flask. A 10 ml. sample should require the addition of exactly 20.00 ml. of 0.1000 N hydrochloric acid to give a pH of 4.5. A Beckman pH meter was used to help attain this concentration of phosphate solution.

Solution B: Dissolve 42.0 g. of C.P. citric acid monohydrate in distilled water and dilute to exactly 2 liters as above. A 10 ml. sample should require the addition of exactly 30.00 ml. of 0.1000 N sodium hydroxide for neutralization to a phenolphthalein end point.

The solutions of the required pH were prepared by combining different volumes of Solution A and Solution B as shown

in Table 9.

The media were prepared by dissolving 1.0 g. of glucose and 0.125 g. of yeast extract in 50 ml. of the desired buffer solution. Each portion of medium was then distributed to five flasks to give 10 ml. of medium per flask. The

Table 9. Preparation of buffer solutions over a pH range of 3 to 8.

pH desired	ml. Solution A.	ml. Solution B.	pH obtained
3.0	20.5	79.5	3.04
4.0	38.5	61.5	4.00
5.0	51.5	48.5	5.01
6.0	63.2	36.8	6.00
7.0	82.4	17.6	6.95[a]
8.0	97.3	2.7	7.94[a]

[a]The pH of these solutions was adjusted to the exact values of 7.00 and 8.00 by the addition of Solution A, using a Beckmann pH meter.

contents of the flasks were designated by a key illustrated below. The lead compound referred to here is triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

3-M pH= 3; medium only; 1 ml. water; sterile

3-0 pH= 3; medium; org.; 1 ml. water

3-4 pH= 3; medium; org.; 10^{-4} mole of Pb compound

3-6 pH= 3; medium; org.; 10^{-6} mole of Pb compound

3-8 pH= 3; medium; org.; 10^{-8} mole of Pb compound

In this key the initial digit "3" denotes the pH of the media, while the suffix (-M, -O, etc.) designates the critical characteristics of the solution such as the presence of medium only (-M); organism and medium only (-O), or a concentration such as 10^{-4} mole of the compound (-4). The prefix "4" indicates a pH of 4; a "5" represents a pH of 5, and so on.

The media were autoclaved at 17 p.s.i. for 30 minutes and each was inoculated with a "loopful" of an active, 24 hour culture of S. cerevisiae. Following an incubation at 30° C. for 24 hours, the presence of any growth was designated by a plus sign; no growth was shown by a minus sign. Suitable aliquots were removed and analyzed for the presence of glucose. These results are given in Table 10.

Autoclaving the alkaline solutions of glucose caused a decrease of the pH in the range of 6.00 to 8.00. Table 11 shows the pH of the media after sterilization.

The values for the glucose utilized by the microorganism at any given pH were based on the total glucose present in the control flask of sterile medium at the respective pH of the solutions being analyzed.

Although the high pH values of 7 or 8 tended to impose a negative effect upon the successful growth of S. cerevisiae,

Table 10. Growth of S. cerevisiae at different pH levels.

Flask No.	Growth in 24 hrs.	Per cent glucose utilized
3-M	-	0.0
3-0	+	90.4
3-4	-	0.97
3-6	+	79.7
3-8	+	35.7
4-M	-	0.0
4-0	+	100.0
4-4	-	0.0
4-6	+	100.0
4-8	+	100.0
5-M	-(-)[a]	0.0
5-0	-(+)	98.0
5-4	-(-)	0.0
5-6	-(+)	52.6
5-8	-(+)	90.0
6-M	-[b]	0.0
6-0	-[b]	7.6
6-4	-	0.0
6-6	-	0.0
6-8	+	19.2
7-M	-[b]	0.0
7-0	-[b]	5.4
7-4	-[b]	0.0
7-6	-[b]	7.2
7-8	-[b]	4.8
8-M	-	0.0
8-0	-	0.0
8-4	-	0.0
8-6	-	0.0
8-8	-	0.0

[a]Values in parentheses were based on further observation after flasks had stood at room temperature for 48 hours. Glucose values were based on media analyzed after this period for all flasks.

[b]Small amount of growth as evidenced by glucose utilization values.

an enhancement of the bacteriostatic effectiveness of the organolead cationic detergent appeared to be demonstrated at a pH of 6 as compared with a pH of 5. A pH of 4.0 to 4.5 is most conducive to the proper growth of S. cerevisiae.

Table 11. Change of pH during sterilization of buffer solutions.

Original pH	pH after sterilization
3.04	3.14
4.00	4.09
5.01	5.01
6.00	5.90
7.00	6.69
8.00	7.86

The effect of British antilewisite, hereafter referred to as BAL, upon S. cerevisiae was studied from the viewpoint of its action against a different type of organometallic compound than the organolead detergent used heretofore. However, the action of BAL was tested on S. cerevisiae when the organolead compound was also present. There appeared to be no beneficial effect to the growth of the S. cerevisiae as there was no enhanced growth nor signs of any depressed growth when BAL was present in concentrations of 10^{-5} mole or

10^{-7} mole per ml. of medium.

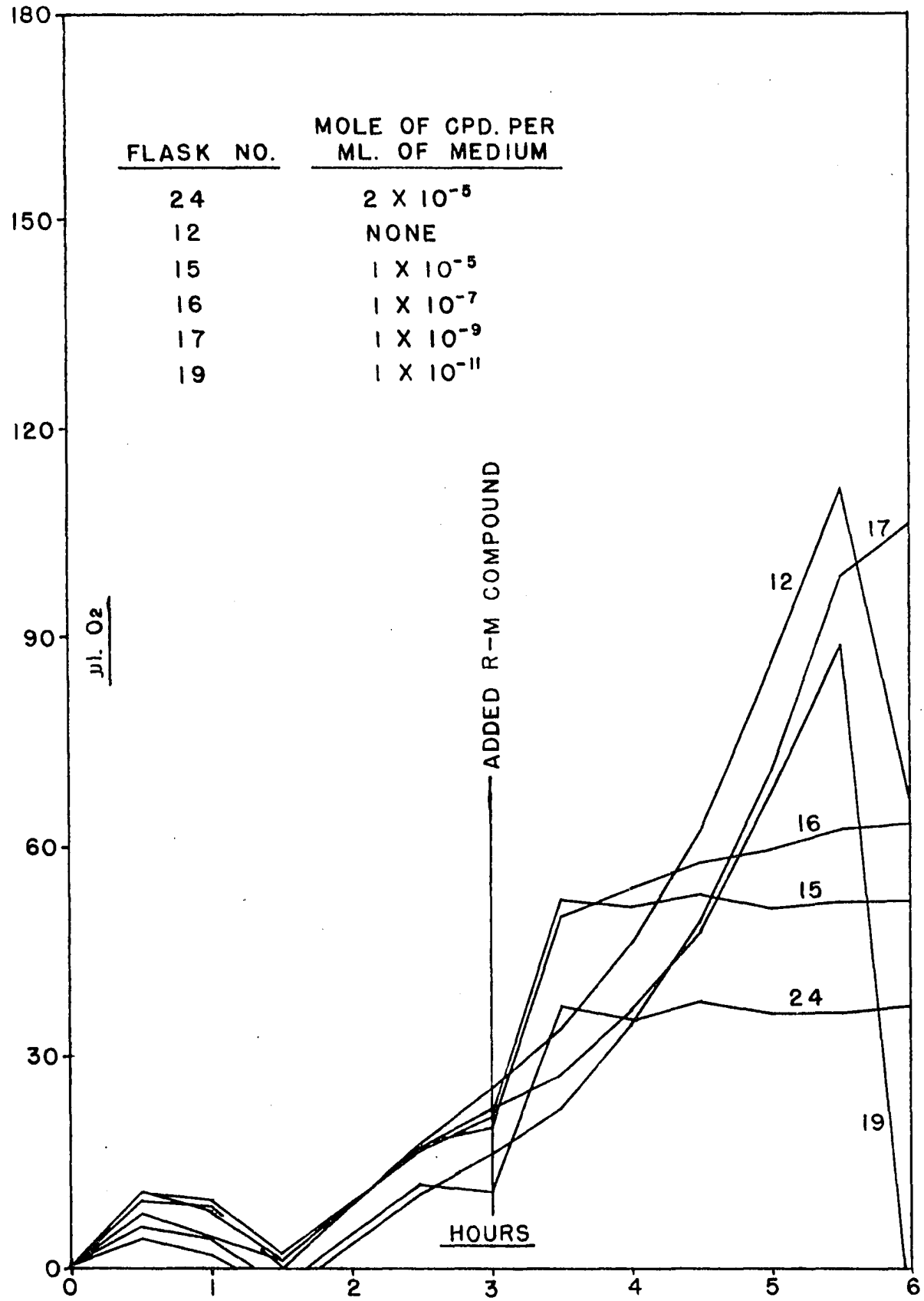
A study of the effect of organometallic compounds on the uptake of oxygen by aerobic microorganisms were made, using the Warburg apparatus. All determinations were made at 30° C. and involved a measurement of the uptake of oxygen in an atmosphere of air. All compounds to be added to the medium were placed in the sidearms of the small Warburg flasks as described by Umbreit et al. (139) and were added by removing a manometer and its attached flask as a unit, tipping the flask sufficiently to cause the contents to spill into the medium, and replacing the manometer and flask.

Readings were taken every 30 minutes generally, by halting the shaking bar, adjusting the right arm of the manometer to the original reading, then reading the left manometer arm. A thermobarometer flask containing water only was shaken along with all other flasks. Its purpose was to cancel any changes in the manometer readings due to temperature changes in the room or to barometric pressure differences; hence its name, thermobarometer.

The effect of triphenyl-Y-(diethylmethylammonium)-propylead methosulfate on the respiration of S. cerevisiae is shown by the plot in Figure 5 in which the microliters of oxygen are plotted vs. the time expressed in hours. The vertical line indicates addition of the organolead compound occurred 3 hours after the first readings were taken.

The uptake of oxygen by S. cerevisiae cells treated with

Figure 5. The respiration of Saccharomyces cerevisiae in the presence of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.


FLASK NO.
MOLE OF CPD. PER ML. OF MEDIUM
24
2 X 10⁻⁵
12
NONE
15
1 X 10⁻⁵
16
1 X 10⁻⁷
17
1 X 10⁻⁹
19
1 X 10⁻¹¹
µl. O2
ADDED R-M COMPOUND
HOURS
180
150
120
90
60
30
0
0
1
2
3
4
5
6
17
12
16
15
24
19

2×10^{-5}, 1×10^{-5} and 1×10^{-7} mole of organolead compound per ml. of medium appeared to have been halted rather abruptly after an initial sudden increase in the rate of oxygen uptake. The addition of the more dilute concentrations appeared to have no effect upon the cell respiration, as the uptake of oxygen paralleled that of the control to which no compounds were added.

The behavior exhibited by the respiration of the yeast cells can be correlated with the results of the utilization of glucose by this organism. The data in Table 6 shows the concentration of the organolead compound (see page 94) in terms of mole of compound per ml. of medium; these results should be compared with the plots in Figure 5. It will be seen that those concentrations which inhibit the respiration are the same as those which inhibit glucose metabolism except for the use of 10^{-7} mole of compound per ml. of medium. The inhibition of the respiration in this case is apparently overcome, as the organism can utilize all of the glucose in a test medium. It might be expected that S. cerevisiae would demonstrate an uptake of oxygen due to endogenous metabolic activity even if the diffusion of glucose into the cell were suddenly inhibited, but no oxygen appears to be taken up. This may point to an inhibition of the respiratory enzymes by the organolead compound, in addition to an action whereby the metabolic enzymes are also prevented from acting upon the glucose molecules.

e. 4,4,4-Triphenyl-n-butyldiethylmethylammonium methosulfate. In order to ascertain the effect of the lead atom of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate upon microorganisms, the same compound had to be synthesized in which a carbon atom replaced the lead atom. The synthesis and characterization of this compound has been described previously and will not be repeated here. The compound will be referred to as "444T" in future references to its activity.

An evaluation of 444T based upon its activity in preventing the metabolism or uptake of glucose shows that it approached the toxicity of its organolead analog, although it is not inhibitory in certain concentration ranges as is the latter compound. The data shown below in Table 12 concerning 444T should be compared with those of Table 6; it will be seen to corroborate the activity ascribed above to this compound.

An interesting contrast of this data above to that found for the organolead analog of 444T is found in the toxicity shown by the latter compound in the concentration range of 10^{-6} mole per ml. of medium. The organolead compound is toxic at such a concentration whereas the 444T does not completely inhibit growth. Such differences as these may possibly be ascribed in part to a dual nature of the organolead detergent arising from its action as a detergent as well as its effect as a toxic compound of a heavy metal, lead.

The phenomenon of clumping of the yeast cells first

noticed with the organolead detergent was also seen here; solutions of 444T when added in concentrations of 10^{-4} mole of the compound per ml. of solution, caused coagulation of the yeast cells. These clumps were examined microscopically

Table 12. The effect of 444T upon the utilization of glucose by Saccharomyces cerevisiae.

Mole of 444T per ml. of medium	Per cent glucose utilized
None	98.0
10^{-5}	0.0
10^{-6}	11.1
10^{-7}	96.0
10^{-9}	95.2
10^{-10}	91.4
10^{-11}	92.9

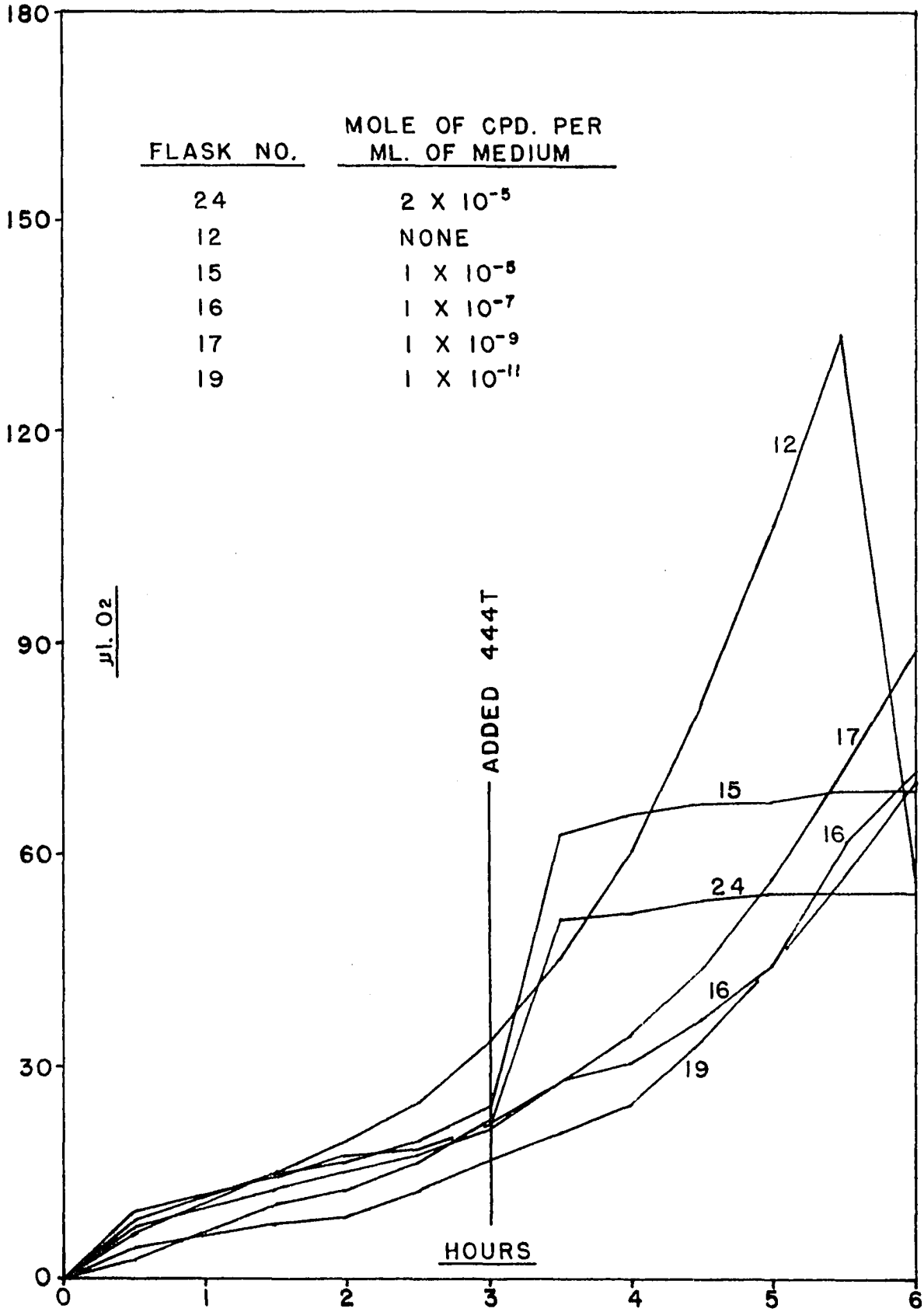
and were found to consist of nothing other than large masses of cells.

