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The Cellulosome of *Clostridium thermocellum*

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I. Introduction

The accelerated interest in microbial cellulases stems from their potential industrial application. This area is an excellent example in which basic and applied science are closely interlinked: the better understood the mechanism of cellulase action, the better will be the applicative value as a biotechnological process. In spite of significant advances in the area, we are still far away from both the scientific and practical goals.

Although current concepts of cellulase action explain to a reasonable extent the phenomenon of cellulose degradation, new hypotheses have recently been introduced. These have been postulated due to the necessity to account for various physical features of cellulolytic organisms that cannot be explained by the nature of the purified extracellular enzymes. These include the observed adherence of various microbes and their cellulases to cellulose, and the extremely high affinity of the cell-associated system versus the cell-free system. In particular, the rate of cellulose catabolism by intact cells is in most systems significantly higher than that of the cell-free cellulase system. These observations have stimulated the formulation of new ideas on the mechanism of cellulose degradation.

In this review, we present a new concept concerning the mode of cellulose degradation. According to our findings (using the anaerobic thermophilic bacterium *Clostridium thermocellum* as a model organism), many of the required enzymes for efficient degradation of crystalline cellulose form a defined, multicomponent, high molecular weight complex which we have termed the *cellulosome*. In the model organism, the cellulosome is anchored to the cell surface in polycellulosomal centers, which also mediate the adherence of the bacterium to the insoluble substrate.

Biomass in the form of cellulose is the major constituent of plant matter, thereby comprising the most abundant organic resource in the world. As such it provides a very appealing renewable raw material for the production of food and energy. However, before using this resource, it is necessary to convert it into a more usable form such as gaseous (e.g., methane) or liquid (e.g., ethanol) feedstock by means of an economically feasible technological process.

One of the potential industrial approaches to biomass utilization is the enzyme-catalyzed hydrolysis of cellulose to soluble sugars that can in turn serve as substrates for fermentation to fuels and chemicals. This particular approach has been the subject of intensive research over the past several decades, and although some of the biochemistry concerning the enzymatic hydrolysis of cellulose has been clarified, the development of a viable biotechnological process has thus far been stymied. In order

to better understand the considerations involved in the design of such a process, it is instructive to review briefly some of the major chemical and structural features of cellulose as well as some of the important characteristics and historical findings concerning its degradation by cellulase enzymes.

A. Cellulose and Cellulases

Cellulose is composed of repeating units of cellobiose, 4-*O*-(β-D-glucopyranosyl)-D-glucopyranose, a simple disaccharide (Fig. 1). Despite its relatively simple primary structure, the tertiary (or quaternary) structure of cellulose is extremely complicated (Cowling and Kirk, 1976). The basic molecular structure is a linear polymer consisting of up to 10^4 D-glucose moieties which are arranged in fibrils. The fibrils consist of several parallel cellulose molecules stabilized by hydrogen bonds. Although individually the hydrogen bonds are relatively weak, collectively they become a strong associative force as the degree of polymerization increases. The fibrils are organized into a "paracrystalline" state, thus adding to the structural rigidity of cellulose. Over three-quarters of the cellulose structure is considered to exist in these enzymatically resistant, crystalline regions and the remainder comprises the relatively easily hydrolyzable "amorphous" areas.

The utilization of cellulose as a substrate for bioconversion processes is determined by its susceptibility to cellulase enzymes, and the availability of high activity enzymes is the basis for the design of a successful process for the enzymatic conversion of cellulose. However, the action of cellulases on cellulose is not a straightforward phenomenon, and the quality of high activity with reference to cellulase must be further discussed.

The enzymatic hydrolysis of cellulose is a heterogeneous reaction system which involves either water-soluble, particulate, or cell-bound enzymes attacking a water-insoluble substrate. Due to the complicated

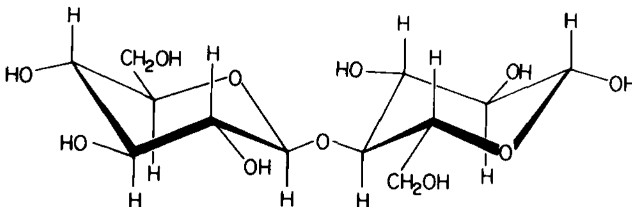

FIG. 1. The structure of cellobiose, the repeating unit of the cellulose polymer.

substrate structure, enzymatic hydrolysis of cellulose to simple sugars is equally complicated. A single enzyme cannot accomplish the task of extensive cellulose degradation, and multiple enzyme systems are required. Consequently, microorganisms that successfully grow on cellulose as a substrate are capable of doing so by producing a collection of different cellulases [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4] including endoglucanases and exoglucanases [exo-cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)] which act cooperatively to hydrolyze the intricate structure of the cellulose matrix.

The overall facility with which a particular enzyme system acts on cellulose may depend upon many factors, including the following:

1. The recognition and binding of the soluble, particulate, or cell-bound enzyme onto cellulose.
2. The diffusion into or motility of the cellulases on the solid cellulosic matrix.
3. The hydrolytic reactions necessary to form soluble sugars.
4. The relative efficiency of hydrolysis of crystalline regions or the capacity to converty crystalline regions to amorphous regions.
5. Product inhibition characteristics of the various cellulase enzymes.

The final industrial design may take into account other factors, which are not directly related to cellulose hydrolysis, such as the following:

1. Subsequent diffusion of the soluble degradation products from the cellulose matrix.
2. Catabolic conversion of soluble sugars to other desirable fermentation products, e.g., ethanol, acetone, acetic acid, butanol.
3. Tolerance of these enzymes and/or of the cells to high levels of these products.

Thus, in considering an appropriate enzyme system for potential industrial saccharification of cellulose in the classical sense, a stable cell-free enzyme preparation with adequate levels of all essential components of the enzyme is required.

B. Mechanism of Cellulase Action

A mechanism for the conversion of native cellulose to soluble sugars was first suggested by Reese and colleagues (1950). Their model was based on a two-step sequential process which was characterized by a C_1 component and a group of components collectively referred to as C_x.

The C_1 component was considered to consist of a "defibrillating factor" which severed the hydrogen bonds necessary to form the crystalline

structure of cellulose. Initially, the hydrolysis of covalent bonds was not attributed to the C_1 component. Although evidence has been accumulated for the existence of this component type in the multimembered cellulase enzyme system of "true" cellulolytic microorganisms, the specificity of the C_1 component(s) is still in controversy. Various laboratories (Halliwell and Griffin, 1973; Wood and McCrae, 1979) have isolated C_1 activity which was identified as a cellobiohydrolase. On the basis of these findings, Reese's original position concerning the C_1 was modified, and in later publications the C_1 component was also considered to have the capacity to degrade portions of cellulose (Reese, 1976). This particular controversy has not been settled and may eventually prove superfluous.

In any event, in the original hypothesis, the C_1 component was envisaged to cause first the alteration or "activation" of the cellulose structure, which then enabled further hydrolysis by the C_x enzyme(s). The latter were considered to comprise cellulolytic enzymes (both endo- and exoglucanases) in the conventional sense where covalent bonds are broken.

The original hypothesis was important for many reasons. First, it served as a working model which stimulated a wide variety of subsequent studies. It also served to describe on the molecular level many of the observed phenomena associated with cellulase hydrolysis of cellulose. For example, the interplay between the C_1 and the C_x components offered a reasonable explanation for the synergy among cellulase components. Also, according to the hypothesis, microorganisms that grow on soluble cellulose derivatives would synthesize only C_x components, whereas microorganisms that grow on highly ordered forms of cellulose would produce both C_1 and C_x.

More recent evidence strongly supports the mechanism of enzymatic degradation first suggested by Eriksson (1969). According to this mechanism, amorphous regions in the cellulose fiber are first attacked by endoglucanases, thereby exposing free chain ends. Exoglucanases then come into action by hydrolytically removing cellobiose from the chain ends. The free cellobiose is hydrolyzed further to glucose through the action of β-glucosidase(s).

