Microbial Inhibition by Food Preservatives

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CONTENTS

I. Introduction

Bacteria and related forms are essentially masses of protoplasm consisting largely of enzymes so organized as to permit the orderly progress of metabolic processes. This organization involves the following factors: (1) a controlling genetic mechanism regulating cell division and insuring that reproduction results in new cells essentially identical with the parent, (2) the cell contents separated from direct contact with the environment by means of a living membrane, and (3) the contents consisting of enzymes and enzyme systems with definite spacial orientation in regard both to each other and to the geography of the cell. Severe disturbances of any of these factors result in death of the cell and trivial disturbances may result in partial or complete inhibition of cellular activity. Disruptive influences may vary from profound effects such as the actual destruction of the cell by physical forces, *e.g.*, supersonic waves, to the slowing of metabolic activity by the presence in excess of some necessary food constituent. A vast literature has accumulated on each detail of the subject. This review will be limited to a discussion of the mechanism of the antimicrobial action brought about by chemical substances and possible applications of this knowledge in the food industries.

Most *in vitro* tests of the activity of chemical agents on microbial cells concentrate the attention of the investigator on the limiting cases. The actual measurements made and reported are either in terms of the lowest concentration of a chemical agent required to bring about an effect, or in terms of the shortest time of action required for a certain concentration of the chemical to exhibit its antimicrobial action under stated environmental conditions. By such methods one presumably studies the inhibition of that one mechanism in the cell most sensitive to the chemical under test.

In actual practice, however, the concentration employed will be many times in excess of that minimal quantity required for inhibition; or the time permitted for action will be many times in excess of that measured in the laboratory experiment. It is likely that under such circumstances several cell-destroying mechanisms may be exhibited by a single chemical agent and the effectiveness of the chemical will be a summation of the several actions. Nevertheless, since the antimicrobial action will fail only when the inhibition of the most sensitive function in the most resistant organism is no longer operative, a study of such limiting mechanisms is essential to our understanding of the basic problem.

II. Interference with the Genetic Mechanism

Our knowledge of chemical interference with the genetic mechanism of the cell is based largely on indirect evidence and analogy. As pointed out by Rahn (1945) the microbial cell is generally regarded as dead when it has lost its power to reproduce. Under many practical conditions, if microorganisms are prevented from reproducing or are even slowed down in their reproductive capacity the objective of the chemical inhibitor is attained. This is due to the fact that in most circumstances the metabolic activity of the organisms makes their presence undesirable only when large numbers of cells are present. Consequently, interference with reproduction is usually an adequate control measure. Strong evidence for the theory that chemical substances destroy bacteria by interference with the genetic mechanism can be inferred from the shape of curves obtained by plotting data from experiments on bactericidal agents. In many instances it will be observed that when the log of the surviving bacteria is plotted against the time of exposure to a chemical agent, a straight line results. Based on analogy with monomolecular chemical reactions Rahn offers a clear statement of the case for gene interference:

"The conclusion is that a logarithmic order of death can be obtained only if the death of the bacterium is brought about by the reaction of one single molecule. This conclusion is absolute. The logarithmic order of death is entirely impossible if more than one molecule must be inactivated to produce death of the cell . . . an approximation of the logarithmic order is obtained if death is caused by inactivation of a very few molecules but if this number is higher than 4 or 5 the order is very definitely not logarithmic. This eliminates denaturation of enzymes or cytoplasm as the cause of death, wherever the logarithmic order prevails because it does not seem possible that the inactivation of a very few of numerous identical molecules can kill the organism. It would probably exclude also the disruption of the cell membrane as a cause of death for this is likely to

require inactivation of a considerable number of molecules before damage becomes irreparable."

"Let us now consider the bacteriologists' definition of death: a bacterium is dead when it has lost the power to reproduce. Cell division is linked with chromosome division, the chromosomes contain the genes, and the inactivation of certain genes is known to cause "lethal mutations", *i.e.*, to prevent the cell from multiplying. While chromosomes and genes have not been definitely found in bacteria, these organisms undoubtedly have some hereditary mechanism, and hereditary units. As a rule there are two genes of the same kind in a diploid cell and one in a haploid cell. According to Fricke and Demerec (1937): "we may assume a gene contains about 2500 atoms . . . This would indicate an average gene diameter of about 25Å." This is the size of a small protein molecule. A gene, then, would consist of only one or two molecules. Each gene is different from the others and has its own rate of denaturation. If one vitally essential gene is denatured, the cell can no longer divide, the bacterium is sterile, that is, dead, according to the bacteriological definition."

