

AN ABSTRACT OF THE THESIS OF

TOMMY KIYOSHI SHIKASHIO for the Ph. D.  
(Name of student) (Degree)

in Microbiology presented on March 17, 1969  
(Major) (Date)

Title: SOME ASPECTS OF THE MECHANISM OF ACTION OF  
IODOPHOR AND OTHER GERMICIDES ON MICROORGANISMS

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Abstract approved: \_\_\_\_\_  
William E. Sandine

The concentrations of iodophor necessary to reduce the activity of several metabolic enzyme systems 50 percent was used as the basic approach in attempts to elucidate the mechanism of germicidal action of iodine. For comparisons, a chlorine-releasing germicide (Trichlorocide) and a quaternary ammonium compound were also tested. It was found that on parts per million basis, Trichlorocide inhibited the activity of most of the enzymes 50 percent in smaller concentrations than either iodophor or the quaternary ammonium compound. However on a molar basis iodophor was the most effective in inhibiting the activity of a majority of the enzymes. Conversely, the activity of most enzymes was not inhibited by the quaternary ammonium compound. In all tests where there was at least 50 percent inhibition of enzyme activity, the concentration of the germicide was well below that normally used for disinfection and sanitation purposes, providing further evidence on the sensitive nature of certain enzyme systems to inactivation by small concentrations of germicide.

Alcohol dehydrogenase and glucose-6-phosphate dehydrogenase when partially inactivated by iodophor were completely reactivated by glutathione and cysteine respectively. Likewise, cysteine was able to partially reverse iodophor inactivated lactic dehydrogenase. This evidence indicated that iodophor inactivated these enzymes, at least in part, through oxidation of their essential sulfhydryl groups.

In addition, the activity of catalase was reduced 60 percent when the enzyme was exposed to 60 ppm iodophor under alkaline conditions indicating that the inactivation occurred as a result of iodination reactions.

Only partial success was achieved in efforts to demonstrate the in vivo oxidation of yeast glucose-6-phosphate dehydrogenase sulfhydryl groups by iodophor.

In comparing the effects of iodophor and Trichlorocide on glucose oxidation and viability of Saccharomyces cerevisiae cells, it was found that with an exposure time of 30 seconds at one and two ppm, iodophor inhibited both glucose oxidation and viable cell count to a greater extent than Trichlorocide. However when the exposure time was increased to five minutes, Trichlorocide was a more effective inhibitor. Cysteine and dithiothreitol were able to partially reverse the inhibitory effects of iodophor on glucose oxidation and completely restore viable cell counts.

With both Pseudomonas fragi and Pseudomonas aeruginosa

cells, as little as 0.75 ppm of iodophor was able to substantially reduce glucose oxidation and cell viability. Dithiothreitol was able to partially reverse these inhibitory effects.

From these results it was concluded that the activity of iodophor on the organisms studied was due, at least in part, to the oxidation of sulfhydryl groups located on proteins and other constituents essential for glucose oxidation and maintenance of cell viability.

Some Aspects of the Mechanism of Action of Iodophor  
and Other Germicides on Microorganisms

by

Tommy Kiyoshi Shikashio

A THESIS

submitted to

Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Doctor of Philosophy

June 1969

APPROVED:

Redacted for privacy

\_\_\_\_\_  
Professor of Microbiology  
in charge of major

Redacted for privacy

\_\_\_\_\_  
Chairman of the Department of Microbiology

Redacted for privacy

\_\_\_\_\_  
Dean of Graduate School

Date thesis is presented March 17, 1969

Typed by Opal Grossnicklaus for Tommy Kiyoshi Shikashio

## ACKNOWLEDGMENTS

I wish to express my sincere and profound appreciation to Dr. W. E. Sandine, major professor, for his advice, guidance, encouragement, and patience during this time of study. I am also very grateful to Dr. P. R. Elliker for his very helpful advice, guidance, and for the privilege of studying in the Department of Microbiology.

The advice and assistance of Mrs. Helen Hays is greatly appreciated.

Grateful acknowledgment is made to the Klenzade Products, Division of Economics Laboratory, Inc., New York, New York for research support and to the National Institute of General Medical Sciences for training grant 5T1 GM 704-04.

An appreciation for the continual encouragement and understanding of my parents is expressed.

This thesis is affectionately dedicated to Hiroko.

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# SOME ASPECTS OF THE MECHANISM OF ACTION OF IODOPHOR AND OTHER GERMICIDES ON MICROORGANISMS

## INTRODUCTION

The importance of iodine as a germicide has long been recognized, and its usefulness for this purpose was enhanced a great deal by the relatively recent introduction of iodophor sanitizers. One of the advantages of iodophors is that the iodine solubilized in surface active substances retains its desirable germicidal activity but not its undesirable irritation and staining properties. The germicidal activity of an iodophor is principally due to the free iodine present, though other ingredients are important depending on the formulation and use conditions.

There have been numerous studies on the germicidal effectiveness of iodine against a wide variety of organisms; however, relatively few investigations have been concerned with the mechanism of activity of iodine, especially iodophors, on microorganisms. The action of iodine on proteins is known to involve the oxidation of sulfhydryl groups and substitution into tyrosyl and histidyl components. From this, it seems that the germicidal effectiveness of iodine would depend on the disruption of structural integrity resulting from substitution or oxidation of proteins whose native conformation is vital for their intended function in a cell.

The present study was undertaken to gain further insight into the mechanism of action of iodophors on microorganisms, the results of which could hopefully be used to improve the effectiveness of germicides. The primary research emphasis was to determine the effect of iodophor on certain metabolic enzymes. Many enzymes such as triose phosphate dehydrogenase require sulfhydryl groups for their activity and oxidation of these groups have been shown to reduce activity. With this and the amino acid iodination reactions in mind, the present work was undertaken. In addition, the comparative effects of quaternary ammonium compound and chlorine on the activities of the various enzymes were examined.

## REVIEW OF LITERATURE

### Iodine

#### History of Iodine

For about 150 years, iodine has been used for various purposes in medicine (Sykes, 1965). It was first used for the treatment of wounds in 1839 and was so used in the American Civil War. As early as 1895, the effectiveness of iodine against a number of pathogenic bacteria was recognized, and in the early 19th century, its usefulness as a skin disinfectant was reported. Today iodine serves in many capacities and in various forms as a germicide. It has been used in the sterilization of air and inanimate objects such as catgut and surgical instruments; as a prophylactic and therapeutic agent in diseases caused by bacteria, viruses, and fungi; for the disinfection of drinking water and swimming pool water; and for sanitization of dairy and brewing equipment (Lawrence and Block, 1968).

#### Physical and Chemical Properties of Iodine

Chemically, iodine is classed as a halogen along with fluorine, chlorine, and bromine. It is a heavy, bluish-black solid at ordinary temperatures, and is only slightly soluble in water. However the solubility in water can be greatly increased by the presence of iodides

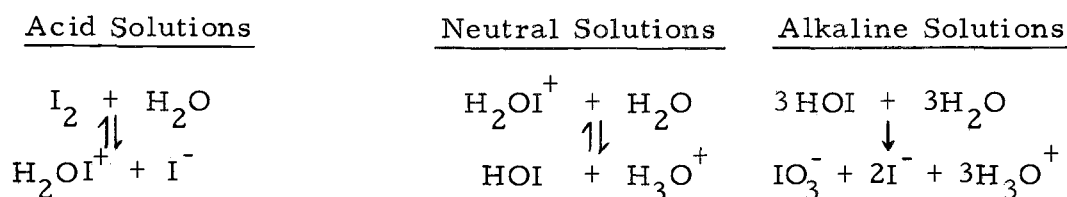
and by any one of a number of organic solvents. Iodine is less active chemically than chlorine. For example, it will not react with ammonia to form amines under conditions in which active chlorine is converted to the less active chloramines. Nevertheless it does have a high chemical activity. It is this high activity which is believed to give iodine its biocidal value. In solid form, it is corrosive to tissue. Iodine concentrations may be determined iodometrically by titration with a standard solution of sodium thiosulfate using starch as an indicator. Since iodine itself gives in dilute solutions a characteristic yellowish color, this may be used for the qualitative and semiquantitative estimation of iodine in solutions of the strength now generally used in sanitation (Wilson, Mizuno, and Bloomberg, 1960).

#### Reactions of Iodine with Water

Iodine reacts with water in solution to form various kinds of iodine compounds and ions, the proportion of the total iodine in the various possible forms depending upon the pH of the solution. For instance, in highly acidic solutions, most of the iodine is in the form of the hydrated iodine cation ( $\text{H}_2\text{OI}^+$ ). As the pH increases, this form of iodine gradually changes into hypiodous acid ( $\text{HOI}$ ), hypiodous acid ion ( $\text{IO}^-$ ), and the iodate ion ( $\text{IO}_3^-$ ). In addition there is the presence of the triiodide ion ( $\text{I}_3^-$ ) from the reaction of diatomic iodine with iodide ions ( $\text{I}^-$ ) (Hughes, 1957). The hydrated iodine

cation has the highest biocidal activity, and the activity decreases greatly for each of the new forms as they appear as the pH is increased. The hypiodous acid has only slight activity and the iodate and triiodide ion no activity. Therefore the pH of the solution is an important factor in the use of iodine for germicidal purposes (Wilson et al., 1960). These reactions may be summarized accordingly:

#### Forms of Iodine Present as a Function of pH in Aqueous Solutions



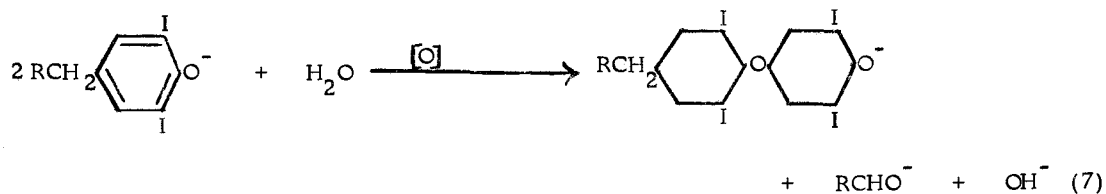
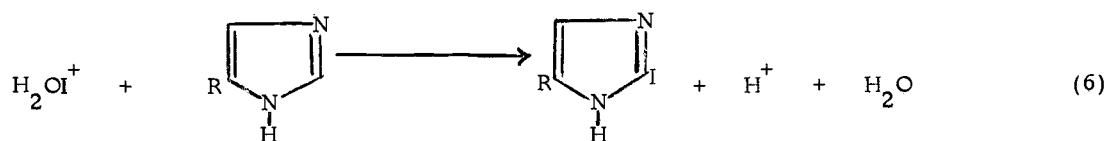
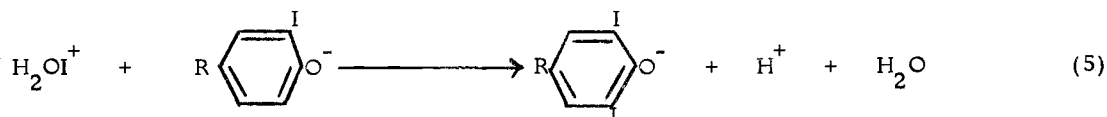
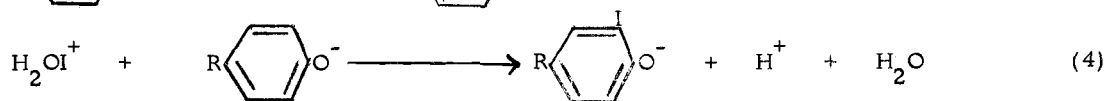
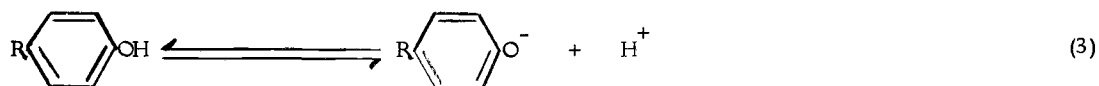
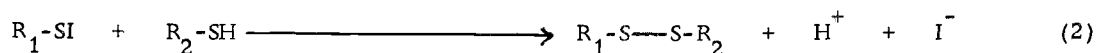
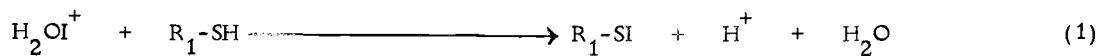
With the production of the active species, the hydrated iodine cation, the iodide ion is also produced, and the rate of reaction of this species varies inversely as the square of the concentration. It is apparent then that the control of the iodide concentration becomes an important factor in regulating the amount of the hydrated iodine cation present. Furthermore the reaction of the iodide ions with the diatomic iodine not only reduces the concentration of the diatomic iodine, but the production of the inactive triiodide ions also occurs (Hughes, 1957).

#### Reactions of Iodine with Protein

The reactions of iodine with protein fall principally into two

categories: oxidation and substitution reactions. The primary reactions are:

Reactions of Iodine with Proteins



The primary components of protein involved are the sulfhydryl groups of cysteine and the tyrosyl and histidyl moieties. Reaction one occurs initially with the formation of the sulfenyl iodide and continues into reaction two whenever stereochemically possible. In the case of monothio proteins such as serum albumin in which the formation of a disulfide link is sterically difficult, two equivalents of iodine are consumed, but the sulfenyl iodide must hydrolyze rapidly since the

final product is devoid of iodine (Hughes, 1957). Iodine may also oxidize the sulfhydryl groups beyond the disulfide stage to produce the sulfenate ( $\text{SO}^-$ ), the sulfinic acid ( $\text{SO}_2^-$ ), and the sulfonate ( $\text{SO}_3^-$ ) groups. The oxidation of the sulfhydryl groups by iodine occurs very rapidly and is favored by an acid pH (Webb, 1966).

According to Hughes (1957) the reaction of iodine with tyrosine takes place in two steps (reactions four and five). He found that by increasing the concentration of the hydrogen ion that the iodination of tyrosine was correspondingly inhibited. It was reasoned, therefore, that the hydrated iodine cation will readily attack the phenolate ion of tyrosine,  $\text{R}-\text{C}_6\text{H}_4\text{O}^-$ , or the quinoid form  $\text{R}-\text{C}_6\text{H}_4=\text{O}$  rather than the phenol form  $\text{R}-\text{C}_6\text{H}_4\text{OH}$ . Therefore a deprotonation of the tyrosine is necessary for any appreciable iodination to occur. The introduction of the second iodine atom takes place more readily than the first. This also may be explained by assuming that the reaction involves the phenolate ion since iodination increases the acidity of the phenolic hydroxyl, thereby increasing the abundance of the substituted phenolate ions concomitantly.

Evidence for reaction six, the iodination of histidyl residues in proteins, has long been suspected of occurring, but only recently substantiated by Covelli and Wolff (1966, 1967). They have reported that extensive iodination of bovine insulin leads to the formation of substituted tyrosyl and histidyl residues, the iodohistidines

consistently appearing later than the iodotyrosines. Li (1942) has measured the rate of iodination of free histidine, and found the rate to be about 30 to 100 times slower than iodination reactions with tyrosine. No other amino acids are likely to undergo substitution reactions although tryptophan may be destroyed by oxidation reactions (Hughes and Straessle, 1950).

Thyroxine formation (reaction seven) also occurs in the later stages of iodination. However both iodination of histidine and thyroxine formation take place appreciably only upon the introduction of large amounts of iodine into the protein molecule (Hughes, 1957).

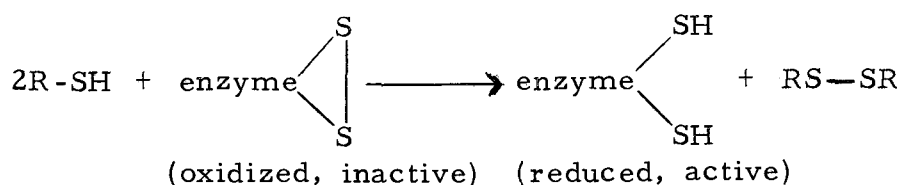
#### Effects of the Reaction of Iodine with Protein

Cellular components containing sulfhydryl groups may, in general, be classified as: (1) low molecular weight thiols, such as the cofactors lipoate, coenzyme A, etc., (2) nonenzyme proteins including cytoplasmic and structural proteins, and (3) enzymes of all types and catalyzing a variety of reactions.

The importance of sulfhydryl groups in certain metabolic reactions is well known, e.g., the conversion of coenzyme A to acetyl coenzyme A at the beginning of the Krebs cycle. The sulfhydryl groups of enzymes have been considered to bind cofactors or coenzymes to the apoenzyme or to form acyl or phosphoryl complexes with intermediates derived from substrates, or to function directly

as redox couples in electron transfer. Whatever the role sulfhydryl groups play in enzyme catalysis their modification often abolishes activity and since metabolism depends on sulfhydryl enzymes, it is evident that most important metabolic pathways would be sensitive to sulfhydryl reagents such as iodine. Even reactions with nonenzyme protein sulfhydryl groups may disturb metabolism because of the role such proteins may play in the structural organization of the metabolic units or in the permeabilities of cells.

