IET's EcaFlo® Anolyte
A Two-edged Biocide

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All living cells depend upon on the chemistry of oxidation/reduction (redox) reactions to regulate energy storage and utilization during cellular metabolism. In addition, the integrity of cellular membrane/wall functions and structure also depend on these chemical reactions. Since these reactions involve the movement of electrons between molecules, compounds and compounds within macromolecular structures, these reactions may affect the structure and functions of macromolecules. Therefore, the electrical charge involved with atoms, molecules, cellular structure and cellular functions is a major aspect of life itself.

The loss of electrons (oxidation) and the gain of electrons (reduction) in a reaction can be measured in half reactions in which a standard potential (E°) is generated. Measurable in millivolts, the standard potential is based on a standard [the hydrogen ion/hydrogen redox couple (H^+/H_2 = 0 millivolts)]. Based on the charge of electrons (-), an oxidizing solution yields positive oxidation/reduction potential (ORP) values in millivolts whereas a reducing solution yields negative ORP values. These redox couples in a living cell are subject to electron flow changes and, when measured as a group, may provide an approximation of the redox state of the entire cell. This redox state has been described by focusing on the major redox couples known to function in living cells such as the following couples:

NAD^+/NADH NAD^+ collects electrons in the Krebs cycle and carries them to the electron transport chain where they support energy synthesis (oxidative phosphorylation-ATP production)

NADP+/NADPH NADPH is a major source of electrons for reductive biosynthesis and is a product of photosynthesis

GSSG/2GSH Glutathione is the major sulfur redox molecule in the cell (present in concentrations as high as 1-11 mM)

TrxSS/$Trx(SH)_2$ Thioredoxin is another sulfur system in cells, about 100-1000 fold lower than the concentration of the glutathione couple

Several points to be emphasized are that the concentrations involved in the ratio of the couple's compounds determine the ORP of the couple. In addition, the major redox couples mentioned above are interrelated so that major changes in these couples would be expected to change the overall ORP of the cell (redox environment of the cell).

During normal metabolism requiring oxygen, various free radicals are produced in the cell that are removed by naturally occurring antioxidants. These reactive oxygen species (ROS), such as superoxide (0_2^-) and the resulting hydrogen peroxide (H_2O_2) are molecules with unpaired electrons in search of other electrons. Regulation of H_2O_2

concentrations then becomes crucial to the cell and is accomplished by enzymes such as peroxidases and catalases. Removal of electrons from the macromolecules of the cell may change their structure and function and result in severe damage to the cell. Since oxygen is the most import oxidant in metabolism, oxidative stress in the cell may result from the reactions that use oxygen. This stress occurs when the equilibrium between the oxidant/anti-oxidant systems are altered. When additional oxidants are added to the cell, they increase in concentration over the anti-oxidants. Although cells have anti-oxidant molecules which include vitamins and anti-oxidants from fruits and vegetables, an increase in the level of oxidants may result in damage to many cellular components including carbohydrates, proteins, lipids and nucleic acids. Such severe oxidative stress will result in the death of the cell by overcoming the cell's anti-oxidant compounds and enzymes, both naturally synthesized and dietary.

Focusing on the GSSG/2GSH couple, since it is the major redox couple in a cell, changes in the half cell reduction potential of this couple are correlated with the biological status of the cell (1). Because of the importance of these redox couples to the redox environment within the cell, it becomes apparent that any compounds entering the cell with a high positive ORP will certainly alter the redox environment.

During the last ten years due to advances in technology and knowledge about the intracellular environment, much attention has been paid to the overall redox state of living cells. These studies have focused on a wide range of cellular functions that are controlled by the redox state of the cell. These include, but are not limited to:

Apoptosis in which a redox signal mechanism induces cell death (2)
Calcium channel activity in cardiac muscle (3)
Regulation of gene expression in cellular inflammation (4)
Cell cycle regulation via bypass of a restriction point (5)
Regulation of gene expression by alteration of transcription proteins (6, 7)
Stem cell fate and oxidative stress in central nervous system disease (8)
Balance between self renew and differentiation in stem cells (9) A redox cycle that regulates the cell cycle (10, 11)
A redox-inducible antioxidant protein (12)
Regulation of circadian rhythm (13)
Binding affinity, activity and expression of proteins (14, 15, 16)

Therefore, an increase in the internal concentration of compounds that are oxidants will drastically and quickly change the cell's external and internal structures and numerous biochemical functions within the cell.