Mutations induced by chemical agents do not necessarily result in death. They may result in the loss of some function of the cell which may definitely retard its growth or stop growth entirely under certain environmental conditions. For example, by ultraviolet and x-ray radiations it is possible to induce mutations involving the loss of ability to synthesize a vitamin or amino acid. This has been done with a number of bacterial species (see Ryan *et al.*, 1946) and a tremendous amount of work has been done with fungi (Beadle, 1945). The recent work of Stone, Wyss, and Haas (1947) indicates that certain mutations may result from the irradiation of the substrate, which is evidence that mutations may be induced in the bacteria by definite chemical agents. It is a common experience among bacteriologists who work with antibacterial agents to observe "small colony" variants which result from experiments on chemical inhibition. These are undoubtedly biochemical mutants which exhibit a nutritional deficiency when growing on the medium which was satisfactory for the production of large normal colonies by their parent strain. Nucleotides interfere with the action of basic dyes in a manner suggesting that part of the inhibition by a dye may be a result of its union with acid groups in the nuclear apparatus. The inhibition of cell division by penicillin and the recent work by Krampitz and Werkman (1947) showing that penicillin inhibits the dissimilation of ribonucleic acid suggests that this inhibition involves the nuclear apparatus of the cell.

It is true that in the few cases which have been carefully studied thus far, the rate of induced mutation to any one particular mutant has been

extremely low and would not account for any degree of inhibition of activity of the cell population. It must be borne in mind, however, that when mutations are produced the majority are lethal to the organism. This is the expected result since a mild chemical treatment may produce a few single gene transformations, but more drastic treatment would undoubtedly produce multiple genetic disturbances in the cell and these are extremely unlikely to be other than deleterious to the welfare of the organism in its accustomed environment.

This is emphasized in the review on mustard gas by Gilman and Philips (1946). They have summarized the evidence that high concentrations of mustard produce many chromosomal breaks which ultimately result in death. Mild treatment also results in chromosomal breaks and if these are not too numerous they may be transmitted to daughter cells in the subsequent mitosis as heritable chromosome abnormalities. Although these experiments were carried out with higher forms, they probably apply to microorganisms since exposure of yeast to mild doses of mustard reduces the growth rate in a manner which is inherited by several succeeding generations of daughter cells. It follows, therefore, that even if minor, specific genetic changes are brought about in a fraction of the organisms present in a population, the ultimate result may be an inhibition of the microbial growth. In addition to mustard gas other chemicals are known to bring about mutations. High concentrations of $BaCl_2$ (Schnitzer *et al.*, 1943), nitrite and a number of other agents (Steinberg and Thom, 1940) added to the medium may result in mutant forms. Some such mutations may arise by selection of pre-existing spontaneous mutants, but others appear to be the result of induced hereditary changes in the population.

A mechanism for induced mutations might be developed based on Delbrück's (1941) theory of autocatalytic synthesis of polypeptides. If the assumption is made that all primary gene actions involve one general mechanism, then since the reproduction of the gene is the only primary gene action of whose general occurrence we are certain, it would appear that the gene acts primarily because and while it is reproducing itself. The gene probably reproduces several times during each cell cycle and the extra replicas diffuse into the cytoplasm where they control the observable characters of the cell. Delbrück offers the following explanation as to how the autocatalytic reproduction of the gene proceeds. He assumes with Linderstrøm-Lang that proteins are synthesized not from amino acids but from the aldehydes uniting first to form an imide bond which is then oxidized to a peptide bond. In the oxidation involving the removal of *two* hydrogen atoms, the first step requires the most energy because it leads to the formation of a radical with a free valency (Michaelis and

Schubert, 1938). It is the function of the catalyst to reduce the size of the first step so that it may be accomplished by the available hydrogen acceptors in the cell and not require some much more powerful oxidant. The size of the first step can be reduced by lessening the energy of the radical and this can be done by having the radical form an intimate complex with a molecule that has already lost both hydrogens. The complex would be a structure that is stabilized by resonance between the two equivalent electronic configurations. The amount of resonance energy involved will depend on, first, the closeness of approach of the two molecules, and second, the near equality of the energy of the two resonating states. Both of these conditions are most easily fulfilled if the catalyzing molecule is identical in structure (including side chains) with the oxidized product of the substrate molecule, *i.e.*, if it reproduces itself exactly. The introduction of a nonphysiological chemical into the system might easily disturb the progress of the autocatalytic reproduction. If the chemical does not prevent the process entirely it might, by uniting with the substrate or the catalyst, introduce distortions which would result in inexact replication. In this manner mutants may arise.

III. Interference with the Cell Membrane

The cell membrane is the outermost layer of highly reactive protoplasmic material of the cell. Naturally, reactive chemical compounds diffusing into the cell will unite with the components of the membrane. Chlorine is an example of a highly reactive chemical which reacts indiscriminately with many substances. Therefore, it is unlikely, in its active state, to get beyond the cell membrane without satisfying the groups present there which can react with it. Chang (1944) reviews the evidence and presents data obtained with cysts of *Entameba histolytica*. Using the indicator, *o*-tolidine, which forms a yellow color with chlorine, he was able to determine that when the cysts were killed by free chlorine the halogen was present inside the cyst, a phenomenon that may have been a result rather than a cause of death. On the other hand, with low concentrations of some less active chloramines, the cysts survived because the chloramine did not, or was unable to, penetrate into the protoplast.