The reigning concept now states that endoglucanases act randomly over the cellulose chain and that exoglucanases act on exposed chain ends, the endo- and exoglucanases having a strong synergistic action. Though synergism between exo- and endoglucanases has clearly been established, extensive hydrolysis of more crystalline forms of cellulose has been shown to be accomplished only by certain pairs of hydrolytic enzymes (Wood and McCrae, 1979). These pairs of endo- and exoglucanases have been suggested to form loose complexes on the cellulose surface. Such a phenomenon might be necessary to allow for rapid sequential action of

these enzymes, although simultaneous action of exo- and endoglucanases may also occur. Complex formation and consequent degradation within crystalline regions would prevent potential reformation of the glycosidic bond after single-point cellulase-induced cleavage. Despite attempts to demonstrate directly by electron micoscopy the presence of such complexes attached to cellulose fibers (White and Brown, 1981), definitive proof awaits further experimentation.

The phenomenon of product inhibition is noteworthy to mention in this context. Cellobiose has been implicated as a potent inhibitor of many cellulolytic systems. The operational activity of these systems can be increased by increasing the cellobiase (β-glucosidase) activity which converts cellobiose to the less inhibitory glucose.

C. Microbial Degradation of Cellulose

The mechanism described above was derived mainly from studies on aerobic fungi such as *Trichoderma* and *Sporotrichum* species. Relatively little has been done with other cellulolytic systems, notably those of anaerobic bacteria, although it has been assumed (perhaps without sufficient basis) that the same principles hold.

Indeed, many fungi and bacteria are known that can degrade cellulose (Table I). However, in many cases, the products of growth are microbial cells and various metabolic products which at present are not industrially applicable.

Simple growth of a successful cellulolytic organism on cellulose will produce a variety of products, some of which are potentially useful industrially and others are not. For example, among the preferred bacterial products are soluble sugars, ethanol, acetone, butanol, butanediol, and the like. Among the undesirable products are acetic acid, lactic acid, and other organic acids which are difficult to separate and process further. In addition, only a few fungal and bacterial species have been reported that produce high activity cellulases capable of extensively degrading insoluble cellulose to soluble sugars *in vitro*. In this context, many organisms are known to degrade only soluble forms of cellulose such as carboxymethylcellulose. Moreover, many organisms do not produce large amounts of cellulases in the soluble form despite the fact that they grow effectively on insoluble cellulose, the degradation of which must take place extracellularly.

One microorganism which has been considered seriously for potential industrial application is *Clostridium thermocellum*, an anaerobic thermophilic cellulolytic bacterium, which we have used as a model organism in our investigations. In the remaining sections of the review, we trace the historical and contemporary studies that have led to the

TABLE I

SOME REPRESENTATIVE CELLULOLYTIC MICROORGANISMS

Microorganism	Reference
BACTERIA	
Anaerobic strains	
Gram positive	
Ruminococcus albus	Sijpesteijn (1949); Hungate (1947)
Ruminococcus flavefaciens	Sijpesteijn (1951)
Eubacterium cellulosolvens	Holdeman and Moore (1972)
Clostridium cellulovorans	Sleat *et al.* (1984)
Clostridium stercorarium	Madden (1983)
Clostridium thermocellum	Viljoen *et al.* (1926)
Gram negative	
Bacteroides cellulosolvens	Guiliano and Khan (1984); Murray *et al.* (1984)
Bacteroides succinogenes	Hungate (1950)
Acetivibrio cellulolyticus	Patel *et al.* (1980); Saddler and Khan (1980)
Aerobic strains	
Gram positive	
Bacillus sp.	Robson and Chambliss (1984)
Streptomyces flavogriseus	Ishaque and Kluepfel (1980)
Cellulomonas fimi	Whittle *et al.* (1982)
Thermomonospora sp.	Henssen (1957)
Gram negative	
Cellvibrio gilvus	Cole and King (1964); Carpenter and Barnett (1967); Breuil and Kushner (1976)
Cellvibrio fulvus	Mullings and Parish (1984); Berg *et al.* (1972a,b)
Pseudomonas fluorescens var. *cellulosa*	Yamane *et al.* (1965)
FUNGI	
Anaerobic strain	
Neocallimastix frontalis (Phycomycetes)	Orpin (1975); Bauchop (1979); Mountfort and Asher (1985)
Aerobic strains	
Trichoderma reesei	Montenecourt (1983)
Sporotrichum pulverulentum	Eriksson (1979)
Myrothecium verrucaria	Halliwell (1961)
Penicillium iriense	Boretti *et al.* (1973)
Phanerochaete chrysosporium	Eriksson (1981)

formulation of the cellulosome concept. The characterization of the cellulosome in C. *thermocellum* is treated in detail, and recent advances using other cellulolytic systems are discussed within the framework of this model.

II. *Clostridium thermocellum*

A. Isolation of the Organism and Catabolic Steps

A species of C. *thermocellum* capable of fermenting cellulose to ethanol was first described by Viljoen *et al.* (1926). However, stable pure cultures of anaerobic thermophilic cellulolytic bacteria were initially isolated by McBee (1948, 1950) and later by several other investigators. Although the cellulolytic properties of several of the latter strains remain poorly defined, many are similar to the C. *thermocellum* strains described by McBee (1950). Certain physiological features of C. *thermocellum* have been sources for conflicting reports. For example, McBee (1948, 1950) was unable to grow C. *thermocellum* on glucose while Patni and Alexander (1971) obtained good growth of the same organism on either glucose or xylose. These authors also demonstrated the presence of an inducible hexokinase along with some other glycolytic enzymes. Later, Lee and Blackburn (1975) described the cellulolytic properties of an isolate similar to C. *thermocellulaseum* (this strain is currently considered a derivative of C. *thermocellum*) that proliferated on cellulose, glucose, and numerous other mono- and disaccharides.

Ng *et al.* (1977) described several isolates which grew on cellulose and its degradation products, but not on glucose. Lamed and Zeikus extensively studied the catabolic pathways of C. *thermocellum* and identified some unusual enzymes including a regulatory alcohol dehydrogenase (Lamed and Zeikus, 1980) and an ammonium-activated malic dehydrogenase (Lamed and Zeikus, 1981).

One of the distinctive features of C. *thermocellum* is its production of very high levels of an extracellular cellulase system. The extracellular endoglucanase activity was found to be constitutive, namely, independent of whether the cells were grown on cellulose, cellobiose, or glucose (Garcia-Martinez *et al.*, 1980; Lamed and Zeikus, 1980). Growth of C. *thermocellum* on a soluble substrate such as cellobiose was more rapid than growth on the insoluble substrate (cellulose), indicating that the solubilization of cellulose is a rate-limiting step for growth. Of note is a report (Ng *et al.*, 1977) that the specific activity of both endoglucanase and exoglucanase remains constant throughout the course of fermentation, suggesting the growth-linked production of extracellular cellulase.

Transport systems for both cellobiose and glucose are present in *C. thermocellum*. Glucose is apparently transported in this organism by an ATP-dependent permease only in glucose-adapted cells (Hernandez, 1982), whereas cellobiose is incorporated by both glucose- and cellobiose-grown cells (Ng and Zeikus, 1982). One plausible explanation for the observed phosphorylation of the residual glucose moiety is that cellobiose transport may be linked to phosphorylation by membrane-bound enzymes (both cellobiose phosphorylase and a hypothetical hexokinase, for example) which together funnel the phosphorylation reaction to completion.

The enzymatic pathways in *C. thermocellum* responsible for the catabolism of cellulose degradation products have not been entirely elucidated. The lack of clarity, especially regarding the initial steps of transport and activation of soluble sugars, may be due to the fact that, among the published works, a variety of different strains were used. In some cases, the cultures may not have been entirely pure.

In most cellulolytic microorganisms, cellobiose is cleaved by β-glucosidases to glucose. In some cellulolytic bacteria, however (e.g., *Cellvibrio gilvus* and in particular *C. thermocellum*), cellobiose is converted into glucose 1-phosphate and glucose by the enzyme cellobiose phosphorylase (Alexander, 1968, 1972; Swisher *et al.*, 1964). The latter mechanism causes the bacterium to exhibit preferential growth on cellobiose (versus glucose) as an energy source, perhaps due to conservation of the energy contained in the glycosidic linkage of cellobiose. Another possible explanation for the preference for cellobiose is the low level (Hernandez, 1982) or lack (Lamed and Zeikus, 1980) of soluble glucokinase in this organism. If hexokinase is indeed absent, it remains unclear how one of the glucose units in cellobiose becomes phosphorylated (Ng and Zeikus, 1982), since cellobiose phosphorylase activity which has been demonstrated in *C. thermocellum* gives glucose 1-phosphate and glucose as products (Alexander, 1968). An isomerase converts glucose 1-phosphate to glucose 6-phosphate. Both glucose units, however, were shown to be metabolized via glucose 6-phosphate, in spite of the absence of demonstrable levels of hexokinase (Ng and Zeikus, 1982) and phosphoenolpyruvate phosphotransferase system (Hernandez, 1982; Lamed and Zeikus, 1980).