Many enzymes have been found to be readily inhibited, often by low concentrations of iodine. In most cases it is impossible to know whether the inhibition is due to reaction with sulfhydryl groups or to iodination reactions. One way of determining if sulfhydryl group oxidation is responsible for enzyme inhibition is to attempt reversal with thiols such as cysteine, glutathione, and dithiotheitol. This reversal may occur as:



A complete reversal certainly implies such a mechanism, but the failure to reverse can be interpreted in various ways. Disulfide groups may not be reversed by thiols if steric factors prevent this reaction, and under these conditions oxidation past the disulfide stage would not be reversible (Webb, 1966). For example Knopfmacher

and Salle (1941) found no reactivation of beta-galactosidase by thiols although they had some reason for believing sulfhydryl groups were involved. Weill and Caldwell (1945) achieved partial reactivation of beta-amylase indicating that some sulfhydryl group oxidation was responsible for the inhibition. Thiols have been reported to completely reactivate urease (Hellerman, 1939), papain (Hellerman and Perkins, 1934), and cardiac lactic dehydrogenase (Nygaard, 1955) so that a sulfhydryl mechanism seems assured for these.

Several investigators have reported the inactivation of enzymes by iodine by mechanisms other than sulfhydryl group oxidation. The iodination of the tyrosyl residues of fructose-1,6-diphosphatase results in the complete loss of catalytic activity (Rosen and Rosen, 1967). Similar results were reported by Wassarman and Kaplan (1968) who found that fructose-1,6-diphosphate aldolase was inactivated upon iodination. Covelli and Wolff (1966) showed that with an increased iodination of tyrosyl and histidyl components of ribonuclease A, there was a progressive loss in activity. Also, the oxidation of a tryptophanyl residue near or part of the active site of lysozyme by iodine was reported to be likely responsible for loss of activity of the enzyme (Hartdegen and Rupley, 1964).

Studies on the reaction of iodine with microorganisms have yielded some interesting and fruitful results. Anson and Stanley (1941) reported that when the sulfhydryl groups of the coat protein

of tobacco mosaic virus were oxidized by iodine the virus remained viable; however when iodination of tyrosine occurred, the virus was inactivated. A later report (Fraenkel-Conrat and Sherwood, 1967) indicated that this iodination resulted in a conformational change of viral protein and ability to form virus rods was lost. Studies by Hsu et al. (1966) have shown that the apparent iodination of tyrosyl moieties in poliomyelitis virus and f2 bacteriophage resulted in the inactivation of the viruses while oxidation of the viral sulfhydryl groups had no effect on viability. However when bacteria were treated with iodine, the loss of viability appeared to be due to oxidation of essential sulfhydryl groups. Brandrick and Newton (1967) have recently shown that the reaction of bacteria with iodine is primarily an oxidation-reduction reaction with only little halogenation occurring. Increased iodination of bacteria by raising the pH does not result in higher bactericidal effect, but rather a decrease (Klebanoff, 1967; Hugo and Newton, 1964).

Despite its strong oxidative action, iodine is unable to inactivate either infectious RNA or DNA (Hsu, 1964). However an interesting report by Goehler and Doi (1966) has shown that a reversible conformational change and inactivation occurs upon iodine oxidation of lysyl-tRNA. The inactivation could be completely reversed by sodium thiosulfate indicating the involvement of sulfhydryl groups either directly or indirectly with the binding efficiency of the

lysyl-tRNA. These results suggest the possibility that iodine may exert part of its germicidal action by inhibition of protein synthesis.

### Some Compounds and Preparations of Iodine

The oldest known compound of iodine used for antiseptic purposes is probably iodoform,  $\text{CHI}_3$ . The unpleasant odor and lack of antibacterial properties in vitro are among its disadvantages, but it does exhibit good antiseptic action in vivo.

An iodine compound especially lethal to bacterial spores is iodine trichloride. A one percent solution is generally marketed as a disinfectant for external application (Sykes, 1965).

Two percent iodine solutions such as alcoholic iodine tincture and aqueous iodine solution are commonly used antiseptics. Tincture of iodine is the most powerful and reliable antiseptic routinely applied to the human skin. However when it is applied to moist mucous membranes, the alcohol may be diluted beyond its capacity to hold the iodine in solution with a resulting precipitation of minute amounts of very caustic metallic iodine (McCulloch, 1945). Thus a solution of iodine-iodide is often preferred since most of the objections of alcoholic solutions are avoided (Lawrence and Block, 1968).

### Germicidal Activity of Iodine

Iodine is effective against a wide variety of organisms including

bacteria, spores, viruses, yeasts, fungi, rickettsiae, and protozoa (Wilson et al., 1960). One of its most striking characteristics is that the concentration of iodine necessary to disinfect does not vary greatly with different organisms and that the organisms are killed rather than held in a condition of bacteriostasis. Furthermore there have been no instances of microorganisms developing a resistance to iodine (McCulloch, 1945).

Gershenfeld and Witlin (1949) found that a one ml solution of two percent free iodine to be bactericidal within one minute against 24-hour-old F. D. A. broth cultures of Staphylococcus aureus (20 ml), Pseudomonas aeruginosa (20 ml), and Bacillus mesentericus (10 ml). In comparing with similar concentrations of free bromine and chlorine over a wide pH range, they also reported that the iodine solutions were more effective antibacterial agents. Chang and Fair (1941) have found that vegetative bacteria suspended in water are killed by five ppm iodine while Carroll (1955) has reported as little as two ppm to be lethal to E. coli in ten minutes and 33 ppm in one minute. These different results are likely due to the various testing methods employed as well as to the amount of organic matter present.

Concentrations of free iodine of 62.5 ppm or higher have been found to be tuberculocidal after a five minute exposure at room temperature (Gershenfeld et al. as cited by Lawrence and Block, 1968).

If spores are in suspension, they show little increased

resistance to iodine in comparison to vegetative bacteria. Both spores of Bacillus species and Clostridium tetani are readily killed by dilute solutions of iodine. Among the halogens, iodine is the most effective against the spores of B. anthracis (Lawrence and Block, 1968).

There are variations of opinions as to the lethal concentration of iodine against fungi, but there is general agreement that it is an effective fungicidal agent. Many of the pathogenic fungi are killed in concentrations of less than 12 ppm iodine (Sykes, 1965).

High dilutions of iodine are also effective against viruses. Solutions as dilute as 60 ppm will inactivate washed influenza virus (Knight and Stanley, 1944). Gershenfeld (1955) has presented in a review the effective inactivation by iodine and certain iodine compounds of the viruses of poliomyelitis, herpes, rabies, vaccinia, and others.

Preparations of iodine, its derivatives and combinations have received wide use as protozoacidal and metazoacidal agents, especially in their effectiveness against amoeba, trichomonads, strongyloides, trichuris, and oxyuris (Lawrence and Block, 1968).

#### Advantages and Disadvantages of Iodine as a Germicide

Although iodine undergoes a marked depressant effect by the presence of organic matter, it is not as great as chlorine and is

actually negligible when used in high concentrations. The temperature coefficient of bactericidal activity is relatively low compared to other germicides being more effective at lower temperatures. Water hardness generally does not have any effect upon the germicidal efficiency of iodine, but should be considered since the water may have other contaminants other than calcium and magnesium salts and thus provide a buffering capacity. Finally iodine has a low toxicity when used as a germicide in comparison to other agents. Some of its disadvantages are its low solubility, objectionable odor, high vapor pressure, skin staining, precipitation upon dilution, and instability.

### Iodophors

Despite the advantages of iodine over other germicides, preparations of iodine were not extensively used before 1950 because of its undesirable properties (Wilson et al., 1960). However with the introduction of the iodophors by Shelanski (as cited by Davis, 1962), a revolution occurred in the use of iodine for disinfecting and sanitation purposes. Using polyvinyl pyroidone and other surface active substances, Shelanski found that large quantities of iodine could be dissolved while keeping the iodine in solution when diluted with water and modifying its undesirable properties. In solution the carriers are able to solubilize up to nearly 30 percent of their weight of

iodine, of which from 70 to 80 percent may be released as available iodine when the concentrated solution is diluted. The constitution of the iodophor is not exactly known, but it is clear that the iodine is bound in the form of micellar aggregates and that on dilution the micelles are dispersed and the linkage of the iodine is progressively weakened (Davis, 1962). A number of anionic, cationic, and non-ionic surfactants may act as carriers; however they vary in their ability to solubilize iodine, viscosity, foaming, detergency, and wetting action. Almost all commercial iodophors use nonionic detergents since they provide better solubility, stability, and other desirable properties than the ionic surfactants. In general the alkylphenoxypolyglycol ethers have proved to be the most useful. Since iodine is more active in acid solutions, almost all commercial iodophors contain a buffering agent to provide the desired pH in solution. Phosphoric acid is commonly used for this purpose.

The advantages of iodophors over other iodine preparations are numerous and include possession of detergent properties, enhancement of germicidal action, little or no odor, elimination of staining and skin irritation, lowering of vapor pressure, and unlimited dilution possible without precipitation (Wilson et al., 1960). In addition because the iodine is brought into solution with a solubilizer other than the iodide ion, there is little or no loss of iodine by its conversion to the inactive triiodide ion, thus probably accounting

for the reports of iodophor activity being higher than with similar iodine solutions. With the combined effect of the phosphoric acid, as little as one ppm iodine is 99.9 percent effective against staphylococci and E. coli in one minute (Sykes, 1965). Using a variety of organisms often found on dairy and food equipment, Hays, Sandine, and Elliker (1967) reported that iodophors and hypochlorites demonstrated a high rate of germicidal activity. Hypochlorites were more effective at acid pH for the bacterial species tested; however the iodophor was more effective against yeasts. In comparing iodophors, chlorine, and quaternary ammonium germicides using Staphylococcus aureus, E. coli, and Pseudomonas aeruginosa as test organisms, Johns (as cited by Davis, 1962) found that iodophors destroyed more increments of test organisms than chlorine or quaternary ammonium compounds. In addition to the vegetative bacteria, Mycobacterium tuberculosis was killed by 50 ppm in two to ten minutes and pathogenic fungi by 40-100 ppm iodine liberated from iodophor (Ortenzio as cited by Davis, 1962). Against the Newcastle disease virus suspended in allantoic fluid Bartlett and Schmidt (1957) have reported that 25 ppm is effective in 30 seconds and that 50 ppm will inactivate the poliomyelitis virus in five minutes. In testing the virucidal activity of hypochlorites, quaternary ammonium compounds, and iodophors, Watkins, Hays, and Elliker (1957) reported that 12.5 ppm hypochlorite and 100 ppm QAC completely inactivated the

bacteriophage of Streptococcus cremoris; however concentrations of iodophor as high as 200 ppm were ineffective.

### Uses of Iodophors

Iodophors are widely used in food processing plants, dairies, brewing industries, hotels, restaurants, schools, and hospitals. The concentrations of iodophors to be used depend upon the purposes desired. In general the following minimum strengths of iodophors are recommended as sanitizing agents: In hospitals for all uses, 75 to 150 ppm; in restaurants, breweries, and food processing plants, 25 to 50 ppm; and for dairy equipment, 12.5 ppm (Lawrence and Block, 1968). Properly formulated iodophors are useful not only as germicides for final sanitization of equipment but also for cleaning purposes as well. Thus a one step cleaning and sanitizing process is possible which is of economic importance (Wilson et al., 1960). Iodophors also stain soiling matter such as milk solids and milk stone so automatically indicate if equipment is physically clean. They do not stain metals, as reported by some users, who apparently have never succeeded in cleaning their equipment properly (Davis, 1962).

Some of the marketed iodophors commonly used include Wescodyne, Betadine, Isoprep, Surgidine, and Klenzade Iodophor.

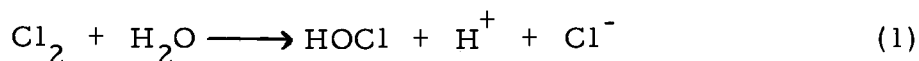
## Chlorine

Soon after its discovery in 1774, the remarkable ability of chlorine to arrest putrefaction and destroy odors was recognized and its use met with almost immediate approval. Chlorine is now one of the most widely used chemical disinfectants largely due to its almost universal application in the disinfection of questionable water supplies.

### Physical and Chemical Properties

Chlorine is a heavy, green-colored gas (McCulloch, 1945). Because of its electronic configuration, it possesses a strong tendency to acquire extra electrons changing to inorganic chloride ions making chlorine a very strong oxidizing agent. It is this property of chlorine which is believed to give it its high germicidal activity. However this strong oxidizing characteristic also enables chlorine to be very corrosive, and thus it attacks many metals as well as organic matter. In chlorination of water a certain portion of this chlorine will be consumed by water impurities; therefore a sufficient amount of chlorine is generally provided to satisfy the initial water demand and an extra quantity of chlorine is added to provide a slight residual of free available chlorine. The term free available chlorine is usually applied to three forms of chlorine which may be

found in water: elemental chlorine ( $\text{Cl}_2$ ), hypochlorous acid ( $\text{HOCl}$ ), and hypochlorite ion ( $\text{OCl}^-$ ). They are formed according to the following reactions:



The disinfecting efficiency of chlorine decreases with an increase in pH and vice versa and is parallel to the concentration of undissociated hypochlorous acid. This would indicate that  $\text{HOCl}$  must be far stronger in germicidal action than  $\text{OCl}^-$  (Lawrence and Block, 1968).

### Preparations and Uses of Chlorine

Chlorine and chlorine compounds are widely used in water supplies, swimming pools, sewage, dairy and food industries, and medicine for sanitation and disinfecting purposes. A wide range of organisms are susceptible to the germicidal action of chlorine including algae, bacteria, spores, viruses, fungi, and protozoa (Sykes, 1965).

The hypochlorites are the most widely used of active chlorine compounds, and are usually composed of either calcium or sodium hypochlorite solutions ranging up to 70 percent available chlorine. Chlorine dioxide is occasionally employed for purification of water. Its advantages are that it does not react with ammonia and it is unaffected at pH levels from six to ten. However it is an extremely reactive compound and therefore difficult and often dangerous to

use (Lawrence and Block, 1968). There are a number of organic chlorine-releasing compounds which can be used as disinfectants. They have an advantage of releasing their chlorine less readily and so exert a more prolonged germicidal effect. Also they are less irritant and toxic and thus advantageous for use in medicine and surgery. Two such compounds are derivatives of isocyanuric acid, the dichloro and trichloro compounds which have about 72 to 90 percent respectively of available chlorine (Sykes, 1965).

#### Mechanism of Action

Despite much research the mechanism of germicidal action of chlorine or chlorine compounds has not been fully elucidated. It is generally agreed that with the exception of chlorine dioxide, chlorine and its compounds are hydrolyzed in solution to some extent to yield hypochlorous acid, and that the germicidal activity can be associated with the concentration of undissociated molecules of hypochlorous acid. Therefore the prevalent theory is that the mechanism involves hypochlorous acid (Sykes, 1965). It has been speculated that  $\text{HOCl}$  liberates nascent oxygen which in turn supposedly combines with components of cell protoplasm, killing the organism. However this theory has not been substantiated as Chang (1944) has reported that chlorine is bactericidal even under conditions that exclude direct oxidation. Also other oxygen producing compounds such as hydrogen

peroxide which produces larger amounts of nascent oxygen do not kill as rapidly as chlorine (Lawrence and Block, 1968).

It has been suggested that chlorine exerts at least part of its bactericidal action by direct chlorination of bacterial protein replacing the hydrogen in the amino groups. The combination of chlorination with oxidative effects on critical structures such as cell membranes may cause the bacteria to disintegrate (McCulloch, 1945). Friberg (1956) reported that the oxidative effect of chlorine and not its chlorinating ability produces a primary bactericidal effect as evidenced by a lack of radioactive chlorine ( $\text{Cl}^{35}$ ) uptake initially. Later Friberg (1957) found that destructive permeability changes in the bacterial cell wall were caused by chlorine, as evidenced by leakage of  $\text{P}^{32}$  from nucleoproteins of the bacterial cell. Pulvertaft and Lumb (1948) have observed the lysis of certain bacterial cells caused by high concentrations of sodium hypochlorite. The bacteria tested varied in the extent to which lysis occurred; advanced lysis being found with staphylococci, pneumococci, and E. coli; less marked lysis was found with Shigella dysenteriae; and very little lysis occurred with streptococci.

Stumpf and Green (1946) and Knox et al. (1948) have advocated that the bactericidal action of chlorine is produced by the inhibition of certain enzyme systems essential to life. They have reported that the inhibition occurs as a result of the oxidation of sulfhydryl

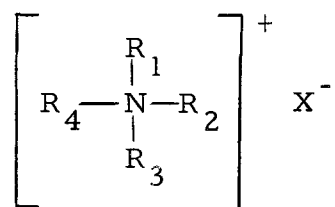
groups of vital enzymes or other enzymes susceptible to oxidation subsequently resulting in inactivation. Furthermore they found an exact parallelism between the minimal concentrations of chlorine at which the oxidation of glucose is completely inhibited and the minimal concentrations at which bacterial growth is suppressed. Therefore they have suggested that inactivation of glucose oxidizing enzymes results in cell death.

Recently Benarde et al. (1967) have concluded from their experimental results that chlorine dioxide does not produce leakage of cell contents. However by using  $C^{14}$ -labeled phenylalanine uptake as an indicator, they found that E. coli cells abruptly stops uptake when exposed to the germicide. No lag phase was observed indicating that a break in protein synthesis was involved rather than inactivation of an enzyme system in the catabolism of glucose.