The respiration of S. cerevisiae was shown to be inhibited by the organolead analog of 444T. Figure 6 shows the effect of 444T on the respiration of S. cerevisiae when various concentrations were used identical to those employed with this same organism and the lead analog. A similarity

in the respiratory inhibition is seen here, as there is a preliminary, sharply increased rate of oxygen uptake, followed by an inhibition of the respiration of the yeast cells. A very apparent change in the respiration occurred with the addition of 1×10^{-7} mole of organolead compound per ml. of medium (see Figure 5) to S. cerevisiae but when 444T was added in the same concentration, the respiratory curve followed that of the control and also the more dilute concentrations, showing no inhibition whatsoever. This behavior is in agreement with the glucose utilization data which show no toxicity for the corresponding concentration of 444T.

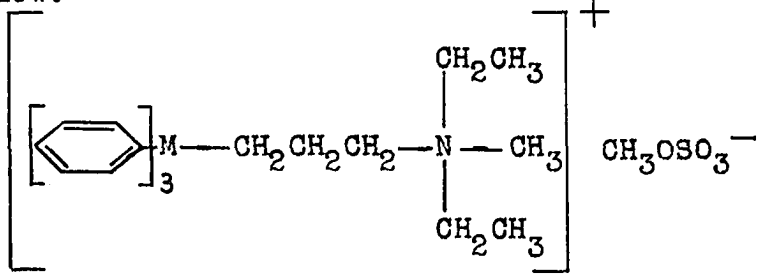
An attempt was made to correlate the effect of 444T on the growth of S. cerevisiae with the length of the period of contact. Ten ml. samples of glucose-yeast extract media were inoculated with one ml. each of a 24 hour culture of S. cerevisiae and incubated for 3 hours. Then inocula of 0.2 ml. from each tube were transferred to tubes of glucose and yeast extract in 1.5 per cent liquid agar medium at different periods following the prior introduction of 10^{-4} mole of 444T to each 3 hour old culture of yeast cells. The inoculated agar media were transferred aseptically to sterile Petri dishes and incubated for 3 days at 30° C. No growth occurred in any of the samples from an exposure time of 10 minutes to 120 minutes. From the observations with the organolead analog, this compound is apparently too toxic to allow viable

Figure 6. The respiration of *Saccharomyces cerevisiae* in the presence of 4,4,4-triphenyl-n-butyl-diethylmethylammonium methosulfate.

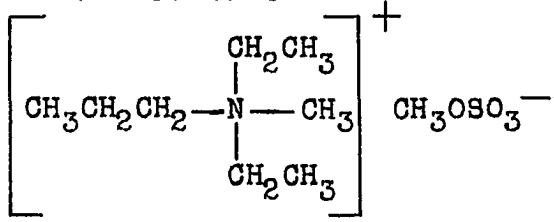

FLASK NO.
MOLE OF CPD. PER ML. OF MEDIUM
24
2 X 10⁻⁵
12
NONE
15
1 X 10⁻⁵
16
1 X 10⁻⁷
17
1 X 10⁻⁹
19
1 X 10⁻¹¹
µl. O2
ADDED 444T
HOURS
180
150
120
90
60
30
0
0
1
2
3
4
5
6
12
17
15
16
24
16
19

cells to remain as such for any extended period greater than 30 to 45 minutes.

f. n-Propyldiethylmethylammonium methosulfate. The preceding two compounds discussed in this thesis were identical in their structure except where a triphenylmethyl group replaced a triphenylplumbyl group. A compound which embodied only the alkyl chain bonded to this lead (or carbon) atom and the same quaternary methosulfate part of these molecules was synthesized. Their relationships are shown by the structures below.



M = Pb or C



n-propyldiethylmethylammonium methosulfate

A test of this compound for its effect on the growth of microorganisms involved a 10 minute exposure to the air of 10 ml. of glucose-yeast extract media in each of two Petri dishes. One dish also contained 10^{-4} mole of the alkyl

quaternary methosulfate. Observation of the plates after two days revealed the growth of a scummy pellicle over the entire surface of the untreated medium, and one mold colony 9 mm. in diameter on the treated medium. A day later, the treated medium had three mold colonies; the untreated medium had one, in addition to the scum. This observation demonstrates a decreased toxicity of this compound towards molds in contrast to the compounds having the triphenyl groups present. The latter inhibited all microorganisms at the same concentration (10^{-4} mole addition). However, the presence of this compound did prevent the growth of the scummy pellicle material, so some resistance to certain types of microorganisms seems to be associated with this molecule.

This observation was borne out by an experiment in which the glucose utilization of S. cerevisiae was studied. Inoculation of sterile media with 0.1 ml. of an active, 24 hour culture of this microorganism, followed by addition of decreasing quantities of the compound being tested gave results as shown here in Table 13.

Incubation for 24 hours at 30° C. produced no glucose utilization; an unexpected behavior for this compound. No reports of any tests of the toxicity of tertiary amines or quaternary alkyl ammonium compounds could be found in the literature, so no basis for comparison is available. It is of interest, however, that if the part of the organolead detergent molecule (see page 116, M = Pb)

Table 13. Glucose utilization of Saccharomyces cerevisiae in the presence of n-propyldiethylmethylammonium methosulfate.

Mole of compound per ml. of medium	Per cent glucose utilized
None	100.0
10^{-5}	3.0[a]
10^{-6}	100.0
10^{-7}	100.0
10^{-8}	100.0

[a]Reinoculation of this sample with a one ml. inoculum of a 24 hour culture of S. cerevisiae resulted in the formation of clumps of cells noted previously with the organolead detergent and with 444T.

represented by this alkyl quaternary ammonium compound can act in an inhibitory manner at this concentration, then there may be at least three types of inhibitory activity associated with the organolead detergent:

1. The physical effect exerted by the molecule acting as a surface tension reductant.
2. The toxic effect of the heavy metal atom, lead, present in the molecule.
3. The physical-chemical inhibitory activity imparted to the molecule by:
   (a) The alkyl groups about the nitrogen atom.
   (b) The nature of the methosulfate anion. Dimethyl sulfate is very toxic to humans.

Thus it appears that a multiplicity of factors may be involved individually or collectively in the inhibition produced by this compound, triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate on the growth of microorganisms. Other compounds of a similar nature may demonstrate like properties.

g. Diphenylmercury. Toxic effects of detergents might possibly arise chiefly from the presence of a quaternary ammonium structure of a molecule and the often consequent water solubility. However, the structure of diphenylmercury possesses neither of these properties; it is water insoluble, contains a heavy metal, mercury, which is bound to two aromatic hydrocarbon radicals by essentially covalent bonds (61), and might be said to resemble a hydrocarbon in which the mercury atom replaces the methylene group in diphenylmethane.

The toxicity of organic compounds of mercury is well known, and many mercury compounds have been evaluated for their inhibition of microorganisms. The use of such compounds has nearly always entailed that type of organomercury compound where an inorganic anion is bonded to the organomercuric cation. Diphenylmercury is not such a compound.

The diphenylmercury was dissolved in ethyl lactate and either 0.1 ml. or 0.2 ml. of a diphenylmercury solution was used at any one time. The action of this organometallic

compound against Saccharomyces cerevisiae was evaluated in terms of glucose utilization by the organism, as shown in Table 14.

The results tabulated above show that the toxic effects of this compound are manifested at very low concentrations and point to a strongly inhibitory activity of the diphenylmercury against S. cerevisiae.

The use of ethyl lactate as a solvent was not wholly satisfactory as the solvent appeared to act in a toxic manner when it was used in certain concentrations approaching 0.2 ml. of lactate per 10 ml. of medium. An inhibition of growth appeared when a concentration of 3×10^{-9} mole of diphenylmercury in 0.2 ml. of ethyl lactate was used, whereas concentrations of 4×10^{-9} mole of the same compound in 0.1 ml. of solvent gave a 26.3 per cent value for utilization of glucose. Masui (96) has shown that D,L-ethyl lactate was hydrolyzed by an enzyme of the pancreas, liver, spleen and kidney of rabbits, and by an enzyme found in fat-free pumpkin seeds; no asymmetric products were found, however. The D,L-ester was hydrolyzed into optically-active products by enzymes of the liver and pancreas of swine, and by enzymes from fat-free castor beans. The D-ethyl lactate appeared to be the more readily hydrolyzed, giving a greater amount of D-lactic acid in the medium. The possibility that the ethyl lactate may be hydrolyzed by an enzymatic attack to give a toxic or inhibitory amount of free D- or L-lactic acid is thus to be

Table 14. The effect of diphenylmercury on the utilization of glucose by Saccharomyces cerevisiae.

Mole of compound per ml. of medium	Per cent glucose utilized
0.1 ml. ethyl lactate	100.0
1×10^{-10}	97.4
2×10^{-10}	95.8
4×10^{-10}	26.3; (74.7)[a]
6×10^{-10}	2.5
8×10^{-10}	2.2
1×10^{-9}	2.2
2×10^{-9}	0.0
2×10^{-8}	0.0
1×10^{-7}	0.0
2×10^{-7}	3.2[b]
2×10^{-6}	7.1[b] (24.9)[b]

[a]Used 0.1 ml. of broth inoculum instead of a small loopful of broth.

[b]Unexplained values indicating an unaccounted growth at these concentrations were obtained in preliminary trials of this compound.

considered. Free acid thus produced would then cleave diphenylmercury to give other inhibitory organomercury derivatives. Such a course of events might be partially responsible for any deleterious effect of ethyl lactate upon the growth of S. cerevisiae. The higher value obtained for glucose utilized when a larger inoculum was used may possibly point to a decreased toxicity of diphenylmercury in the presence of an increased number of cells, indicating that a decreased ratio of the number of molecules to the number of yeast cells will greatly affect the resultant inhibition.

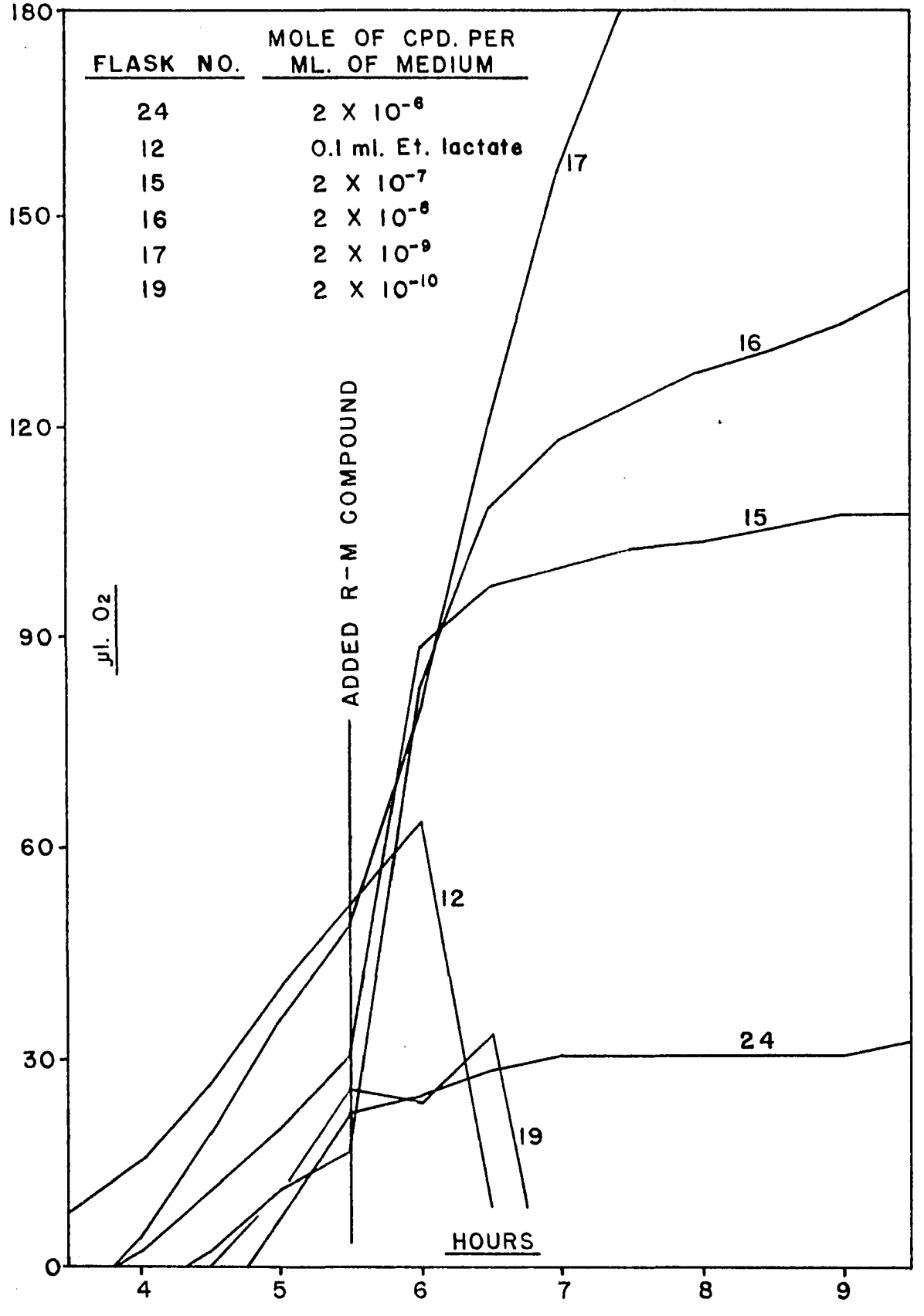
A knowledge of the limits of toxicity for diphenylmercury with S. cerevisiae should be correlated with its action against the respiratory system of the yeast cells. Cook et al. (30) showed that phenylmercuric nitrate in concentrations of 1.7×10^{-5} molar to 5.0×10^{-5} molar solutions displayed a depression of cytochrome in the oxidation of ascorbic acid when cytochrome oxidase was present also. The degree of inhibition increased with an increase in the concentration of phenylmercuric nitrate. Complete inhibition (no oxygen uptake) resulted with succinoxidase. An active inhibition of succinic and of lactic dehydrogenases occurred while only a depression of activity for glucose dehydrogenase was found. Catalase activity underwent a 36 per cent decrease in activity with a 4.5×10^{-5} molar concentration. If the mode of activity of diphenylmercury is similar to that of phenylmercuric nitrate, it might be expected to be demon-

strated by a depression of the respiration of S. cerevisiae. Such an effect was demonstrated using different concentrations of diphenylmercury solutions. A determination employing the Warburg apparatus was performed. The concentrations of diphenylmercury used and a plot of the oxygen uptake vs. time (in hours) are given in Figure 7.

It is noted here that one concentration of diphenylmercury which has been shown to be inhibitory to glucose metabolism has not inhibited the respiration of S. cerevisiae cells; i.e. the addition of 10^{-7} mole of the organomercury compound which gave 2×10^{-9} mole of compound per ml. of medium. At this concentration, the metabolism of glucose was inhibited 100 per cent, although the respiration of the cells suffered no such fate. Thus it appears that the compound is more active against the metabolic enzymes than those concerned directly with respiration. The 10^{-5} mole of added mercury compound (2×10^{-6} mole of compound per ml. of medium) appears to have caused a complete cessation of oxygen and glucose uptake. The behavior of the "control" culture will be discussed later with regard to the apparent decrease of oxygen uptake. Such behavior in other Warburg determinations has been noticed also, and will be dealt with later.

Perkins (109) has recently studied the effect of amino acid structure on the stabilities of complexes formed by Group II metals, and has said that mercury appears to chelate specifically with sulfhydryl (-SH) groups. Albert (1)

Figure 7. The respiration of *Saccharomyces cerevisiae* in the presence of diphenylmercury.


FLASK NO.
MOLE OF CPD. PER ML. OF MEDIUM
24 2 X 10⁻⁶
12 0.1 ml. Et. lactate
15 2 X 10⁻⁷
16 2 X 10⁻⁸
17 2 X 10⁻⁹
19 2 X 10⁻¹⁰
ADDED R-M COMPOUND
µl. O2
HOURS
180
150
120
90
60
30
0
4
5
6
7
8
9
17
16
15
12
24
19

undertook a similar study and demonstrated a reaction of copper with ethylenediamine type of structures. Perkins stated that mercury might also be expected to react in this manner.

Commenting on the reaction of sulfhydryl groups, Barron and Kalnitsky (13) stated that inorganic mercury is not believed by Haarmann to be specific for sulfhydryl groups although it is said by him to combine with peptide groups in proteins primarily and with the nitrogen of imidazole groups, secondarily.