B. Industrial Potential

The advantages and disadvantages of anaerobic thermophilic fermentation, with particular relevance to ethanol production, have been reviewed recently (Sonnleitner and Fiechter, 1983; Esser and Karsch, 1984; Slapack *et al.*, 1986). On the positive side, the application of a thermophilic organism would reduce energy costs required for refrigeration of fermentors during culture of mesophilic organisms; circulating tap

water is sufficient for temperature maintenance in thermophilic cultures. In addition, fermentation under anaerobic conditions would obviate problematic and costly aeration procedures. Moreover, thermophiles are capable of fermenting a broad range of substrates, product distillation costs are less expensive, the danger of contamination (particularly by pathogens) is low, and the thermophilic enzymes produced are unusually stable to a variety of harsh conditions.

These advantages are offset by a spectrum of limiting factors which have curbed the industrial application of anaerobic thermophilic strains. These organisms usually exhibit relatively low productivity and low tolerance to high concentrations of desired products (such as ethanol). Fermentation at high temperatures is deleterious to biotechnological equipment, causing extensive deterioration of biosensors and the leaching of inhibitory trace metals from fermentation vessels. Consequently, there are serious problems in scale-up procedures and in the industrial applicability of these relatively uncharacterized strains.

Despite these limitations, various potential strategies have been considered in order to obtain desired products from cellulose. One approach would be to apply either a crude or purified cellulase preparation to obtain soluble sugars which can, in turn, serve as substrates for further fermentations. A recently described variation of this approach was the use of a resting cell suspension capable of cellulose degradation (Giuliano and Khan, 1985). An alternative approach would be the direct fermentation of cellulose to preferred products. This approach would theoretically use a cellulolytic organism that effectively produces high levels of a desired product under optimal conditions. Such a strain has yet to be described, and for this reason the application of cocultures has been considered.

Coculture fermentation involves the growth of two or more compatible and complementary organisms, one capable of effective cellulolysis and the second (or additional strains) capable of competing successfully for the resulting sugars, thereby producing a preferred product(s) which predominates (Zeikus *et al.*, 1983). A combination of the two above-described approaches has recently been described and has been called simultaneous saccharification fermentation (Ooshima *et al.*, 1985). Using this procedure, cellulose is hydrolyzed enzymatically via a given cellulase system, and the resultant sugars are then fermented by a suitable microorganism (i.e., bacteria or yeast). A related, but potentially more elegant, approach would be to genetically incorporate the essential cellulase enzymes into the sugar-fermenting bacterium or yeast.

C. Strategies Involving *C. thermocellum*

The concept of direct cellulose fermentation to ethanol has been studied actively by several research groups. As mentioned above, in this approach soluble sugars produced by extracellular cellulolysis would be

assimilated secondarily by a high ethanol-yielding organism which is grown either in coculture or in succession with the cellulolytic species (the latter having an unfavorable product pattern). The idea behind this approach is based on the successful modification of the product pattern of *C. thermocellum* from mixed acid–ethanol production to almost strictly ethanolic fermentation when another clostridial species with high ethanol yield is cocultured.

In this context, *C. thermocellum* has been studied specifically together in culture with either *Clostridium thermohydrosulfuricum* (Ng *et al.*, 1981) or *Clostridium thermosaccharolyticum* (Avgerinos and Wang, 1980; Wang *et al.*, 1983; Saddler and Chan, 1984). Despite the fact that theoretical ethanol yields were attained in these systems, the approach was hampered by the problem of adapting *C. thermocellum* to high ethanol concentrations (4–5%). In addition, difficulties were encountered in fermenting cellulose levels greater than a few percent of the insoluble substrate. This was even more striking when natural substrates (corn stover, wood shavings, etc.) were fermented. Furthermore, toxic substances were produced which adversely affected growth and product pattern in pretreated corn stover. The latter was studied as a substrate in the "MIT process" with genetically improved *C. thermocellum* and *C. thermosaccharolyticum* (Leuschner *et al.*, 1983). Attempts to recycle cellulase also failed to improve the process significantly, perhaps due to the "large molecular nature" of the cellulase of this organism (see following section).

There have also been attempts to isolate wild-type or mutant strains of *C. thermocellum* exhibiting improved ethanol production or improved adaptability to high concentrations of ethanol (Herrero and Gomez, 1980). Unfortunately, several of these strains (which were selected during growth on pure cellulose and which produced lower levels of undesirable organic acids in small-scale experiments) behaved differently upon fermentation in larger vessels or upon growth on "natural" substrates. For example lignin, which is not degradable under anaerobic conditions, severely interferes with cellulose degradation. In addition, the property of resistance to ethanol concentrations greater than 2% is difficult to acquire and the trait is usually genetically unstable.

D. "TRUE" CELLULASE IN *C. thermocellum*

The ability of *C. thermocellum* to grow efficiently on α-crystalline cellulose has long been recognized (McBee, 1948). "True" cellulase activity, the ability to dissolve crystalline cellulose completely (Johnson *et al.*, 1982b), has also been demonstrated in cell-free preparations, and this feature is probably the major reason why *C. thermocellum* remains such an attractive organism. Such activity has been shown to depend

upon the presence of Ca^{2+} and thiols and has been shown to be comparable on a volume-to-volume basis to the growth supernatant of *Trichoderma reesei*. Moreover, the specific activity of *C. thermocellum* true cellulase, as measured by the degradation of native cotton cellulose (a substrate representative of high crystallinity), was 50 times higher than that of *T. reesei*. However, the rate of bacterial degradation of swollen cellulose (phorsphoric acid treated, yielding a cellulose preparation of low crystallinity) was slower than that of *T. reesei* cellulase. These results indicate that the cellulase system of *C. thermocellum* is superior to that of *T. reesei* when "native" or highly crystalline cellulose is used as a substrate, but the action of the fungal enzyme system on cellulose of low crystallinity excels that of the bacterial cellulase.

As in other cellulolytic organisms, the cellulase system from *C. thermocellum* is strongly inhibited by cellobiose when acting on microcrystalline cellulose (Johnson *et al.*, 1982a). The observed product inhibition was shown to be less pronounced for amorphous substrates, and the enzyme complex was essentially resistant to inhibition when trinitrophenylcarboxymethylcellulose was used as a substrate (Shinmyo *et al.*, 1979). Glucose is much less inhibitory for the microcrystalline substrate.

E. Purification and Cloning of Endoglucanases

Although for many years various researchers have tried to resolve biochemically the components of the cellulase complex in *C. thermocellum*, attempts in this direction have not been particularly successful. Following separation of crude cellulase by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), many protein bands have been observed consistently, several of which were reported to express endoglucanase activity (Ait *et al.*, 1979; Beguin, 1983). However, the purification of only two endoglucanases from the fermentation broth of *C. thermocellum* has been described. In these studies, much emphasis has been devoted to the isolation of separate component(s), preferably a defined polypeptide chain.

Ng and Zeikus (1981) purified an endoglucanase responsible for part of the cellulolytic activity with an apparent molecular weight of 94,000. Their preparation contained 11% carbohydrate. The activity of the purified enzyme was characterized using cellodextrins. The activity decreased twofold upon reducing the length of the substrate from cellohexaose to cellopentaose and about 15-fold from cellopentaose to cellotetraose. The cellulase activity on carboxymethylcellulose was three times higher than that observed for cellohexaose as substrate.

The difficulties encountered in isolating to purity single polypeptide chains that possess cellulolytic activity prompted the application of harsh

chromatographic conditions aimed at diminishing aggregation of multiple protein components. Thus, one of the major *C. thermocellum* endoglucanases was purified to apparent purity by Petre *et al.*, (1981) using a procedure involving ion-exchange chromatography in the presence of 8 *M* urea. The molecular weight of this endoglucanase was 56,000. The major product obtained from the action of the purified endoglucanase on acid-treated cellulose was cellotriose. Antibodies against this purified cellulase were prepared and have been used as a tool to screen *Escherichia coli* clones that express the corresponding *cel A* gene encoding for the 56-kDa endoglucanase (Cornet *et al.*, 1983a,b). The nucleotide sequence of the *cel A* gene was determined and compared with the NH_2-terminal amino acid sequence of the purified enzyme (Beguin *et al.*, 1985). A signal sequence of 32 amino acids was proposed. A duplication of a sequence of 23 amino acids at the COOH terminal was suggested to relate to sugar binding.