### Quaternary Ammonium Compounds

#### Structure and Function

Basically the quaternary ammonium compounds (QAC) are organically substituted ammonium compounds in which the nitrogen atom has a covalence of five. Since they are structurally related to amines, the general formula is:



The X represents a halide, sulfate, or similar radical, and the substituents  $R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$  each represents an alkyl, araalkyl or heterocyclic radical of a given size or chain length. The QAC are quite desirable as germicides since they are colorless, odorless, nonirritating, possess detergent action, quite stable, and have low toxicity.

#### Antimicrobial Activity

The antimicrobial activity of the QAC is quite good; however there is a variation in effectiveness among different organisms. In general the QAC are more selectively active against Gram-positive organisms than against the Gram-negative types, Pseudomonas aeruginosa being the most resistant (Sykes, 1965). Virulent strains of Mycobacterium tuberculosis were reported by Smith et al. (1950) to be resistant to the bactericidal action of QAC, and spores of bacilli were found by Davies (1949) to be quite resistant. The anti-fungal activity of the QAC has been reported (Dunn, 1938; Joslyn, Yaw, and Rawlins, 1943) as well as their effectiveness against protozoa (Fair et al., 1945; Quisno and Foter, 1946). Viruses, in general, appear to be somewhat more resistant than other organisms

to the lethal effects of the QAC. Armstrong and Froelich (1964) have reported that examples of viruses which are inactivated by QAC include influenza, measles, rabies, and vaccinia while poliomyelitis, Cocksackie, and ECHO were resistant.

### Mechanisms of Action

There have been several explanations for the germicidal action of the quaternary ammonium compounds. Hotchkiss (1946) has reported the release of nitrogen and phosphorus containing compounds from QAC treated bacteria. Salton (1951) similarly noted the release of cellular constituents from bacteria and attributed that part of the germicidal action was due to this action. Damage and denaturation of the cytoplasmic membrane by the action of QAC have been reported by Tomcsik (1955). Recently Washam (1968) has shown in electron micrographs the lysis of sensitive P. aeruginosa cells exposed to high concentrations of QAC. Contrary to earlier concepts, Dawson, Lominski, and Stern (1953) have reported that the QAC split lipoprotein complexes throughout the cell resulting in the release of autolytic enzymes which act within the cells. They found that when low ratios of organisms to QAC were used, cell lysis occurred; however at higher concentrations, lysis did not occur presumably due to denaturation of the autolytic enzymes.

As a mechanism of action, the relationship between the

inactivation of enzymes and denaturation of essential cell protein is quite close. Indeed enzyme inactivations follow much more closely cell death than lysis (Sykes, 1965). For instance Baker, Harrison, and Miller (1941) reported that the loss of viability of bacterial cells roughly paralleled the suppression of respiratory and glycolytic activity. Sevag and Ross (1944) have advocated that the inhibition of cytochrome C oxidase by zephiran results in the inhibition of yeast cells. One of the strongest pieces of evidence in support of enzyme inactivation over lysis is that of Stedman, Kravitz, and King (1957). They reported a less than two percent loss of cell contents at QAC levels which would inhibit glucose oxidation by 50 percent. When the germicidal concentration was increased ten fold resulting in a 1,000 fold difference in lethal action, there occurred only a 0.3 percent change in the amount of lysis. Roberts and Rahn (1946) determined the activity of all the energy producing enzymes of E. coli grown on acetate medium in the presence of growth-retarding, growth-inhibiting, and lethal concentrations of cetyl pyridinium chloride. They found that growth inhibition occurred at a concentration of the germicide which had little or no effect on dehydrogenase, catalase, and oxidase activities. Therefore they expressed the opinion that the bacteriostatic condition results from the QAC preventing multiplication and not through inactivation of enzymes. However with lethal concentrations of QAC, they found inactivation of

practically all dehydrogenases and oxidases, but not catalase. The lethal action was not reversible whereas the bacteriostatic reaction was reversible. Knox et al. (1949) reported that zephiran kills E. coli cells parallel with the inhibition of certain metabolic reactions of the cells. These effects of killing and inhibition were proportional to the zephiran bacterial ratio and not to the zephiran concentration; therefore it was strongly suggested that enzyme inhibition accounts for the metabolic inhibition, increased permeability, and subsequent cell death.

## MATERIALS AND METHODS

### Enzymes

All except four of the enzymes were obtained from Sigma Chemical Company, Inc., St. Louis; catalase was supplied by Dr. M. Hiraga, Science Research Institute, Oregon State University. Bacterial glucose-6-phosphate dehydrogenase was isolated from Pediococcus cerevisiae according to the method of DeMoss (1955). The beta-galactosidases of Streptococcus lactis and Lactobacillus helveticus were obtained from G. A. McFeters and L. L. McKay respectively, both of the Department of Microbiology, Oregon State University. Protein concentrations were determined by the method of Lowry et al. (1951). Previously reported methods were used for the enzyme assays as follows: Alcohol dehydrogenase (Racker, 1955); Beta-galactosidase (McFeters, Sandine, and Elliker, 1967); Lactic dehydrogenase (Kornberg, 1955); Catalase (Beers and Sizer, 1952); Cytochrome c reductase (Mahler, 1955); Hexokinase (Darrow and Colowick, 1962); Glucose-6-phosphate dehydrogenase (Worthington Enzymes, Enzyme Reagents Manual, 1966a); Malic dehydrogenase (Ochoa, 1955); Glyceraldehyde-3-phosphate dehydrogenase (Worthington Enzymes, Enzyme Reagents Manual, 1966b; with no cysteine used in buffer).

### Microorganisms

Pseudomonas aeruginosa, Pseudomonas fragi, Pediococcus cerevisiae P-60, and Saccharomyces cerevisiae were obtained as stock strains from the collection maintained in the Department of Microbiology, Oregon State University. P. aeruginosa, P. fragi, and P. cerevisiae were maintained on tryptone, glucose, yeast extract (TGY) agar (tryptone 5 g; yeast extract 5 g; glucose 1 g;  $K_2HPO_4$  1 g; agar 20 g in 1 liter of distilled water). S. cerevisiae was maintained on a special TGY agar (glucose 20 g; tryptone 10 g; yeast extract 5 g; agar 20 g in 1 liter of distilled water).

### Germicides

Iodophor, formula ID-10; Trichlorocide, sodium dichloroisocyanurate, formula XP-100; and alkyl dimethyl ethyl benzyl ammonium chloride were obtained from the Klenzade Products, Division of Economics Laboratory, Inc., Beloit, Wisconsin. The available iodine in iodophor was determined by titration to a colorless endpoint with a standard solution of sodium thiosulfate. Available chlorine was also determined by standard sodium thiosulfate titrations according to the method described by the American Public Health Association (1960). Quaternary ammonium compound concentrations were determined by the method of Furlong and Elliker

(1953).

### Effect of Germicides on Enzymatic Reactions

The effect of each germicide on an enzymatic reaction was measured by adding the enzyme to the assay system containing the inhibitor. Such a method provides some similarity to what may be occurring in vivo. It also prevents the effects of dilution which occurs in instances where the enzyme is preincubated outside of the assay system with the inhibitor and then tested. By varying the concentration of the inhibitor and repeating the assays several times, it was possible to determine the concentration of germicide to cause 50 percent inhibition of each enzyme. All calculations were based on initial velocity reactions of zero order. A Beckman DU spectrophotometer attached to a Gilford multiple channel absorbancy recorder was used in all assays. Catalase was also assayed manometrically using a Gilson Differential Respirometer.

### Iodine Inactivation and Reactivation of Certain Metabolic Enzymes

Three different enzymes were selected which were believed to depend upon their intact sulfhydryl groups for their activity. These enzymes were subjected to mild oxidation by iodine and attempts were made to reactivate the inhibited enzyme by use of

reducing agents. In addition, a study was done on the possible mechanism of action of iodine on catalase, a sulfhydryl independent enzyme.

### Sulfhydryl Dependent Enzymes

Alcohol dehydrogenase (yeast). To a solution consisting of 2.7 ml 0.01M, pH 7.5 sodium phosphate buffer containing 1.2 ppm iodine at 25 C, 0.3 ml of the enzyme (0.84 mg protein per ml) was added with mixing. After two min, 0.1 ml aliquots were removed and assayed. Reactivation was attempted by adding various concentrations of cysteine or glutathione after the two min with continued incubation for an additional two min before assaying.

Lactic dehydrogenase (rabbit muscle). To a solution consisting of 1.8 ml of 0.1M, pH 7.4 potassium phosphate buffer containing 0.5 ppm iodine at 25 C, 0.2 ml of the enzyme (31  $\mu$ g per ml) was added with mixing. After one min, 0.1 ml aliquots were removed and assayed. Reactivation was attempted by adding various concentrations of cysteine after one min with continued incubation for an additional two min before assaying.

The method of Boyer (1954) was used to follow the reaction of parachloromercuribenzoate (PCMB--Sigma Chemical Co.) with protein sulfhydryl groups. To determine the extent of oxidation of sulfhydryl groups in the lactic dehydrogenase solution, 0.1 ml of the

enzyme (0.58 mg per ml) was added to 2.8 ml of 0.1M, pH 7.4 potassium phosphate buffer containing 42 ppm iodine with mixing, the solution was then incubated for two min at 25 C. PCMB [0.1 ml at  $9.42 \times 10^{-4}$  M prepared according to the method of Neilands (1954)] was then added and the change in optical density followed at 250 m $\mu$  in a Beckman DU spectrophotometer fitted with a Gilford absorbancy recorder. This procedure was repeated with noniodine treated enzyme to measure the normal reaction of PCMB with sulfhydryl groups in the enzyme solution.

Glucose-6-phosphate dehydrogenase (yeast). To a solution consisting of 0.6 ml of 0.1M, pH 7.4 Tris buffer containing one ppm iodine at 25 C, 0.2 ml of the enzyme (0.15 mg per ml) was added with mixing. After two min, 0.1 ml aliquots were removed and assayed. Reactivation was attempted by adding various concentrations of cysteine after the two min with continued incubation for an additional two min before assaying.

#### Sulfhydryl Independent Enzyme

Catalase (beef liver). The effect of iodine on catalase activity was determined by adding 0.2 ml of catalase (35  $\mu$ g per ml) to 1.8 ml of 0.1M, pH 7.0 potassium phosphate buffer containing 60 ppm iodine. The solution was then incubated at 4 C and assayed at various time intervals. A similar experiment was carried out at pH 8.5.

The retarding effect of iodide ions on the reaction between iodine and catalase was measured by adding 0.2 ml of various concentrations of potassium iodide to 1.4 ml of 0.1M, pH 8.5 potassium phosphate buffer containing 60 ppm iodine. Catalase (0.2 ml at 35  $\mu$ g per ml) was then added with mixing and the solution incubated at 4 C. Assays were performed on aliquots removed at various time intervals.

Isolation and Partial Purification of Glucose-6-phosphate  
Dehydrogenase from Iodophor-treated and  
Noniodophor-treated Yeast Cells

A modification of the method of Kornberg and Horecker (1955) was employed for isolation and partial purification of glucose-6-phosphate dehydrogenase from yeast. A unit of enzyme was defined as that amount which causes an initial change in optical density of 1.000 per min at room temperature (23-25 C) under the previously described assay conditions. Specific activity was expressed as units per mg of protein. Protein concentrations were determined spectrophotometrically according to the method of Kalchar (1947).

Culturing of Cells and Preliminary Treatment

S. cerevisiae cultures were grown on TGY agar slants for 18 hr at 30 C. A broth suspension of a culture was then inoculated into 1800 ml of TGY broth and incubated with shaking for 20 hr at 30 C. The cells were harvested by centrifugation at 2500  $\times$  g for 20 min,

washed once in 170 ml saline, and resuspended in saline to an optical density of 0.47 at 650 m $\mu$ . For the iodophor treatment of cells, 100 ml of the cells were added to 900 ml of pH 7.0, 0.2M sodium phosphate buffer containing 20 ppm iodophor with mechanical mixing at 25 C. After 30 sec, 25 ml of 0.1N sodium thiosulfate were added as a neutralizer. As a control the same procedures were followed except that the iodophor was omitted.

#### Autolysis and Sonication of Cells

Since there appeared to be little lysis in earlier trials with sodium bicarbonate treatment, a combination of autolysis and sonication was used. The iodophor-treated cells were collected by centrifugation at  $2500 \times g$  for 20 min and washed once with 150 ml 0.1N sodium bicarbonate. Partial autolysis was achieved by incubating the cells at 40 C for five hours in 150 ml of the 0.1N sodium bicarbonate. They were then sonicated by the Raytheon 10KC Sonic Oscillator at 100 percent tuned voltage for 30 min.

#### Fractionation with Ammonium Sulfate

All procedures from this step were continued with the preparations at or near 4 C. The autolyzate was centrifuged at  $5800 \times g$  for 20 min, and the sediment was discarded. The supernatant was diluted to about 140 percent of the original volume with 0.1N sodium

bicarbonate. Ammonium sulfate was then slowly added with mechanical mixing to 55 percent saturation. The mixture was left for about 20 min and then centrifuged at  $5000 \times g$  for 20 min. The sediment was discarded. To the supernatant the ammonium sulfate concentration was further increased to 70 percent. This mixture was left standing for 20 min and then centrifuged at  $5000 \times g$  for 20 min. The supernatant was discarded, and the sediment was dispersed in 50 ml of cold distilled water.

#### Calcium Phosphate Gel Adsorption

Calcium phosphate gel was added to the 50 ml preparation with slow mechanical mixing up to a 1:1 ratio by weight of gel to protein. The adsorption was allowed to proceed for 20 min; then the solution was centrifuged at  $4100 \times g$  for 20 min and the supernatant volume noted and discarded.

#### Elution of the Partially Purified Enzyme

The partially purified enzyme was eluted from the gel by adding 0.1M, pH 7.4 sodium phosphate buffer in an amount equal to the volume of the original supernatant. Elution was allowed to continue with mechanical mixing for 25 min. The solution was then centrifuged at  $5000 \times g$  for 20 min. The sediment was discarded and the supernatant was used as the enzyme preparation.

Effect of Iodophor or Trichlorocide on Glucose Oxidation  
and Viability of *S. cerevisiae*

Yeast cells were grown on TGY agar slants for 18 hr at 30 C; then inoculated into 100 ml TGY broth and incubated for 18 hr at 30 C with shaking. Cells were then harvested by centrifugation and washed twice in cold saline. Stock solutions of cells were prepared at a concentration of one mg nitrogen per ml which was equivalent to about  $12 \times 10^8$  viable cells per ml. The cells were exposed to the appropriate germicide with mechanical stirring in 0.2M, pH 7.0 sodium phosphate buffer at 27 C for either 30 sec or five min periods. In a typical experiment three ml of cells were added to 297 ml of buffer containing germicide. An excess of 0.1N sodium thiosulfate was then added at the appropriate time to stop the reaction and the cells were collected by centrifugation. Cells were then resuspended in three ml of saline. A Gilson Differential Respirometer was used to measure glucose oxidation in which microliters of oxygen uptake were read directly on the volumometer. One ml of the cells, 1.4 ml distilled water, and 0.3 ml of 0.1M, pH 7.0 sodium phosphate buffer were added to a respirometer flask; 0.3 ml of 0.05M glucose was added to the side arm. Two drops of 20 percent potassium hydroxide were introduced in the center well holding a piece of fluted filter paper to adsorb carbon dioxide. After 15 min equilibration, the glucose solution was tipped in and the oxygen uptake measured at

30 C. Appropriate controls were included in all experiments. The number of viable cells was determined by removing cell samples after neutralization of the germicide by sodium thiosulfate and performing dilution plate counts. Plates were incubated at 30 C for 48 hr. In experiments where reducing agents were added to the reaction flask, samples were removed after addition of the reductant. Attempts to reverse the inactivating effects of iodine on glucose oxidation and viability were done by adding the reducing agent after the cells had been exposed to iodine. With dithiothreitol (Cleland's reagent, Calbiochem Corp.), 0.8 ml of a 0.2M solution was added 30 sec after the reaction with iodine had been terminated with sodium thiosulfate. No additional incubation or washing of cells was done. With cysteine, 0.5 ml of a 1M solution was added 30 sec after the reaction with iodine had been terminated with sodium thiosulfate. The solution was then incubated for 15 min at 37 C. The treated cells were then collected by centrifugation and washed once with cold saline.

Effect of Iodophor on Glucose Oxidation and  
Viability of *P. fragi* and *P. aeruginosa*

Effect on *P. fragi*

*P. fragi* cells were transferred at least three times in seven ml of TGY broth (modified to contain three grams glucose per liter) at 12-hr intervals with shaking at 30 C. A drop of the culture was then inoculated into four 250-ml flasks each containing 50 ml of the

modified TGY broth and incubated with shaking for 12 hr at 30 C. The cells were then harvested by centrifugation at  $2500 \times g$  for 20 min, washed twice in 100 ml cold saline, and resuspended in 40 ml of saline. One ml of the culture was then removed and diluted with saline to yield an optical density of 1.15 at 650 m $\mu$  equivalent to about  $10 \times 10^8$  viable cells per ml. For the exposure of cells to iodophor, three ml of stock cells were pipetted into 37 ml of 0.2M, pH 7.0 sodium phosphate buffer containing 0.75 ppm iodine at 27 C with mechanical stirring. After 30 sec, 0.3 ml of 0.1N sodium thiosulfate was added. A similar procedure was followed for attempts to reverse any inhibitory effects of iodine by adding 0.4 ml of 0.2M dithiothreitol 30 sec after addition of the sodium thiosulfate. A buffer solution containing no iodine was used as a control. The cells were then collected by centrifugation at  $10,000 \times g$  for 20 min and resuspended in three ml saline. Glucose oxidation was measured by the Gilson Differential Respirometer by the method previously described for yeasts. Colony counts were done by plate dilution techniques by use of modified TGY agar. Counts were done after incubation at 30 C for 48 hr.