With these studies in mind, it was decided to observe any possible interaction between a diphenylmercury solution and a solution of an amino acid hydrochloride. A suspension of about 10 mg. of lysine monohydrochloride in 2 ml. of ethyl lactate was prepared, and up to 14 drops of distilled water added with shaking until all the hydrochloride was in solution. There was then added 0.6 ml. of an ethyl lactate solution of diphenylmercury which contained 10^{-4} mole of the organomercury compound per ml. Crystalline material began to precipitate from the homogeneous solution approximately 1.5 minutes later, continuing to do so until sufficient material was available to fill the bottom portion of the small six inch test tube.

A blank test was made in which no lysine monohydrochloride was present. Excess water was added to see if the precipitate was only diphenylmercury coming out of solution; no precipitation occurred, however. An excess of ethyl

lactate was then added to the tube with the original precipitate to see if lysine monohydrochloride would be forced out of solution; no additional precipitation occurred. The addition of water in this case, however, unlike that above, caused immediate precipitation to occur, showing that this material was neither lysine monohydrochloride (very soluble in water) nor diphenylmercury.

It was desired to perform the same experiment using valine hydrochloride instead of lysine monohydrochloride as the former compound has an amine hydrochloride only, while the latter has both an amine hydrochloride and a free amino group. The positive charge of the amine hydrochloride might possess a degree of attraction for the mercury in diphenylmercury, as Hampson (61) has indicated that the negative part of the dipole between mercury and the ring carbon of an adjoining phenyl ring lies toward the mercury atom.

As in the case with lysine monohydrochloride, the same results were found to occur when valine hydrochloride was used. This hydrochloride was prepared from valine by preparing a thin paste of valine with concentrated hydrochloric acid, adding sufficient distilled water to cause solution, and evaporating the solution to dryness <u>in vacuo</u>, using a vacuum desiccator containing pellets of sodium hydroxide in two open Petri dishes. Dry crystalline valine hydrochloride was obtained in 2 hours, and this was used in the same manner as was lysine monohydrochloride.

The free amino acid (valine) was also tested as were the hydrochlorides but no precipitate was produced. It was thought that either the presence of the hydrogen chloride in the amine hydrochloride or the positively-charged nitrogen was responsible for this phenomenon. Therefore, an amount of hydrochloric acid, equivalent in its concentration of hydrogen chloride to the hydrogen chloride present in valine hydrochloride, was tested with diphenylmercury. Again a precipitation occurred, even though the quantity of hydrochloric acid was only 1 drop (ca. 0.05 ml.) of a 1.3 molar solution, or 0.065 milliequivalent of acid, although the amount of diphenylmercury was even less. The addition of hydrochloric acid to the solution of free valine used above also caused precipitate formation when diphenylmercury was added.

All of these precipitates were found to melt at 250-251° C. An authentic sample of phenylmercuric chloride was then prepared by treating an alcoholic solution of diphenylmercury with dilute aqueous hydrochloric acid. There were obtained white crystals of the product, M.P. 251-251.5° C. A mixed melting point of the phenylmercuric chloride and the precipitated material described above melted at 250-250.5° C., showing that in all these cases, the presence of an amino acid hydrochloride will readily cleave diphenylmercury to form phenylmercuric chloride.

Because of the possibility of diphenylmercury cleavage to produce phenylmercuric chloride, it was thought that the

latter compound might readily react with essential sulfhydryl groups of enzymes as reported with phenylmercuric nitrate previously. Therefore, the addition of BAL (British antilewisite) should perhaps reduce or possibly eliminate the toxic effect of diphenylmercury on S. cerevisiae.

A preliminary check of this theory on the growth of S. cerevisiae was made when 0.1 ml. of a solution of BAL containing 10^{-3} mole per ml. of 35 per cent ethanol was added to 10 ml. of glucose-yeast extract containing 0.2 ml. (2×10^{-5} mole) of diphenylmercury in ethyl lactate. After 17 hours, a control culture had used only 60.7 per cent of the glucose, but the BAL test culture showed only 4.5 per cent utilization.

In Table 15 are shown the results of the growth of S. cerevisiae with diphenylmercury when BAL is present and absent. The BAL added to the sterile sample allowed for the effect of the compound upon the reducing sugar analyses.

It will be recalled that the addition of 4×10^{-10} mole of diphenylmercury gave a value of 26.3 per cent and also a higher value of 74.7 per cent when the larger inoculum was used. In this experiment, however, a value of 96.1 per cent was obtained; apparently this concentration is responsible for a variable degree of inhibition because of a marginal toxicity at such a value (4×10^{-10} mole per ml. of medium). The use of 1×10^{-9} mole of diphenylmercury per ml. of medium led to a 5 per cent glucose utilization; addition of 10^{-7} mole of BAL per ml. of medium gave 7.1 per cent, while 10^{-6}

mole of BAL per ml. of medium gave a 10.3 per cent uptake of glucose. This indicates to a small degree some possible interaction of the BAL in aiding to overcome the toxicity of the diphenylmercury.

Table 15. Glucose utilized by Saccharomyces cerevisiae when BAL is present with diphenylmercury.

Mole of compounds per ml. of medium		Per cent glucose utilized
Diphenylmercury	BAL	
None	10^{-7}	0.0
None	10^{-7}	99.0
4×10^{-10}	None	96.1
4×10^{-10}	10^{-7}	100.0
4×10^{-10}	10^{-7}	98.6
1×10^{-9}	10^{-6}	10.3
1×10^{-9}	10^{-7}	7.1
1×10^{-9}	None	5.0

The speed with which the cell viability of S. cerevisiae was destroyed with diphenylmercury was tested in the same manner as that used for the organolead detergent (see page 100). Each tube of an active, 14 hour old culture of S. cerevisiae was inoculated with 0.1 ml. of a solution of 10^{-5} mole per ml. of diphenylmercury in ethyl lactate. The tubes

were shaken and 0.1 ml. aliquots transferred to fresh medium after a period of 5 to 60 minutes. The fresh media were then incubated at 30° C. for one week. No growth was found in any of the new media, indicating that even with a 5 minute exposure, the diphenylmercury destroyed the viability of the cells.

h. Mercuric chloride. A comparison of the action of mercuric chloride on cells of S. cerevisiae was desired, using concentrations which were known to be toxic when employing diphenylmercury against this microorganism. Accordingly, the addition to 10 ml. of medium of the amounts of mercuric chloride required for inhibition were determined as set forth in Table 16.

The value for the 1×10^{-10} mole concentration appears to be out of line with the others, and inasmuch as more concentrated solutions were not inhibitory to essentially complete growth, it is believed that this value should probably be 100 per cent also. It is seen by comparing these results with those of diphenylmercury in Table 14 that the organomercury compound is more toxic to S. cerevisiae than is mercuric chloride, as a concentration of 6×10^{-10} mole of diphenylmercury per ml. of medium is inhibitory to growth whereas a concentration of 1×10^{-8} mole of mercuric chloride per ml. of medium is not toxic.

i. Sodium benzoate. A preliminary check of the toxicity of sodium benzoate against S. cerevisiae was made using the same concentrations as those of other compounds found to be toxic against this organism. Handler and Bernheim (62)

Table 16. Glucose utilization by Saccharomyces cerevisiae in the presence of mercuric chloride.

Mole of compound per ml. of medium	Per cent glucose utilized
None	100.0
1×10^{-5}	0.0
1×10^{-7}	0.0
1×10^{-8}	99.0
2×10^{-9}	100.0
1×10^{-10}	90.0
2×10^{-11}	100.0

found that benzoic acid inhibits the D-amino acid oxidase of D (†)-methionine, so it was felt that some knowledge of the action of sodium benzoate on S. cerevisiae might be of use to this problem. However, concentrations of 10^{-5} mole of benzoate per ml. of medium and of 10^{-7} mole per ml. of medium showed essentially no inhibition of growth.

j. Triphenyl-p-tolyllead. Dioxane, the most suitable

solvent found for this compound, was so compatible to the normal growth of S. cerevisiae that it was used safely in concentrations up to four per cent of the total volume of the medium. A study of the action of this compound on S. cerevisiae was made following preliminary trials which indicated it to be toxic to the organism. Inocula were 0.1 ml. of an actively-growing culture of S. cerevisiae, and the incubation was continued for 24 hours at 30° C. The results are tabulated in Table 17. The BAL was added to give 10^{-7} mole per ml. of medium.

The use of higher concentrations of BAL in the preliminary trials appeared to be inhibitory, but the addition of 10^{-6} mole of BAL aided the yeast cells to overcome the toxicity of the triphenyl-p-tolyllead when the latter was present in 4×10^{-7} mole per ml. of medium. This lead compound is not as toxic to S. cerevisiae as was the diphenylmercury, and its mode of action is not known. A test was conducted identical with that used for diphenylmercury in which triphenyl-p-tolyllead was added to an amino acid hydrochloride. No precipitated material was found after standing for 2 to 3 days. However, it is an unsymmetrical organolead compound, and as such, it is more readily cleaved than is a symmetrical organolead compound such as tetraphenyllead. No suitable solvent for tetraphenyllead could be found so it was not possible to test this compound.

The mediation of BAL points to a relationship of the

Table 17. Utilization of glucose by Saccharomyces cerevisiae in the presence of triphenyl-p-tolyllead and BAL.

Mole of compounds per ml. of medium		Per cent glucose utilized
Lead cpd.	BAL	
None	10^{-7}	0.0
None	10^{-7}	99.0
1×10^{-6}	None	19.6
4×10^{-7}	None	17.6
4×10^{-7}	10^{-7}	89.8
1×10^{-7}	None	89.4 (8.5)[a]
1×10^{-7}	10^{-7}	96.6 (4.9)[a,b]
1×10^{-8}	None	96.1
1×10^{-8}	10^{-7}	98.6
1×10^{-9}	None	100.0
1×10^{-10}	None	99.2

[a]Preliminary trial value; used one loopful of inoculum.

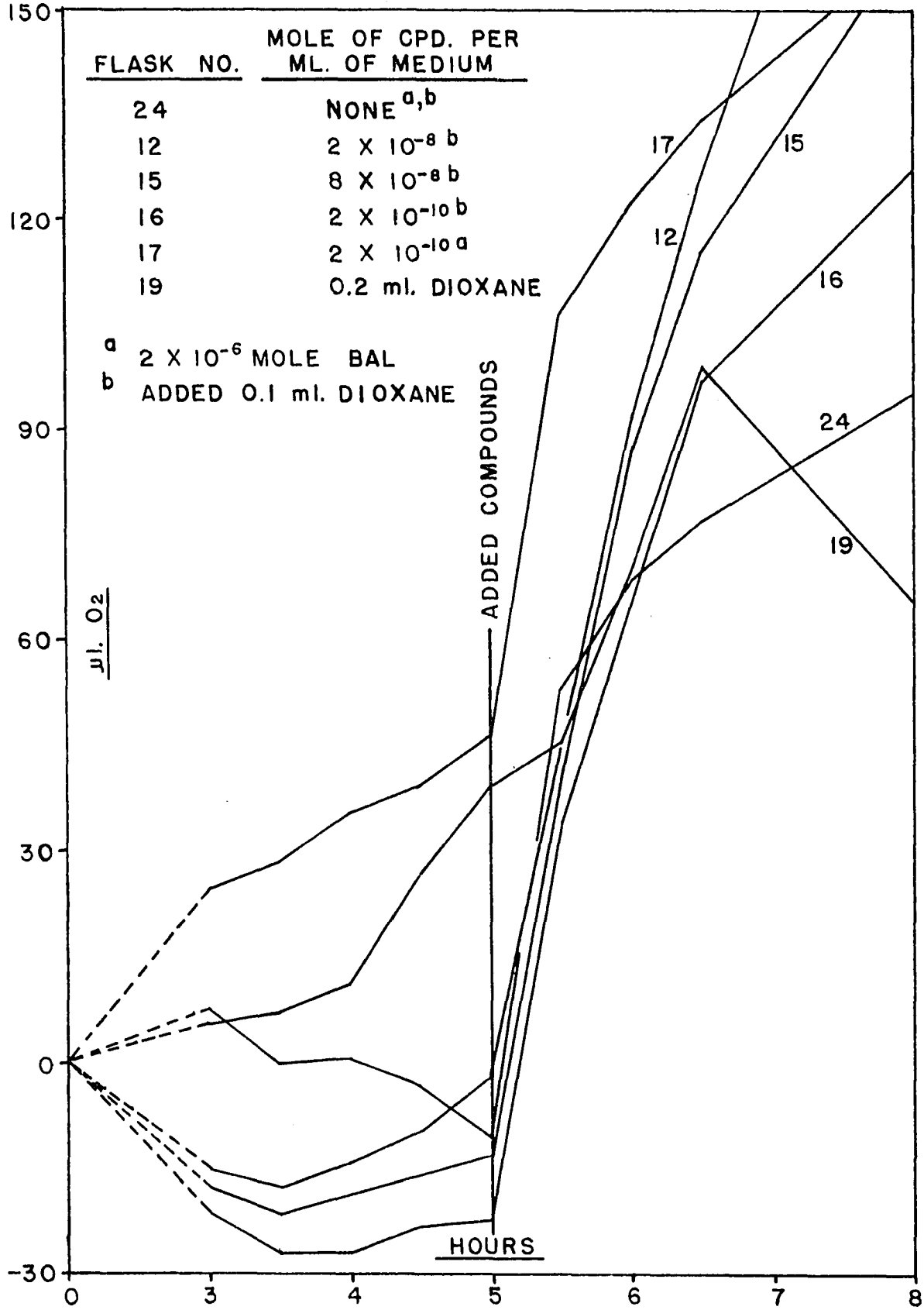
[b]Preliminary trial value; 10^{-5} mole of BAL per ml. of medium.

activity of triphenyl-p-tolyllead against sulfhydryl groups of essential enzyme systems in S. cerevisiae.

The respiration of S. cerevisiae in the presence of triphenyl-p-tolyllead was also studied for comparison with the compounds previously examined. No definite inhibition of respiration could be seen except for a slight decrease in the rate due, probably, to the large amount of BAL in the control sample. All cells experienced a sharp and sudden increase in their respiratory rates, including the control to which only dioxane was added. This was therefore attributed to the action of the dioxane on the yeast cells; i.e., it acted as a stimulant to their respiration. Glucose analyses were made on the media in the flasks following the termination of this experiment. The results, shown below in Table 18, show that the concentration of BAL used proved to be very inhibitory, causing complete inhibition of glucose metabolism. The inhibited metabolism of glucose in the two cases for flask 12 and 15 did not decrease the respiration to any extent, so the triphenyl-p-tolyllead did not inhibit the respiratory enzymes. The Warburg plots are shown in Figure 8.

k. A study of the endogenous respiration of S. cerevisiae. In some of the experiments in which the respiration of microorganisms was studied, the rates of oxygen uptake, which were measured manometrically in terms of the internal pressure change due to an increase or a decrease in the volume

Figure 8. The respiration of *Saccharomyces cerevisiae* in the presence of triphenyl-p-tolyllead and BAL.


FLASK NO.
MOLE OF CPD. PER ML. OF MEDIUM
24 NONE a,b
12 2 X 10-8 b
15 8 X 10-8 b
16 2 X 10-10 b
17 2 X 10-10 a
19 0.2 ml. DIOXANE
a 2 X 10-6 MOLE BAL
b ADDED 0.1 ml. DIOXANE
ADDED COMPOUNDS
µl. O2
HOURS
150
120
90
60
30
0
-30
0
3
4
5
6
7
8
17
15
12
16
24
19

of the flask and manometer system, showed abnormal behavior. Sudden pressure changes were observed in a negative direction which could only arise from a sudden increase of the gaseous phase in the flasks. It was thought that this behavior

Table 18. The utilization of glucose by Saccharomyces cerevisiae in the presence of triphenyl-p-tolyllead and BAL following a study of the respiration of this microorganism.

Warburg flask No.	Mole of compounds per ml. of medium		Per cent glucose utilized
	Lead cpd.	BAL	
24	None[a]	2×10^{-6}	0.0
12	2×10^{-8}[a]	None	10.7
15	8×10^{-8}[a]	None	11.1
16	2×10^{-10}[a]	None	100.0
17	2×10^{-10}	2×10^{-6}	0.0
19	None[b]	None	100.0

[a]Added 0.1 ml. dioxane to each original flask of medium.
[b]Added 0.2 ml. dioxane to the medium.