A second endoglucanase, the *cel B* gene product, was detected in another *E. coli* clone by screening for endoglucanase activity (Cornet *et al.*, 1983a,b; Beguin *et al.*, 1983). The enzyme was purified from the *E. coli* cells; two active bands were obtained having molecular weights of 53,000 and 55,000. Antibodies raised against the *cel B* gene product reacted with components in the fermentation broth of *C. thermocellum*.

This general approach can thus serve as a complementary strategy to conventional biochemical studies of the cellulase system in *C. thermocellum*. Indeed, partial genomic banks have been constructed in *E. coli* and screened for either endoglucanase or cellobiohydrolase-like activity using carboxymethylcellulose and methylumbelliferyl-β-cellobioside, respectively. Five additional endoglucanase clones were found as well as three others that appear to encode for cellobiohydrolases (Millet *et al.*, 1985). From these studies it is evident that *C. thermocellum* DNA is very efficiently expressed in *E. coli*. Moreover, a very intriguing finding is that about a third of the *C. thermocellum* genome appears to code for cellulases. There is no doubt concerning the potential applicative value in cloning cellulase genes either into *C. thermocellum* or into other suitable host organisms which would then become very effective cellulase producers.

III. The Cellulosome Concept

A. Culture Stirring

During the course of our previous studies on the direct fermentation of cellulose to ethanol by *C. thermocellum* cultures and cocultures, much effort was invested in exploring the various parameters affecting the product pattern, i.e., the relative amounts of the various products. The

goal of these studies was to develop a system whereby very high concentrations of ethanol would be produced by a stable cell system in a reproducible manner.

Another approach we and others have taken was to select for wild-type or mutant strains that produce a higher proportion of ethanol compared to acetic and lactic acids. At the General Electric Research Laboratories, the *C. thermocellum* YS strain was selected, since this isolate produced several times more ethanol than acetate as final product. The YS strain was originally isolated from soil samples obtained at the hot springs of Yellowstone National Park.

At the time, small-scale laboratory growth procedures were carried out in sealed anaerobic serum bottles without stirring. When these cultures were transferred to controlled fermentation conditions (with stirring), it was somewhat disappointing to discover that the ratio of ethanol to acetate decreased dramatically (sometimes four- to five-fold). In light of the above finding, it was suspected that stirrring the *C. thermocellum* culture may interfere with cellulose degradation. Surprisingly, the opposite effect was found (Su *et al.*, 1981a,b) as can be seen in Table II. The end product formation rate was increased by stirring, as reflected by the enhanced formation of acetate. This result was quite puzzling since it could not result from detachment of cells loosely associated with cellulose, nor by the "dilution" of the extracellular cellulase in the entire volume of the broth. The increased fermentation rate generated by stirring, however, was consistent with a very tight binding of both the cells and their cellulases to the particulate substrate. Another potential cause for the commonly observed stimulation in fermentation rates could have been the enhanced diffusion (by stirring) of toxic products away from the bacterial cells.

The reason for the increase of the acetate to ethanol ratio in stirred growth was eventually identified (Su *et al.*, 1981a,b). It was independent

TABLE II

EFFECT OF STIRRING ON CELLULOSE FERMENTATION BY *C. thermocellum*

Time of culture (hours)	Stirred (μmol)			Unstirred (μmol)		
	Acetate	Ethanol[a]	H_2	Acetate	Ethanol[a]	H_2
10	120	70	210	70	70	130
13	150	110	300	90	110	170
16	180	160	350	100	150	200
22	200	180	380	110	200	220

[a]Lactate formed corresponded to about 10% of the ethanol formed at each point on a molar basis. The initial amount of cellobiose was 290 μmol.

of whether soluble cellobiose or insoluble cellulose served as carbon source. Stirring interferes with the buildup of supersaturation levels of catabolically secreted molecular hydrogen which is stimulatory for production of ethanol (the more reduced product). The required catabolic enzyme machinery to account for the effect was previously shown to be present in the cells (Lamed and Zeikus, 1980). The stirring effect was found to be a general feature in several other fermentations (Su *et al.*, 1981b) including acetone-butanol fermentations (Doremus *et al.*, 1985).

B. ADHERENCE OF *C. thermocellum* TO CELLULOSE

One of the interesting microscopic observations to come out of the study described in the previous section was that cells of *C. thermocellum* adhere closely to the cellulose substrate prior to extensive cellulolysis. An electron micrograph of adherent *C. thermocellum* cells to amorphous cellulose is shown in Fig. 2A. Indeed, the adherence of a wide variety of bacteria to various insoluble target substrates (including host cell

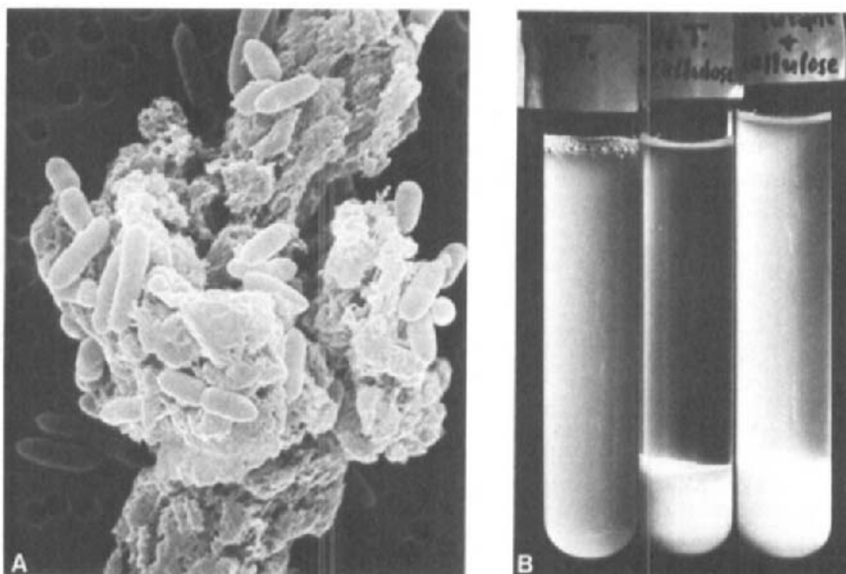

FIG. 2. Adherence of *C. thermocellum* to cellulose. (A) Scanning electron micrograph of *C. thermocellum* cells attached to a particle of amorphous cellulose. (B) Three adjacent test tubes showing (from left to right) a suspension of wild-type *C. thermocellum* strain YS cells, a similar suspension to which insoluble cellulose has been added, and a suspension of cellobiose-grown mutant AD2 cells with added cellulose. Note that the cellulose has largely cleared the solution from wild-type cells (middle tube) but has *failed to do* so with the mutant (right tube). (Reproduced by permission from Bayer *et al.*, 1983.)

tissues) bears general physiological and ecological significance and is considered to be a key factor in the further utilization of these substances (Ofek and Beachey, 1980; Sharon *et al.*, 1981). In this context, Minato and Suto (1978) have used the property of adherence to cellulose as a criterion for the fractionation of rumen bacteria, a phenomenon which was later studied by electron microscopy in more detail (Akin and Barton, 1983). Using our initial observation as a focal point, we decided to investigate further the adherence phenomenon with the intention of eventually isolating an adherence factor (Bayer *et al.*, 1983).

The adherence phenomenon was found to be specific for cellulose, since cells of this organism failed to attach specifically to various other uncharged polymers, including insoluble polysaccharides (Table III). Adherence to cellulose was unaffected by the presence of salts, mild detergents, and changes in pH (between 4.0 and 9.5). On the other hand, positively charged insoluble polymers adsorbed the cells, probably due, for the most part, to electrostatic interaction which could be counteracted by salt.