#### Effect on *P. aeruginosa*

*P. aeruginosa* cells were transferred at least three times in seven ml of modified TGY broth at 12 hr intervals with shaking at

30 C. A drop of the culture was then inoculated into 200 ml of the modified TGY broth contained in a one liter flask, and incubated for eight hr at 30 C with shaking. The cells were harvested by centrifugation at  $2500 \times g$  for 20 min, washed once in 100 ml saline, and resuspended in 40 ml saline. One ml of the culture was removed and diluted with saline to yield an optical density of 1.15 at 650 m $\mu$  equivalent to about  $5 \times 10^8$  viable cells per ml. The cells were exposed to 0.75 ppm iodine by the identical procedures described for P. fragi except that 0.2 ml sodium thiosulfate and 0.2 ml dithiothreitol were used. The cells were collected by centrifugation at  $10,000 \times g$  for 20 min and resuspended in 2.5 ml saline. Measurement of glucose oxidation and colony counts were done as previously described.

## RESULTS

Comparative Effects of Germicides  
on Metabolic Enzymes

The comparative effects of iodophor, Trichlorocide, and the quaternary ammonium compound on the activities of certain metabolic enzymes are shown in Table 1. On a parts per million basis the activity of most of the enzymes was inhibited 50 percent by a smaller concentration of Trichlorocide than either iodophor or the quaternary ammonium compound. However on a molar basis, iodophor inhibited the activity of a majority of enzymes in the smallest concentration. In general the quaternary ammonium compound was the least effective inhibitor. It may be noted, however, that the alcohol dehydrogenase, beta-galactosidase (liver), and cytochrome c reductase systems were quite sensitive to inhibition by the quaternary ammonium compound in small concentrations indicating the susceptibility of some enzyme systems to this compound.

Certain enzymes, such as glucose-6-phosphate dehydrogenase, were quite sensitive to iodophor but resistant to Trichlorocide. Others, such as catalase, were sensitive to Trichlorocide but resistant to iodophor. It would seem then that in these two cases iodophor and Trichlorocide are exerting their effects by two different mechanisms. In the effect of QAC on beta-galactosidase of different

sources, the QAC inhibited the activity of the enzyme from liver, stimulated the activity of the enzyme from Lactobacillus helveticus, and had no effect with the concentration used on the activity of the enzyme from Streptococcus lactis.

On a molar  $I_{50}$  value per milligram enzyme protein per ml it may be noted that several of the enzymes were sensitive to very small concentrations of the germicide, e.g., alcohol dehydrogenase, while others were only sensitive comparatively to large amounts of the germicide, e.g., lactic dehydrogenase. This disparity may be due to differences in purity of the enzyme as well as accessibility of the active sites of the enzyme to the germicides.

In all instances where there occurred inhibition of enzyme activity of at least 50 percent, the concentrations of germicide causing such inhibition was well below concentrations normally used for sanitation and disinfection purposes.

#### Mechanism of Iodophor Inactivation of Certain Metabolic Enzymes

Since many of the enzymes in Table 1 were quite sensitive to iodophor, it was thought that a more detailed study of certain enzymes might help in determining the mechanism of the germicidal activity of iodophors. Three enzymes which were either known or suspected to depend upon their sulfhydryl groups and one shown to be independent

Table 1. Comparative effects of germicides on metabolic enzymes.

Enzyme	Germicide <sup>a</sup>	I <sub>50</sub> Value <sup>b</sup> (ppm)	Molar I <sub>50</sub> Value <sup>c</sup> (X10 <sup>-5</sup> )	Specific I <sub>50</sub> Value <sup>d</sup> (X10 <sup>-3</sup> )
Alcohol dehydrogenase (yeast)	IOD	1.1	0.86	0.11
	TCC	0.5	1.40	0.19
	QAC	8.2	1.86	0.26
β-galactosidase (liver)	IOD	1.5	1.17	0.24
	TCC	7.0	19.70	4.10
	QAC	8.4	1.89	0.39
β-galactosidase ( <i>Lactobacillus helveticus</i> )	IOD	2.0	1.56	1.13
	TCC	2.0	5.63	4.08
	QAC	--- <sup>e</sup>	----	----
β-galactosidase ( <i>Streptococcus lactis</i> )	IOD	2.1	1.64	0.14
	TCC	2.0	5.63	0.49
	QAC	20.0 <sup>f</sup>	----	----
Lactic dehydrogenase (Rabbit muscle)	IOD	12.0	9.38	936.00
	TCC	6.0	16.90	1690.00
	QAC	512.0 <sup>g</sup>	----	----
Catalase (liver)	IOD	128.0 <sup>f</sup>	----	----
	TCC	14.0	39.40	475.00
	QAC	640.0 <sup>f</sup>	----	----
Cytochrome-c-reductase (pig heart)	IOD	24.0	18.70	2.80
	TCC	13.0	36.60	5.50
	QAC	10.0	22.50	3.36
Hexokinase (yeast)	IOD	--- <sup>h</sup>	----	----
	TCC	115.0 <sup>f</sup>	----	----
	QAC	115.0	25.90	25.90
Glucose-6-phosphate dehydrogenase (yeast)	IOD	1.5	1.17	9.40
	TCC	256.0 <sup>f</sup>	----	----
	QAC	512.0 <sup>f</sup>	----	----
Glucose-6-phosphate dehydrogenase ( <i>Pediococcus cerevisiae</i> )	IOD	4.0	3.12	0.07
	TCC	256.0 <sup>f</sup>	----	----
	QAC	512.0 <sup>f</sup>	----	----
Malic dehydrogenase (pig heart)	IOD	8.0	6.25	51.10
	TCC	6.0	16.90	138.00
	QAC	512.0 <sup>f</sup>	----	----
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	IOD	1.0	0.78	6.32
	TCC	0.1	0.28	2.26
	QAC	512.0 <sup>f</sup>	----	----

a IOD = Iodophor; TCC = Trichlorocide; QAC = quaternary ammonium compound.

b Concentration of germicide required for 50% inhibition of the enzyme.

c Calculated using 127.9, 35.5 and 444.0 as molecular weights for iodine, chlorine and QAC respectively.

d Molar I<sub>50</sub> values per mg enzyme protein per ml.

e QAC stimulated activity in concentrations up to 1800 ppm.

f Concentrations of germicide up to this amount had no effect.

g This concentration inhibited by about 30%.

h Unable to assay because of the immediate decolorization by iodophor.

of these groups for activity were selected for study. The results in Table 2 show that alcohol dehydrogenase inhibition (31%) is completely reversed by glutathione. The importance of using an appropriate reducing agent became apparent when it was found that cysteine in lower concentrations failed to produce the same effect. High concentrations inactivated the enzyme further, presumably by disruption of disulfide bonds necessary for the conformational integrity of the enzyme.

Table 2. Effect of glutathione and cysteine additions on the activity of alcohol dehydrogenase (ADH) inhibited by iodophor (1.2 ppm iodine).

Experimental conditions	Initial velocity $\Delta$ O.D. per min	Percent inhibition
Control (ADH in buffer)	0.052	0
Control (ADH in buffer); later 0.1 ml 0.25M glutathione	0.052	0
ADH with iodophor; later 0.1 ml 0.25M glutathione	0.052	0
ADH with iodophor; later 0.1 ml 0.05M cysteine	0.036	31
ADH with iodophor; later 0.1 ml 0.125M cysteine	0.036	31
ADH with iodophor	0.036	31
ADH with iodophor; later 0.1 ml 0.25M cysteine	0.018	66

Similarly, partially inactivated lactic dehydrogenase (65%) was reactivated to only 25 percent inhibition by cysteine (Table 3). It is suspected in this case that some of the essential sulfhydryl groups were oxidized further than the disulfide stage, thus could not be reduced.

With glucose-6-phosphate dehydrogenase, a 23 percent inhibition was completely reversed by cysteine (Table 4).

To provide further evidence that iodophor readily oxidizes particular enzyme sulfhydryl groups, parachloromercuribenzoate (PCMB) was used. This compound reacts specifically with sulfhydryl groups; therefore, by first exposing the enzyme to iodophor and then to PCMB an indication of the extent of oxidation may be obtained by measuring the decrease in reaction between PCMB and the iodophor-treated enzyme. In such experiments, it was found that treatment of the enzyme with iodophor for one minute greatly reduced the amount of PCMB taken up by the enzyme. The reaction of the enzyme with iodophor from two to four minutes completely prevented subsequent uptake of PCMB (Figure 1). Since the non-treated enzyme reacted as expected towards the PCMB, it was concluded then that a majority of the sulfhydryl groups had been oxidized by the iodophor resulting in the minimal reaction of the PCMB with the iodophor-treated enzyme.

Table 3. Effect of cysteine addition on the activity of lactic dehydrogenase (LDH) inhibited by iodophor (0.5 ppm iodine).

Experimental conditions	Initial velocity $\Delta$ O.D. per min	Percent inhibition
Control (LDH in buffer)	0.060	0
Control (LDH in buffer); later 0.1 ml 0.5M cysteine	0.060	0
LDH with iodophor; later 0.1 ml 0.5M cysteine	0.045	25
LDH with iodophor; later 0.1 ml 1M cysteine	0.025	60
LDH with iodophor	0.021	65
LDH with iodophor; later 0.1 ml 0.1M cysteine	0.007	88
LDH with iodophor; later 0.1 ml 0.01M cysteine	0.002	97

Table 4. Effect of cysteine addition on the activity of glucose-6-phosphate dehydrogenase (G6PD) inhibited by iodophor (1.0 ppm iodine).

Experimental conditions	Initial velocity $\Delta$ O.D. per min	Percent inhibition
Control (G6PD in buffer)	0.110	0
Control (G6PD in buffer); later 0.05 ml 0.1M cysteine	0.110	0
G6PD with iodophor; later 0.05 ml 0.1M cysteine	0.110	0
G6PD with iodophor	0.085	23

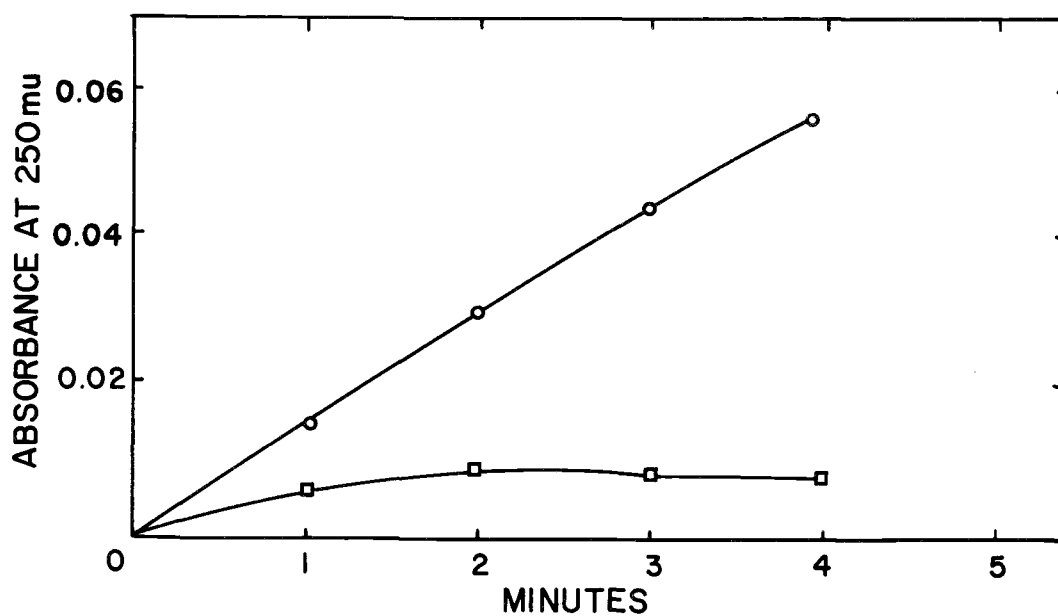


Figure 1. The effect of the treatment of lactic dehydrogenase with iodophor (42.0 ppm iodine) on the reaction of PCMB with the enzyme. Symbols:  $\circ$ — $\circ$  before treatment with iodophor;  $\square$ — $\square$  after treatment with iodophor.

Previous evidence shown in Table 1 has indicated that exposure of catalase for a short time to iodophor concentrations as high as 128 ppm had no effect on its activity. It appears then that the sulfhydryl groups in catalase are not essential for its activity. However, inactivation of catalase by iodophor may also occur through iodination reactions. On the basis of this possibility, experiments were conducted to determine the effects of iodination. As deprotonation of tyrosyl and probably histidyl groups is the first step toward iodination, an experiment was designed to control the extent of deprotonation and thus iodination by varying the pH of the reaction mixture containing the iodophor and enzyme. As can be noted in Figure 2 there was no additional inactivation of catalase after prolonged incubation with iodophor at neutral pH in comparison to the control. Here little iodination has occurred because the deprotonation was largely suppressed by the neutral pH. However when deprotonation and iodination were encouraged by the alkaline conditions, inactivation of catalase up to 60 percent was promoted.

Since iodide ions suppress the formation of hydrated iodine cations, the active ion forms in iodination reactions, as well as suppressing iodination, addition of these ions would be expected to inhibit enzyme inactivation. Evidence for the iodide inhibition is shown in Figure 3. It can be noted that there was a concomitant increase in catalase activity when increasing concentrations of

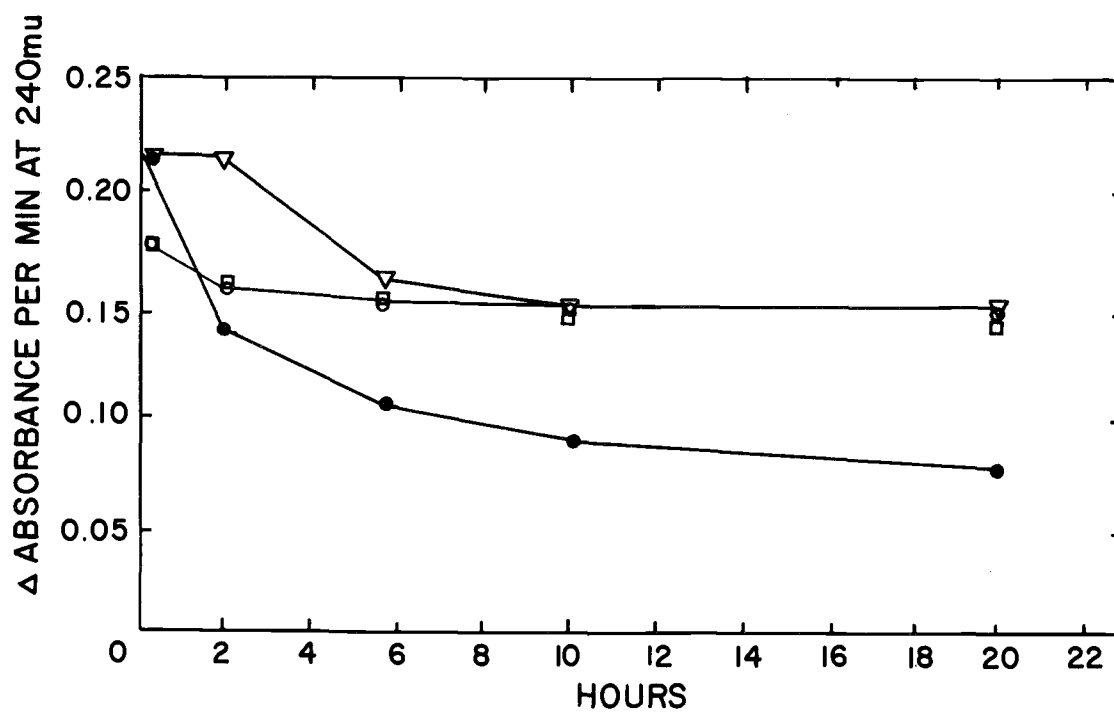


Figure 2. Effect of pH on the inactivation of catalase by iodophor (60 ppm iodine). Symbols: ○—○ pH 7.0, no iodophor; □—□ pH 7.0, with iodophor; ▽—▽ pH 8.5, no iodophor; ●—● pH 8.5, with iodophor.