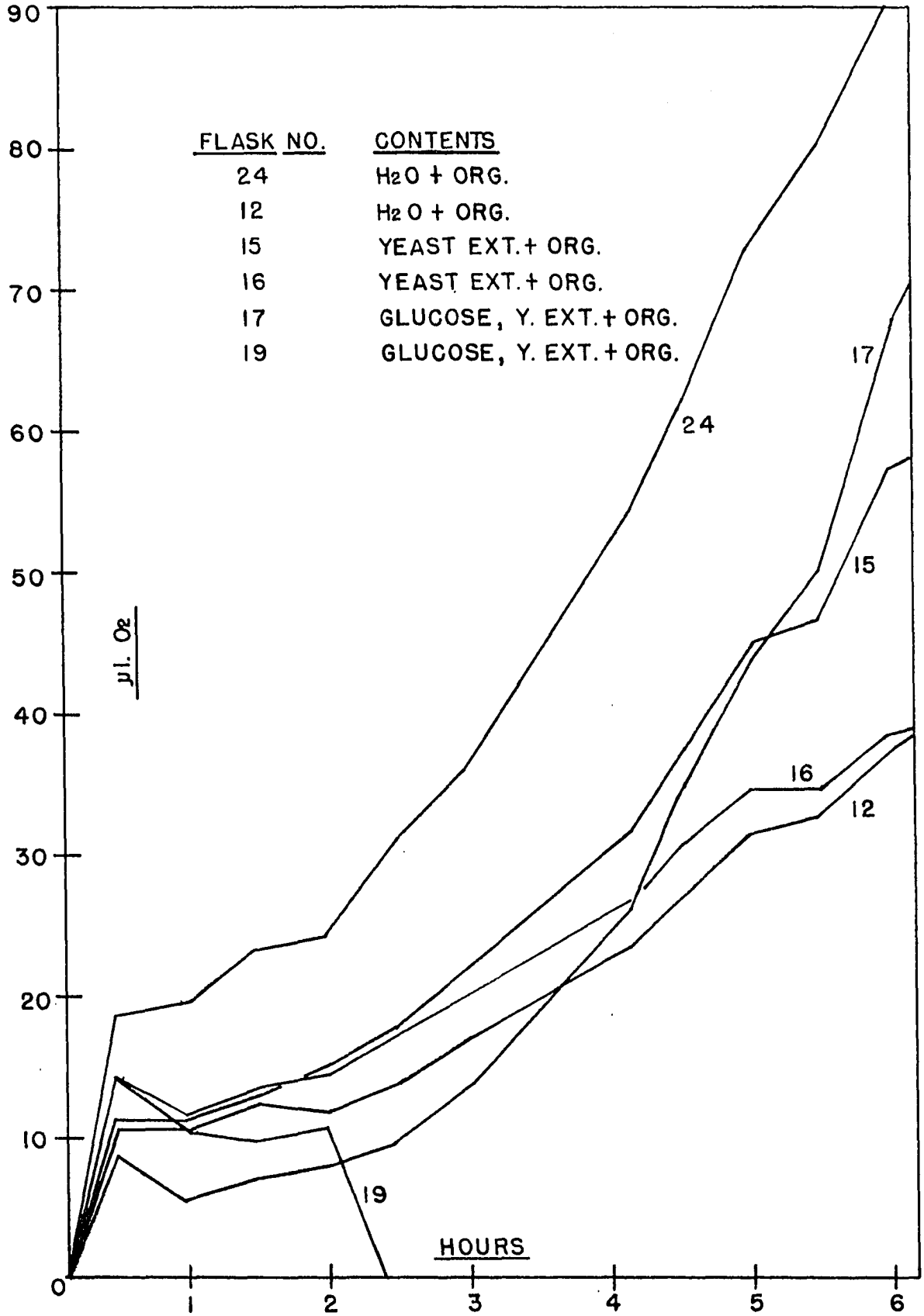
resulted from the rapid production of carbon dioxide in the flask. Each flask contained a center well in which 0.2 ml. of a 10 per cent potassium hydroxide solution was placed for the removal of carbon dioxide produced by the cells, and this was felt to be sufficient for the calculated amount of

carbon dioxide evolved during the time required for an experiment.

An experiment was therefore carried out using two flasks containing a suspension of S. cerevisiae cells, harvested from a 23 hour old culture, in distilled water. A 0.1 ml. inoculum was transferred aseptically to the sterile water. The cells were not washed as previous analyses had shown that no more sugar was present in the inocula used. Two other flasks were prepared which contained a suspension of S. cerevisiae cells in 5 ml. of a 0.25 per cent solution of Difco yeast extract, while two more flasks had a 0.1 ml. inoculum of cells added to 5 ml. of glucose (2 per cent) and yeast extract (0.25 per cent) medium in each flask. The media having no glucose would not be able to supply an energy source for the cells, so the latter would respire only because of metabolic activity originating and sustained within themselves.

The plot of the oxygen uptake in Figure 9 shows a normal pattern with the exception of flask 19, which suddenly registered a decrease in rate of oxygen uptake. Its duplicate, flask 17, showed no such activity during the experiment (6 hours duration) but ultimately reversed its direction also. All the remaining flasks continued to exhibit oxygen uptake due to endogenous metabolism and then stopped. Only the flasks containing the usual glucose-yeast extract media reversed the direction of the curve for utilization of oxygen.

Figure 9. The respiration of *Saccharomyces cerevisiae* in water, in aqueous yeast extract, and in glucose and yeast extract media.


FLASK NO. CONTENTS
24 H2O + ORG.
12 H2O + ORG.
15 YEAST EXT.+ ORG.
16 YEAST EXT.+ ORG.
17 GLUCOSE, Y. EXT.+ ORG.
19 GLUCOSE, Y. EXT.+ ORG.
µl. O2
HOURS
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
24
17
15
16
12
19

It is concluded that the extraordinarily vigorous production of carbon dioxide encountered with some cultures when glucose was available for continued growth was of such magnitude that the potassium hydroxide present was insufficient for its intended purpose.

3. Acetobacter suboxydans

This microorganism was grown on a medium of 10 per cent sorbitol and 0.5 per cent Difco yeast extract; evidences of growth were by the detection of sorbose by paper chromatographic procedures described previously, or by quantitative determination of the sorbose produced. In these analyses, the flask which showed the highest number of mg. of sorbose produced was rated arbitrarily as representing 100 per cent sorbitol utilization. All other flasks were then rated on a relative basis to the 100 per cent sample.

Preliminary tests for production of sorbose from sorbitol were made using several different compounds and solvents, so the results will be given in one table. The presence of growth was detected by observing a black spot on the chromatogram which indicated the presence of sorbose in the medium. A small loopful of the medium was placed at the bottom of the paper along with controls of sorbitol and sorbose.

The chromatograms were made by using 3:4:6 solvent containing n-butyl alcohol, pyridine and water as explained

previously in the section entitled "Methods". A ten hour exposure to the solvent mixture was sufficient for resolution of the sorbitol and sorbose. It was by this means that the presence of sorbose was ascertained.

An inoculum of 0.1 ml. per flask was used for the first determination of the effect of organometallic compounds and solvents upon the growth of A. suboxydans. The inocula were taken from a 24 hour old culture; in the second determination, 0.1 ml. inocula from a 48 hour old culture were employed. The cultures were all grown at 30° C.

Following a 48 hour period of incubation, chromatograms were prepared from the culture media by previously described methods; these are shown in Figures 10 and 11. The changes of behavior of the cultures in a period of 24 hours vs. 48 hours are to be seen in Table 19 in those instances in which growth did not occur in the shorter period of time, but did occur after 48 hours had elapsed.

Of interest is the behavior of dioxane and ethyl lactate which were present only as 1 per cent solutions, but which apparently were responsible in part, perhaps, for all of the negative reports of growth in those instances where either of them was used as a solvent. Such cases are represented by those compounds listed in Table 19 other than the water-soluble organolead detergent and 444T.

A comparison between the column in Table 19 indicating the utilization of sorbitol and the spots in Figures 10 and 11

Table 19. The growth of Acetobacter suboxydans in the presence of organometallic and quaternary ammonium compounds.

Compound[a] added	Mole of compound per ml. of medium	Sorbose present after 24 hrs.	Sorbose present after 48 hrs.	Per cent sorbitol utilized in 48 hrs.
1. None	None	+	+	88.3
2. Pb.cpd.[c]	10^{-5}	-	-	0.0
3. Pb.cpd.	10^{-6}	-	-	0.0
4. Pb.cpd.	10^{-7}	+(sl.)	+	70.5
5. Pb.cpd.	10^{-9}	+(sl.)	+	86.1
6. Pb.cpd.	10^{-11}	+(sl.)	+	89.0
7. Diphenylmercury	10^{-6}	-	-	0.0
8. Diphenylmercury	10^{-8}	-	+(sl.?)	2.5
9. Ethyl lactate	1% concn.	-	+	44.6
10. Dioxane	1% concn.	-	+	93.9
11. Pb.cpd.[d]	4×10^{-7}	-	+(sl.?)	3.7
12. Pb.cpd.	1×10^{-7}	-	+(sl.?)	6.4
13. Pb.cpd.	10^{-9}	-	+	95.0
14. 444T[e]	10^{-5}	-	-	0.0
15. 444T	10^{-7}	+(sl.)	+	100.0
16. 444T	10^{-9}	+(sl.)	+	89.0

[a]All flasks contained sterile medium of 10 per cent sorbitol and 0.5 per cent Difco yeast extract. Volume per flask: 10 ml.

[b]The analyses for sorbose were those described for glucose

Compound[a] added	Mole of compound per ml. of medium	Sorbose present after 24 hrs.	Sorbose present after 48 hrs.	Per cent sorbitol utilized in 48 hrs.
1. None	None	+	+	88.3
2. Pb.cpd.[c]	10^{-5}	-	-	0.0
3. Pb.cpd.	10^{-6}	-	-	0.0
4. Pb.cpd.	10^{-7}	+(sl.)	+	70.5
5. Pb.cpd.	10^{-9}	+(sl.)	+	86.1
6. Pb.cpd.	10^{-11}	+(sl.)	+	89.0
7. Diphenylmercury	10^{-6}	-	-	0.0
8. Diphenylmercury	10^{-8}	-	+(sl.?)	2.5
9. Ethyl lactate	1% concn.	-	+	44.6
10. Dioxane	1% concn.	-	+	93.9
11. Pb.cpd.[d]	4×10^{-7}	-	+(sl.?)	3.7
12. Pb.cpd.	1×10^{-7}	-	+(sl.?)	6.4
13. Pb.cpd.	10^{-9}	-	+	95.0
14. 444T[e]	10^{-5}	-	-	0.0
15. 444T	10^{-7}	+(sl.)	+	100.0
16. 444T	10^{-9}	+(sl.)	+	89.0

[a]All flasks contained sterile medium of 10 per cent sorbitol and 0.5 per cent Difco yeast extract. Volume per flask: 10 ml.

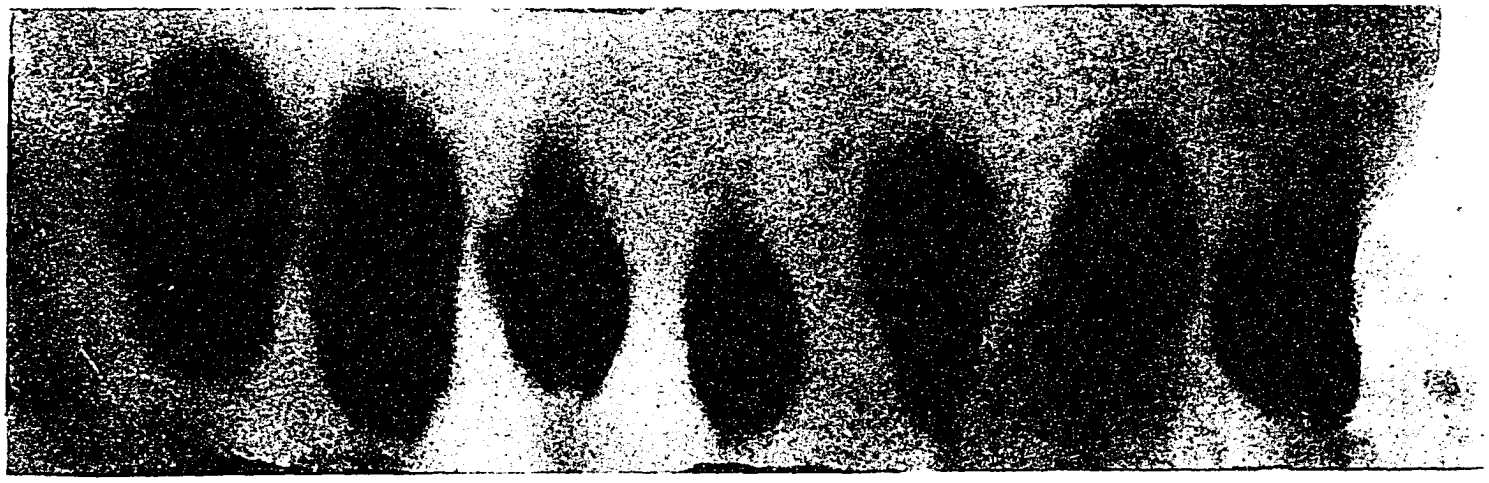
[b]The analyses for sorbose were those described for glucose as given by Underkofler *et al.* (140). Used 20 minute heating time.

[c]Triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.

[d]Triphenyl-p-tolyllead.

[e]4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate.

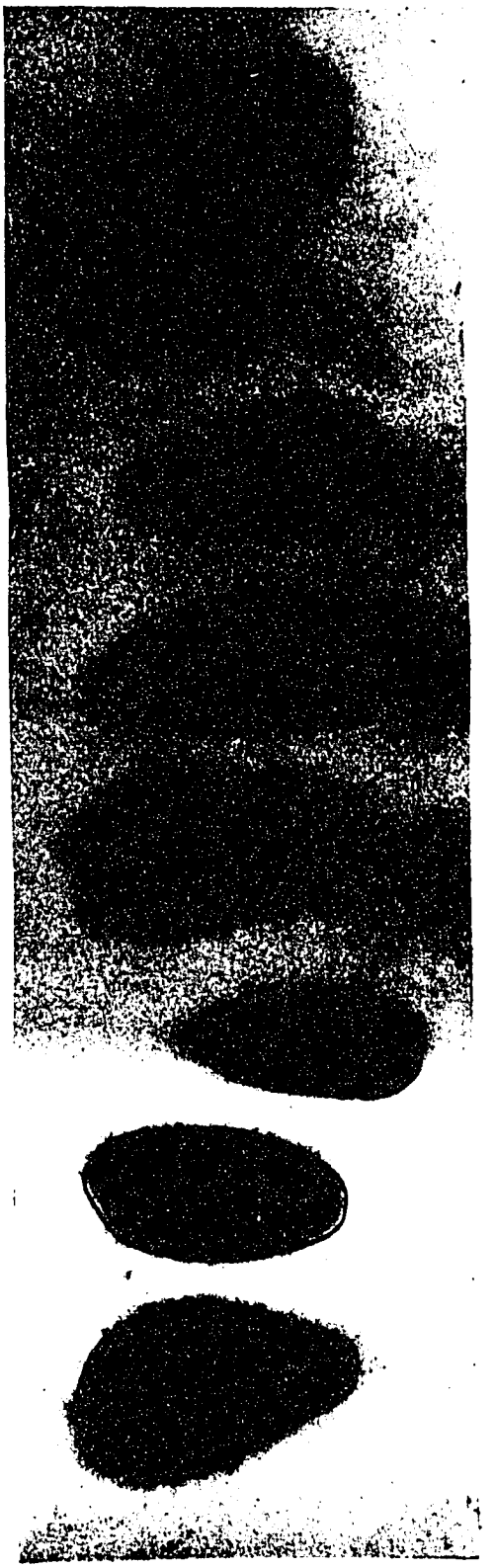
Figure 10. Chromatographic analysis of the production of sorbose by Acetobacter suboxydans in the presence of organometallic and quaternary ammonium compounds. (See Table 19 for corresponding numbers.)



5 4 3 2 1 S'ose S'tol

10 9 8 7 6 S'ose S'tol

Figure 11. Chromatographic analysis of the production of sorbose by Acetobacter suboxydans in the presence of organometallic and quaternary ammonium compounds. (See Table 19 for corresponding numbers.)



shows an agreement in those cultures where a very slight trace of sorbose was detected chromatographically as well as by reducing sugar analyses. These examples may be ascribed to an essentially complete inhibition, but not necessarily an overall bactericidal action.

In a study of the inhibition of A. suboxydans by the compounds given in Table 19, it was noticed that triphenyl-p-tolyllead did not inhibit its growth completely. Instead, a small per cent of sorbitol was oxidized to sorbose and was detected by chromatographic and reducing sugar analyses. Hence a study of the addition of this organolead compound at different periods of elapsed time were made, using identical cultures, and by adding an equal amount of this organolead compound. The inoculum was 0.5 ml. of a 48 hour culture of A. suboxydans; the 0.2 ml. aliquots were added after the inocula at the times shown in Table 20.