Such a compound is hypochlorous acid (HClO). One of the major methods used in generating HClO is by the passage of brine through an electrolytic cell resulting in the production of electrolyzed oxidizing water (EO water). EO water has been used to eliminate bacterial and fungal growth on the surfaces of fruits and vegetables as well as on various other surfaces (17, 18, 19, 20).

As generated by equipment manufactured by Integrated Environmental Technologies, Ltd. (IET), brine is converted into chlorine/oxygen oxidants. At a near-neutral pH (6.3-6.5), the predominant compound is the highly biocidal HC1O (19) with an ORP of 800-900 mV.

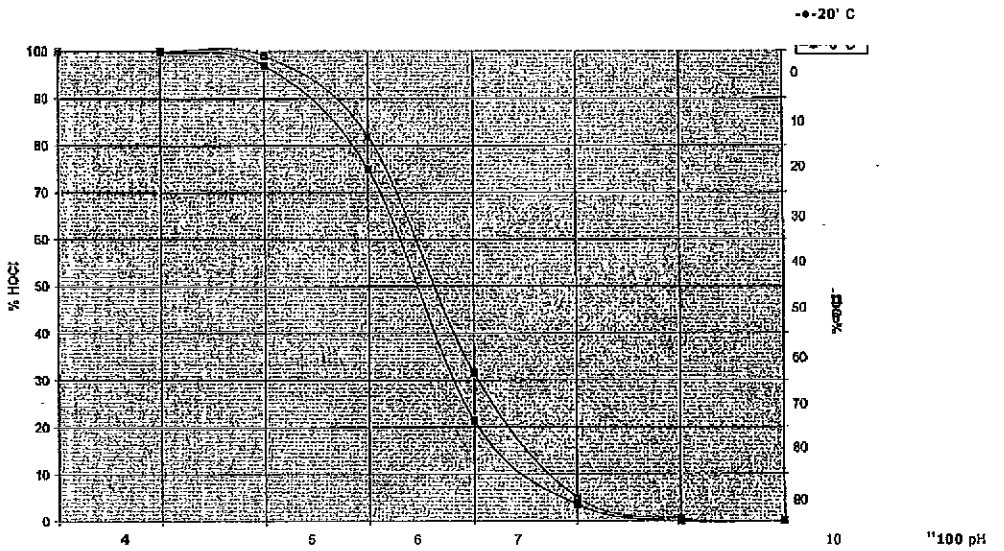


Figure 1. Ratio of HClO to OCL as a function of pH (22)

The higher the ORP, the more likely the compound will take electrons from other compounds and, therefore, act as a strong oxidizer. Most importantly, the ratio of HClO to Na hypochlorite in the solution is determined by pH (Fig. 1, 2). There is an inverse relationship between the pH and the concentration of HClO in solution such that very high ORP values are obtained in solutions that are acidic in applications to maximize biocidal activity. As can be seen when comparing Figures 1 and 2, ORP remains above 650 mV over a wide range of pH whereas the percentage of HC1O over Na hypochlorite rapidly decreases above a pH of 7.0.