Numerous monosaccharides and polysaccharides, including various cellulose derivatives and degradation products, failed to inhibit cellular adherence to cellulose (Table IV). Various other natural and synthetic polymers, detergents, and salts were noninhibitory to adherence even at very high concentrations. Only polyethyleneimine was found to interfere significantly with the adherence. The exact nature of this interference is as yet unknown since polylysine (another positively charged synthetic macromolecule) failed to affect the observed adherence. The only other

TABLE III

ADHERENCE OF *C. thermocellum* TO VARIOUS INSOLUBLE MATRICES

Adhering matrices[a]	Nonadhering matrices
Cellulose	Sephadex G-100
Amorphous cellulose[b]	Sepharose 4B
Polyethyleneiminecellulose	Carboxymethylcellulose
DEAE-cellulose[c]	Carboxymethylcellulose hydrazide
Dowex-1[c]	Cellulose acetate
Polystyrene surfaces	Starch
CAB-O-SIL EH-5	Polyvinyl alcohol
Bentonite	Silica
Hydroxyapatite	Alumina
Charcoal	Polyvinylpyrrolidone
	Celite 545

[a]Adhering matrices were defined as those capable of reducing more than 50% of the initial A_{400} of the cell culture.

[b]Phosphoric acid-swollen cellulose.

[c]Adherence to these substances was salt sensitive (inhibited by 1 *M* NaCl).

TABLE IV

INHIBITION OF ADHERENCE OF *C. thermocellum* TO CELLULOSE

Noninhibiting substances[a]	Highest concentration tested (%)
Glucose	10
α-Methylglucopyranoside	1
β-Methylglucopyranoside	1
Cellobiose	4
Methylcellulose	0.3
Carboxymethylcellulose (low viscosity)	0.5
Hydroxyethylcellulose (low viscosity)	0.5
Glycogen	1
Dextran sulfate	1
Soluble starch	0.5
Polyvinyl alcohol	3
Polyethylenemaleic acid	1
Triton X-100	1
Sodium chloride	8
Poly-L-lysine	1.5
Effective inhibitor[b]	**Inhibitory concentration (%)**
Polyethyleneimine	0.5
Purified cellulosome	0.01

[a]Defined as those substances that had an inhibiting effect of less than 10% on the standard cellulose assay.

[b]At the reported concentration over 90% inhibition of the bacterial adherence to cellulose was observed.

inhibitory substance was the purified cellulosome (see below), which effectively prevented cellular adherence even at very low concentrations.

C. MUTANT SELECTION

In order to study further the interaction of *C. thermocellum* cells with their insoluble cellulosic substrate, a combined genetic–immunochemical approach was undertaken (Bayer *et al.*, 1983). After the initial hypothesis that the observed adherence of the organism to cellulose is an important primary event, we proceeded to isolate an adherence-defective mutant.

A spontaneous mutant was isolated from the wild-type strain YS by an enrichment procedure which included repetitive cycles of growth on cellobiose and selective removal of adhering bacteria. A single colony isolated by a roll bottle technique was defined as adherence-defective mutant AD2. As shown in Fig. 2B, cellobiose-grown mutant cells fail to adhere to cellulose, in contrast to the wild-type cells. A turbidity-based adherence assay was developed based upon this observation.

Rabbits were then immunized intravenously with washed intact wild-type cells that had been cultured on cellobiose-containing medium to the mid-exponential phase of growth. The antiserum was precipitated with ammonium sulfate to obtain the immunoglobulin fraction.

In order to render the antibody preparation specific for the putative adherence factor, antibodies common to the cell surfaces of both the mutant and wild type were removed from the reference (anti-whole cell) antibody preparation by adsorption onto cellobiose-grown mutant cells. Using this method, antigens common to the surfaces of both cell types should be removed from the preparation; only the antigen(s) specific to the wild-type cell should be related to the cellular function present in the wild type but defective in the mutant (i.e., in this case, the attachment of the cells to cellulose). One of the major assumptions in using this technique is that the antigen is associated with the cell surface of the wild-type cell. It is also desirable that the antigen in question is missing on the mutant cell surface although there are other possibilities. For example, the antigen can be present but masked physically by other surface components (such as a capsule). For practical purposes, however, the fact that adherence between *C. thermocellum* and cellulose is (perhaps by definition) a surface phenomenon emphasizes the validity of this approach. In any event, this was proven experimentally as shown later.

Interaction of the antibody preparations with the cells caused agglutination of the latter. The antibody-induced agglutination was followed turbidometrically as shown in Fig. 3, where the agglutinability characteristics of the parent strain are compared with those of the

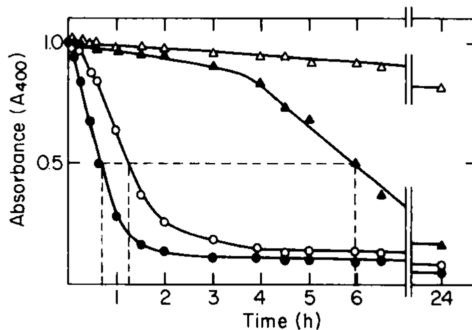

FIG. 3. Antibody-induced agglutinability of wild-type (YS) and mutant AD2 cells. Exponential-phase cells of the wild type (•,▲) and mutant (○,△) were incubated with 0.2 mg/ml [final protein concentration of either the reference antibodies (circles) or the cellulosome-specific antibodies (triangles)].

adherence-defective mutant (AD2). As expected, the anti-whole cell antibody preparation presumably recognized a variety of agglutinogenic species on both the parent and the mutant surfaces, since both cell types agglutinated strongly in the presence of this antibody. Agglutinogenic components are also located on the cell surface of mutant AD2, although to a lesser extent as expressed by the different rates of agglutination. As expected, the mutant-adsorbed antibody failed to agglutinate mutant AD2 cells.

D. The Cellulose-Binding Factor (CBF)

Several arguments support the existence of a distinct CBF in *C. thermocellum*. The isolation of an adherence-defective mutant and production of a specific antibody (which distinguishes between cells of the mutant and wild-type strains) suggest the existence of a cell-associated factor responsible for the specific adherence of the bacterium to cellulose. This contention was corroborated further by crossed and rocket immunoelectrophoretic analysis of sonic extracts of both wild-type and mutant cells. Eventually the CBF was isolated and characterized as presented in the ensuing sections. As mentioned in Section III,B, the purified CBF binds tightly to cellulose and effectively inhibits cell adherence.

E. Association of Endoglucanase Activity with the CBF

To determine the possible connection between the anti-CBF antigen activity and cellulolytic activity, rocket immunoelectrophoresis was combined with a gel-overlay technique for qualitative examination of enzymatic carboxymethylcellulase activity directly on the gel (Fig. 4). The CBF peak appeared in the intermediate gel precipitated by the CBF-specific (AD2-adsorbed) antibody. Enzymatic activity was observed as a clearing where the carboxymethylcellulose substrate was hydrolyzed (and therefore not precipitated by isopropanol treatment of the gel). Most of the cellulolytic activity was associated with the CBF "rocket."

F. The Cellulosome

Various lines of evidence led to the recognition that the CBF is more than just an adherence factor (Lamed *et al.*, 1983b). The fact that the CBF antigen appeared in large quantities both in the culture supernatant and on the cell surface hinted that its involvement may not simply be in mediation between the surfaces of the cell and its substrate. This conjecture was further strenghthened by the tight association with

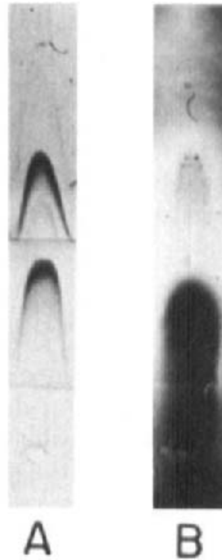

FIG. 4. Rocket immunoelectrophoretograms of wild-type sonic extracts stained for protein (lane A) or treated for carboxymethylcellulase activity (lane B). The intermediate gel contained the CBF-specific antibody, and the upper gel contained the reference antibody preparation. Samples were applied to the well in the bottom gel, and electrophoresis was performed with the anode at the top. (Reproduced by permission from Bayer et al., 1983.)

endoglucanase activity and eventually by the demonstration that the molecular composition of the cell-bound and cell-free forms is almost identical. We therefore renamed the CBF and chose *cellulosome* as a descriptive term to indicate more clearly the central role in the general process of cellulose degradation.

IV. Characterization of the Cellulosome

A. ISOLATION OF CELL-FREE AND CELL-ASSOCIATED FORMS

Crossed or rocket immunoelectrophoresis of the supernatant fluids or cell sonicates derived from either wild-type YS or mutant AD2 strains revealed a single precipitin peak with the cellulosome-specific antibodies (Fig. 5). This precipitin peak was present in both the parent and mutant supernatant fluids but only on the parent cell surface.