Membrane damage can also be attributed to such agents as the fat solvents which, if present in sufficient concentration, dissolve out the lipoidal constituents and thus destroy the functioning membrane. This can be demonstrated readily by placing yeast cells under ether and observing the rapid and complete dissolution of the cells. High concentrations are required since the action depends on differential solubilities. The enzyme, lysozyme, attacks specifically the acetylaminopolysaccharide in the cell

envelope of certain susceptible microorganisms. A number of species are inhibited by the action of the enzyme but a very few species are completely lysed by it.

The large group of compounds known as surface active agents produce their antibacterial effect, at least in part, by action on the cell membrane (Hotchkiss, 1946). Experiments with compounds of this type show that within a few minutes after application of a lethal dose there appears in the surrounding medium various phosphate esters and nitrogen compounds which were an integral part of the functioning protoplasm of the living cell. Such effects occur at low temperature, indicating that they are not the result of a disturbed autolytic mechanism which often functions when death has been brought about under conditions favorable for enzyme action. The surface active agents effect the destruction of the semipermeable nature of the cell membrane. That these phenomena are the cause rather than the result of death is proved by experiments with a wide variety of nonsurface active killing agents which bring about death but do not result in the rapid diffusion of the cell contents into the medium. It is assumed that surface active substances not only may disturb the permeability of the cell membrane, but that they also may destroy the orientation of various constituents within the cell, an orientation that is preserved only as the surface tension is maintained within definite limits. When a disturbance is introduced, certain enzymes come in contact with substrates from which they are ordinarily restricted by means of cell geography or cell architecture, and the resulting action is disastrous to the cell.

An example of a substance bringing about membrane damage is tyrocidine, a surface active polypeptide having a molecular weight of 2500, which can be extracted from culture fluids of *Bacillus brevis*. The action described for this compound in damaging the cytoplasmic membrane does not appear to differ greatly from other antibacterial substances with surface active properties. These include not only such simple compounds as fatty acids, alcohols, and aldehydes, but also most of the hundreds of synthetic wetting and emulsifying agents and detergents which have been so extensively studied over the past ten years. The activity of the phenols appears to belong to this group, and substitution on phenolic compounds to give a greater heteropolar character to the molecule results in stronger activity. In keeping with all compounds of this type a hydrophobic group is attached to one or more hydrophilic groups. Increase in the hydrophobic group results in greater water insolubility and this is often accompanied by greater antibacterial activity when tested by conventional methods. The total activity depends on two factors: (1) the tendency to concentrate in or about the bacterial cell thus giving a higher concentra-

tion than is present in the menstruum, a property governed by the water solubility and the surface activity and (2) the actual toxicity of the compound when at the site of action. Attempts to prepare the most active germicidal compound of a series of surface active agents are complicated by the fact that modifications in the molecular structure may result in an increase in absolute toxicity without this fact being evident from the antibacterial tests. The advantage gained by increased toxicity may be masked by a simultaneous decrease in the tendency of the modified molecules to concentrate on or in the bacterial cells. Surface active ions also have a strong affinity for proteins and combine readily with them. The results of Anson (1941) indicate, however, that the amounts required for denaturation of protein are generally far in excess of those required for killing bacteria. From this and other experimental evidence, Hotchkiss (1946) concludes that when limiting concentrations of surface active agents are considered, the effect on the membrane is the phenomenon which causes death or inhibition of the organism.

Confirmation of this view is offered by the analytical work of Gale and Taylor (1947) and the electron micrographs of Mitchell and Crowe (1947). The former workers show that the glutamic acid and lysine from the internal environment of bacteria and yeast rapidly leaks out of the cells which are treated with detergent substances. This did not occur after the application of inhibitory concentrations of substances which were not surface-active. The substances employed included phenol, the antibiotic, tyrocidine, the cationic detergent, cetyltrimethylammonium bromide and the anionic agent, Aerosol O. T. Gale and Taylor believed the lytic action of these compounds to be sufficient to account for their antibacterial effe ct. Mitchell and Crowe found the cell *wall* disrupted in streptococci treated with surface active agents. Presumably the cell wall is itself the amino acid-confining barrier, or it may be attached to the cell membrane or some other confining structure, which is disrupted with it.

On gram positive bacteria there appears to be a definite layer of a specific chemical substance which absorbs basic dyes such as gentian violet (Henry and Stacey, 1946). The exact location of the material is in doubt, but in many organisms it appears to be a part of, or layered on, the cytoplasmic membrane. This dye absorbed in the surface layer is probably harmful to the cell, since organisms having the dye-absorbing layer are inhibited by dyes to a greater extent than are those in which the layer is absent. It seems likely, therefore, that at least part of the bacteriostatic effect of dyes is due to their interference with proper functioning of the membranes of the cell.

IV. Interference with Enzyme Activity

Interference with the activity of enzymes results in microbial inhibition or death. Such interference is readily accomplished. Unfortunately, our studies on enzymes have been largely limited to those exerting katabolic activity since most anabolic enzymes can be studied only through measurements on the over-all life process. A beginning has been made in the elucidation of the enzymes involved in phosphorylation which link the energy-yielding and the energy-consuming reactions in the cell. The enzymes involved in synthesis are undoubtedly similar in many respects to those involved in katabolic reactions and inhibitors will affect them in an analogous manner.