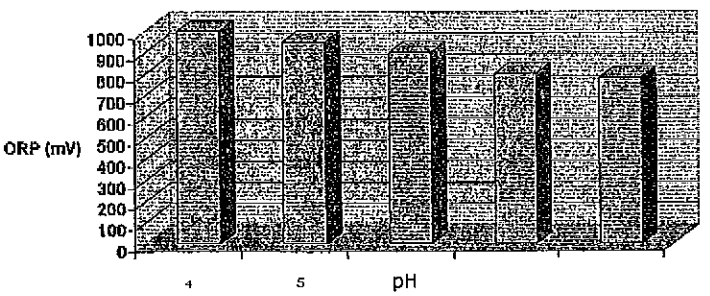


Figure 2. Relationship of ORP to changes in pH. (23)

As produced by IET, the EcaFlo® anolyte solution (pH = 6.3) contains a 0.046% solution of HC1O (99.5% H_20, 0.45% NaCl and 0.004% trace compounds). EO water similar to the above solution has been shown by UV-Vis absorbance to disrupt internal membranes of Escherichia coli and the cell wall (determined by electron microscopy) in 4 and 5 seconds, respectively (20). Cell death has been shown to occur with a change in reduction potential of +72 mV in HL-60 cells and a change of +186 mV in. HT 29 cells (21). A level of 650 mV has been determined by the World Health Organization to instantly kill E. coli regardless of the free available chlorine level (24). The disruption of internal membranes and macromolecules in bacteria, fungi and viruses would stop biochemical processes involved in the production of numerous important molecules including lipids and proteins. Perhaps, more importantly, disruption of reactions involving electron transport and oxidative phosphorylation will eliminate the production of ATP, the cell's source of energy.

Another major reason for the use of HC1O as a biocide, in addition to the ORP alternations that occur when HC10 enters the cell, is its chemical interaction with other compounds of the cell. The presence of HC1O in a living cell is known to chemically interact with all four of the major types of biomolecules: proteins, carbohydrates, lipids and nucleic acids. HC1O is a weak acid, colorless in aqueous solution with a pKa = 7.5 (Fig. 1). It is a strong oxidizer, stronger than chlorine or bleach (sodium chlorite) and is generated naturally in the immune system by the following reaction catalyzed by the enzyme myeloperoxidase.

H2O2 + Cl^- + H} HC1O + H2O

This generation of HC1O results in the immune system's ability to destroy contaminating bacteria and viruses. The use of HClO as a biocide on a large scale in many applications is based on this natural process. In terms of reactive oxygen species (ROS) and their toxic effects on the macromolecules of the cell, NO from nitric oxide synthase, superoxide, and HC1O have been rated the "good, bad and the ugly", respectively (25).

Effect on Proteins

The correct formation of the three-dimensional structure of proteins involves the interaction of the chemical side groups of the component amino acids with each other and with the surrounding environment. Two of these amino acid side groups that are quite susceptible for chemical interaction with HC1O is the sulfhydral group (-SH) of the amino acid cysteine and the amino group ($-NH_2$) of several amino acids. At high intracellular concentrations, HC1O will interact with the sulfhydral groups of adjacent cysteine residues and form disulphide bridges (S-S) which can severally alter the conformation of the protein and possibly result in a nonfunctional or inhibited protein. It should be pointed out that at physiological concentrations, HC1O produced in cells as described above, can function in destroying S-metal linkages in inactive proteins (zymogens) thereby resulting in the activation of that protein. For example, the zymogen pro-matrix metalloproteinase (pro-MI$^-$ VIP) is activated by the interaction of HC1O with a S-Zn linkage which results in a cleavage of the zymogen forming an active catalytic domain (26). It might be expected that enzymes that involve the SH group in their catalytic sites such as cysteine proteases are susceptible to HC1O modification.

The number of SH groups in a protein's amino acid sequence may also determine the total effect of HClO, especially since each SH may be involved with more than one molecule of HClO, depending on the concentration of HC1O. As illustrated in the following set of equations, if the concentration of HC1O is sufficiently high, several acids of sulfur are formed.

R-SH + HC1O ---> R-SOH (sulfenic acid)

R-SOH + HC1O - $R\text{-}SO_2H$ (sulfenic acid)

$R\text{-}SO_2H$ + HC1O -i $R\text{-}S0_3H_2$ (sulfurous acid)

This series of reactions with one SH group may contribute to the decrease in the efficacy of HC1O as a biocide when treating solutions or surfaces that have a high protein load.