From the data of Table 20 it is apparent that dioxane is inhibitory to the growth of A. suboxydans, so such behavior of the solvent should be taken into account when the effect of this organolead compound is evaluated. A study of the data would lead one to the conclusion that triphenyl-p-tolyllead may even aid the organism to overcome a part of the inhibition of the solvent.

The action of BAL in competing with sulfhydryl groups of essential enzyme proteins for mercaptide formation was investigated, using A. suboxydans and diphenylmercury. It was

desired to see if BAL would decrease the demonstrated toxicity of diphenylmercury, thus giving a possible clue to its mode of action. Data are shown in Table 21.

The results of these analyses show diphenylmercury to be

Table 20. The effect of the addition of triphenyl-p-tolyllead to growing cultures of Acetobacter suboxydans.

Addition to medium at start of experiment	Hours elapsed after inoculation till addition of organolead cpd.	Per cent sorbitol utilized in hours
8×10^{-7} mole cpd.[a,c]	0 (control)	0.0 in 60
8×10^{-7} mole cpd.	0	16.5 in 60
None	12	99.3 in 60
None	None added	76.3 in 24[b]
None	24	84.8 in 60
None	36	100.0 in 60
0.2 ml. dioxane	None added	33.6 in 24[b]
0.2 ml. dioxane	None added	49.3 in 60

[a]This concentration was attained in all media to which the organolead compound was added.

[b]Analysis performed on sample of medium removed prior to addition of organometallic compound.

[c]Sterile medium.

very toxic to A. suboxydans, and the use of BAL does not reduce this action of the compound as might have been expected. The BAL appeared to exhibit some inhibition of its own, as

Table 21. The effect of BAL on the toxicity of diphenylmercury with Acetobacter suboxydans.

Compound added	Mole of diphenylmercury per ml. of medium	Per cent sorbose formed in 72 hours
None	None	100.0
Ethyl lactate	1 per cent	94.0 (44.6)[b]
BAL[a]	None	64.3[c]
Hg cpd.; BAL	1×10^{-6}	0.7
Hg cpd.	1×10^{-8}	2.3
Hg cpd.; BAL	1×10^{-8}	1.1
Hg cpd.	1×10^{-9}	3.8
Hg cpd.; BAL	1×10^{-9}	1.1
Hg cpd.	8×10^{-10}	97.3
Hg cpd.; BAL	8×10^{-10}	93.2
Hg cpd.	6×10^{-10}	100.0
Hg cpd.; BAL	6×10^{-10}	94.4

[a]All additions of BAL were 1 ml. of an aqueous solution containing 10^{-6} mole of BAL per ml.

[b]Incubation time, 48 hours.

[c]Incubation time, 32 hours.

did also the solvent, ethyl lactate. The control cultures showed a 100 per cent production of sorbose within 32 hours, so the partial values for BAL and ethyl lactate show that these two compounds cause a delay in the oxidative capacity of the microorganism by some kind of inhibition.

Concentrations of less than 10^{-9} mole of diphenylmercury per ml. of medium are shown to be non-toxic to A. suboxydans although they may demonstrate an initial inhibition in the early stages of growth of the microorganism.

The supposition that the diphenylmercury is cleaved to give phenylmercuric chloride and thus react with essential sulfhydryl groups is not upheld by these experimental results. Nakamoto and Nagayama (108) have said that perhaps the mercury compounds (among them, diphenylmercury) which they studied exert their toxic activity in the undissociated state, and not as an ion. They assumed that the dissociation product, RHgOH, was responsible for the bactericidal action of mercury compounds. The inhibition of catalase was shown by organomercury compounds, but as A. suboxydans lacks this enzyme, no such activity is possible here.

A study of the effect of diphenylmercury on the oxygen uptake was attempted; however, the characteristics of the culture gave erratic growth in one flask, and no growth in the control. The flasks which contained media to which were added concentrations of 10^{-5}, 10^{-6} and 10^{-7} mole of diphenylmercury, respectively, showed a rapid and sharp halt in oxygen

uptake.

From these findings, it can be seen that these quantities of diphenylmercury are able to interact rapidly with biochemical centers in a manner which halts the uptake of oxygen almost immediately. This demonstrates a high toxicity of this compound against A. suboxydans as well as against S. cerevisiae.

4. Clostridium acetobutylicum

This organism is an anaerobe and cultures were grown on a 7 per cent concentration of ground corn. All media were boiled to cause gelatinization of the corn starch before distribution to individual flasks and tubes unless the contents of any one flask were of sufficient size to warrant its individual treatment. An alternative to boiling was to steam the larger flasks in the autoclave for 30 minutes, followed by the usual sterilization at 15 to 17 p.s.i. for 1 to 1.5 hours.

a. Triphenylsilanol and triphenylbenzyllead. Solutions of triphenylsilanol and of triphenylbenzyllead were prepared, using Butyl Cellosolve as the solvent, in concentrations of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-8} mole of organometallic compound per ml. of solvent. These were used in the following experiments with Cl. acetobutylicum, using 300 ml. quantities of 7 per cent corn mash as the substrates.

A small sample of a spore culture of Cl. acetobutylicum was introduced into 20 ml. of sterile mash, the mash and spores were then "heat-shocked" for 2 minutes, cooled, and incubated at 37° C. Successive transfers were made every 24 hours until the sixth transfer, which was made into flasks containing 300 ml. of sterile 7 per cent corn mash. These were the main fermentation media. The organometallic compounds were added to the media before sterilization; this led to a gray color in those flasks containing the highest concentration of the organolead compound and was probably due to some decomposition.

In Table 22, the flasks were designated by the prefix "P" if the organolead compound was present, by a "C" if they were controls with no compounds added, and by an "S" if the organosilicon compounds was used. The solvents were obtained as aqueous solutions in a total volume of 100 ml. of distillate, following distillation of the mashes. Calcium carbonate was added to prevent the distillation of the volatile acids.

The per cent yields were not calculated as it was not believed that these results were of any special significance. There did not appear to be any valid deductions which could be made from these values other than a negative nature; i.e. the addition of the organosilicon and the organolead compounds did not appear to alter or inhibit the fermentations to any noticeable degree. However, the value for the grams of total solvents per 100 ml. of distillate assumes a ratio of

Table 22. The effect of triphenylsilanol and of triphenylbenzyllead on the growth of Clostridium acetobutylicum.

Flask No.	Mole of compound added to media	Specific gravity of distillate	Aver. g. solvents per 100 ml. distillate[a]
C-1	None	0.9948	3.33
C-2	None	0.9951	
S-4	2×10^{-4}	0.9951	3.16
S-4	2×10^{-4}	0.9953	
S-6	2×10^{-6}	0.9935	3.92
S-6	2×10^{-6}	0.9946	
S-8	2×10^{-8}	0.9946	3.43
S-8	2×10^{-8}	0.9950	
P-4	2×10^{-4}	0.9950	3.43
P-4	2×10^{-4}	0.9946	
P-5	2×10^{-5}	0.9952	3.33
P-5	2×10^{-5}	0.9947	
P-6	2×10^{-6}	0.9950[b]	3.29
P-6	2×10^{-6}	0.9967	
P-8	2×10^{-8}	0.9950	3.29
P-8	2×10^{-8}	0.9950	

[a]Overall average: 3.40 g. per 100 ml. solvent

[b]Solvent lost by leakage of distillation apparatus. Value for solvents is that of one determination only, not an average.

60:30:10 for 1-butanol, acetone and ethanol, respectively. This ratio might have been altered by these compounds which would indicate a possible interaction with the hydrogenases responsible for the reduction of some of the aliphatic acid intermediates such as butyric or acetic acid. If the solvent ratio was unchanged, it might be due to an insufficient amount of the organometallic compound being present to exert any inhibition, or it may indicate that these compounds are not effective against Cl. acetobutylicum.

A determination of acetone by Messinger's method as given by Goodwin (57) was undertaken on the control and on a sample of distillate from sample P-4. The control contained 1.22 g. of acetone per 100 ml. of distillate, while the P-4 samples were found to have 1.05 g. of this ketone in 100 ml. of distillate. There is very little difference in these values, which points to an essentially unchanged solvent ratio. The 1.22 g. of acetone represents 36.6 per cent of 3.33 g. of total solvents, which is about normal for this fermentation.

b. Triphenyl-Y-(diethylmethylammonium)-propyllead methosulfate. A preliminary trial growth of Cl. acetobutylicum in the presence of 444T indicated that poor growth resulted, so the organolead compound containing the triphenylplumbyl group instead of the triphenylmethyl group was used in tests with this microorganism.

Long, narrow fermentation tubes were used for growth of

the cultures, using 15 ml. of a 7 per cent corn mash in each tube. Inocula of 2 ml. were employed, and a 1 ml. portion of organolead solution was added to each flask, giving a total of 18 ml. of mash per tube. After 72 hours at 37° C., the tubes were removed. The growth of the organism was observed and recorded after 24 hours, and is shown below in Table 23.

Table 23. The effect of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate on the growth of Clostridium acetobutylicum.

Mole of compound added	Growth in 24 hours	Milliequivalents of volatile acid per ml. of medium
0	good	0.009
10^{-4}	sluggish	0.015
10^{-5}	sluggish	0.033
10^{-6}	fair	0.015
10^{-7}	fair	0.018
10^{-8}	good	0.011

The volatile solvents were removed by steam distillation, first adding excess powdered calcium carbonate to prevent the distillation of the volatile acids. After collecting 50 ml. of distillate, the contents of the distilling flasks were cooled, then made acidic by the addition of

excess concentrated sulfuric acid, and the volatile acids distilled. One hundred ml. of distillate was collected for each determination, and the sample titrated with standard sodium hydroxide (0.1010 N) to a phenol red endpoint. The amount of acid present was determined as milliequivalents of acid per ml. of medium as the size of samples used differed although their volumes were known.

In a normal fermentation of this type, the amount of acid present in the medium attains a maximum and then decreases when the reduction of these acids produces alcohols. Therefore, it may be possible to block the reduction of these acids or inhibit the rate of reduction in such a way that a determination of the acids present will demonstrate an excess of them in the media. The data from Table 23 appear to show that in the fair or sluggish fermentations there resulted an increase in the milliequivalents of acid present. This seems to be due to a general decrease in the rate of fermentation because of an inhibition imposed by the organolead compound on Clostridium acetobutylicum. Pett and Wynne (110) have reported up to 100 per cent inhibition of the phosphatases of Cl. acetobutylicum by heavy metal ions of mercury, silver and lead. The media in the cases where sluggish growth occurred did not appear to have lost their original viscous character which results from the presence of the gelatinized starch; hence it seems that the breakdown of this starch by the microorganisms has been delayed by the organolead detergent.

c. Diphenylmercury and triphenyl-p-tolyllead. The work initiated on Cl. acetobutylicum with triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate was extended to the use of the organomercury compound, diphenylmercury, and the organolead compound, triphenyl-p-tolyllead.

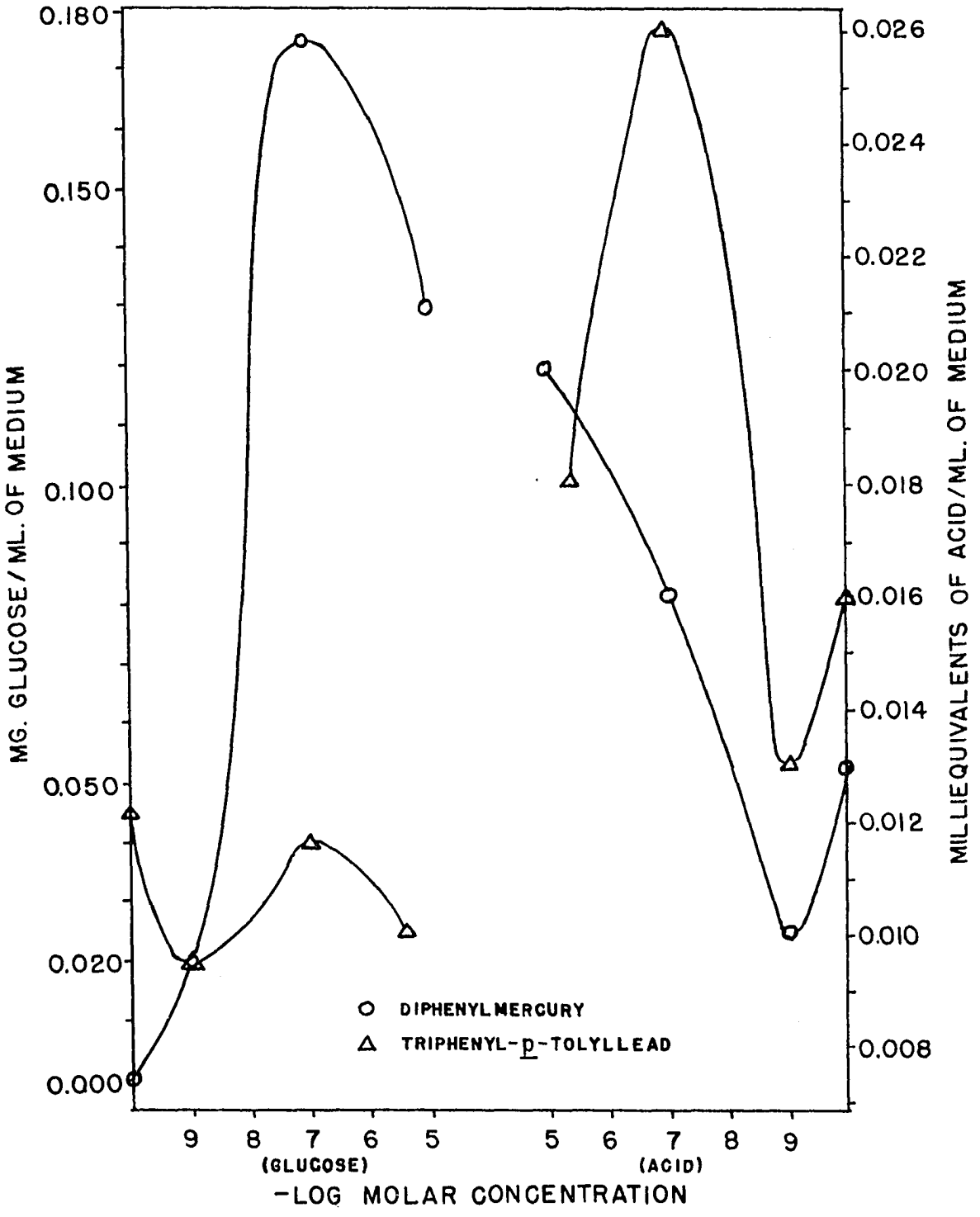
The procedure for the analyses and the preparation and inoculation of the mashes were identical to the work on the organolead detergent. The corn mash was 7 per cent, and was gelatinized by boiling for about one minute. Each tube contained 15 ml. of medium, and was inoculated with 2 ml. of a 24 hour culture of Cl. acetobutylicum.

Table 24 gives data showing that here one can differentiate between the diphenylmercury and the triphenyl-p-tolyllead in their effect on Cl. acetobutylicum.

The organomercury compound produced some increase in the milliequivalents of volatile acid when 10^{-5} mole of diphenylmercury was added to the fermentation. The same results were found with the addition of 4×10^{-6} mole and 1×10^{-7} mole of triphenyl-p-tolyllead. These results are plotted in Figure 12.

The glucose analyses demonstrated an inhibition of glucose utilization when diphenylmercury was present in the two highest concentrations, but no such activity was seen with triphenyl-p-tolyllead at any concentration. As further evidence of this, the observed growth after 24 hours was generally poorer in the fermentations to which the

Figure 12. The formation of glucose and production of acid by Clostridium acetobutylicum in the presence of an organomercury and an organolead compound.


0.180
0.150
0.100
0.050
0.020
0.000
MG. GLUCOSE / ML. OF MEDIUM
0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
MILLIEQUIVALENTS OF ACID/ML. OF MEDIUM
DIPHENYLMERCURY
TRIPHENYL-p-TOLYLLEAD
9 8 7 6 5
(GLUCOSE)
5 6 7 8 9
(ACID)
-LOG MOLAR CONCENTRATION

diphenylmercury was added. This may be evidence for a different mode of action on the part of diphenylmercury toward enzymes concerned with glucose metabolism, such as the phosphorylases.

Table 24. The formation of glucose and production of acid by Clostridium acetobutylicum in the presence of an organomercury and an organolead compound.