Enzymes are protein colloids which contain reactive groups that unite with a substrate molecule. For activity, the colloidal nature of the protein must be maintained, the prosthetic group, if there be one, must be properly attached and functioning and various functional groups on the protein moiety must be maintained in their free and active state. Any interference with these properties results in inactivation. A wide variety of agents is known to be protein denaturant. They may act by rendering the protein molecules incapable of maintaining themselves as a colloidal suspension. For example, wetting agents may irreversibly denature enzymes by so altering the colloidal properties and the surface of the molecules as to interfere with the enzyme action. Enzymes, like other colloids, are sensitive to marked changes in the ionic content of the medium. This may be due partly to the effect on reactive groups, but more often is concerned with maintenance of the colloid. High concentrations of salts precipitate proteins or so alter the colloids as to drastically curtail their biological activity. The effect of some ions is more marked than others, *e.g.*, NaCl inhibits some proteinases at concentrations where most other sodium salts are not effective. From the data of Ingram (1947) it appears that the action of concentrated salt solutions on bacterial respiration may be partly explained in terms of "salting out" the enzymes. The limiting action is probably on a dehydrogenating enzyme. There are some factors of salt action not yet explained, for although the data on halophiles bear out this conception, the enzymes of nonhalophiles are more sensitive in the intact cell than when isolated.

One of the most powerful chemical agents for inhibiting enzyme action is the hydrogen ion. When large departures in pH from the optimum for enzyme action are maintained, irreversible denaturation results, which is undoubtedly due to disturbance of the colloidal nature of the enzyme. The bacteriostatic effect obtained by adjusting the pH slightly above or below that which permits growth is an example of bacteriostasis which is

due to enzyme inhibition. The assumption is usually made that only uncharged or slightly charged particles of enzymes are active. The adjustment of the hydrogen ion concentration away from the isoelectric point reduces enzyme action. The pH activity curves of enzymes, however, are complicated by the effect of the pH on the substrate, and when living cells are involved there are other factors, such as cellular pH and the relative permeability of the membrane to ions *vs.* molecules, to be considered.

The enzyme chemist studies the active groups on the enzyme proteins by noting the effect of mildly reactive inhibitors. For example, an enzyme which is inactivated by very mild oxidizing agents and which can be reactivated by reducing agents is thought to contain sulfhydryl groups which are essential for activity. The studies on the active groups of enzyme proteins by means of inhibitors have been reviewed by Barron and Singer (1945) and Singer (1946). From such studies we are able to obtain a clearer picture of the mechanism of action of many inhibiting agents since the experimental data indicate the relative susceptibilities of enzymes and their active groups.

Of the reactive groups attached to enzyme proteins, the —SH groups have received widest attention. The development of the concept of the active sulfhydryl group as an essential part of the functioning enzyme is summarized by the work of Hellerman (1937). In a series of experiments he brought about the inactivation and reactivation of enzymes by alternating the application of selected oxidizing and reducing agents. Hellerman suggested that the disulfide form of the enzyme (Enzyme—S—S—Enzyme) was inactive and that reduction of the disulfide group to active sulfhydryl groups activated the enzyme thus:

$$\underset{\text{(inactive)}}{\text{Enzyme—S—S—Enzyme}} + 2\text{H} \longleftrightarrow \underset{\text{(active)}}{2\ \text{Enzyme—SH}}$$

Of a list of about 40 enzymes tabulated by Singer (1946), it can be noted that for over ⅔ of the enzymes studied, functioning sulfhydryl groups are essential for activity. These include respiratory enzymes as well as hydrolases. Obvious poisons for sulfhydryl enzymes are the oxidizing agents, *e.g.*, the halogens, ozone, hydrogen peroxide, and permanganate which in high dilution may destroy cellular activity by oxidation of sulfhydryl enzymes. Many microorganisms do not maintain the enzymes in the active reduced state during inactive periods, and it is necessary to supply the proper reducing material in order to restore active —SH groups. The activity of the enzyme may be indefinitely postponed by maintaining a high oxidation-reduction potential and it is believed that the bacteriostatic action of certain dyes is due in part to their ability to poise the O—R potential of the medium at such a level that the sulfhydryl enzymes

of the inoculum have difficulty in getting under way. There are a great many inhibitory substances which are mild oxidizing agents and they may function in this manner.

Another common poison of the sulfhydryl groups on the protein is of the heavy metal type. Copper, mercury and silver ions, compounds of bismuth and arsenic, and other mercaptide forming agents combine with the sulfhydryl groups on the protein and the result is "poisoning" of the enzyme. This inhibition of enzyme activity may require only trace quantities of the heavy metals. In some instances the amount required may be so small that the inactivation is regarded as the oxidation of the essential —SH catalyzed by a trace of metal, but in most cases the poisons form more or less tightly bound compounds with the —SH group in the enzyme. In such cases the inactivation can be reversed by removing the toxic agent with adsorbents or by selectively precipitating them with lower molecular weight thiols, but this must be done before the cell dies from lack of the functioning enzyme.