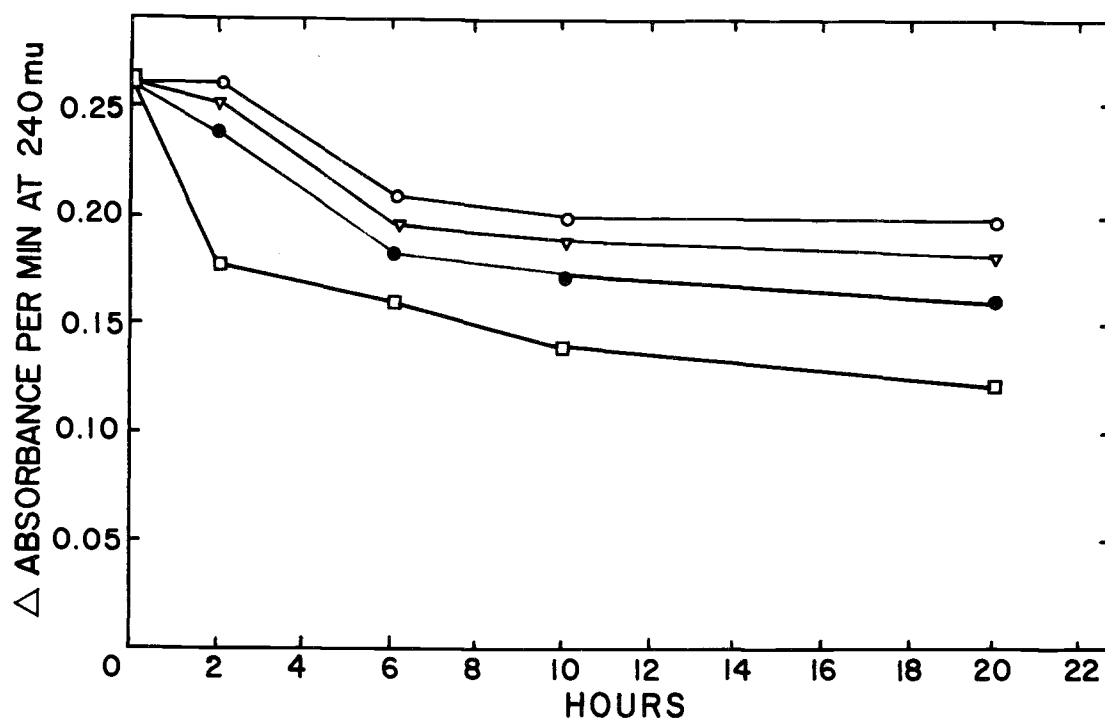


Figure 3. Effect of iodide ions on the inactivation of catalase by iodophor (60 ppm iodine) at pH 8.5. Symbols: ○—○ no iodophor or iodide; □—□ with iodophor only; ▽—▽ with iodophor plus iodide, 0.1M; ●—● with iodophor plus iodide, 0.01M.

iodide ions were introduced. It was therefore concluded that the mechanism of iodophor inactivation of catalase was through iodination of tyrosyl and histidyl moieties which altered the structural integrity necessary for enzyme activity.

The Isolation of Partially Purified Glucose-6-phosphate  
Dehydrogenase from Iodophor-treated and Nontreated  
*S. Cerevisiae* Cells and Attempts to Reverse  
any Inhibition with Cysteine

Since it was shown that yeast glucose-6-phosphate dehydrogenase is partially inactivated by iodophor and that this partially inactivated enzyme can be completely reactivated by cysteine, the question may be asked if this enzyme inactivation by iodophor can occur in vivo. If such an inactivation is occurring, it may account for the decrease in glucose oxidation and subsequent contribution to the lethal action of iodophor. In an effort to gain insight into this possibility, yeast cells were treated with iodophor and then subjected to isolation and purification of the glucose-6-phosphate dehydrogenase. If the enzyme isolated from iodophor-treated yeast cells exhibits a reduced activity in comparison to the enzyme isolated from noniodophor-treated cells and can be reactivated by cysteine, then such evidence would support the theory that iodine permeates the cells causing oxidation of essential sulfhydryl groups of glucose-6-phosphate dehydrogenase and most likely that of other enzymes.

Unfortunately the experiments were only partially successful. As can be noted in Table 5, the specific activity of the partially purified enzyme isolated from iodophor-treated cells was much higher than from the nontreated control cells. For some reason yet unknown, the iodophor had some effect on the protein fraction containing the enzyme to be more selectively adsorbed to the calcium phosphate gel, whereas with the nontreated yeast cells the adsorption was slightly less than expected. It is felt that in order to make a meaningful comparison, the partially purified enzymes from the treated and nontreated cells should possess similar specific activities and ideally the enzyme from the treated cells with slightly less such that similar concentrations of reducing agents may be used. However repeated attempts to prepare such partially purified enzymes failed as the enzyme from the treated cells had in all cases much higher specific activities than from the nontreated cells.

Nevertheless, attempts were made to determine the effects of cysteine on the enzymes isolated from the iodophor-treated and nontreated cells. The results as expressed in Table 6 indicate that cysteine in test number four was able to stimulate the activity of the enzyme about 27 percent over the activity of the enzyme not exposed to cysteine (test number one). In a control experiment cysteine did not stimulate the activity of the enzyme isolated from nontreated cells (Table 7, test number two). However it was found that upon

Table 5. Partial purification of glucose-6-phosphate dehydrogenase from nontreated and iodophor-treated S. cerevisiae cells.

Type of <u>S. cerevisiae</u> cells	Yeast autolyzate		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction		Ca <sub>3</sub> PO <sub>4</sub> eluate	
	Protein mg/ml	Specific activity	Protein mg/ml	Specific activity	Protein mg/ml	Specific activity
Nontreated	5.20	0.20	2.30	0.52	1.1	1.1
Iodophor-treated	1.00	1.00	2.25	0.55	0.18	17.9

Table 6. Effect of cysteine addition on the activity of partially purified glucose-6-phosphate dehydrogenase (G6PD) from iodophor-treated S. cerevisiae cells.

Test no.	Experimental conditions	Initial velocity O.D. /min	Percent activation
1	Control (G6PD* in buffer)	0.0110	0
2	G6PD plus 0.04 ml 0.5M cysteine, incubated 2 min then assayed	0.0115	4
3	G6PD plus 0.04 ml 1.0M cysteine, incubated 2 min then assayed	0.0110	0
4	G6PD plus 0.04 ml 0.5M cysteine, incubated 4 min then assayed	0.0140	27
5	G6PD plus 0.04 ml 1.0M cysteine, incubated 4 min then assayed	0.0115	4

\*Assay procedures and conditions were those previously described with the above modifications. Concentration of G6PD was 0.18 mg/ml.

Table 7. Effect of cysteine addition on the activity of partially purified glucose-6-phosphate (G6PD) from noniodophor-treated S. cerevisiae cells.

Test no.	Experimental conditions	Initial velocity	Percent inhibition	Percent activation
1	Control (G6PD* in buffer)	0.035	0	0
2	G6PD plus 0.05 ml 0.2M cysteine, incubated 2 min then assayed	0.030	14	--
3	G6PD plus 1.5 ppm iodophor, incubated 2 min then assayed	0.025	29	--
4	G6PD plus 1.5 ppm iodophor, incubated 2 min, added 0.05 ml 0.2 M cysteine, incubated 2 min then assayed	0.025	29	--

\*Assay procedures and conditions were those previously described with the above modifications. Concentration of G6PD was 0.30 mg/ml.

upon partially inactivating this enzyme by iodophor and attempting to reactivate by various concentrations of cysteine, no reversal occurred (Table 7, tests numbers 3 and 4). Therefore the efficacy of stimulating the enzyme from nontreated cells by cysteine is somewhat open to question since the partially inactivated enzyme would be expected to be reactivated, at least partially, by cysteine. Previously (Table 4) it was shown that the commercial glucose-6-phosphate dehydrogenase from yeast cells which had not been partially inactivated by iodophor did not undergo stimulation of activity when exposed to cysteine. The results of these experiments suggested that iodophor was able to partially oxidize the essential sulfhydryl groups of glucose-6-phosphate dehydrogenase in the yeast cells leading to reduction of enzyme activity and subsequent decrease in metabolism of glucose.

#### Effect of Iodophor and Trichlorocide on Glucose Oxidation and Viability of *S. cerevisiae*

Since both iodophor and Trichlorocide were able to inactivate enzymes involved in the metabolism of glucose (Table 1), a study was conducted in which the effects of these germicides on glucose oxidation and suppression of growth of yeast cells could be compared. Also attempts were made to reverse inhibition by use of the reducing agents cysteine and dithiothreitol.

With an exposure time of 30 sec and a concentration of one and two ppm, iodophor inhibited both glucose oxidation and viable cell count to a greater extent than Trichlorocide. At one ppm of both germicides there was an accelerated rate of oxygen uptake, especially after 30 min, indicating that the yeast cells were beginning to recover from the inhibitory effects possibly through resynthesis of enzymes. However at two ppm of both germicides, oxygen uptake was linear, illustrating the apparent absence of any recovery (Figure 4). With an exposure time of five min and a concentration of one ppm, Trichlorocide inhibited glucose oxidation and viable cell count to a greater extent than iodophor (Figure 5). There was a close correlation between percent inhibition of glucose oxidation and percent inhibition of viable cell count (Table 8) with both germicides suggesting that the inhibition of glucose oxidation resulted in the inhibition of growth.

There was an average reversal effect of 18 percent in glucose oxidation when the yeast cells were exposed to cysteine after iodophor treatment (Table 9). The reversal response was immediate and linear suggesting that the reversal was due, in part at least, to a partial reactivation of inhibited glucose metabolizing enzymes (Figure 6). It is unlikely that this reversal effect was due to the metabolism of cysteine since appropriate controls were included during the respirometer experiments. The addition of cysteine to the

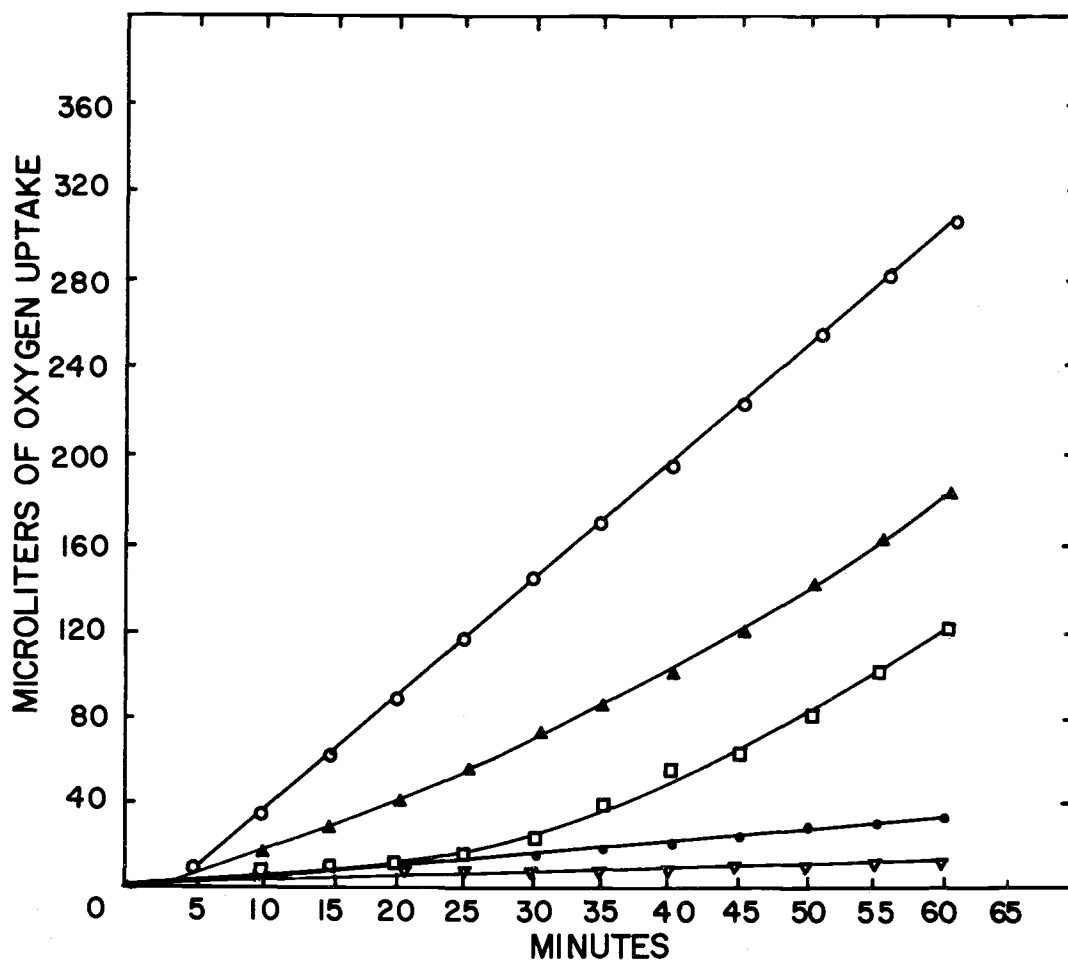


Figure 4. Effect of iodophor and Trichlorocide on inhibition of glucose oxidation by *S. cerevisiae* after exposure time of 30 seconds. Symbols: ○—○ no germicide; □—□ after exposure to 1 ppm iodophor; ▽—▽ after exposure to 2 ppm iodophor; ▲—▲ after exposure to 1 ppm Trichlorocide; ●—● after exposure to 2 ppm Trichlorocide.

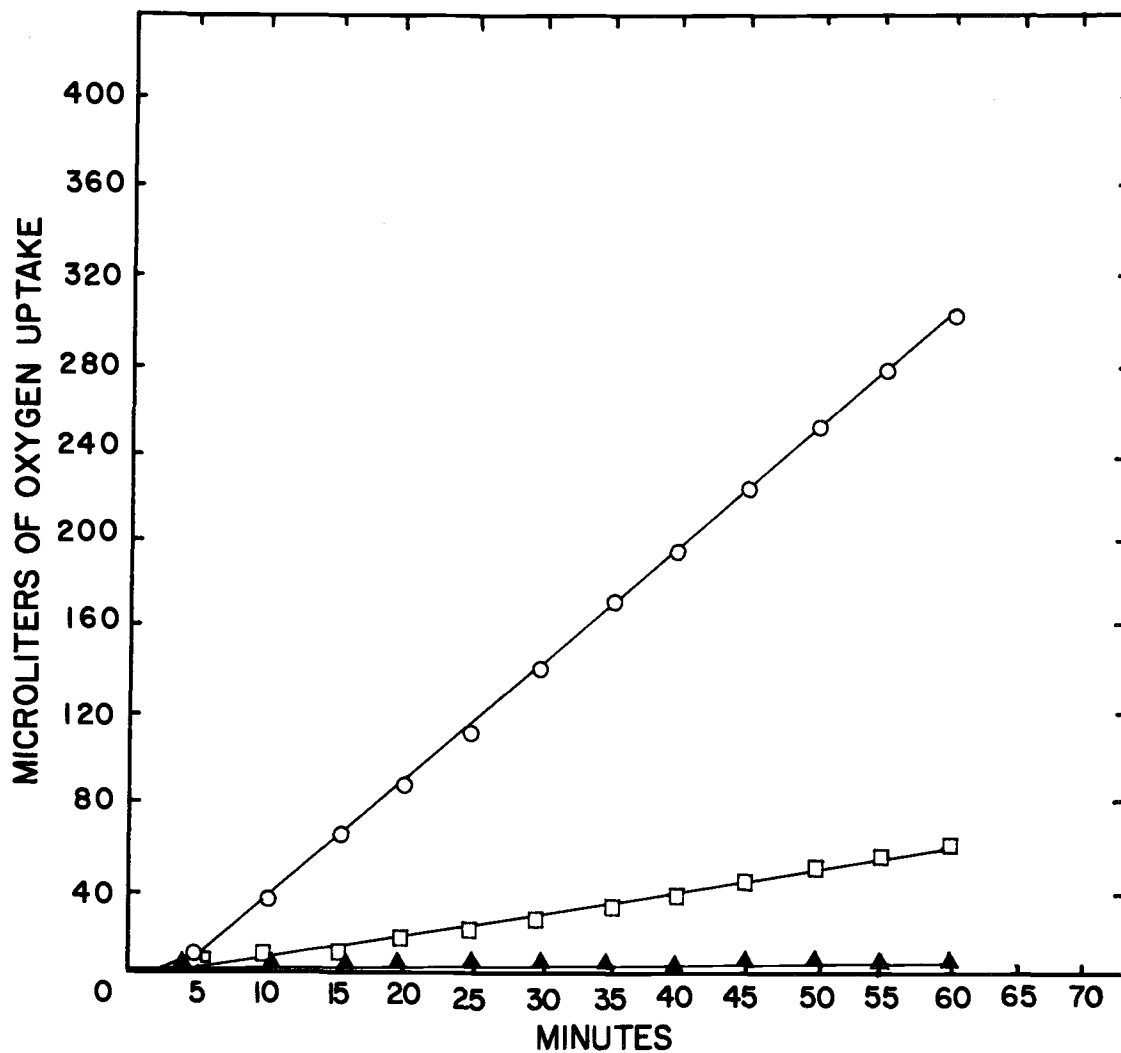


Figure 5. Effect of iodophor and Trichlorocide on inhibition of glucose oxidation by *S. cerevisiae* after exposure time of 5 min.  
Symbols: ○—○ no germicide; □—□ after exposure to 1 ppm iodophor; ▲—▲ after exposure to 1 ppm Trichlorocide.