The cell-associated and cell-free forms of the cellulosome were isolated from sonicated cell extract and from the various cell-free supernatant fluids (of cellulose- or cellobiose-grown cells) by almost identical procedures (Fig. 6). The procedure involved batchwise adsorption of the cellulosome onto cellulose and its elution either by distilled water or by

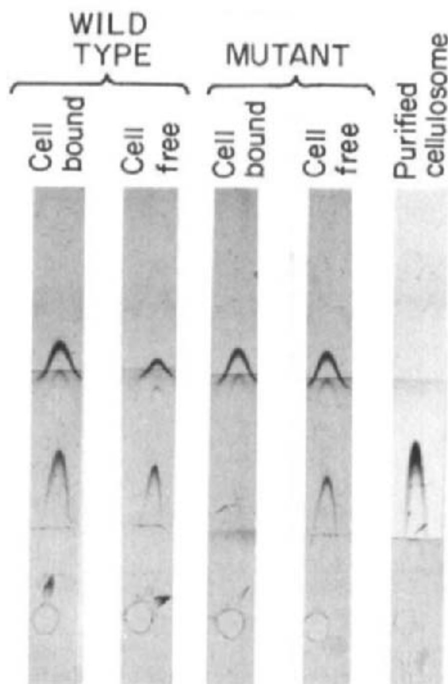

FIG. 5. Immunochemical analysis of cell-associated and extracellular antigens derived from wild-type YS and mutant AD2. Whole-cell sonicates and spent medium from growing cells were subjected to rocket immunoelectrophoresis. The upper gel contained the reference (anti-whole cell) antibody and the intermediate gel contained the cellulosome-specific antibody. Note that the major difference is in the lack of a cell-associated cellulosome peak in the mutant.

a 1% solution of triethylamine. After neutralization and concentration by acetone precipitation, the material was further fractionated according to molecular size by gel filtration using agarose columns.

Both the extracellular and cell-bound forms of the cellulosome were characterized by several peaks which exhibited endoglucanase activity; those indicated in Fig. 7A as peaks I, II, and III were shown to contain cellulosome-associated material. The respective polypeptide compositions of all three peaks were very similar. All reacted strongly with the cellulosome-specific antibody.

Peak I represents very high molecular weight, turbid (perhaps vesicular) material which appeared at the void volume on Sepharose 2B columns. The vesicular fraction was more pronounced in cell sonicates than in cell-free supernatant fluids (Lamed *et al.*, 1983b). It was postulated that peak I represents polycellulosomal clusters, and that the cellulosome is

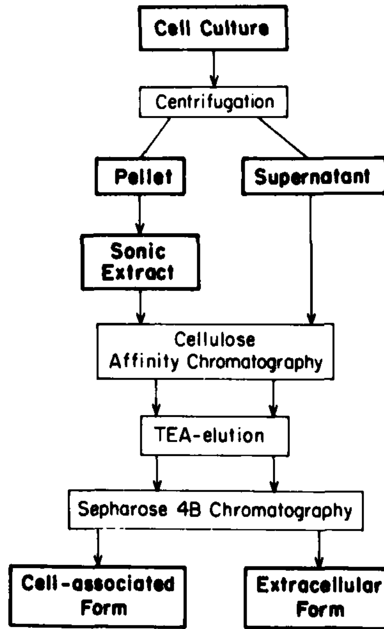

FIG. 6. Schematic description of the strategy used to isolate cell-associated and cell-free forms of the cellulosome. In some cases, Sepharose 2B chromatography was used for the gel filtration step.

arranged on the cell surface in such polycellulosome centers. This contention was eventually corroborated by ultrastructural studies and is discussed in Section VI.

Peaks II and III were distinguished mainly by differences in their apparent molecular weights. One major difference was observed in their polypeptide pattern and is mentioned in Section IV,C.

In the extracellular form, the major cellulosome type was associated with peak II (Lamed *et al.*, 1983a). Sedimentation velocity studies of the cellulose eluate (see Fig. 7B) revealed one major and two minor peaks, the latter of which were removed by the gel filtration step (Fig. 7C). The resultant preparation, which corresponded to peak II, afforded a single

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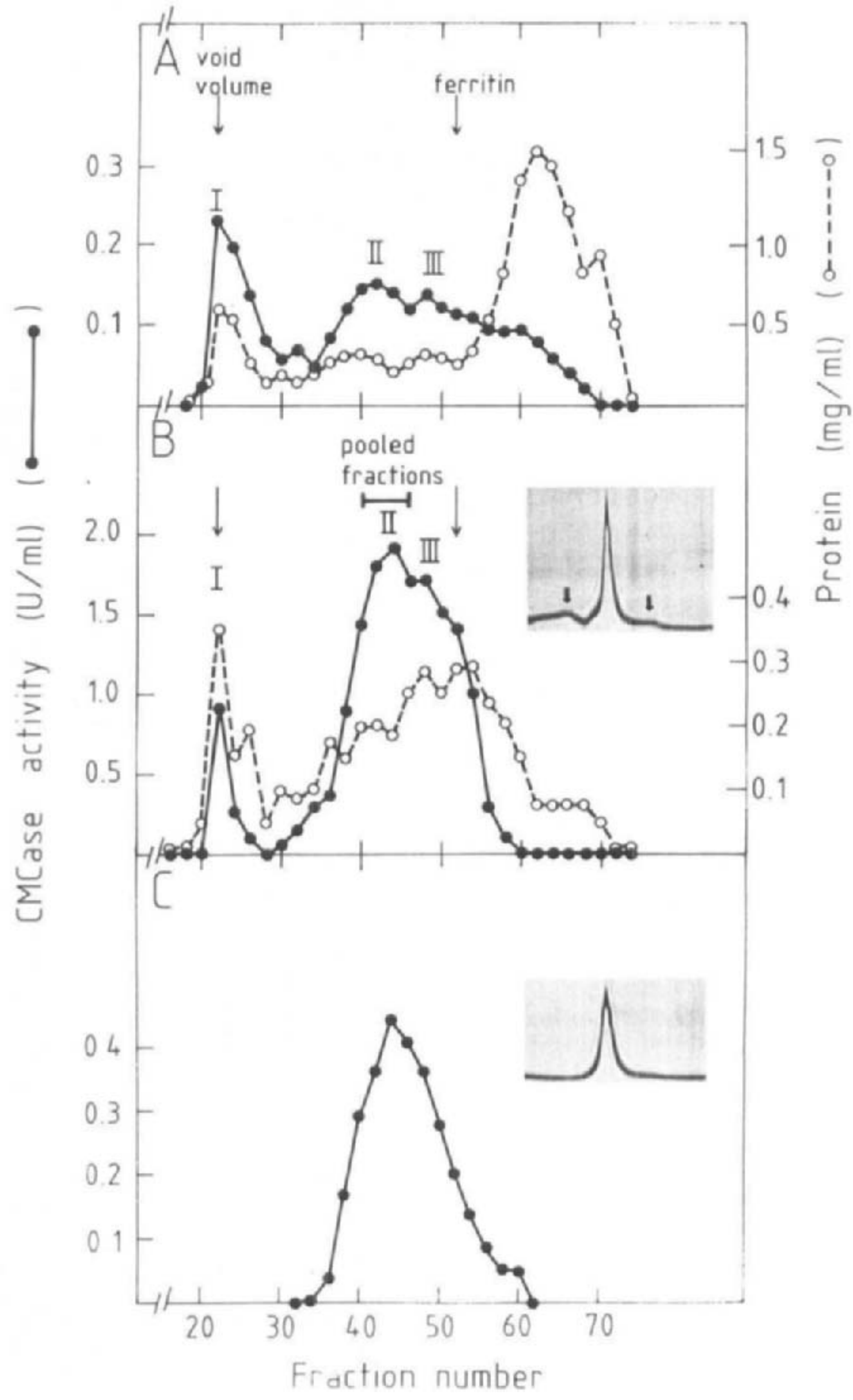
FIG. 7. Gel filtration analysis of purification steps for cell-associated cellulosome. Sepharose 2B gel chromatography was performed on (A) sonicates of washed *C. thermocellum* cells, (B) the partially purified cellulosome following cellulose (affinity) chromatography, and (C) rechromatography on Sepharose 2B of the pooled fractions designated peak II in (B). Insets in B and C show sedimentation pattern of the respective material. Note that the extraneous peaks (arrows) in B were eliminated after rechromatography. The lower pattern is characterized by a single symmetrical peak.