Substance added	Mole of compound added to medium	Growth in 24 hours	Mg. of glucose per ml. of medium	Milliequivalents of acid per ml. of medium
Ethyl lactate	0.1 ml.	very good	0.00	0.013
Hg cpd.[a]	1×10^{-5}	slight	0.13	0.020
Hg cpd.	1×10^{-7}	fair	0.175	0.016
Hg cpd.	1×10^{-9}	good	0.02	0.010
Dioxane	0.1 ml.	good	0.045	0.016
Pb cpd.[b]	4×10^{-6}	good	0.025	0.018
Pb cpd.	1×10^{-7}	fair	0.04	0.026
Pb cpd.	1×10^{-9}	good	0.02	0.013

[a]Diphenylmercury

[b]Triphenyl-p-tolyllead

5. Lactobacillus casei

This organism was grown on a medium composed of the following materials:

10 per cent glucose
1 per cent Difco peptonized milk
1 per cent Difco yeast extract
Distilled water

Each culture flask contained 100 ml. of medium to which was added 6 grams of sterile calcium carbonate after the flasks were inoculated. The inoculum for each flask was 10 ml. of a culture of L. casei which had grown for 24 hours at 30° C. The flasks were incubated at 30° C. with shaking for 84 hours and removed for analysis of the glucose utilized, and for the lactic acid produced. Because no utilization of glucose would result in no lactic acid being produced, only those cultures which showed definite glucose metabolism were analyzed for acid production.

Concentrations of the various compounds used were those previously demonstrated to be inhibitory and toxic to other microorganisms. The data obtained are found in Table 25.

The control flask value in Table 25 is the same used in Table 26 below. The use of the organolead detergent and the carbon atom analog, 444T, show that the degree of toxicity towards L. casei cells is very similar to that demonstrated previously to other microorganisms. There seems to be a slightly lessened inhibition with 444T than with its lead atom analog, however.

An interesting aspect of this trial of compounds resulted from the addition of 10^{-5} mole of BAL to the medium where 1×10^{-5} mole of triphenyl-p-tolyllead was present. The

striking difference in the glucose utilized and the lactic acid produced appears to point out the effect of BAL against the lead compound inhibition which normally occurs.

The use of dioxane as a solvent does not appear to have any adverse effects on the growth of L. casei; this is in agreement with the effect of this solvent on S. cerevisiae.

Table 25. The effect of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate and 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate on the growth of Lactobacillus casei.

Compound added	Mole of compound added to medium	Total volume of medium	% of total glucose utilized	% of total glucose changed to lactic acid
	none	110 ml.	64.0	27.5
Pb cpd.[a]	1×10^{-4}	111 ml.	0.0	0.0
Pb cpd.	1×10^{-6}	111 ml.	70.9	55.2
444T[b]	1×10^{-4}	111 ml.	3.1	0.0
444T	1×10^{-6}	111 ml.	77.8	64.2

[a]Triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate.
[b]4,4,4-Triphenyl-n-butyldiethylmethylammonium methosulfate.

The total volume of each culture was included in order to notice the effect of the overall concentration of organometallic compound when added to such a medium. The dilution which occurred tended to reduce the concentration of organometallic compound per ml. of medium; thus the toxic

Table 26. The effect of triphenyl-p-tolyllead, dioxane, BAL and diphenylmercury on the growth of *Lactobacillus casei*.

Compounds added	Mole of compound added to medium[a]	Total volume of medium	% of total glucose utilized	% of total glucose changed to lactic acid
None	None	110 ml.	64.0	27.5
Pb. cpd.[b]	4×10^{-5}	111 ml.	0.0	0.0
Pb.cpd.BAL	4×10^{-5}	112 ml.	0.0	0.0
Pb. cpd.	1×10^{-5}	111 ml.	4.2	----
Pb.cpd.BAL	1×10^{-5}	112 ml.	86.8	53.6
Dioxane	1 ml.	111 ml.	90.5	59.6
Hg cpd.[c]	1×10^{-4}	111 ml.	0.0	0.0
Hg cpd.BAL	1×10^{-4}	112 ml.	0.0	0.0
Hg cpd.	1×10^{-5}	111 ml.	0.0	0.0
Hg cpd.BAL	1×10^{-5}	112 ml.	2.0	----
Hg cpd.BAL[d]	1×10^{-5}	112 ml.	2.6	----

[a] Added 10^{-5} mole (1ml.) of BAL in all cases except as noted.

[b] Triphenyl-p-tolyllead.

[c] Diphenylmercury.

[d] Added 10^{-4} mole (1ml.) of BAL to medium.

levels of these compounds are much less than indicated at the first glance. This appears to demonstrate a high toxicity for the triphenyl-p-tolyllead and the diphenylmercury molecules when added to L. casei.

VI. DISCUSSION AND CONCLUSIONS

An investigation of the effect of organometallic compounds on microorganisms would be greatly simplified by the use of water-soluble molecules which can be used as simple aqueous solutions. With this as a goal, the search for such compounds which might be toxic and yet possess a sufficiently high solubility revealed the dearth of possibilities for achievement of this aim. A few such compounds were obtained, however, and subjected to preliminary trials.

One such compound was the sodium salt of triphenyl-2-(p-carboxyphenylazo)-5-dimethylaminophenyltin, which was found to show no toxicity toward S. cerevisiae or L. delbrueckii. The decreased toxicity is expected in relation to its more toxic Group IV neighbor, lead, as the toxicity of these elements is known to decrease as one goes toward the lighter members of this group. Hence, a search for a water-soluble lead compound was responsible for the trial of tetrakis-(p-dimethylaminophenyl)-lead tetramethiodide(for its structure, see page 85). Its solubility in water was too low to permit the preparation of concentrated solutions greater than 3×10^{-6} mole per ml. Such concentrations were not toxic to S. cerevisiae cells, however.

The preparation of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate has been described. Its solubility

in water allowed the preparation of aqueous solutions of the compound which contained 10^{-4} mole per ml. (0.1 molar), so this material was used in conjunction with different types of microorganisms. In all cases, the use of 10^{-4} mole in 10 ml. of medium was found to be bactericidal in a general sense of the meaning, to yeasts, molds or bacteria. Concentrations below this, as 10^{-6} mole per ml. of medium, were usually toxic also, although lesser values from 10^{-7} to 10^{-9} mole per ml. of medium usually had little, if any, effect.

When molds were exposed to the action of this compound, the same results were found to be true as for bacteria, except that the use of lower concentrations as 10^{-10} and 10^{-11} mole per ml. of medium gave a stimulation of the growth as evidenced by increased metabolism and uptake of glucose.

The mode of action shown in the inhibition of glucose uptake by S. cerevisiae was also seen in the inhibition of respiration by the organolead detergent. It is recognized that this compound may possess a dual nature, exercising its effect as a surface tension reductant in one instance, while exerting a toxic action as a result of its normally toxic heavy metal atom, lead, which is present in the molecule.

The effect of the lead in this molecule was not known, so the compound possessing the same structure as this organolead detergent was synthesized, except that the lead atom was replaced by a non-toxic carbon atom. Neither this carbon analog nor the free amine from which it was prepared have

been reported in the literature so the methosulfate was analyzed qualitatively and quantitatively for the presence and the percentages, respectively, of carbon, hydrogen, nitrogen and sulfur. Infrared spectra of the known organolead compound and its carbon analog completed the proof of structure. These compounds are shown on page 70 of this thesis. The carbon analog was called 444T from its complete name; i.e. 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate.

The tests for evaluating the toxicity of 444T were the same as those for its lead-containing analog; it appeared to possess very similar physical and biochemical properties. The solution of 10^{-4} mole per ml. formed a thixotropic gel at room temperatures which indicated that a true solution was not formed, but rather a lyophilic sol. No such behavior was noted with the organolead compound, showing that it is more soluble than the carbon atom analog, as a true solution is obtained instead.

A difference in toxicity between 444T and the lead compound was found at concentrations of 10^{-6} mole of these compounds per ml. of medium. There was partial growth of S. cerevisiae when 444T was present, but none when the organolead compound was used. Similar results were found in the study of the respiration of this yeast in the presence of these compounds in individual experiments. The differences alluded to by these examples are due to replacement of the lead atom by a carbon atom, and therefore demonstrate that the heavy

metal does exert a very definite influence in the toxicity of the detergent molecule.

The effect of the presence of the lead is not to be taken at face value; that is, there may be a simple alteration of the physical properties of the molecule as a whole when lead is replaced by carbon which will result in a different degree of adsorption of 444T on a bacterial cell, giving a change in the physical interaction of this compound with the cell surface. Such an indication can be seen by the difference in the solubility relationships between the two compounds.

The greatly enhanced tendency for cleavage of the unsymmetrical organolead detergent cation suggests the added possibility that the formation of a triphenylplumbyl ion may occur which would be expected to react in its own way and possibly enhance the number of toxic reactions which can occur with reactive groups present in bacterial proteins.

Valko and DuBois (142) believe that a close relationship exists between the antibacterial behavior of metallic and dye cations, and that of surface-active cations because their reactions with bacterial cells can all be considered as phenomena of great importance because mercuric ions and dye ions are strongly adsorbed but possess very little surface activity.

Okamoto and Nagayama (108) agree with Beck (16) that the compounds possessing a large surface activity are the most

readily adsorbed on bacterial surfaces; however, Beck (16, 17) believes that the quaternary ammonium detergents react only by adsorption in the case of yeast cells, and that there is a correlation between the decrease in surface tension exhibited by a detergent, and its effect in decreasing the respiration of the cells. Okamoto and Nagayama (108) have shown that phenylmercuric hydroxide, phenylmercuric acetate and diphenylmercury show no surface activity yet have a high antibacterial activity. Furthermore, diphenylmercury is not believed to undergo hydrolytic dissociation.

Cause for more investigation of the reactivity of these compounds has been given by the experiments in this thesis which show that diphenylmercury is cleaved to phenylmercuric chloride by valine hydrochloride or by lysine monohydrochloride. This cleavage product may react as such with essential groups in proteins or may hydrolyze to form phenylmercuric hydroxide which in itself is very bactericidal.

The alkyl group which is joined at one end to the central lead atom of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate, contains the quaternary nitrogen at the other end. This portion of the organolead detergent was synthesized and its activity tested against S. cerevisiae. It is called n-propyldiethylmethylammonium methosulfate. The highest concentration prepared was a solution containing 10^{-4} mole of compound per ml., and the use of 1 ml. of this solution in 10 ml. of medium caused complete inhibition of

growth. This conduct shows that the surface activity alone is not responsible for bactericidal action as the molecular weight is considerably less here than with the lead compound just cited or its carbon analog, and no surface tension reduction of an aqueous solution was noted.

Another similarity between this smaller methosulfate and its related higher molecular weight types was found in the action of these compounds in causing a clumping of the yeast cells. Large clumps of cells were formed which could be seen with the naked eye when 10^{-4} mole of any of these three compounds was added to 10 ml. of a culture of S. cerevisiae. These clumps might result from the removal of lipids from the cell membrane by the action of the detergent, causing an attraction of positive centers on one cell to negative centers in another, although the smaller compound would not be expected to do this. However, the counteraction of the zeta potential of a cell surface by the addition of the positive quaternary ammonium cation might result in a decreased repulsion between the cells which would bring about their ultimate agglomeration.

The possible toxicity of the uncommon methosulfate anion might contribute to the effect of these compounds also. An attempt to produce the quaternary chloride by the addition of barium chloride caused formation of a thick gel with both 444T and the lead atom analog, so no further attempts were made. Dimethyl sulfate, however, is a toxic molecule and

should these microorganisms assimilate the methosulfate anion, it is believed that these ions might displace sulfate ions in a way such that an inhibition of cellular activity would result.

The rapidity with which the triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate would inactivate the cells of S. cerevisiae to lead to a loss of their viability was determined by exposing the cells to a known lethal quantity of the compound for a measured length of time. After intervals of from 5 to 60 minutes, the cells were transferred to excess fresh media and incubated. Growth was inhibited temporarily up to an exposure time of 30 minutes, and no growth resulted after a 45 minute treatment of the cells. This demonstrates that the mechanics of the antibacterial activity require a given length of time and are not instantaneous. This does not appear to involve an adsorption alone as the ratio of the number of molecules to the number of yeast cells is of such magnitude that the centers where adsorption occurs would certainly be surrounded at all times by a large number of molecules, and adsorption of a lethal nature would be expected to occur more rapidly.

Of more likelihood is the possibility of a cytolytic injury to the cells as stated by Hotchkiss (67) who has demonstrated a "leakage" of essential nitrogen and phosphorus cell components from the cells of S. cerevisiae which results in very low metabolic activity of the cells. A relationship

to the 45 minutes required here for complete loss of the viability of the cells seems more plausible. Adsorption may play an important role, however, in causing this action to occur.

The lack of water-soluble compounds for continuing these tests made it necessary to use water-insoluble types. It was decided that the best and most convenient method for adding an insoluble compound to an aqueous medium would be to add a solution of the compound, dissolved in a water-miscible solvent, to the medium. The solvent would be thoroughly compatible with the water, thereby allowing the compound to form a suspension in the medium. The use of solutions would allow an accurate measure of the amount of compound added.

Tests were conducted to evaluate the suitability of a solvent; three criteria were essential. First, the solvent had to be miscible with, or very soluble in water; second, the organometallic compound had to be sufficiently soluble in this solvent so as to allow enough of it to be dissolved for practical usage. The practicality of this second criterium was limited by the third; i.e., the solvent must not be toxic to the microorganism when a quantity of it was added to the medium sufficient to introduce a toxic amount of the organometallic compound. Butyl Cellosolve, ethyl lactate and dioxane were the most successful in fulfilling these requirements, although each solvent was not satisfactory in itself for all given microorganisms, as the toxicity of any

one solvent varied with the organism used.

The first tests were conducted with triphenylsilanol in Butyl Cellosolve using Clostridium acetobutylicum. This was found to exert no inhibition of the production of solvents from the fermentation of corn. The same results were obtained by the use of triphenylbenzyllead in the same solvent. Use of Butyl Cellosolve for S. cerevisiae was unsatisfactory as a concentration of solvent as low as one per cent caused a partial inhibition of growth while higher concentrations were completely inhibitory. Butyl Cellosolve is ethylene glycol monobutyl ether: ($HOCH_2CH_2OCH_2CH_2CH_2CH_3$).

It was shown that diphenylmercury will be cleaved to produce phenylmercuric chloride when either valine hydrochloride or lysine monohydrochloride is present. The possibility of reaction with sulfhydryl groups seemed to be logical so tests with a compound known as BAL were conducted. Fildes (41) has shown that the presence of sulfhydryl groups (-SH) in compounds added to a medium will prevent toxic effects of mercuric ions when the latter are present in the medium. BAL is 2,3-dimercaptopropanol, and it can react with those compounds which normally react with essential -SH (sulfhydryl) groups in enzymes, thus protecting the latter from the inhibition which often results from mercaptide formation with heavy metals.

It was found that BAL did not prevent the inhibition of microorganisms by diphenylmercury, but seemed to enhance the

toxicity instead. Control fermentations were also suppressed when BAL alone was used, the same being true for the respiration of microorganisms. Waters and Stock (145) have reported this enhancement of toxicity in the case of arsenical-produced inhibitions, and have also shown that BAL will interfere with cytochrome oxidase activity because it is a strong reducing agent, and so keeps cytochrome c in the reduced state.

Studies of the respiration of microorganisms were conducted in which diphenylmercury was added to the respiring cells. This compound, which was very toxic to the growth of the cells of Acetobacter suboxydans and S. cerevisiae, was equally inhibitory to their respiration.

The use of triphenyl-p-tolyllead was surprising in its high antibacterial activity as it was not expected to undergo any decomposition, nor does it possess any ionic lead. All four valences of the lead atom are employed as covalent bonds to hydrocarbon groups.

Triphenyl-p-tolyllead was soluble in dioxane; tetraphenyllead, which differs only by a single para methyl group on one benzene nucleus, was quite insoluble. An unexpected behavior with regard to this solvent was its low toxicity to the growth of S. cerevisiae, because ethyl ether has been been shown to be toxic to S. cerevisiae in low concentrations above one per cent, but stimulatory at concentrations of 0.25 per cent (79). Kerr and Young (73) reported that

toluene, xylene and ethyl ether cause a marked reduction in the fermentation of glucose by S. cerevisiae and cells of this organism are ultimately killed by toluene or ether. Dioxane was not toxic to any appreciable extent in concentrations as high as 4 per cent when added to a glucose fermentation employing yeast.

When BAL was added to cultures in which triphenyl-p-tolyllead was present, a very marked decrease in inhibition resulted. This effect is evidence in favor of the reaction of this organolead molecule with -SH groups as the mode of action by which it is toxic. The addition of BAL gave improved yields in 3 cases where inhibition by the compound was otherwise noted when no BAL was used.

A study of the respiration of S. cerevisiae showed that although glucose metabolism was inhibited 90 per cent by triphenyl-p-tolyllead as indicated by analyses performed on the media after the respiration studies, there had not been any corresponding inhibition of the uptake of oxygen. This finding seems to indicate that triphenyl-p-tolyllead will depress glucose metabolism without halting the respiration, contrary to other cases where both glucose metabolism and respiration are halted.