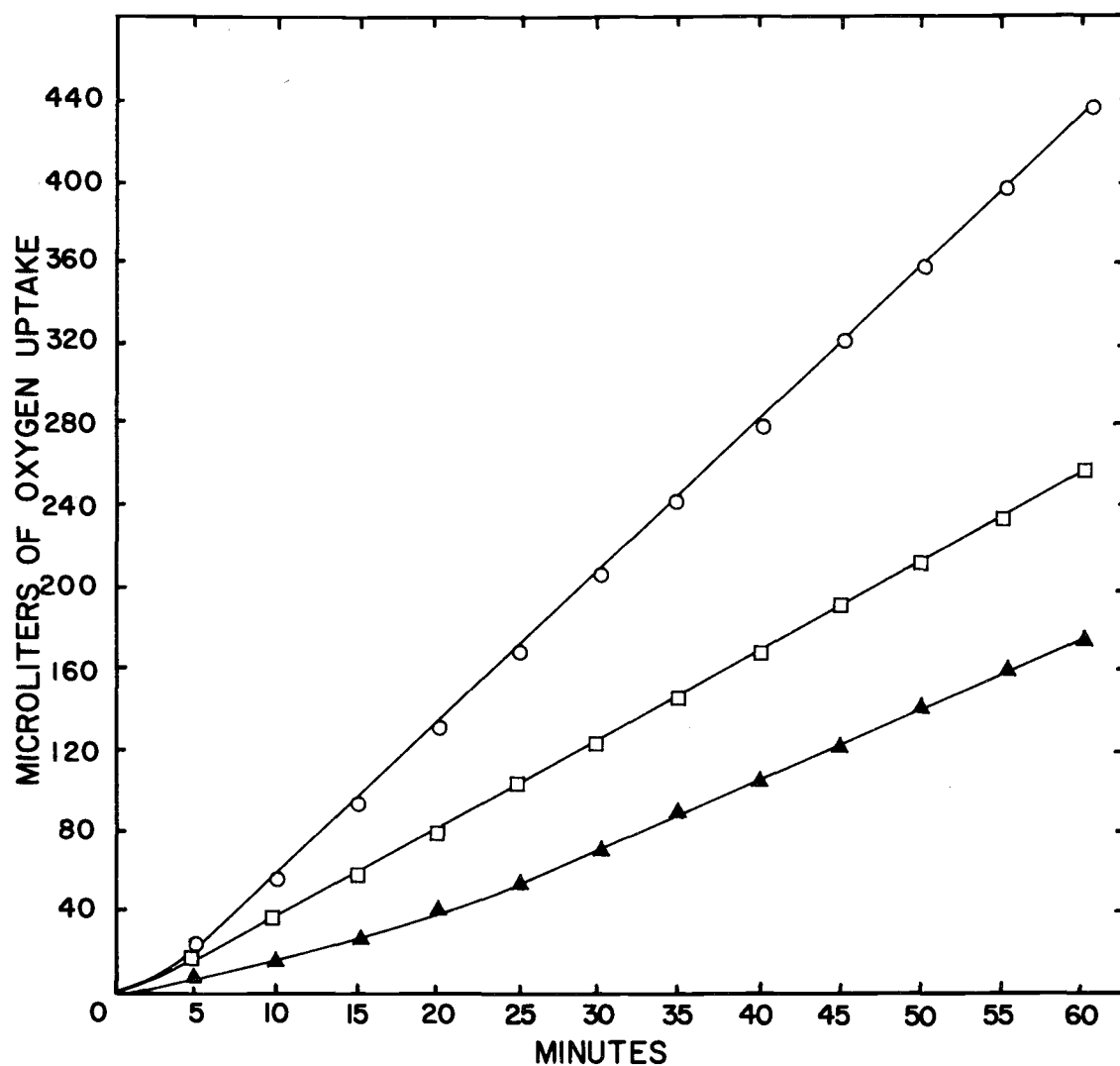


Figure 6. Partial reactivation of glucose oxidation in *S. cerevisiae* by cysteine. Symbols: o—o control, cysteine-treated and washed cells; ▲—▲ 1 ppm iodophor-treated cells; □—□ 1 ppm iodophor, then cysteine-treated cells.

Table 8. Percent inhibition by iodophor and Trichlorocide of glucose oxidation and viable cell count.

Measurement	Germicide conc.	Iodophor		Trichlorocide	
		30 sec	5 min	30 sec	5 min
Glucose oxidation	1 ppm	61	80	49	98
	2 ppm	96	--	89	--
Viable cell count	1 ppm	63	75	45	99
	2 ppm	99	--	84	--

Table 9. The reversal effects of cysteine and dithiothreitol on iodophor inhibition of glucose oxidation and viable cell count in S. cerevisiae.

Measurement	Percent inhibition		
	1 ppm iodophor only	1 ppm iodophor, then cysteine	1 ppm iodophor, then dithiothreitol
Glucose oxidation	60	42	40
Viable cell count	47	0	0

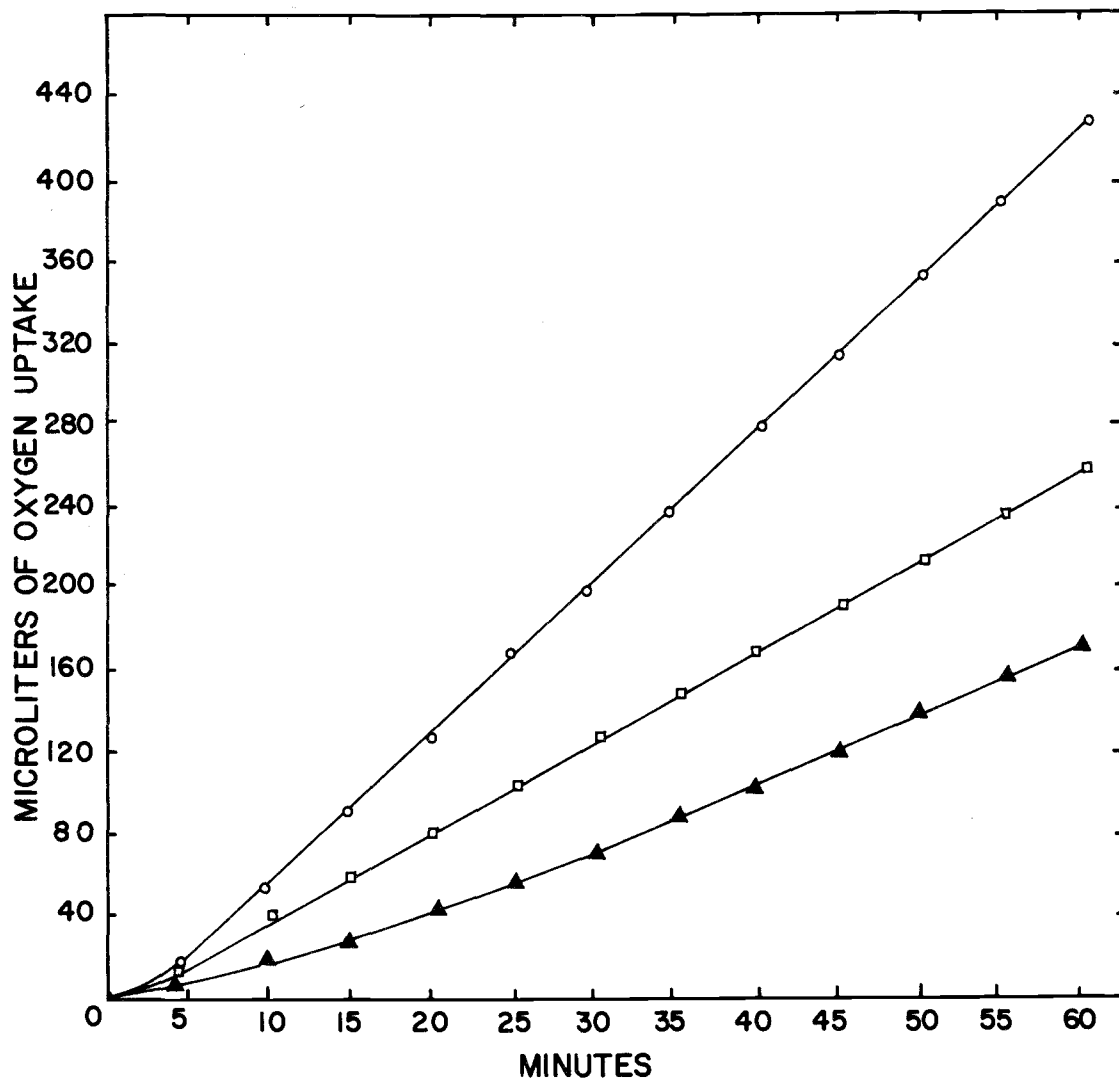


Figure 7. Partial reactivation of glucose oxidation in *S. cerevisiae* by dithiothreitol. Symbols: o—o control, dithiothreitol-treated cells; ▲—▲ 1 ppm iodophor-treated cells; □—□ 1 ppm iodophor, then dithiothreitol treatment of cells.

iodophor-treated cells after they had begun to oxidize glucose was found to have no reversing effect. Similar results were obtained with dithiothreitol (Figure 7; Table 9). Both cysteine and dithiothreitol were able to completely reverse the effects of iodophor when growth was inhibited by 47 percent. Although similar reversing effects did occur even after washing the cells previously exposed to the reducing agents, it was not known whether or not this reversal was due to the metabolism of the reducing agents.

Effect of Iodophor on Glucose Oxidation and  
Viability of *P. fragi* and *P. aeruginosa*

When cells of *P. fragi* were exposed to 0.75 ppm iodophor for 30 sec, there was a 44 percent inhibition of glucose oxidation. However dithiothreitol was able to reverse this inhibition 30 percent to 14 percent inhibition. Cell viability was inhibited 97 percent, but could be reversed by the dithiothreitol to 65 percent inhibition, a reversal of 32 percent (Table 10). Dithiothreitol was able to completely reverse the inhibited glucose oxidation during the first 30 min, but the reversal became partial after there was accelerated oxidation by the control cells (Figure 8).

When cells of *P. aeruginosa* were exposed to 0.75 ppm iodophor for 30 sec, there was a 54 percent inhibition of glucose oxidation. However dithiothreitol was able to reverse this inhibition to

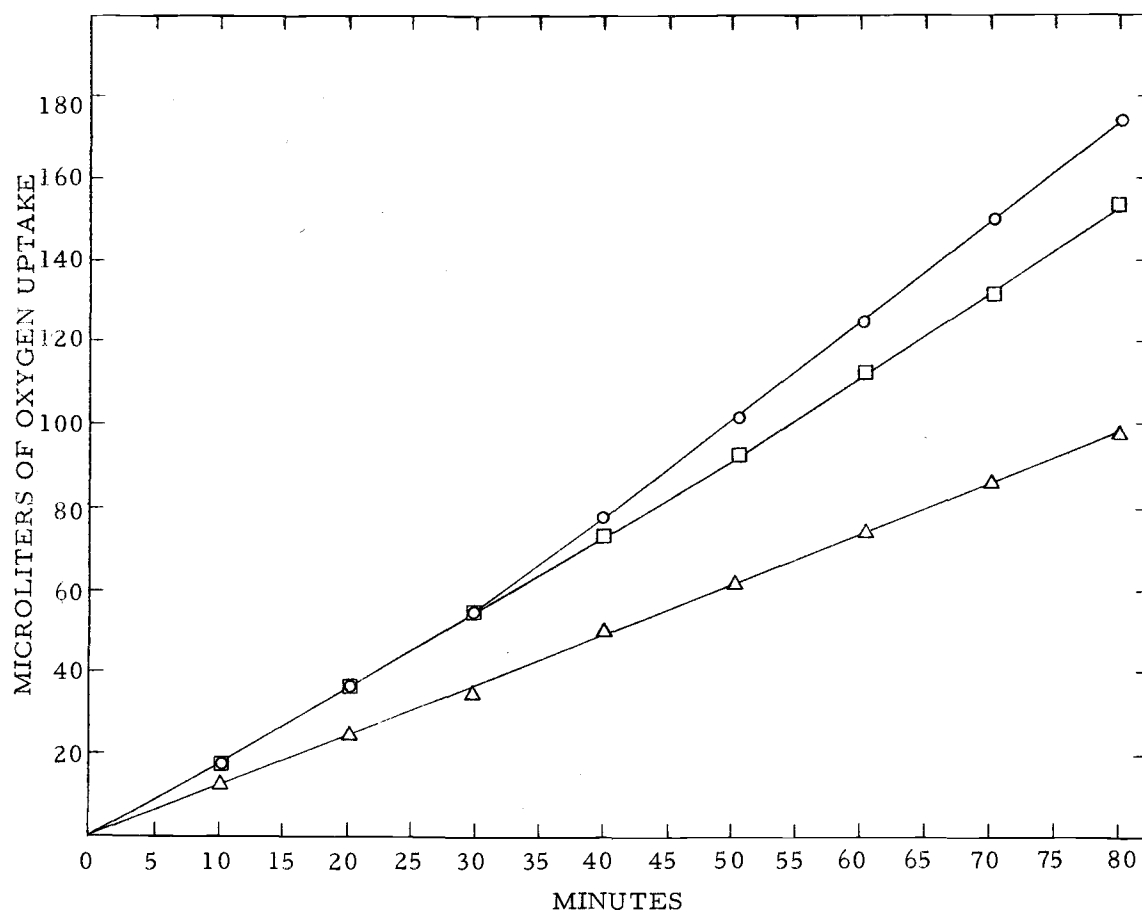


Figure 8. Reactivation of glucose oxidation in *P. fragi* by dithiothreitol  
Symbols: o—o control; Δ—Δ 0.75 ppm iodophor-treated cells; □—□ 0.75 ppm iodophor, then dithiothreitol-treated cells.

25 percent, a 29 percent reversal effect. Cell viability was inhibited 68 percent, but was reversed by the dithiothreitol to 38 percent inhibition, a reversal of 30 percent (Table 10). As with P. fragi there was a complete reversal of glucose oxidation by dithiothreitol during the first 30 min of measurement, but again only a partial reversal was evident during the accelerated glucose oxidation of the control cells (Figure 9).

Table 10. The reversal effect of dithiothreitol on iodophor inhibition of glucose oxidation and viable cell count of P. fragi and P. aeruginosa.

Measurement	Percent inhibition			
	0.75 ppm iodophor		0.75 ppm iodophor then dithiothreitol	
	<u>P. fragi</u>	<u>P. aeruginosa</u>	<u>P. fragi</u>	<u>P. aeruginosa</u>
Glucose oxidation	44	54	14	25
Viable cell count	97	68	65	38

Attempts to Develop and Isolate an Iodophor  
Resistant Strain of P. aeruginosa

In view of the ability of P. aeruginosa to develop resistance to high concentrations of QAC, an effort was made to demonstrate a similar resistance by the organism to iodophor by selection of survivors among a large population of cells exposed. No increase in

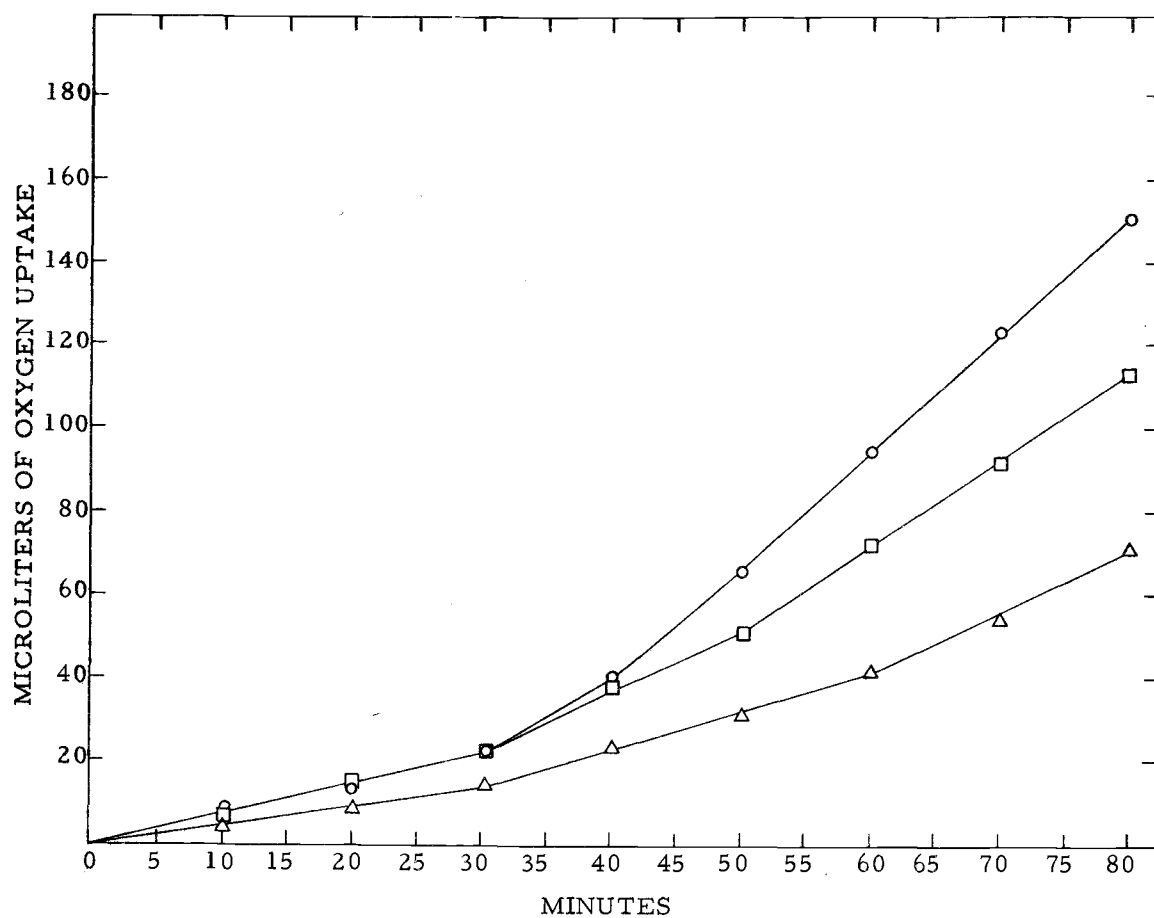


Figure 9. Reactivation of glucose oxidation in *P. aeruginosa* by dithiothreitol. Symbols: ○—○ control; △—△ 0.75 ppm iodophor-treated cells; □—□ 0.75 ppm iodophor, then dithiothreitol-treated cells.

resistance could be demonstrated when survivors were retested by repeated treatment.