A
void volume
ferritin
I
II
III
B
pooled fractions
C
CMCase activity (U/ml)
Protein (mg/ml)
Fraction number

symmetrical peak in the analytical ultracentrifuge ($s^{o}_{20,w}$ = 22.0, $D^{o}_{20,w}$ = 1.02 × 10^{-7} cm^2/sec). A molecular weight of 2.1 million was calculated, which was in good agreement with the observed gel filtration properties. Pooling the peak III fractions yielded a lower molecular weight material ($s_{20,w}$ = 12.7). Due to the complexity of the polypeptide pattern of the cellulosomes (see Section IV,C), it is difficult to assess whether a given cellulosome type (i.e., peak II or peak III) represents a "basic unit" which may exist in a larger multimer form(s) (i.e., peak I).

B. Electron Microscopy of the Cellulosome

The apparent homogeneity exhibited by ultracentrifuge analysis and the relatively large dimensions that were calculated for the cellulosome types prompted an electron microscopic study of the purified complex (Lamed *et al.*, 1983a,b). Negatively stained preparations of the isolated cellulosome revealed particulate structures (see Fig. 8) of relatively uniform size (approximately 18 nm in diameter). These particles appeared to be composed of multiple subunits. The varying shapes of the molecule in the picture appear to reflect the different faces of the complex and/or to reflect a flexibility in its structure. Larger structures, composed perhaps of higher aggregates which contain several 18-nm particles, were also visible. The larger aggregates were particularly evident in the turbid fractions derived from cell-asssociated material (see peak I, Fig. 7A).

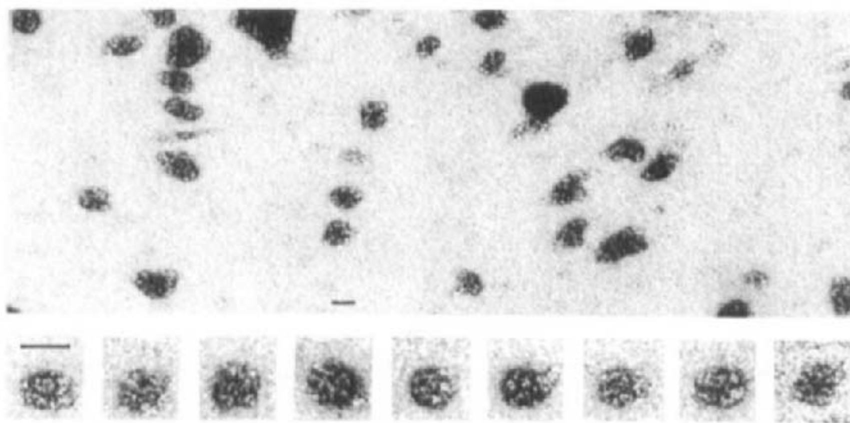


FIG. 8. General view of a negatively stained preparation of the extracellular cellulosome. Structures are particulate in nature and appear to be composed of several smaller subunits. In some instances, higher order aggregates can be observed. A variety of different particle forms are visible. Higher magnification of some characteristic particle images are shown in the insets at the bottom of the figure. Bars represent 20 nm. (Reproduced by permission from Lamed *et al.*, 1983a.)

C. Peptide Composition and Activities

The polypeptide profiles of both cell-associated and extracellular forms of the cellulosome (isolated from the Peak II fractions as demonstrated in Fig. 7) were compared (Lamed *et al.*, 1983b). When the cells were grown on cellulose as the growth substrate, the patterns were strikingly similar. Densitometry tracings of stained SDS–polyacrylamide gels indicated that the relative amounts of each subunit are also similar. The major peptides in terms of relative content in the complex were S8 (28%) and S1 (24%) (Fig. 9). In the cellulosome obtained from cells grown on cellobiose, the S8 subunit was much less dominant (Bayer *et al.*, 1985). In addition, the relative levels of endoglucanase activity, sugar content,

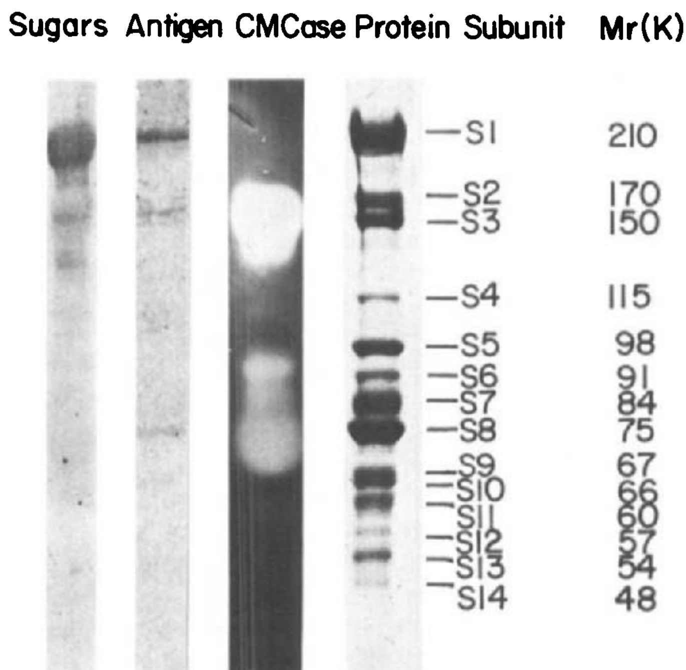


Fig. 9. Subunit composition and biochemical characterization of the cellulosome. Cellulosome samples were subjected to SDS–PAGE and stained for various activities. For protein content, gels were stained with Coomassie brilliant blue R250. For endoglucanase activity, gels were processed using a carboxymethylcellulose-overlay technique. For antigenic activity, blot transfers of the gel were labeled successively with biotinylated cellulosome-specific antibody and avidin–biotinyl peroxidase complexes. For sugar content, blots were oxidized with periodate, and the resultant aldehydes were labeled with enzyme hydrazide (Gershoni *et al.*, 1985). Note that the S1 subunit appears to be a glycoprotein which contains most of the antigenic activity. In contrast, no endoglucanase activity is associated with this band.

and immunochemical activity in each subunit were measured by zymogram and blotting techiques (see Fig. 9).

It is interesting to note that carboxymethylcellulase activity was recovered under the described renaturing conditions despite the harsh treatment involved (boiling in SDS prior to electrophoresis). Subunits S2 and S8 exhibited the highest levels of renatured cellulolytic activity. Subunits S5, S11, S13, and S14 also appear to be cellulases, and some cellulase activity may be associated with subunits S3, S4, S6, or S7. In short, with the exception of S1 most of the other protein bands identified with the cellulosome complex seem to be cellulases (Lamed *et al.*, 1983a).

As already mentioned, the polypeptide patterns of all three cellulosome-containing peaks (see Fig. 7A) were very similar. Following SDS–PAGE, densitometry tracings of the pooled fractions corresponding to peaks II and III revealed that the relative amounts of most of the bands other than S1 in these two peaks were also similarr. The amount of S1 in peak III, however, was much less than that observed in peak II. It is not yet known whether the apparent heterogeneity reflects an inherent multiplicity in cellulosome structure or whether these results are a consequence of the purification procedure employed.

More recent results (Lamed *et al.*, 1985) demonstrated that previous data accumulated for the crude cellulase system in *C. thermocellum* essentially reflect the contribution of the cellulosome. Similar to the crude enzyme system, true cellulolytic activity was also obtained for the purified cellulosome. As with crude enzyme preparations, the cellulolytic activity of the purified cellulosome was enhanced both by calcium ions and by thiols and was inhibited by cellobiose (the major end product of the cellulosome-mediated cellulose degradation). In addition, at low ionic strength cellulose-adsorbed cellulosome was detached intact from the cellulose matrix. Using controlled conditions, maximal enzymatic activity was shown to correpsond to suboptimal conditions of cellulosome adsorption to cellulose. Under the conditions of the assay, maximal cellulase activity was observed at 1 m*M* sodium acetate, which corresponded to about 70–80% adsorption of the cellulosome to the cellulosic substrate. At low salt concentrations, the low levels of enzymatic activity are most likely due to the lack of sufficient adsorption of the cellulosome to the substrate, whereas at higher ionic strengths, the lowered activities observed may be due to the restricted mobility of the cellulosome, which is bound very tightly to its substrate.