BAL was used in a higher concentration in the respiratory studies above than in the glucose metabolism experiments, and proved to be very toxic at such a level. No glucose metabolism occurred when BAL was used, while dioxane caused

stimulated respiration in all cultures as seen by the sharp increase in the oxygen uptake. This behavior is like that noted for very low concentrations of ethyl ether (79).

With Acetobacter suboxydans, dioxane was inhibitory, suppressing the oxidation in which sorbitol is converted to the keto sugar, sorbose. A yield of approximately 50 per cent of sorbose was obtained when a 2 per cent concentration of this solvent was in the medium. The triphenyl-p-tolyllead was also inhibitory to this organism.

The investigation of these compounds thus leads to the conclusion that when heavy metals, which are known to be toxic in the free metallic or the ionic state, are incorporated in organometallic compounds where all the carbon to metal bonds are covalent, they probably act in a toxic manner by dissociation or by cleavage to form more reactive moieties. These more reactive products then combine chemically with other chemical groups to produce an inhibition of essential biochemical reactions necessary for the metabolism and/or respiration, or reproduction of the cell.

This problem can be extended by investigating the use of phosphorylated sugars as additives to inhibited cultures to see if phosphorylation is being prevented. The use of enzyme preparations in conjunction with the solvents and the organometallic compounds would be desirable from the standpoint of observing any specific enzymic inhibition or denaturation at variable pH values. The introduction of these water-

insoluble compounds might be accomplished in a better way; a method which eliminated the use of solvents would be very beneficial, as larger cultures could be used with greater ease, thus allow better analyses of the products of the fermentations.

VII. SUMMARY

1. The effect of organometallic compounds upon various microorganisms was studied by means of their introduction into cultures of a yeast, some molds, and some bacteria. The organisms were grown at their optimum temperatures using ideal conditions whenever possible.

2. Extensive use was made of the yeast, Saccharomyces cerevisiae, because of its importance as a microorganism, its ease of cultivation and its availability. Bacteria used included Acetobacter suboxydans, Lactobacillus delbrueckii, Lactobacillus casei and an anaerobe, Clostridium acetobutylicum.

3. A preliminary study was conducted on water-soluble compounds in order to allow optimum contact of the cells with the molecules. An impure sodium salt of an organotin dye was found to possess no toxicity to Saccharomyces cerevisiae at a concentration of approximately 0.0001 g. per ml. A water-soluble organolead tetramethiodide was found to possess no toxicity for S. cerevisiae when 3×10^{-6} mole was added to 10 ml. of medium. These two compounds were not investigated further because of poor solubility characteristics and difficulty in attaining sufficient quantities of pure material in an aqueous solution.

4. Preliminary investigation of triphenyl-γ-(diethylmethylammonium)-propyllead methosulfate showed it to be a suitable water-soluble organolead compound for these studies, as it was possible to prepare a 0.1 molar solution in distilled water. Such a concentration gave 10^{-4} mole of the lead compound per ml. of solution, and was found to completely inhibit the growth of all microorganisms when one ml. of solution was added to 10 ml. of a medium.

This compound was synthesized and was studied from two viewpoints; as a detergent, and as an organolead compound. Studies were conducted on air-borne molds by exposing 10 ml. samples of semi-synthetic media containing glucose, yeast extract and 10^{-4} mole of this compound to the atmosphere of the laboratory.

5. The growth-inhibition studies were next extended to a pure strain of the mold, Aspergillus niger, and the complete inhibition which was noted previously upon adding 10^{-4} mole of this organolead detergent to a 10 ml. culture of an air-borne mold was also found to occur here. Lower concentrations of the compound, such as 10^{-10} mole quantities, caused an enhanced glucose uptake by A. niger, leading to more rapid growth.

6. Inhibition which caused a complete cessation of the growth of S. cerevisiae within 45 minutes was found to result when 10^{-5} mole of organolead detergent per ml. of medium was

present. The presence of 10^{-7} mole of compound per ml. of medium was still toxic to S. cerevisiae. No cell formation nor glucose uptake was observed, and in addition, the cells were seen to form clumps when 10^{-4} mole of the compound was used in 10 ml. of medium.

7. The synthesis of the carbon analog of the water-soluble organolead compound was accomplished. As this was a new compound, it was analyzed quantitatively and the structure proven by the results of these analyses and by a comparison of the infrared spectra of the compound with that of its lead analog. It was called 4,4,4-triphenyl-n-butyldiethylmethylammonium methosulfate. This name was shortened to 444T for reference purposes.

8. The compound, 444T, was used in tests which compared its activity with that of the lead analog. An analysis of the toxicity of 444T showed it to be somewhat less toxic to the growth and respiration of cells than its heavy metal analog, although both compounds appeared to act in an identical manner in the inhibition of the metabolic and respiratory activities of microorganisms.

9. A discussion of the activity of these quaternary ammonium organolead compounds against microorganisms was given. The alkyl side chain portion of the 444T molecule; i.e., n-propyldiethylmethylammonium methosulfate, was synthesized and tested for its antibacterial activity as a contribution

to the overall study of the 444T and its lead analog. It was found to possess less activity against S. cerevisiae and air-borne bacteria, and was completely ineffective for preventing the growth of air-borne molds. An interesting behavior was its ability to cause clumping of the cells of S. cerevisiae in the same manner as the 444T and its analog containing lead. No growth of S. cerevisiae occurred when 10^{-4} mole of this alkyl compound was added to 10 ml. of a medium.

10. A number of solvents, as Butyl Cellosolve, ethanol, acetal, dioxane, ethyl lactate and water were individually evaluated for their (a) ability to dissolve a useful quantity of an organometallic compound, (b) their high solubility or miscibility with water, and (c) their toxicity when any one solvent was added to a normal growing culture of a microorganism in amounts which would be sufficient to introduce a toxic quantity of a water-insoluble organometallic compound to that culture. Portions of from one to five per cent were added to media and tested for their inhibitory effect toward the microorganism present by means of determining the uptake of glucose which occurred.

11. The mode of action of certain water-insoluble organometallic compounds; i.e., diphenylmercury, triphenylbenzyllead, and triphenyl-p-tolyllead, was studied and theories were presented in an attempt to explain their inhibitory

activities. Support for these theories was obtained from the results of experiments which were conducted with BAL (British antilewisite) and with the organometallic compounds when both were present simultaneously in a culture. The BAL supplied -SH groups which might react with some of the more reactive degradation products of the organometallic compounds should such occur. Diphenylmercury was shown to be cleaved by valine hydrochloride or lysine monohydrochloride to give benzene and phenylmercuric chloride, but no such effect was found for triphenyl-p-tolyllead.

12. The action of these organometallic compounds on an anaerobic microorganism, Clostridium acetobutylicum, was studied. Possible interference with the metabolism of glucose and/or the reduction of acids to alcohols was demonstrated by the results of analyses which showed changes in the amount of glucose present and in the presence of volatile acids which had not been reduced to alcohols.

13. The respiration of the aerobic microorganisms mentioned previously was studied by adding organometallic compounds to their growing cultures following a preliminary three to six hour growing period. The Warburg apparatus was standardized and used in these investigations for following the respiratory activity of the microorganisms. In general, when growth was halted, the respiration was found to be inhibited also. With triphenyl-p-tolyllead, however, the respiration of S.

cerevisiae did not halt when glucose metabolism was inhibited completely.

14. Suggestions for extending and enlarging the scope of this type of investigation have been given, based upon the work done in this study.

VIII. LITERATURE CITED

1. Albert, A. Quantitative studies of the avidity of naturally occurring substances for trace metals. II. Amino-acids having three ionizing groups. Biochem. J. 50: 690 (1952).

2. _____. Avidity of terramycin and aureomycin for metallic cations. Nature. 172: 201 (1953).

3. Alexander, E. R. Principles of Ionic Organic Reactions. Chap. 5. New York. John Wiley and Sons, Inc. 1950.

4. Amantea, F. Magnesium in experimental uremia. Arch. farmacol. sper. 60: 353 (1935). (Original not examined; abstract read in Chem. Abst. 30: 762. 1936)

5. Anantakrishnan, S. V. The nature of the metal-carbon bond in organoalkali compounds. Proc. Indian Acad. Sci. 34A: 299 (1951).

6. Archdeacon, J. W., Nash, J. B., and Wilson, G. C. The biliary excretion of gallium^{72}, gold^{198}, and hafnium^{181} in the rat. Texas Repts. Biol. Med. 10: 281 (1952).

7. Asai, T., Ikeda, Y., and Sasaki, T. Pseudooxidative bacteria. V. Glucose oxidase and gluconic acid oxidase of various oxidative bacteria. J. Agr. Chem. Soc. Japan. 24: 147 (1951).

8. Bachmann, W. E. Triphenylmethane. Org. Syn. 23: 100. New York. John Wiley and Sons, Inc. 1943.

9. _____, and Wiselogle, F. Y. The reaction of sodium with triphenylchloromethane and with triphenylmethyl in organic solvents. J. Am. Chem. Soc. 58: 1943 (1936).

10. Baetslé, R. The yeast and bacterial growth-inhibition effect exhibited by humulone and lupulone. Rept. Proc. 4th Intern. Congr. Microbiol. 1947: 535 (1949).

11. Baker, Z., Harrison, R. W., and Miller, B. F. Action of synthetic detergents on the metabolism of bacteria. J. Exptl. Med. 73: 249 (1941).

12. Ball, E. G., Anfinson, C. B., and Cooper, O. Inhibitory action of napththoquinones on respiratory processes. J. Biol. Chem. 168: 257 (1947).

13. Barron, E. S. G., and Kalnitsky, G. The inhibition of succinoxidase by heavy metals and its reactivation with dithiols. Biochem. J. 41: 346 (1947).

14. _____, and Singer, T. P. Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism. J. Biol. Chem. 157: 221 (1945).

15. Baughan, E. C., Evans, M. G., and Polanyi, M. Covalency, ionization and resonance in carbon bonds. Trans. Faraday Soc. 37: 377 (1941).

16. Beck, G. E. Physikalisch-chemische Untersuchunger zur wirkung von Desinfizienzien. Schweiz. Z. Path. u. Bakt. 11: 66 (1948).

17. _____, and Meier, R. Mechanismus der Reaktion von Invertseifen mit isolierten Zellen. Experientia. 3: 371 (1947).

18. Becker, D. E., Terrill, S. W., Meade, R. J., and Edwards, R. M. The efficacy of various antibacterial agents for stimulating the rate of gain in the pig. Antibiotics and Chemotherapy. 2: 421 (1952).

19. Bischoff, F., Maxwell, L. C., Evans, R. D., and Nuzum, F. R. Studies on the toxicity of various lead compounds given intravenously. J. Pharmacol. Exptl. Therap. 34: 85 (1928).

20. Bonrath, W., and Klös, H. Mono (organomercury) acetylides. U. S. Patent, 2, 251, 778. 1941 (Original not examined; abstract read in Chem. Abst. 35: P7638. 1941)

21. Brewster, R. Q. Organic Chemistry. p. 177, 178. New York. Prentice-Hall, Inc. 1949.

22. Burger, A. Medicinal Chemistry. Vol. 2, Chap. 41. New York. Interscience Publishers, Inc. 1951.

23. Calingaert, G. The organic compounds of lead. Chem. Revs. 2: 43 (1925).

24. Carlson, C. E. Über das verschiedene verhalten organischer und anorganischer Arsenverbindungen Reagenzien gegenuber, sowie über ihren Nachweis und ihre Bestimmung im Harn nach einfuhrung in den Organismus. Z. physiol. Chem. 49: 410 (1906).

25. Cella, J. A., Eggenberger, D. N., Noel, D. R., Harriman, L. A., and Harwood, H. J. The relation of structure and critical concentration to the bactericidal activity of quaternary ammonium salts. J. Am. Chem. Soc. 74: 2061 (1952).

26. Chaplin, C. E. Bacterial resistance to quaternary ammonium disinfectants. J. Bact. 63: 453 (1952).

27. Christensen, L. M., and Fulmer, E. I. Analysis of n-butanol, acetone, and ethanol in aqueous solution. Ind. Eng. Chem. 7: 180 (1935).

28. Clarke, R. L., and Mooradian, A. Pyrolysis of some amino acids. J. Am. Chem. Soc. 71: 2825 (1949).

29. Coleman, G. H., Weed, L. A., and Myers, C. D. Bactericidal properties of certain organomercuric acetates. J. Am. Chem. Soc. 59: 2703 (1937).

30. Cook, E. S., Kreke, C. W., McDevitt, M. of L., and Bartlett, M. D. The action of phenylmercuric nitrate. I. Effects on enzyme systems. J. Biol. Chem. 162: 43 (1946).

31. Cook, E. S., and Perisutti, G. The action of phenylmercuric nitrate. III. Inability of sulfhydryl compounds to reverse the depression of cytochrome oxidase and yeast respiration caused by basic phenylmercuric nitrate. J. Biol. Chem. 167: 827 (1947).

32. Deley, J., Peeters, G., and Massart, L. The influence of acridine compounds on baker's yeast. Biochem. et Biophys. Acta. 1: 393 (1947).

33. Demis, D. J., Rothstein, A., and Meier, R. The relationship of the cell surface to metabolism. X. The location and function of invertase in the yeast cell. Arch. Biochem. Biophys. 48: 55 (1954).

34. Domagk, G. Eine neue Klasse von Desinfektionsmitteln. Deut. med. Wochschr. 61: 829 (1935).

35. Eagle, H. The effect of sulfhydryl compounds on the antispirochetal action of arsenic, bismuth and mercury compounds *in vitro*. J. Pharmacol. Exptl. Therap. 66: 436 (1939).

36. Ehrlich, P. Über den jetzigen Stand der Chemotherapie. Ber. 42: 17 (1909).

37. Elderfield, R. C., Kreysa, F. J., Dunn, J. H., and Humphreys, D. D. A study of the synthesis of plasmochin by the reductive amination method with Raney nickel. J. Am. Chem. Soc. 70: 40 (1948).

38. Fels, I. G., and Cheldelin, V. H. Methionine in selenium poisoning. J. Biol. Chem. 176: 819 (1948).

39. _____, and _____. The role of sulfate in selenate toxicity in yeast. Arch. Biochem. 22: 323, 402 (1949).

40. Fieser, L., and Fieser, M. Organic Chemistry. 2nd ed. p. 1058. Boston, Mass. D. C. Heath and Co. 1950.

41. Fildes, P. Mechanism of the antibacterial action of mercury. Brit. J. Exptl. Path. 21: 67 (1940).

42. Fisher, H. L. Laboratory Manual of Organic Chemistry. 4th ed. p. 151. New York. John Wiley and Sons, Inc. 1938.

43. Frankland, E. Notiz über eine neue Reihe organischer Körper, welche Metalle, Phosphor u.s.w. enthalten. Ann. 71: 213 (1849).

44. French, D., and Knapp, D. W. The maltase of *Clostridium acetobutylicum*. J. Biol. Chem. 187: 463 (1950).

45. Frobisher, M. Fundamentals of Bacteriology. 4th ed. p. 4. Philadelphia, Pa. W. B. Saunders Co. 1950.

46. Gassner, G. The toxic action of mercury alkyls. Phytopathol. Z. 14: 385 (1943). (This issue not available because of wartime conditions; abstract read in Chem. Abst. 38: 6481. 1944.)

47. Gastrock, E. A., Porges, N., Wells, P. A., and Moyer, A. J. Gluconic acid production on pilot-plant scale. Ind. Eng. Chem. 30: 782 (1938).

48. Gates, R. L., and Sandstedt, R. M. Effect of a cationic detergent on the digestion of raw cornstarch *in vitro*. Science. 116: 482 (1952).

49. Gilman, H. Some biological applications of organometallic compounds. Science. 93: 47 (1941).

50. _____. Organic Chemistry. 2nd ed., Vol. I. p. 490-491. New York. John Wiley and Sons, Inc. 1943.

51. _____, and Bailie, J. C. Relative reactivities of organometallic compounds. XXI. Organolead radicals and derivatives. J. Am. Chem. Soc. 61: 731 (1939).

52. _____, and Robinson, J. D. The preparation of triphenyllead chloride and diphenyllead dichloride. J. Am. Chem. Soc. 51: 3112 (1929).

53. _____, and Schulze, F. A qualitative color test for the Grignard reagent. J. Am. Chem. Soc. 47: 2002 (1925).

54. _____, and Summers, L. The preparation of organolead compounds containing water-solubilizing groups. J. Am. Chem. Soc. 74: 5924 (1952).

55. _____, Summers, L., and Leeper, R. W. The reaction of aryllithium compounds with lead dichloride. Triphenyllead lithium. J. Org. Chem. 17: 630 (1952).