## DISCUSSION

The primary emphasis of this research to determine the mechanism of germicidal action of iodine has been to study the inhibitory or inactivating effects of iodophor on certain metabolic enzymes. For comparative purposes, the effects of Trichlorocide and a quaternary ammonium compound on the activities of the various enzymes were also examined. If the treatment with iodophor was able to inactivate one or more essential enzymes of the microbial cell, then a break in the life-processes would occur such that the cell would be unable to reproduce and so, by definition, be presumed dead (Sykes, 1965).

The results of the present study provide further evidence on the sensitive nature of enzymes to inhibition by germicides. In general lower quantities of Trichlorocide inhibited by 50 percent more enzymes than iodophor. However iodophor was generally more effective on a molar basis. This would indicate that on a weight basis Trichlorocide is a more effective inhibitor. On a molar concentration basis, however, it appears that fewer iodine molecules are required to inhibit the identical concentration of enzyme than chlorine. A possible explanation for these results is the requirement of additional chlorine molecules as the sulfhydryl group oxidation proceeds. In this regard, it is believed that more chlorine molecules are reduced as the sulfhydryl groups are oxidized through the  $\text{SO}^-$ ,  $\text{SO}_2^-$ , and  $\text{SO}_3^-$  stages:

Under normal conditions during usual disinfecting and sanitizing operations, higher concentrations of iodine-containing germicides

are employed than were used in this study. Practical experience has dictated this condition, and it is well known that several hundred ppm concentrations are necessary to insure safety from a contamination standpoint. However, the use of such high concentrations in a study designed to define the present effects of iodine on enzyme systems would have yielded meaningless results. Only by using low amounts of germicide was it possible to measure the effect of iodine on specific enzymes. Also if germicidal activity were to occur in stages due to different effects caused by the disinfectant as the concentration increased, challenging the enzyme systems with high amounts of inhibitor would have been improper. Moreover, partly due to permeability factors, it is likely that enzyme inactivation within the cell by germicides occurs at low concentrations. With the present results, in all tests where the enzyme activity was inhibited at least 50 percent the germicide concentration was far below that normally used for disinfecting and sanitation purposes.

The enzymes which were most susceptible to small concentrations of iodophor or Trichlorocide were those catalyzing key metabolic reactions and included beta-galactosidase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, glucose-6-phosphate dehydrogenase, cytochrome c reductase, and malic dehydrogenase. These are indispensable, energy-linked

enzymes involved in lactose hydrolysis, glycolysis, the pentose cycle, oxidative phosphorylation, and the Krebs cycle.

Because of the essential link between cell functions and enzyme activities, several other workers also have investigated the survival of bacteria as related to the inhibition of one or more specific enzymes treated with disinfectants. For instance, Sykes (1939) after investigating the effects of alcohols and phenols on the dehydrogenases of E. coli, suggested that the amount of inhibition of succinic dehydrogenase activity might be used as an index of degree of cell death. Furthermore, Knox et al. (1949) showed that the death of E. coli in the presence of cationic detergents paralleled the loss of certain metabolic functions as a consequence of the inhibition of a number of enzymes, particularly, lactic acid oxidase.

From the experimental results of the present study in which a majority of the enzymes tested were found to be very sensitive to low concentrations of iodophor or Trichlorocide, the inference may be made that the inactivation of one or more of these critical enzymes by the germicide would be sufficient to inhibit growth or, more likely, to kill the cell. Since the concentrations of germicides normally used for disinfection are several times greater than the concentrations that were found to cause enzyme inhibition, it is quite likely that other factors such as protein denaturation and permeability changes occur along with enzyme inactivation effects to rapidly kill

the cells. In addition, it is clear that there are important differences in resistance of enzymes to the inhibitory effects of iodophor and Trichlorocide. For example, catalase was sensitive to inhibition by Trichlorocide but resistant to short iodophor exposure. By comparison, glucose-6-phosphate dehydrogenase was quite sensitive to iodophor but resistant to Trichlorocide. These results indicate that there are differences in the mechanism of enzyme inactivation between iodophor and Trichlorocide. Since Trichlorocide is chemically more reactive than iodophor, this may explain its effect on catalase; however, the resistance of glucose-6-phosphate dehydrogenase to inhibition by Trichlorocide remains unexplained.

Hays et al. (1967) have reported that yeast cells exhibited a greater resistance to hypochlorite than iodophor. They believe that possibly the larger cell mass of the yeast created a greater chlorine demand that had to be satisfied before chlorine could destroy the yeast cells. Another possibility which occurred as a result of the present study is that the chlorine resistance of a key enzyme may contribute significantly to the resistance of the yeast cells. In this regard, yeast glucose-6-phosphate dehydrogenase was found to be resistant to Trichlorocide but sensitive to iodophor. To help determine if this chlorine-resistant enzyme was unique in yeast cells and thus a factor in chlorine resistance, the enzyme was isolated from P. cerevisiae, an organism more sensitive to destruction by

Trichlorocide than iodophor. However the P. cerevisiae enzyme was also found to be resistant to chlorine and sensitive to iodophor indicating the unlikelihood that the resistance of glucose-6-phosphate dehydrogenase to chlorine is the only contributing factor to the yeast cells in their resistance to chlorine.

Since the majority of enzymes tested were resistant to inactivation by the quaternary ammonium compound, this may partially explain the greater resistance of microorganisms to QAC in comparison to Trichlorocide and iodophor. On the other hand, Albin (1966), in her studies on bacteria resistant to QAC, indicated that QAC inhibited a number of enzymes including those involved in carbohydrate fermentation. It is conceivable that the inhibition of even one essential enzyme in a metabolic scheme by QAC would be sufficient to prevent carbohydrate fermentation; however, it is felt that more definitive studies both in vivo and in vitro would be in order since the present results show that a majority of the glycolytic enzymes tested were resistant to inhibition by QAC.

During the present study it was found that there was no apparent distinction between effects of QAC on different classes of enzymes. For example alcohol dehydrogenase was inhibited by QAC while glyceraldehyde-3-phosphate dehydrogenase was not, yet both are believed to depend upon their sulfhydryl groups for activity (Barron, 1951).

From the results section (Tables 2, 3, and 4), it can be seen that additional evidence is provided indicating that the mechanism of iodophor inactivation of certain enzymes involves oxidation of their essential sulfhydryl groups; both alcohol dehydrogenase and glucose-6-phosphate dehydrogenase, partially inactivated by iodophor, were completely reactivated by reducing agents while inhibited lactic dehydrogenase was partially reactivated. Apparently, with alcohol and glucose-6-phosphate dehydrogenases, the oxidative effect of iodophor is the only inhibitory mechanism involved because complete reactivation was possible; however, with lactic dehydrogenase, possibly iodination in addition to sulfhydryl oxidation leads to inactivation of the enzyme. Further evidence that iodophor does oxidize sulfhydryl groups was provided (Figure 1) when it was found that lactic dehydrogenase, previously exposed to iodophor, failed to react with PCMB, a sulfhydryl group specific reagent (Boyer, 1954). It is likely that the number of enzymes which can be inactivated by oxidation caused by small concentrations of iodophor are numerous, and this would further substantiate one aspect of the mechanistic effect of iodophors on enzymes.

Other enzymes which do not depend upon their sulfhydryl groups for their activity may still be susceptible to inactivation by iodophor by virtue of direct iodination reactions. In this regard, it was found that catalase, which was resistant to inactivation by iodophor

concentrations as high as 128 ppm at a neutral pH, was quite susceptible when treated in an alkaline solution. Since it has been indicated by Nicholls and Schonbaum (1963) that the tyrosyl and histidyl moieties probably contribute to the tertiary structure of the catalase protein, it is feasible that the iodination of these moieties result in a conformational change affecting the active site of the enzyme. Similarly, iodination reactions have been reported to cause the inactivation of fructose-1,6-diphosphate aldolase (Wassarman and Kaplan, 1968), fructose-1,6-diphosphatase (Rosen and Rosen, 1967), and ribonuclease A (Covelli and Wolff, 1966). The critical nature of tyrosyl and often histidyl residues in maintenance of catalytic activity has been well documented in these reports, and it was found in all cases that there was an immediate loss of catalytic activity upon iodination of these amino acid residues.

Since it is now known that the pH and iodide concentration of an iodophor solution greatly influence its germicidal activity when iodination reactions are involved, the results of Watkins et al. (1957) seem less surprising. They reported that iodophor was ineffective against Streptococcus cremoris bacteriophages in concentrations as high as 200 ppm during a 60 second exposure time. Since a pH of 7.0 was used in their studies, it is likely that iodination reactions were not sufficient to inactivate the bacteriophages. By increasing the alkalinity of the solution and allowing further deprotonation of

tyrosyl and histidyl groups with subsequent iodination, inactivation of bacteriophages would be expected to occur readily. In this respect, Hsu et al. (1966) reported that f2 bacteriophages of E. coli were quite resistant to iodine inactivation at pH 4.0; however as the pH became alkaline there was increased inactivation of the viruses which they attributed to increasing iodination of tyrosyl residues.

In experiments designed to determine if iodophor was actually oxidizing sulfhydryl groups of glucose-6-phosphate dehydrogenase in S. cerevisiae cells, difficulties were encountered in obtaining an enzyme preparation of similar specific activity to that obtained from control cells; the enzyme obtained from iodophor-treated cells exhibited several times more specific activity than the control preparations. The increased selective adsorption of the enzyme from iodophor-treated cells, apparently caused by some component in the iodophor, remains unexplained. Nevertheless there was a 27 percent stimulation of activity of the enzyme isolated from iodophor-treated cells by cysteine, suggesting that the iodophor caused partial oxidation of its sulfhydryl groups in vivo.

When S. cerevisiae cells were exposed to one and two ppm of iodophor or Trichlorocide for 30 seconds, iodophor was more inhibitory for both glucose oxidation and viability. However with a five minute exposure, Trichlorocide was more inhibitory. Since Trichlorocide is generally considered to be chemically more

reactive than iodophor, over an extended period, it undoubtedly was able to inflict a greater degree of inactivation of the glucose oxidizing system and indirectly those systems necessary for viability.

It may be noted from Figure 4 that after about 30 minutes there was an accelerated rate of glucose oxidation by the cells previously exposed to one ppm of either germicide but not with two ppm. It appeared that the additional one ppm was critical enough to prevent any recovery from the detrimental effects of the iodophor. This may be caused by the irreversible damage to one or more cellular enzymes involved in glucose catabolism.

When glucose oxidation by yeast cells was inhibited 60 percent by iodophor, cysteine was able to reverse this inhibition by 18 percent and dithiothreitol by 20 percent. Also yeast cell viability, which was inhibited 47 percent by iodophor, was completely restored by both cysteine and dithiothreitol. It was concluded from these results that the mechanism of action of iodophor on yeasts was due in part to the oxidation of essential sulfhydryl groups. These groups are most likely connected with glycolytic enzymes, other proteins essential for glucose oxidation and transport, and with those proteins whose structural integrity is necessary for cell viability.

When both P. fragi and P. aeruginosa cells were first exposed to 0.75 ppm iodophor and then to dithiothreitol, it was found that

during the initial 30 minutes the cells oxidized glucose, there were identical rates of oxidation for both control and test cells (Figures 8 and 9). This indicated that the dithiothreitol was able to completely reverse the inhibitory effects of iodophor. Therefore it was concluded that during the initial 30 minutes, the exclusive mechanism for inhibition of glucose oxidation was oxidation of essential sulfhydryl groups by iodophor. The accelerated glucose oxidation after this time may be due to the differential rates of growth of the variously treated cells.

It has been reported that iodine reacts with bacteria by an oxidation-reduction process with little iodination occurring (Hsu et al., 1966; Brandrick and Newton, 1967). Based on this, it was expected that the extent of restoration of iodophor-inhibited glucose oxidation and viability would depend upon several factors, including the effectiveness of the reducing agent and the extent of oxidation of the essential sulfhydryl groups. With yeast cells, the correlation of glucose oxidation inhibition with reduction in cell viability suggested that the iodophor and Trichlorocide acted specifically by inhibiting one or more enzymes of glucose oxidation--an inhibition which caused cell death. However, with the Pseudomonas there was no such correlation, indicating that inhibition of glucose oxidation in this case had little or no direct effect on cell viability.

It is realized from the overall results that the mechanism of

germicidal action of iodophors is only partly due to oxidation of essential sulfhydryl groups and iodination reactions. Since a majority of the tests were conducted with concentrations of iodophor well below that normally used for disinfectant and sanitation purposes, the mechanism of action described would be primarily applicable to the low concentrations stated. At higher concentrations of iodophor, other factors such as nonspecific protein denaturation, destruction of cell permeability, and leakage of cell contents likely enhance the effects of sulfhydryl oxidation and iodination to kill the cell. However an examination of the results with the organisms tested at low concentrations of iodophor indicates the extreme sensitivity of the organisms to destruction by iodophor concentrations as low as one and two ppm. Since it has been shown that at these concentrations the primary mechanism of action of iodophor is oxidation of essential sulfhydryl groups, this action by itself would be sufficiently germicidal.

It has been shown by Hsu et al. (1966) that inactivation of poliovirus and f2 bacteriophage by iodine is inhibited considerably by low concentrations of iodide ion. Also at low pH values where iodination was inhibited, they found little inactivation of the viruses. These results along with the present ones further emphasize the consideration which will often be necessary in the use of iodophors and other iodine-containing compounds against a particular class

of microorganisms. If the destruction of viruses is the primary goal, and if it is assumed that iodination of viruses leads to inactivation, then pH values above neutral for the disinfecting solution as well as low iodide concentrations would be desirable. However if the destruction of organisms such as bacteria and yeasts is the primary goal, a low pH is desirable to favor sulfhydryl group oxidation. Iodide ion concentration would not be as significant since oxidation of sulfhydryl groups is not materially retarded by its presence.

It would seem then that further studies on the effectiveness of iodine and iodine-containing compounds would be in order. For example, the Globaline tablet, containing tetraglycine hydroperiodide (Hsu et al., 1966), is used by the United States Armed Forces as a universally effective water disinfectant for emergency purposes. A study of the effectiveness of this tablet against microorganisms especially viruses at various pH and iodide concentrations may well prove significant.

It also becomes evident from the present and other studies, the important role the constituents of an iodophor especially its iodide concentration play in its germicidal action. In this regard, iodophors have an advantage in that iodine is brought into solution with a solubilizer other than the iodide ion resulting in a minimum amount of these ions. However because of the differences in formulation of various iodophors, it may well be fruitful to examine the

formulation of different types of iodophors in regard to the relationship between iodide concentration and their effectiveness against microorganisms especially viruses. The application of the spectrophotometric method of determining iodide concentration (Brandrick and Newton, 1967) may well be feasible here.

Though there are good indications that iodination reactions lead to the inactivation of f2 bacteriophage and poliovirus (Hsu et al., 1966) and in the present studies the inactivation of catalase, the evidence is still somewhat presumptive. To obtain conclusive evidence as well as gaining further insight into the mechanism of action of iodine, radiotracer work with  $I^{131}$  would be desirable. The method would involve exposing the viruses to iodine for varying periods in an aqueous solution, centrifuging, and measuring for radioiodine in the supernatant as well as the sediment. In addition, hydrolysis of the protein coat of the viruses or enzyme protein followed by radiochromatogram scanning of the chromatographed amino acids should permit identification of any iodinated tyrosine or histidine residues. If inactivation of the viruses or enzyme is occurring by iodination, then there should be a decrease in activity in proportion to the extent of iodination.

Although it has been known for many years that iodine is sporidical, very little work has been done on the mechanism of action. It has been suggested that the dormant state of a bacterial spore is

maintained by a protein coat stabilized by disulfide linkages, and that the reduction of these linkages by treatment with a reducing agent leads to activation and subsequent germination of the spore (Keynan, Issahary-Brand, and Evenchik, 1964). Since the germinating spore is more likely to be susceptible to the action of iodine than a dormant one, the sporicidal action of iodine may be enhanced by first treating the dormant spore with a reducing agent, allowing germination, and then exposing to iodine.