Numerous other compounds will react with the —SH group and consequently destroy the activity of enzymes where the free group is necessary for functioning. Among those which have been useful tools in biological research are iodoacetate and bromoacetate which form alkyl derivatives with the enzyme at the —SH linkage. Though their reaction is slow it is irreversible and at low concentrations they produce strong inhibition of certain enzymatic processes. It must be pointed out here that a number of agents which are powerful enzyme inhibitors when tested on isolated enzyme systems, fail to show great inhibition of the intact microbial cell since at normal pH values they pass only with great difficulty through the cell membrane. In some cases this is the effect of the dissociation as described by Jacobs (1940). He points out that most undissociated molecules pass through the membrane and through protoplasm with great rapidity, limited only by the diffusion rate, but that the permeability of cells to ions is a more complex phenomenon and considerably more limited in its extent. The work of Anderson (1945) showing that pyruvic acid can pass into cells only in its undissociated form is good confirmation of this view.

Reducing agents also inhibit enzyme action. There is a class of enzymes known as disulfide enzymes which require for functioning the group S—S. Such enzymes are inactivated by reducing agents such as SO_2, H_2S, HCN, dithionate or formaldehyde. The unit in the enzyme protein sufficiently labile to reduction to account for the effect is probably in the amino acid, cystine. Reducing agents act by splitting the S—S linkage into S—H groups. The inactivation is generally not reversible since the split S—S groups would, upon mild oxidation, probably not unite in the

original positions to restore the enzyme activity. At least this was found to be the case in studies on the biological activity of insulin (du Vigneaud *et al.*, 1931). Inhibition of enzymes by such reducing agents as H_2S or HCN cannot be ascribed to the destruction of disulfide groups unless it has been ascertained that the enzyme in question does not contain a metallic prosthetic group which is sensitive to these reagents.

The phenolic hydroxyl group, essential for the activity of certain enzymes, is also sufficiently reactive to be attacked by various chemical agents. Numerous possibilities exist including any esterifying reagent and acylating agents. Herriott and Northrop (1934) observed that pepsin could be inactivated by acetylation of both the amino and phenolic groups with ketene and that the subsequent liberation of the phenolic hydroxyls by gentle hydrolysis restored the activity of the enzyme. Of the inhibitions caused by iodine, at least part are due to reaction with the hydroxyl in the tyrosine groups in the enzyme proteins. Herriott (1936) was able to isolate the iodinated tyrosine from pepsin inactivated by iodine. Similarly prolonged treatment with nitrite under acid conditions (Philpot and Small, 1938) attacks the OH groups and renders certain enzymes inactive although milder treatment with nitrite appears to bring about a reversible oxidation in addition to its well-known action on primary amino groups.

The chemicals which attack the amine and amide groups in enzyme proteins are usually nonspecific. Formaldehyde owes at least part of its action to union on amino or amide groups. Theis and Lams (1944) have studied the groups on proteins which are involved by formaldehyde and find that pH has an important influence upon the extent and nature of the reaction. Ethylene oxide and other epoxides react with amino groups as well as with sulfhydryl groups and phenolic hydroxyls. The enzyme chemists use ketene and carbon suboxide as reagents to detect the essential nature of amino groups in enzymes. Any amine reagent would presumably be effective. Carboxyl groups are also essential for activity for some enzymes and may be tied up by numerous chemicals. Epoxides such as ethylene oxide, propylene oxide, and epichlorohydrin were found by Fraenkel-Conrat (1944) to be suitable reagents for esterification of protein carboxyl groups in aqueous solutions and at room temperature. From this survey it is evident that the active groups on enzyme proteins react readily with many chemical substances and that in many cases such reactions destroy enzyme activity.

In addition to the inhibitors which react with the functional group of the enzyme proteins, there is a wide variety of compounds which interfere with the prosthetic groups of the enzymes. There may be (1) competition between the protein and the inhibitor for the prosthetic group, or (2) competition between the prosthetic group and the inhibitor for the

protein. Examples of the first method of inhibition include H_2S, HCN, CO, azide or hydroxylamine which react with the copper or iron containing prosthetic groups of the respiratory enzymes. The presence of such substances lowers the amount of active prosthetic groups available for union with the enzyme and consequently lowers the biological activity. Other examples are oxalate and fluoride which inhibit some Ca-requiring enzymes and citrate which inhibits some Mg-requiring enzymes. The second method for interfering with prosthetic groups is exemplified by the inhibition by Mg, of some enzymes requiring Ca as the prosthetic group, or the interference of certain flavin enzymes by acriflavin or atabrine.