Any amino acid side chain of a protein that contains an amino group (NH_2) may react with HCLO to form chloramines.

-NH2 + HC1O -* NH_2C1 + H2O

With higher concentrations of HCLO, once the available NH2 groups in side chains have been modified, it is possible that HC1O may attack the nitrogen involved in the peptide bonds between the amino acids in the peptide chain. In addition, chloramines may also chemically interact with SH groups and continue protein modification and degradation initiated by HClO (27).

Effect on Nucleic Acids

The use of HClO as a biocide provides the opportunity for destroying bacteria, viruses and fungi by not allowing the organism to be viable for natural selection. Although slow relative to other effects, HC1O reacts with DNA and RNA resulting in denaturing double stranded regions of both molecules. This separation of double stranded regions is thought to be a result of the attack of HC1O on the amino groups and heterocyclic NH-groups of the nitrogenous bases in the molecule and not to oxidative fragmentation. This attack would modify the bases to the extent that they would not be able to form the hydrogen bonds and hydrophobic interactions required for and a result of double-strandedness (28).

Nucleotides, the building blocks of nucleic acids, react with HC1O depending on the presence of amino groups and NH groups. HClO reacts faster with the NH groups of GMP, inosine (precursor of AMP and GMP) and TMP than with the amino groups present in AMP, CMP and GMP (28). In general, HC1O reacts with nucleosides (nitrogenous base + sugar) to form chlorinated nucleosides at physiological concentrations and pH. Interestingly, tertiary amines (trimethylamine and nicotine) have been found to enhance the chlorination of these precursors of DNA. This increase in chlorination may play important roles in diseases that involve inflammation, perhaps those caused by the use of tobacco (29).

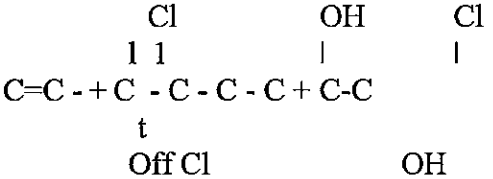
Effect on Carbohydrates

Interaction of HClO with carbohydrates is not as rapid with other molecules in the cell and may depend on chemical groups discussed above that have been attached to the

carbohydrate during synthesis and processing. These groups include NH, amino groups and SH groups. Since HC1O has been shown to be involved in the inflammatory diseases such as rheumatic diseases, studies have been accomplished in which HC1O's effect on synovial fluid complex carbohydrates was observed. HC1O has been shown to damage N-acetylglucosamine, N-acetylgalactosamine, chondroitin sulphate and hyaluronic acid by interacting with the N-acetyl side chains of these molecules (30). The apparent mechanism of attack via the N-acetyl side chains results in the formation of chloramines which eventually degrade to acetate (31).

Effect on Lipids

The major point of attack by HC1O on lipids are the double bonds of unsaturated fatty acids, cholesterol (olefins) and as expected, any modified lipids containing SH or amino groups. The mechanism for the interaction of HC1O with a carbon to carbon double bond is a two step reaction as follows:



In this reaction the Cl+ ion polarizes the double bond and is added to one of the carbon atoms forming a chlorohydrin (32). In the membranes of red blood cells, the formation of chlorohydrins from HC1O have been shown to disrupt the lipid bilayer and initiate cellular events leading to cell lysis (33). Plasmalogens, complex phospholipids containing a double bond, are also degraded by HClO and recognized as specific targets for oxidizing molecules, so much so that they are considered natural antioxidants (34). Action by HCLO on these molecules would decrease the cell's ability to protect other molecules from the degradation of HClO.

HC1O is an effective biocide because of its two-edged attack through disruption of the cell's electrochemistry (ORP) as well as its chemical reactivity with numerous molecules within the cell. IET's ability to produce their anolyte solution (EcaFlo®) at neutral pH provides a powerful biocide that can be employed in numerous applications.