An interesting phenomenon which may be worthy of further investigation was the effect of iodophor in increasing greatly the adsorption of the glucose-6-phosphate dehydrogenase to calcium phosphate gel in the enzyme purification trials. Preliminary experiments were performed with a coagulase fraction to determine if the effect could also occur here; however, negative results were obtained. Nevertheless, experimentation with other enzyme purification procedures may prove fruitful.

## SUMMARY

In studies designed to gain further insight into the mechanism of action of germicides especially iodophor against various microorganisms, several selected metabolic enzyme systems were exposed to concentrations of germicide necessary to reduce their activity 50 percent. It was found that on a parts per million basis, the activity of most of the enzymes was inhibited by a smaller concentration of Trichlorocide than of iodophor or the quaternary ammonium compound. However on a molar basis, iodophor inhibited the activity of a majority of enzymes in the smallest concentration. In general the quaternary ammonium compound was the least effective inhibitor. In all experiments where there was at least 50 percent inhibition, the concentration of germicide was well below that normally used for disinfection and sanitation purposes. These results further emphasize the sensitive nature of certain enzymes to inactivation by germicides; consequently, it is suggested that inactivation of critical enzymes in microorganisms is likely one of the factors responsible for germicidal action.

Certain enzymes, such as glucose-6-phosphate dehydrogenase, were quite sensitive to inhibition by iodophor but resistant to Trichlorocide. Others such as catalase were inhibited by Trichlorocide but resistant to iodophor. These contrasting results intimate a

difference in the mechanism of action of iodophor and Trichlorocide.

In a study of the mechanism of action of iodophor on sulfhydryl group dependent enzymes, it was found that alcohol dehydrogenase inhibition (31%) and glucose-6-phosphate dehydrogenase inhibition (23%) were completely reversed by glutathione and cysteine respectively. Similarly partially inactivated lactic dehydrogenase (65%) was reactivated to 25 percent inhibition by cysteine. Furthermore by use of the sulfhydryl group specific reacting agent parachloro-mercuribenzoate, it was shown that with lactic dehydrogenase, there occurred a general abolishment of sulfhydryl groups after the enzyme was exposed to iodophor. It is concluded from these results that the primary mechanism of inactivation of these particular enzymes by iodophor is by oxidation of their essential sulfhydryl groups.

For the sulfhydryl-independent enzyme catalase, it was found that little or no inactivation occurred when exposed to 60 ppm iodophor at pH 7.0. However when the pH was increased to 8.5, inactivation up to 60 percent occurred. The inactivation thus likely occurred through iodination reactions of tyrosyl and possibly histidyl moieties in the enzyme rather than through oxidation of sulfhydryl groups.

Attempts to demonstrate the in vivo oxidation of yeast glucose-6-phosphate dehydrogenase sulfhydryl groups by iodophor were only partially successful. Although cysteine was able to stimulate the

activity of the enzyme isolated from iodophor-treated yeast cells 27 percent, because of wide differences in specific activity of the enzyme from nontreated and iodophor-treated cells, a valid comparison was not possible.

When S. cerevisiae cells were exposed to one or two ppm iodophor or Trichlorocide for 30 seconds, there was a greater inhibition of glucose oxidation (12%) and viable cell count (18%) by the iodophor than by the Trichlorocide. However with an exposure time of five minutes, Trichlorocide inhibited glucose oxidation and viable cell count to a greater extent (18% and 24% respectively). Cysteine and dithiothreitol were able to reverse the inhibitory effects of iodophor on glucose oxidation up to 18 percent and 20 percent respectively and restore the viable cell counts up to 47 percent.

When P. fragi cells were exposed to 0.75 ppm iodophor for 30 seconds, there was a 44 percent inhibition of glucose oxidation and 97 percent inhibition of viable cells. However dithiothreitol was able to reverse these effects 30 and 32 percent respectively. Comparable results were obtained with P. aeruginosa cells.

These results indicate that the mechanism of action of iodophor on the organisms studied is at least partly due to the oxidation of essential sulfhydryl groups involved in glucose oxidation and the maintenance of viability.

## BIBLIOGRAPHY

- Albin, I. A. S. 1966. Effect of quaternary ammonium compounds on bacteria: comparison of sensitive and resistant strains of the same species. Master's thesis. Corvallis, Oregon State University. 83 numb. leaves.
- American Public Health Association. 1960. Standard methods for the examination of water and wastewater. 11th ed. New York. 626 p.
- Anson, M. L. and W. M. Stanley. 1941. Some effects of iodine and other reagents on the structure and activity of tobacco mosaic virus. *Journal of General Physiology* 24:679-690.
- Armstrong, J. A. and E. J. Froehlich. 1964. Inactivation of viruses by benzalkonium chloride. *Applied Microbiology* 12:132-137.
- Baker, Z., R. W. Harrison and B. F. Miller. 1941. Action of synthetic detergents on the metabolism of bacteria. *Journal of Experimental Medicine* 73:249-271.
- Barron, E. S. G. 1951. Thiol groups of biological importance. *Advances in Enzymology* 11:201-266.
- Bartlett, P. G. and W. Schmidt. 1957. Surfactant-iodine complexes as germicides. *Applied Microbiology* 5:355-359.
- Beers, R. F., Jr. and I. W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* 195:133-139.
- Bernarde, M. A., W. B. Snow, V. P. Olivieri and B. Davidson. 1967. Kinetics and mechanism of bacterial disinfection by chlorine dioxide. *Applied Microbiology* 15:257-265.
- Boyer, P. D. 1954. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. *Journal of the American Chemical Society* 76:4331-4337.
- Brandrick, A. M. and J. M. Newton. 1967. An investigation into the interaction between iodine and bacteria. *Journal of Applied Bacteriology* 30:484-487.

- Carroll, B. 1955. The relative germicidal activity of triiodide and diatomic iodine. *Journal of Bacteriology* 69:413-417.
- Chang, S. L. 1944. Destruction of micro-organisms. *Journal of the American Water Works Association* 36:1192-1206.
- Chang, S. L. and G. M. Fair. 1941. Viability and destruction of the cysts of Endamoeba histolytica. *Journal of the American Water Works Association* 33:1705-1715.
- Covelli, I. and J. Wolff. 1966. Iodohistidine formation in ribonuclease A. *Journal of Biological Chemistry* 241:4444-4451.
- Covelli, I. and J. Wolff. 1967. The histidyl residues of insulin. I. Reactivity toward iodine. *Journal of Biological Chemistry* 242:881-886.
- Darrow, R. A. and S. P. Colowick. 1962. Hexokinase from baker's yeast. *Methods in Enzymology* 5:226-235.
- Davies, G. E. 1949. Quaternary ammonium compounds, a new technique for the study of their bactericidal action and the results obtained from Cetavlon (cetyltrimethyl-ammonium bromide). *Journal of Hygiene (Cambridge)* 47:271-277.
- Davis, J. G. 1962. Iodophors as detergent-sterilizers. *Journal of Applied Bacteriology* 25:195-201.
- Dawson, I. M., I. Lominski and H. Stern. 1953. An electron microscope study of the action of cetyl-trimethyl-ammonium bromide on Staphylococcus aureus. *Journal of Pathology and Bacteriology* 66:513-526.
- DeMoss, R. D. 1955. Glucose-6-phosphate and 6-phosphogluconic dehydrogenases from Leuconostoc mesenteroides. *Methods in Enzymology* 1:328-332.
- Dunn, C. G. 1938. Fungicidal properties of alkyl-dimethylbenzyl-ammonium chlorides. *Proceedings of the Society for Experimental Biology and Medicine* 37:427-429.
- Fair, G. M., S. L. Chang, M. P. Taylor and M. A. Wineman. 1945. Destruction of water-borne cysts of Entamoeba histolytica by synthetic detergents. *American Journal of Public Health* 35:228-232.

- Fraenkel-Conrat, H. and M. Sherwood. 1967. Reactivity of the tyrosine residues of tobacco mosaic virus protein with iodine. *Archives of Biochemistry and Biophysics* 120:571-577.
- Friberg, L. 1956. Quantitative studies on the reaction of chlorine with bacteria in water disinfection. *Acta Pathologica et Microbiologica Scandinavica* 38:135-144.
- Friberg, L. 1957. Further quantitative studies on the reaction of chlorine with bacteria in water disinfection. *Acta Pathologica et Microbiologica Scandinavica* 40:67-80.
- Furlong, T. E. and P. R. Elliker. 1953. An improved method of determining concentrations of quaternary ammonium compounds in water solutions and milk. *Journal of Dairy Science* 36:225-234.
- Gershenfeld, L. 1955. Iodine as a virucidal agent. *Journal of the American Pharmaceutical Association, Science Edition* 44:177, 182.
- Gershenfeld, L. and B. Witlin. 1949. Free halogens: a comparative study of their efficiencies as bactericidal agents. *American Journal of Pharmacy* 121:95-106.
- Goehler, B. and R. H. Doi. 1966. Conformation and binding of lysyl-tRNA to poly-A-ribosome complexes. *Proceedings of the National Academy of Sciences* 56:1047-1050.
- Hartdegen, F. J. and J. A. Rupley. 1964. Inactivation of lysozyme by iodine oxidation of a single tryptophan. *Biochimica et Biophysica Acta* 92:625-627.
- Hays, H., P. R. Elliker and W. E. Sandine. 1967. Microbial destruction by low concentrations of hypochlorite and iodophor germicides in alkaline and acidified water. *Applied Microbiology* 15:575-581.
- Hellerman, L. 1939. Reversible processes in the control of activity of certain enzymes. *Cold Spring Harbor Symposium on Quantitative Biology* 7:165-173.
- Hellerman, L. and M. E. Perkins. 1934. Activation of enzymes. II. Papain activity as influenced by oxidation-reduction and by the action of metal compounds. *Journal of Biological Chemistry* 107:241-255.

- Hotchkiss, R. D. 1946. The nature of the bactericidal action of surface-active agents. *Annals of the New York Academy of Sciences* 46:479-493.
- Hsu, Y. 1964. Resistance of infectious RNA and transforming DNA to iodine which inactivates f2 phage and cells. *Nature* 203:152.
- Hsu, Y., S. Nomura and C. W. Kruse. 1966. Some bactericidal and virucidal properties of iodine not affecting infectious RNA and DNA. *American Journal of Epidemiology* 82:317-328.
- Hughes, W. L. 1957. The chemistry of iodination. *Annals of the New York Academy of Sciences* 70:3-18.
- Hughes, W. L., Jr. and R. Straessle. 1950. Preparation and properties of serum and plasma proteins. XXIV. Iodination of human serum albumin. *Journal of the American Chemical Society* 72:452-457.
- Hugo, W. B. and J. M. Newton. 1964. The adsorption of iodine from solution by micro-organisms and by serum. *Journal of Pharmacy and Pharmacology* 16:49-55.
- Joslyn, D. A., K. Yaw and A. L. Rawlins. 1943. Germicidal efficacy of phemeral. *Journal of the American Pharmaceutical Association, Science Edition* 32:49-51.
- Kalchar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *Journal of Biological Chemistry* 167:461-475.
- Keynan, A., G. Issahary-Brand and Z. Evenchik. 1965. Activation of bacterial spores. In: *Spores III*, ed. by L. L. Campbell and H. O. Halvorson. Symposium held at Allerton Park, Illinois, 1964. (Washington, D. C.), American Society for Microbiology. p. 180-187.
- Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *Journal of Experimental Medicine* 126:1063-1078.
- Knight, C. A. and W. M. Stanley. 1944. The effect of some chemicals on purified influenza virus. *Journal of Experimental Medicine* 79:291-300.

- Knopfmacher, H. P. and A. J. Salle. 1941. Studies on the lactase of Escherichia coli. Journal of General Physiology 24:377-397.
- Knox, W. E., V. H. Auerbach, K. Zarudnaya and M. Spirites. 1949. The action of cationic detergents on bacteria and bacterial enzymes. Journal of Bacteriology 58:443-452.
- Knox, W. E., P. F. Stumpf, D. E. Green and V. H. Auerbach. 1948. The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. Journal of Bacteriology 55:451-458.
- Kornberg, A. 1955. Lactic dehydrogenase of muscle. Methods in Enzymology 1:441-443.
- Kornberg, A. and B. L. Horecker. 1955. Glucose-6-phosphate dehydrogenase. Methods in Enzymology 1:323-327.
- Lawrence, C. A. and S. S. Block (eds.). 1968. Disinfection, sterilization, and preservation. Philadelphia, Lea and Febiger. 808 p.
- Li, C. H. 1942. Kinetics and mechanism of 2,6-Di-iodo-tyrosine formation. Journal of the American Chemical Society 64: 1147-1152.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265-275.
- McCulloch, E. C. 1945. Disinfection and sterilization. 2d ed. Philadelphia, Lea and Febiger. 472 p.
- McFeters, G. A., W. E. Sandine and P. R. Elliker. 1967. Purification and properties of Streptococcus lactis beta-galactosidase. Journal of Bacteriology 93:914-919.
- Mahler, H. R. 1955. DPNH cytochrome c reductase (animal). Methods in Enzymology 2:688-693.
- Neilands, J. B. 1954. Studies of lactic dehydrogenase of heart. III. Action of inhibitors. Journal of Biological Chemistry 208:225-230.
- Nicholls, P. and G. R. Schonbaum. 1963. Catalases. In: The enzymes, ed. by P. D. Boyer, H. Lardy and K. Myrback. Vol. 8. 2d ed. New York, Academic. p. 147-225.

- Nygaard, A. P. 1955. On the reactivity of thiol groups in ox heart lactic dehydrogenase. *Acta Chemica Scandinavica* 9:1048.
- Ochoa, S. 1955. Malic dehydrogenase from pig heart. *Methods in Enzymology* 1:735-739.
- Pulvertaft, J. V. and C. D. Lumb. 1948. Bacterial lysis and antiseptics. *Journal of Hygiene (Cambridge)* 46:62-64.
- Quisno, R. and M. J. Foter. 1946. Cetyl pyridinium chloride. *Journal of Bacteriology* 52:111-117.
- Racker, E. 1955. Alcohol dehydrogenase from baker's yeast. *Methods in Enzymology* 1:500-503.
- Roberts, M. H. and O. Rahn. 1946. The amount of enzyme inactivation at bacteriostatic and bactericidal concentrations of disinfectants. *Journal of Bacteriology* 52:630-644.
- Rosen, S. M. and O. Rosen. 1967. The effect of iodination upon the catalytic and regulatory activities of fructose-1, 6-diphosphatase. *Biochemistry* 6:2094-2097.
- Salton, M. R. J. 1951. The adsorption of cetyl trimethyl ammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *Journal of General Microbiology* 5:391-404.
- Sevag, M. G. and O. A. Ross. 1944. Studies on the mechanism of the inhibitory action of zephiran on yeast cells. *Journal of Bacteriology* 48:677-682.
- Smith, C. R., H. Nishihara, F. Golden, A. Hoyt, C. O. Guss and M. C. Kloetzel. 1950. The bactericidal effect of surface-active agents on tubercle bacilli. *Public Health Reports* 65:1588-1600.
- Stedman, R. L., E. Kravitz and J. D. King. 1957. Studies on cell surface-germicide and enzyme-germicide reactions and their contribution to the lethal effect. *Journal of Bacteriology* 73:655-660.
- Stumpf, P. K. and D. E. Green. 1946. On the mode of action of chlorinating compounds. *Federation Proceedings* 5:157-158.

- Sykes, G. 1939. The influence of germicides on the dehydrogenases of Bact. coli. Part I. The succinic acid dehydrogenase of Bact. coli. Journal of Hygiene (Cambridge) 39:463-469.
- Sykes, G. 1965. Disinfection and sterilization. 2d ed. London, E. & F. N. Spon. 486 p.
- Tomcsik, J. 1955. Effect of disinfectants and of surface-active agents on bacterial protoplasts. Proceedings of the Society for Experimental Biology and Medicine 89:459-463.
- Washam, C. J. 1968. Properties of Pseudomonas aeruginosa resistant to quaternary ammonium compound. Ph. D. thesis. Corvallis, Oregon State University. 142 numb. leaves.
- Wassarman, P. M. and N. O. Kaplan. 1968. Iodination of muscle fructose diphosphate aldolase. Journal of Biological Chemistry 243:720-729.
- Watkins, S. H., H. Hays and P. R. Elliker. 1957. Virucidal activity of hypochlorites, quaternary ammonium compounds, and iodophors against bacteriophage of Streptococcus cremoris. Journal of Milk and Food Technology 20:84-87.
- Webb, J. L. 1966. Enzyme and metabolic inhibitors. Vol. 2. New York, Academic. 1237 p.
- Weill, C. E. and M. L. Caldwell. 1945. A study of the essential groups of beta-amylase. II. Sulfhydryl groups. Journal of the American Chemical Society 67:214-217.
- X Wilson, J. L., G. Mizuno and C. S. Bloomberg. 1960. The role of iodine in food products sanitation. Paper presented at Canadian Manufacturers of Chemical Specialties Association Third Annual Meeting and Conference, Montreal, Canada, October 26, 1960.
- Worthington Biochemical Corporation. 1966a. Glucose-6-phosphate dehydrogenase. In: Worthington enzymes, enzymes reagents manual. Freehold, New Jersey. p. 1.1.1.49.
- Worthington Biochemical Corporation. 1966b. Glyceraldehyde-3-phosphate dehydrogenase. In: Worthington enzymes, enzyme reagents manual. Freehold, New Jersey. p. 1.2.1.12.