Competition with the substrate for place on the active enzyme surface is a type of inhibition that has received much attention in recent years. The specific inhibition of enzyme action on one compound by the presence of a structurally related compound is explained by the existence of two separate phases in the process. First the enzyme combines with the substrate and second, the enzymic change takes place within the enzyme-substrate complex. Substances similar to the substrate in molecular configuration can combine with the enzyme but undergo no further change. Thus they inhibit the process by combining with the active groups of the enzyme. The extent of the inhibition depends on the proportion of the enzyme which thus can be rendered inert; this can be varied from no activity to maximum activity by varying the concentration ratio of inhibitor to substrate. Competitive inhibition is best exemplified by malonic acid ($COOHCH_2COOH$) which interferes competitively with succinic dehydrogenase in its oxidation of succinic acid ($COOHCH_2CH_2COOH$). Another example of competitive inhibition is H_2 gas competing with N_2 gas for space on the surface of the nitrogen-fixing enzyme of Azotobacter (Wyss and Wilson, 1941). Better known are the competitive inhibitions caused by sulfonamides and other analogues of vitamins or amino acids. In some cases enzymes that attack a compound having an *l*-configuration are inhibited by the presence of the optical isomer. A complete review of competitive inhibition by metabolite antagonists is presented by Roblin (1946).

Another type of enzyme inhibition is the interference with the forward progress of an enzyme-catalyzed reaction by the accumulation of the end products of the reaction. The effectiveness of such inhibition depends on the equilibrium conditions. It is possible in some cases to protect effectively a substrate from enzyme activity by the addition of an end product since the forward progress ceases when equilibrium is reached. Such a method is presumably of theoretical interest only, since the achievement of high concentrations of end product at the site of enzyme action would

be difficult to attain with living organisms. From natural substances there has been isolated a group of enzyme inhibitors whose action is not well understood. These substances are called antienzymes. Some of them appear to be protein hydrolysis products, *e.g.*, the anti-tryptic substance from egg white isolated by Balls and Swenson (1934). Others are proteins such as the trypsin inhibitor crystallized by Northrop (1939) and the pepsin inhibitor crystallized by Herriott (1941). These substances occur widely in nature. Mirsky (1944) found that the crystalline "trypsin inhibitor" as well as the antitrypsin of soybean origin (Ham and Sandstedt, 1944) inhibited the fibrinolytic activity of streptococci. Intestinal parasites contain substances which inhibit the action of proteolytic enzymes. A naturally occurring inhibitor of amylase action has been found in a number of cereals by Kneen and Sandstedt (1943). A fat soluble factor found in navy beans and believed to be present in many other foods strongly inhibits the activity of pancreatic amylase (Bowman, 1943).

V. Applications

The naturally occurring inhibitors are of especial interest to the food technologist as they explain the natural resistance of certain materials to autolytic enzymic destruction and undoubtedly offer some protection against the enzyme action of microorganisms. It appears likely that as more of these substances are studied and their action is better understood some may be developed that will prove useful in food preservation. Since such substances are already constituents of some foods, the problem of toxicity to the consumer may not be as formidable as in the case of other chemical agents. The many antibiotic agents which are being isolated and studied must also be considered for their possible usefulness in this field. The specificity of many of these substances does not necessarily exclude them since many food spoilage problems can be solved by restraining a single species. In this connection the synergistic action of combinations of the agents has not been studied.

Of the myriad of synthetic chemical agents known to have antibacterial action only a comparatively small number are of concern to the food technologist. In the interest of sanitation and during cleaning procedures he encounters the soaps and synthetic detergents. As pointed out these compounds destroy microorganisms by virtue of their action on the cell membranes and in high concentrations they also disturb the colloidal nature of the proteins. In this class we must also place the phenols and cresols, although killing curves with these substances offer strong evidence that in the limiting concentrations microbial death may be brought about by interference with the genetic mechanism involving cell division. Unpublished experiments from the author's laboratory indicate that the

chlorphenols also cause membrane destruction. The high activity of these compounds is due in part to their low water solubility and hence their tendency to concentrate in the lipoidal cell membrane. Strong oxidizing agents may also destroy the cell membrane, but it appears likely that

TABLE I

Mechanism of Action of Food Preservatives and Related Chemicals

Compound	Probable Action
Cationic and anionic surface active agents	Destruction of cell membrane Denaturation of enzyme proteins(?)
Phenols, chlorphenols, naphthol sulfonates, cinnamic acid	Destruction of cell membrane Reaction with protein in genetic mechanism(?).
Fatty acids, alcohols, and long chain aldehydes	Destruction of cell membrane Competitive inhibition of enzymes by short chain acids
Chloracetic acids	Membrane action. Competitive inhibition(?)
Benzoates, chlorbenzoate, hydroxybenzoate and its esters	Membrane action Competition with coenzyme
Salicylate	Membrane action. Competition with cozymase. Competitive interference with enzymic utilization of amino acids
Borates	Reaction with enzyme involved in phosphate metabolism
Sulfur dioxide, sodium sulfite or persulfite	Reaction with aldehyde formed in carbohydrate dissimilation. Reduction of S—S links in enzyme protein
Chlorine, chloramines, nitrogen trichloride, peroxides, nitrates, and other oxidizing agents	Destruction of sulfhydryl groups in enzyme protein (or gene protein(?))
Ethylene oxide and other epoxides	Reaction with carboxyl and other active groups on enzyme protein
	Oxidizing action
Fluorides, fluosilicates, and fluoborates	Interference with prosthetic groups Reaction with active groups on enzyme protein
Formaldehyde	Reaction with active groups on protein of enzyme (or gene?)
Salt	Precipitation of enzyme protein

inhibition of organisms by oxidizing agents, excepting such powerful agents as free chlorine, ozone and peroxide, is actually due to the oxidation of —SH enzymes to the inactive S—S form. Patents have been issued involving iodates and bromates for the suppression of the action of proteolytic enzymes.