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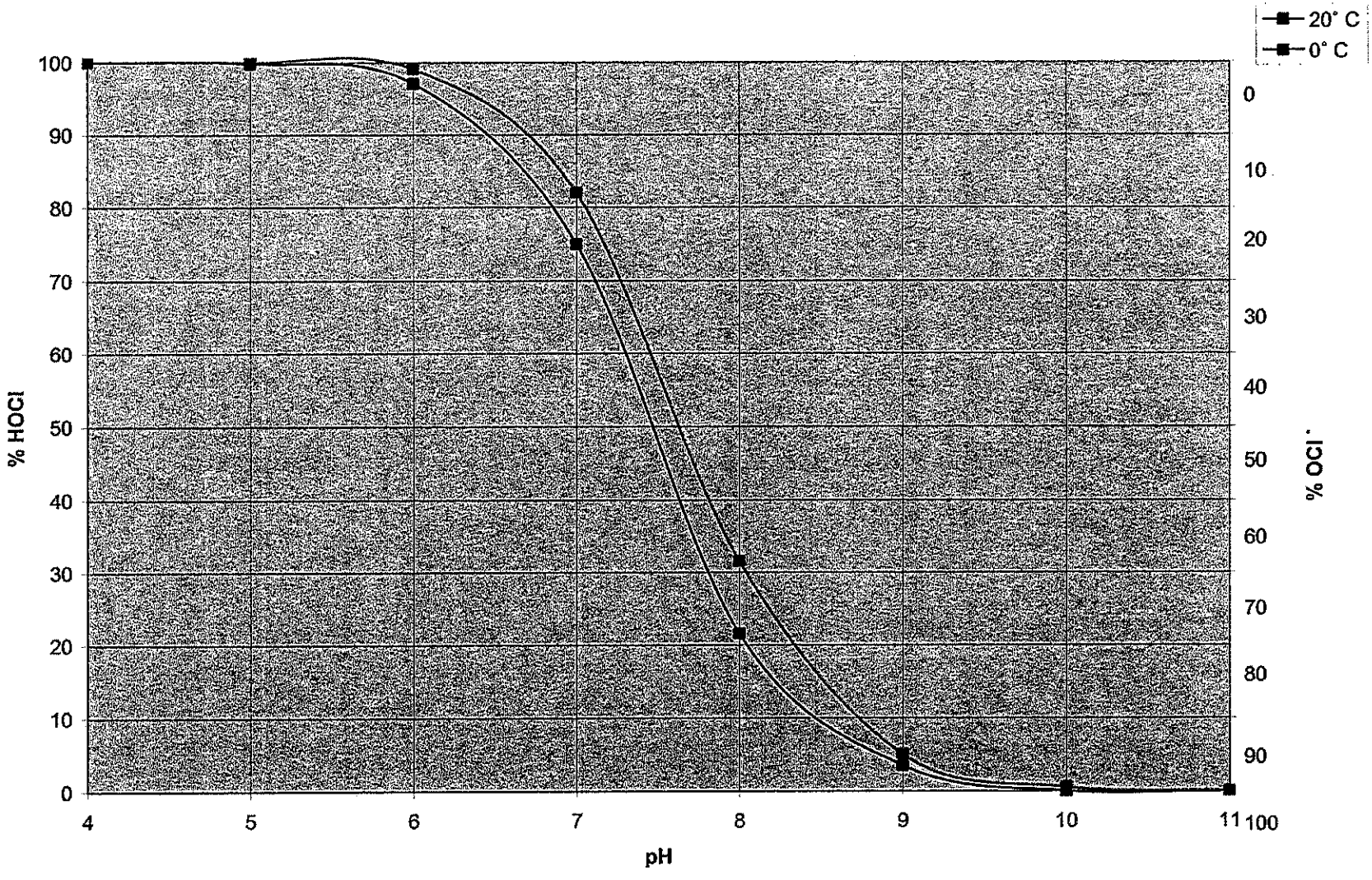
Protolysis of Hypochlorous Acid



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