56. _____, Zoellner, E. A., and Selby, W. M. The yields of some organolithium compounds by the improved procedure. J. Am. Chem. Soc. 55: 1252 (1933).

57. Goodwin, L. F. The analysis of acetone by Messinger's method. J. Am. Chem. Soc. 42: 39 (1920).

58. Gordy, W. A new method of determining electronegativity from other atomic properties. Phys. Rev. 69: 604 (1946).

59. Gruttner, G. Quecksilber-derivate des cyclohexans. Ber. 47: 1651 (1914).

60. _____. Metallverbindungen des cyclohexans. Ber. 47: 3257 (1914).

61. Hampson, G. C. The stereochemistry of mercury and the moment of the mercury-carbon link. Trans. Faraday Soc. 30: 877 (1934).

62. Handler, P., and Bernheim, M. L. C. The specificity of L-(-)-methionine in creatine synthesis. J. Biol. Chem. 150: 335 (1943).

63. Hartmann, H., and Kägi, H. Säure Seifen. Z. angew. Chem. 41: 127 (1928).

64. Hata, S. Über die Sublimathemmung und die Reaktivierung der Fermentwirkungen. Biochem. Z. 17: 156 (1909).

65. Higuti, K. Action of dyes on yeasts. Japan J. Dermatol. Urol. 45: 125 (1939). (Original not examined; abstract read in Chem. Abst. 37: 3787. 1943.)

66. Hopkins, F. G., and Morgan, E. J. LXXXI. The influence of thiol groups in the activity of dehydrogenases. Biochem. J. 32: 611 (1938).

67. Hotchkiss, R. D. The nature of the bactericidal action of surface active agents. Ann. N. Y. Acad. Sci. 46: 479 (1946).

68. Hough, L. Application of paper partition chromatography to the separation of the polyhydric alcohols. Nature. 165: 400 (1950).

69. Ing, H. R. in Gilman, H. Organic Chemistry. Vol. 3. p. 507. New York. John Wiley and Sons, Inc. 1953.

70. Jenkins, G. L., and Hartung, W. H. The Chemistry of Organic Medicinal Products. 3rd ed. Chap. 12. New York. John Wiley and Sons, Inc. 1949.

71. Jerchel, D. Über invertseifen. XI. Phosphonium- und arsonium-verbindungen. Ber. 76: 600 (1943).

72. Johnson, M. J., Peterson, W. H., and Fred, E. B. Intermediary compounds in the acetone-butyl alcohol fermentation. J. Biol. Chem. 101: 145 (1933).

73. Kerr, N. G., and Young, W. J. The action of certain fat solvents on alcoholic fermentation. Australian J. Exptl. Biol. Med. Sci. 3: 176 (1926).

74. King, T. E., and Cheldelin, V. H. Oxidative dissimilation of nonnitrogenous compounds in Acetobacter suboxydans. Science. 115: 14 (1952).

75. Kinsey, V. E., and Grant, W. M. Action of mustard gas and other poisons on yeast cells. VI. Study of the relationship between inhibition of carbohydrate metabolism and inhibition of growth by various poisons, and effects of other toxic agents on yeast. J. Cellular Comp. Physiol. 30:31 (1947).

76. Kitavin, G. S. Stimulating respiration and biosynthesis of vitamin B_2 in Aspergillus niger by action of poisons. Mikrobiologiya. 21: 438 (1952). (Original not examined; abstract read in Chem. Abst. 46: 11328. 1952.)

77. Klotz, I. M., Urquhart, J. M., and Fiess, H. A. Interactions of metal ions with the sulfhydryl group of serum albumin. J. Am. Chem. Soc. 74: 5537 (1952).

78. Knox, W. E., Auerbach, V. H., Zarudnaya, K., and Spirtes, M. The action of cationic detergents on bacteria and bacterial enzymes. J. Bact. 58: 443 (1949).

79. Kostyschew, S., and Berg, V. Über Alkoholgarung. XX. Die Einwirkung von Giftstoffen auf lebende Hefe, Trockenhefe und Macerationsaft. Z. physiol. Chem. 188: 133 (1930).

80. Kreke, C. W., and Nadeau, L. V. Effect of some organic metal salts on yeast growth. Studies Inst. Divi Thomae. 5: 7 (1946).

81. Kuhn, R., and Bielig, H. J. Über invertseifen. I. Die einwirkung von Invertseifen auf Eiweiss-stoffe. Ber. 73: 1080 (1940).

82. _____, and Dann, O. Über invertseifen. II. Butyl-, Octyl-, Lauryl-, und Cetyl-dimethyl-sulfoniumjodid. Ber. 73: 1092 (1940).

83. _____, Jerchel, D., and Westphal, O. Über invertseifen. III. Dialkyl-methyl-benzyl-ammonium chloride. Ber. 73: 1095 (1940).

84. Lederer, K. Vorläufige mitteilung über gemischte bleitetraaryle. Ber. 49: 349 (1916).

85. Levaditi, C., and Lepine, P. Etude de 45 éléments du point de vue de leurs proprietés curatives dans les spirilloses, la syphilis et les trypanosomiases. Compt. rend. 193: 404 (1931).

86. _____, Mezger, J. C., and Schoen, R. Action du tellure sur le virus herpetique. Compt. rend. soc. biol. 109: 1118 (1932).

87. Levine, N. D. Screening tests of organometallic and other heavy metal compounds on horse strongyle larvae. J. Parasitol. 37: 195 (1951).

88. MacIlvaine, T. C. in Lange, N. A. Handbook of Chemistry. 4th ed. p. 940. Sandusky, Ohio. Handbook Publishers, Inc. 1941.

89. Mackworth, J. F. The inhibition of thiol enzymes by lachrymators. Biochem. J. 42: 82 (1948).

90. Malquori, A., and Tizzano, A. Sull'attivita battericida di alcuni derivati mercurio-organici. Ricerca sci. e ricostruz. 16: 1483 (1946).

91. Marxer, A. Grignard-Reaktionen mit Halogen-alkyl-aminen. Helv. Chim. Acta. 24: 209E (1941).

92. Massart, L. The biochemical action of acridine derivatives. Nederland. Tijdschr. Geneeskunde. 91: 3192 (1947). (Original not examined; abstract read in Chem. Abst. 42: 2639. 1948.)

93. _____. Antagonism between chemotherapeutic agents and different ions. Arch. intern. pharmacodynamie. 80: 44 (1949). (Original not examined; abstract read in Chem. Abst. 43: 7134. 1949.)

94. _____, Peeters, G., de Ley, J., and Vercauteren, R. The influence of salts on the inhibition of the respiration of baker's yeast by basic dyes. Experientia. 3: 154 (1947).

95. _____, and Schoon, J. L'influence du bromure d'hexadecyltrimethylammonium sur l'inhibition de la respiration des levures causée par des ions metalliques. Rec. trav. chim. 71: 33 (1952).

96. Masui, S. The reaction of various esterases on D,L-ethyl lactate. Acta Schol. Med. Univ. Imp. Kioto. 13: 339 (1931). (Original not examined; abstract red in Chem. Abst. 25: 3676. 1931.)

97. Meisel, M. N., and Umanskaya, V. P. Mechanism of action of quaternary ammonium compounds on microbe cells. Mikrobiologiya. 18: 11 (1949). (Original not examined; abstract read in Chem. Abst. 43: 6700. 1949.)

98. Miekley, A. Ueber die wirkung des atoxylsauren quecksilbers auf die menschliche syphilis. Deut. med. Wochschr. 35: 1785 (1909).

99. Mikhlin, E. D., and Golysheva, M. G. The effect of catalase on the oxidation of sorbitol by ketogenic microorganisms. Biokhimiya. 17: 91 (1952). (Original not examined; abstract read in Chem. Abst. 46: 6196. 1952.)

100. Moore, F. J. A History of Chemistry. 3rd ed. p. 266. New York. McGraw-Hill Book Co., Inc. 1939.

101. Mudd, S. Changes in the bacterial cell brought about by the action of germicides and antibacterial substances as demonstrated by the electron microscope. Am. J. Public Health. 33: 167 (1943).

102. Müller, D. Studien über ein neues enzym glykoseoxydase I. Biochem. Z. 199: 136 (1928).

103. Neish, A. C. Analytical methods for bacterial fermentations. Prairie Regional Lab. N.R.C. Saskatoon, Canada. 1952.

104. Nelson, R. D. Some derivatives of phenothiazine. Unpublished PhD. Thesis. Ames, Iowa. Iowa State College Library. 1951.

105. Nord, F. F., and Weiss, S. The Enzymes. Vol. 2. Chap. 64. New York. Academic Press, Inc. 1951.

106. Northcote, D. H., and Horne, R. W. The chemical composition and structure of the yeast cell wall. Biochem. J. 51: 232 (1952).

107. Okamoto, G., and Nagayama, M. Inhibition of catalase by mercury compounds. J. Chem. Soc. Japan. 69: 43 (1948). (Original not examined; abstract read in Chem. Abst. 46: 8685. 1952.)

108. _____, and _____. Physicochemical properties of aqueous solutions of mercury compounds. Japan J. Pharm. and Chem. 24: 358 (1952). (Original article translated into English by Dr. Kazuo Nakamoto.)

109. Perkins, D. J. A study of the effect of amino acid structure on the stabilities of the complexes formed with metals of Group II of the periodic table. Biochem. J. 55: 649 (1953).

110. Pett, L. B., and Wynne, A. M. CCXXVI. Studies on bacterial phosphatases. II. The phosphatases of Clostridium acetobutylicum Weizmann and Propionibacterium jensenii van Niel. Biochem. J. 27: 1660 (1933).

111. Pickett, M. J., and Clifton, C. E. The effect of selective poisons on the utilization of glucose and intermediate compounds by microorganisms. J. Cellular Comp. Physiol. 22: 147 (1943).

112. Quagliozzi, E., and Rescigno, A. Cellular permeability of yeast to metallic ions. Nature. 170: 35 (1952).

113. Razuvaev, G. A., and Ol'dekop, Y. A. Photoreactions of metalloorganic compounds of mercury in solution. VII. Reactions of diphenylmercury. Zhur. Obshchei Khim. 21: 1122 (1951). (Original not examined; abstract read in Chem. Abst. 46: 1479. 1952.)

114. Reck, R. A., and Harwood, H. J. Antimicrobial activity of quaternary ammonium chlorides derived from commercial fatty acids. Ind. Eng. Chem. 45: 1022 (1952).

115. Richards, T. Spoilage of industrial materials by microorganisms. Nature. 173: 102 (1954).

116. Ripert, J., and Sisley, J. Biological properties of cation-active soaps. Soaps, Perfumery, Cosmetics. 19: 834 (1946). (Original not available in library; abstract read in Chem. Abst. 41: 2593c. 1947.)

117. Rodd, E. H. Chemistry of Carbon Compounds. Vol. I, part B. p. 786. New York. Elsevier Publishing Co. 1952.

118. Roholt, K. Effect of benzoic acid and benzoic acid derivatives on the growth of Streptobacterium plantarum and Saccharomyces cerevisiae. Compt. rend. trav. lab. Carlsberg, Ser. physiol. 24: 172 (1946).

119. Rosenfeld, G. Studies of the metabolism of germanium. Arch. Biochem. Biophys. 48: 84 (1954).

120. Rothstein, A., Frenkel, A., and Larrabee, C. Certain characteristics of uranium complex with cell surface groups of yeast. J. Cellular Comp. Physiol. 32: 261 (1948).

121. _____, and Larrabee, C. The relationship of the cell surface to metabolism. II. The cell surface of yeast as the site of inhibition of glucose metabolism by uranium. J. Cellular Comp. Physiol. 32: 247 (1948).

122. _____, and Meier, R. The relationship of the cell surface to metabolism. IV. The role of cell surface phosphatases of yeast. J. Cellular Comp. Physiol. 34: 97 (1949).

123. Schmitthenner, F. Action of carbon dioxide on yeast and bacteria. Weinbau, wiss. Beih. 3: 147 (1949). (Original not examined; abstract read in Chem. Abst. 46: 9161. 1952.)

124. Selzer, L., and Baumberger, J. P. The influence of metallic mercury on the respiration of cells. J. Cellular Comp. Physiol. 19: 281 (1942).

125. Sevag, M. G., and Ross, O. A. Studies on the mechanism of the inhibitory action of zephiran on yeast cells. J. Bact. 48: 677 (1944).

126. Sexton, W. A. Chemical Constitution and Biological Activity. p. 91, 253-255. New York. D. Van Nostrand Co., Inc. 1950.

127. Shriner, R. L., and Fuson, R. C. The Systematic Identification of Organic Compounds. 2nd ed. New York. John Wiley and Sons, Inc. 1940.

128. Smith, L. T., and Claborn, H. V. Lactic esters-preparation and properties. Ind. Eng. Chem. 32: 692 (1940).

129. Smith, R. L. Isolation and determination of nutritional factors in food and fodder yeast. Unpublished PhD. Thesis. Ames, Iowa. Iowa State College Library. 1954.

130. Smyth, C. P. Dipole moment and bond character in organometallic compounds. J. Org. Chem. 6: 421 (1941).

131. Snyder, H. R., Smith, C. W., and Stewart, J. M. Carbon-alkylation with quaternary ammonium salts. A new approach to the synthesis of compounds containing the β-indolemethylene group. J. Am. Chem. Soc. 66: 200 (1944).

132. Sollmann, T., Schreiber, N. E., and Cole, H. N. Excretion of mercury after clinical intramuscular and intravenous injections. Arch. Dermatol. Syphilol. 32: 1 (1935).

133. Stephenson, M. Bacterial Metabolism. p. 96. New York. Longmans, Green and Co. 1949.

134. Stoppani, A., Actis, A. S., Deferrari, J. O., and Gonzalez, E. L. The role of sulphhydryl groups of yeast carboxylase. Biochem. J. 54: 378 (1953).

135. Sturm, H., Konermann, E., Aeschbacher, R., and Gradmann, R. Quaternary ammonium compounds with bactericidal properties. Ind. Eng. Chem. 45: 186 (1953).

136. Suhadolnik, R. J. Oxidation of disaccharide alcohols by Acetobacter suboxydans. Unpublished M.S. Thesis. Ames, Iowa. Iowa State College Library. 1953.

137. Tafel, J. Eine merkwürdige Bildungsweise von Quecksilberalkylen. Ber. 39: 3626 (1906).

138. Tria, E. Action of para-aminobenzoic acid on alcoholic fermentations. Boll. soc. ital. biol. sper. 24: 434 (1948). (Original not examined; abstract read in Chem. Abst. 43: 3559. 1949.)

139. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Tissue Metabolism. Minneapolis, Minn. Burgess Publishing Co. 1949.

140. Underkofler, L. A., Guymon, J. F., Rayman, M. M., and Fulmer, E. I. A semi-micro method for the determination of reducing sugars in fermentation media. Iowa State Coll. J. Sci. 17: 251 (1943).

141. Utter, M. F., and Werkman, C. H. Effect of metal ions on the reactions of phosphopyruvate by Escherichia coli. J. Biol. Chem. 146: 289 (1942).

142. Valko, E. I., and Du Bois, A. S. The antibacterial action of surface-active cations. J. Bact. 47: 15 (1944).

143. Voets, J. Sur l'action anti-microbienne des détergents. Parasitica. 6: 98 (1950).

144. von Braun, S. Zur Kenntnis der cyclischen Imine. Ber. 43: 2853 (1910).

145. Waters, L. L., and Stock, C. BAL (British anti-lewisite). Science. 102: 601 (1945).

146. Werkman, C. H., and Wilson, P. W. Bacterial Physiology. New York. Academic Press, Inc. 1951.

147. Winzler, R. J. A comparative study of the effect of cyanide, azide and carbon monoxide on the respiration of baker's yeast. J. Cellular Comp. Physiol. 21: 229 (1943).

148. Wood, H. G., Brown, R. W., and Werkman, C. H. Mechanism of the butyl alcohol fermentation with heavy carbon acetic and butyric acids and acetone. Arch. Biochem. 6: 243 (1945).

149. Work, T. S., and Work, E. The Basis of Chemotherapy. p. 3. London. Oliver and Boyd, Ltd. 1948.

IX. ACKNOWLEDGEMENTS

The writer wishes to express his most sincere gratitude for the excellent cooperation and guidance which was given to him by Dr. L. A. Underkofler throughout this investigation. Thanks are also due to Dr. Henry Gilman, Mr. Jay Curtice, and Mr. Sanders Rosenberg for samples of organometallic compounds which were supplied for the purposes of this study. Appreciation is expressed to Mr. Marvin Margoshes for the infrared spectra of the organic compounds; and to Dr. Kazuo Nakamoto for his translation of a Japanese article into the English language. Last, but not least, the writer is sincerely grateful to his wife, Charlotte, who has given daily encouragement and help throughout the investigation, and to whom he is indebted for typing the first draft of this thesis.