Of the inhibiting agents used for food preservatives the fatty acids, alcohols, and aldehydes have received considerable attention. All these series are surface active and therefore are presumed to act by virtue of their

effect on the membrane. When tested in nutrient broth the activity of the succeeding members of the fatty acid series increases with chain length until their insolubility in water becomes the limiting factor. When tested in a minimal medium, however, without amino acids or vitamins, it is observed that acetic, propionic, and butyric acids are much more active than would be expected (Wyss, 1946; Wright and Skeggs, 1946). This enhanced activity is due to the competition with certain amino acids for space on the active enzymic groups. One of the enzymes competitively inhibited appears to function in the synthesis of pantothenic acid. The competitive substrates may be β-alanine, or in certain cases aspartic acid or glutamic acid or their breakdown products. From this one would infer that propionic acid, *e.g.*, would have a much greater inhibiting effect in bread than would be anticipated from laboratory tests using a nutrient medium and that this enhanced action would be less pronounced in a food material containing free amino acids or pantothenic acid. Wyss *et al.* (1945) observed that with the short-chain fatty acids a decrease in pH enhances activity to a much greater degree than is the case with the acids with longer chain lengths. This fits in with the hypothesis of interference with intracellular enzymes by short chain members of the fatty acid series. As pointed out above, the molecular form of acids passes through the cell membrane more readily than the ionic form. Since the dissociation constant for the 3-carbon propionic acid does not differ greatly from that of the 8-carbon caprylic acid, the differential pH effect indicates that the latter acid apparently does little damage on entering the cell beyond its effect on the cell membrane, while the former exhibits an additional intracellular inhibition if the antagonistic metabolites are not present in high concentration.

A similar situation exists when salicylic acid is employed as an inhibitor. Iványovics (1942a,b) reports that in the presence of protein breakdown products about 0.1 *M* salicylate is required for the inhibition of the growth of an organism which is inhibited by 0.00002 *M* salicylate in the absence of complex nitrogenous food. Under the latter conditions the salicylate, like the short-chain fatty acids, interferes with the use of certain amino acids for the synthesis of pantothenic acid. Stanley and Schlosser (1945) present data which indicate that it is the synthesis of the pantoate portion of the pantothenate molecule which is inhibited. Salicylic acid also inhibits cozymase-conditioned reactions (v. Euler and Ahlström, 1943). The action is not a specific removal of the coenzyme by salicylate but more likely a competition for the protein by cozymase and the inhibitor, or at least an apparent weakening of the affinity of the apozymase for cozymase. The studies with cozymase were done with isolated enzyme systems and it is likely that in the intact cell these enzymes will be pro-

tected except at pH values where the salicylic acid exists largely in its undissociated form (Rahn and Conn, 1944).

Benzoic acid also exhibits several types of action, one of which appears to be a competition with the coenzyme for the enzyme protein. In the case of glucose or lactic dehydrogenase the inhibition by benzoate is alleviated by the addition of coenzyme (v. Euler, 1942). Unpublished experiments from the author's laboratory show that a much greater activity can be demonstrated in a medium low in amino acids than is observed in nutrient broth, and that under certain conditions nicotinic acid interferes with inhibition by benzoate. In a rich medium the high concentrations required for inhibition appear to interfere with the functioning of the membrane, an interference which can be enhanced by certain substitutions in the molecule to increase its heteropolar nature. Some compounds which have been employed as preservatives and function in this manner are β-naphthol, its sulfonate, and various esters of benzoic acid.

Boric acid and borates are known to interfere with phosphate metabolism (Pfeiffer *et al.*, 1945). Borate assimilated in the animal body is tied up with glycerol or other polyhydroxy alcohols. Zittle (1947) demonstrated that at very low concentrations it inhibits the enzyme, phosphomonoesterase, obtained from intestinal mucosa. Whether this is due to the borate ion competing with the phosphate or is due to its union with the polysaccharide portion of a polysaccharide-protein complex is not evident. Fluorides also inhibit enzymes catalyzing the splitting of phosphate esters. Fluoride has been a useful tool in the elucidation of the mechanism of glycolysis as, in limiting concentrations, it is quite specific in action. At higher concentrations it inhibits a wide variety of enzymes probably by forming a reversible, inactive combination with the enzyme protein. Belfanti *et al.* (1935) report that calcium-requiring enzymes are inhibited by fluorides and oxalates which compete with the enzyme protein for the prosthetic group (calcium).

The action of sulfur dioxide in reducing S—S linkages which are essential for enzymatic activity has been mentioned. Under some circumstances the acidity of the sulfurous acid resulting from the solution of the gas is an important factor contributing to the inhibition. The compound is a powerful inhibitor of yeasts since at the low pH it can penetrate the cell and disrupt the normal progress of the alcohol fermentation. There the sulfite reacts with acetaldehyde making a combination which is not attacked by the enzyme and thus exhibiting another type of enzyme inhibition, *viz.*, a competition between the enzyme and the inhibitor for the substrate.

The highly reactive formaldehyde inactivates enzymes by attacking a number of the groups on the protein which may be essential for activity.

At pH values of 5 or below, it is fixed mainly in the acid amide and imino groups. Above pH 6 it attacks the nitrogen in the imidazole ring of histidine and above pH 9.5 it blocks the free amino groups of the lysine residues. At high pH values it also reacts with sulfhydryl groups. The concentrations of formaldehyde that are required for inactivation of organisms and enzyme systems vary widely. It is possible to obtain active invertase from yeast cells treated with 0.5% formaldehyde, a concentration which destroys the fermenting enzymes. In some cases the action appears to be partially reversed by the addition of oxidizing agents.

The chloracetic acids will effectively inhibit microbial growth if they are used at a low pH where they can pass through the cell membrane. There they react with sulfhydryl groups and thus inactivate enzymes. It is likely that they may, also, in a minimal medium, interfere competitively with enzymatic syntheses. Many of the compounds discussed in this section are not at present acceptable as food preservatives in this country, but they have been used recently in other countries and some are being used illegally in the United States.

The reaction of ethylene oxide with carboxyl and other active groups on proteins has been mentioned. Since this is not an acid, pH has little effect upon its penetration into the cell. In water solutions, ethylene oxide hydrolyzes to ethylene glycol which is relatively nontoxic for microbial cells. The longer chain epoxides, such as propylene oxide, hydrolyze even more rapidly. A thorough study of the action of epoxides against yeasts, molds, and bacteria, and their use in food preservation is now in progress (Whelton *et al.*, 1946). The completion of this work, which involves the effect of the agents on metabolic processes as well as its over-all effect on growth, should facilitate the interpretation of the mechanism of action of the epoxides.

VI. Summary and Conclusions

It is evident that the chemicals which have been employed as food preservatives fall into several general classes. The reactive compounds which destroy functioning groups on enzyme proteins must be relatively nonstable so that they do not persist in the food and poison the consumer. Compounds of this class include formaldehyde, sulfur dioxide, ethylene oxide and peroxides, and other oxidizing agents. As preservatives they give a good initial effect but their action soon disappears; they are useful to lower the initial microbial count and thus extend the storage life of food or assist the action of other preservative measures. It is essential that the decomposition products of such compounds be carefully defined under all possible conditions so that the possibility of toxic residues is eliminated. There is a distinct need for new effective agents of this type

to meet special requirements in the fresh food industries. Further research is needed on the epoxides and other types of oxidizing agents, especially gaseous types such as nitrogen trichloride and organic peroxides. A thorough study of reducing agents which decompose to nontoxic products would probably yield new additions to our short list of useful preservatives.

Persistent enzyme poisons such as borates and fluorides find little favor in the food industries since the enzymes which are inactivated are essential to the consumer as well as to the microorganism. The high concentrations required for microbial inhibition indicate that they can be successfully employed only in foods which are consumed as a very small part of the diet. Consideration must be given to the use of such substances under conditions where only small amounts are actually consumed. For example, they might be effectively employed in antiseptic ice. Under some conditions they may find use for impregnating the inedible portion of a food product such as fruit rinds or the superficial layer and even the bone in meat products. Substances which have a cumulative toxicity might be used during emergencies when the time of consumption is limited to a few days or weeks. An example of such a situation might be a military campaign or a camping expedition. The requirement of new agents of this type as well as of those presently available is a thorough understanding of all phases of their toxic action so that they can be employed with the same confidence that many toxic pharmaceuticals are now being used.

The chemicals which interfere with the membrane should prove useful in some instances, although some of the best agents of this type are barred because of obnoxious tastes and odors, their effect on texture, and because of additional physiological action. Certainly, of the hundreds of compounds of this type that have been prepared, some can be selected which are useful even though only under restricted conditions. With the exception of penicillin and related agents most of the chemicals which are said to destroy or inhibit microorganisms by affecting the genetic mechanism, are highly toxic to higher forms. The current emphasis on fundamental research on antibiotics should reveal information required for determining the usefulness of these products for food preservation. The competitive enzyme inhibitors such as propionic and benzoic acids appear to offer the greatest possibilities (salicylic acid is less suitable because of its additional physiological effects such as that on blood clotting). For successful food preservation these competitive inhibitors must be used for preserving foods whose content of the competing metabolite is low. Naturally they must have, at the concentrations consumed, no other marked physiological action, and other foods in the diet must contain sufficient amounts of the competing metabolite to prevent manifestations of the competitive inhibition in the consumer. Regardless of how thoroughly the theoretical as-

pects of the inhibition are understood their actual employment in food can be sanctioned only after extensive pharmacologic tests. From the tremendous number of metabolite antagonists which are being synthesized or studied it may eventually be possible to select other successful preservatives for many kinds of foods. It is possible that the best food preservative will involve the use of combinations of such agents to attack a complex anabolic enzyme system at several steps.

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