D. Structural Stability and Denaturation by Detergent

Of many conditions (including the addition of urea, guanidine hydrochloride, and various detergents) known to interfere with interpeptide bonding, only treatment with SDS effected significant decomposition

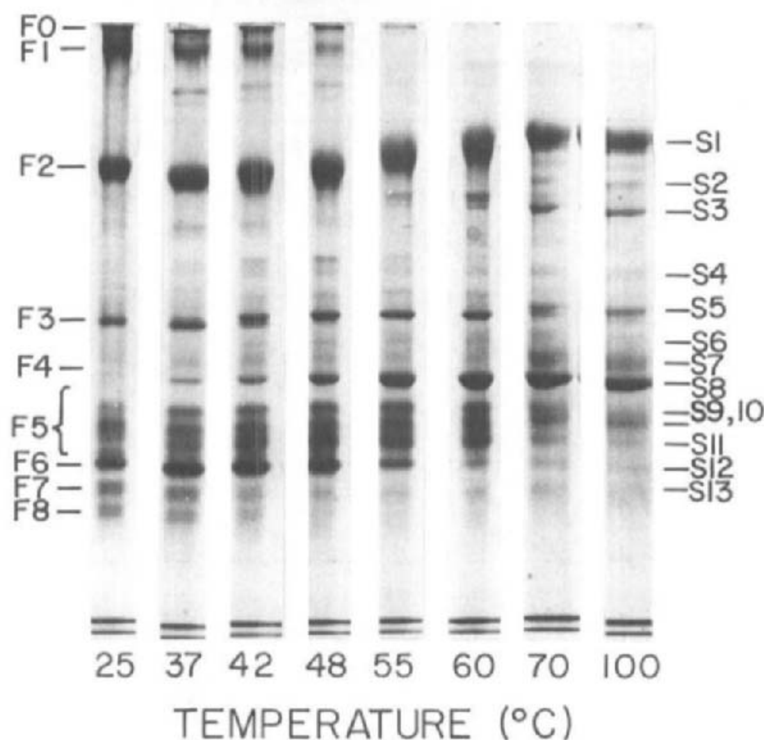


FIG. 10. Graduated heating of cellulosome in the presence of SDS. Samples of the cellulosome were brought to 0.1% with SDS and incubated for 10 minutes at the designated temperature. Note the temperature-dependent alterations in the positions of the cellulosome fractions (F0–F8) to form subunits (S1–S14).

of the cellulosome complex (neutral detergents such as Triton X-100 did not significantly affect cellulosome structure). By incubating the cellulosome with SDS at room temperature (rather than boiling), an altered but reproducible SDS–PAGE pattern was achieved. Upon increasing the temperature of the treatment (see Fig. 10), the pattern gradually reverted to that observed after boiling. The major bands or band clusters obtained (designated F0 through F8) were further studied. The various fractions were extracted from the gel and subjected to SDS–PAGE after total denaturation in boiling SDS. The extracted fractions were also analyzed for different cellulolytic activities and respective distribution of the soluble sugar products (Lamed *et al.*, 1983b). The results are summarized in Table V.

It is interesting to note that the largest subcomplex, the F1 fraction, contains most of the recognized cellulosome subunits. Of the extracted fractions, F1 displayed the highest level of true cellulase activity using

TABLE V

SUBUNIT CONTENT, CELLULASE ACTIVITIES, AND PRODUCT ANALYSIS OF PARTIALLY DENATURED CELLULOSOME FRACTIONS

Cellulosome subcomplexes or fractions	Components		Cellulose activities		Products		
	Major	Minor	Endoglucanase	Avicelase	Glucose	Cellobiose	Cellotriose
Intact cellulosome	S1–S14		+ + +	+ + +	−	+ + +	−
F1	S1–S5, S8	S6, S9–S11	+ +	+ +	−	+ + +	−
F2	S1, S3	S5, S8	±	−	−	−	−
F3	S3, S7	S5, S8	±	−	−	−	−
F4	S7	S4, S5, S8	+ +	−	−	−	−
F5	S5–S10	S11, S13, S14	+ + +	±	−	+ + +	−
F6	S8, S11	S6, S9,10, S13	+ + +	+	+	+ + +	+
F7	S9,10, S13	S7, S11	+ + +	±	−	+ +	+ + +
F8	S13	S8–12	+	−	−	+	−

Avicel, whereas only moderate levels of cellulolytic activity were recorded for F1 using either carboxymethylcellulose of amorphous cellulose as substrate. This further substantiates the apparent requirement of complex formation for enhanced cellulolytic activity.

In contrast to the high cellulolytic activity found in subcomplex F1, the other confirmed subcomplex (contained in fraction F3) exhibited relatively low levels of enzymatic activity. This may indicate that the subunits associated with this particular subcomplex require additional subunits (e.g., S1 or S2) for enhanced cellulolytic activity. Alternatively, the endogenous F3 subunits may themselves play an alternative (other than cellulolytic) functional role(s) in the cellulosome complex.

Fractions F5 through F7 achieved the highest levels of cellulolytic activity with carboxymethylcellulose or amorphous cellulose as substrate. Moderate degradation of Avicel was also observed for these three fractions, although the observed enzymatic activity on this substrate was less in each case than that recorded for fraction F1. Of particular note are the significant levels of cellotriose as a final product in F6 and more markedly in fraction F7. The data suggest that an endoglucanase represents a proponderant cellulase species present in these two fractions, and that cellobiohydrolase is absent. In this regard, careful review of the subunit composition of these fractions indicates a common prominent band at the S13 position and common secondary bands at the S9,10 positions. Since our reported size for the S13 subunit (54 kDa) is striking similar to the published value (56 kDa) for the endoglucanase (the *cel A* gene

product described in Section II,E) purified by Petre *et al.* (1981), it is interesting to speculate whether this particular cellulosome subunit is identical to the enzyme described by those authors.

E. THE S1 SUBUNIT

From the earliest stages of our work on the purified extracellular cellulosome, it was clear to us that the largest component of the cellulosome, the 210-kDa S1 subunit, is unique in its properties and serves as a distinctive, integral, salient part of the cellulosome. Although this subunit appears to constitute about a quarter of the total cellulosomal protein, cellulolytic activity could not be demonstrated (as judged from endoglucanase zymograms and activity measurements following SDS–PAGE and extraction of the S1-containing band). This subunit, however, is highly antigenic, and interacts strongly with the cellulosome-specific antibody (Fig. 9). The diffuse band in SDS–PAGE, the direct staining for sugar, as well as phenol–sulfuric acid determination of total sugars of both intact cellulosome and an enriched S1 fraction (obtained by preparative SDS–PAGE) suggest that this subunit bears the majority of the covalently bound sugar (approximately 10% w/w of the cellulosome or an estimated 40% of the molecular weight of the S1 itself). The high antigenicity may be related to the relatively external position of the S1 subunit on the cellulosome and hence an exposed position on the cell surface. This initial assumption was eventually verified by electron microscopy (see Section VI). Alternatively, the S1 subunit could simply be more antigenic, perhaps due to its sugar content.

The S1 subunit is relatively easily removed from the rest of the cellulosome by low concentrations of SDS (0.02%) without heating. In spite of the relative "ease" of dissociation from the complex, this subunit has never been observed detached from the cellulosome in a free form.

The specific function of the antigenic S1 subunit (which, unlike many of the other cellulosome components, exhibits no detectable cellulolytic activity) is as yet unknown. It may, for example, be responsible for the organization of the component parts into the complex or it may serve to anchor the complex onto the cell surface. We have also considered a cellulose-binding role for the S1 subunit. However, we have been unable to demonstrate whether the S1 or any other single cellulosome component is directly responsible for attachment of the intact complex (and the cell) to cellulose. It is possible that the arrangement of multiple forms of cellulolytic enzymes (each of which possesses an inherent affinity for the substrate) into a large complex would serve to collectively enhance the affinity of the entire complex for the substrate, i.e., adherence may be a function of the separate affinities of the various cellulases in the complex.

F. Reassembly Experiments

We have already mentioned that various harsh conditions failed to break the intersubunit association in the cellulosome. We also have attempted to reconstitute the high molecular weight complex from its components which had been dissociated from the intact cellulosome by boiling SDS.

Various means of removing the ionic detergent were attempted, the most convenient of which was the Bio-Rad ion-exchange resin AG 11A8, known to bind SDS irreversibly and to remove it from proteins (Vinogradov and Kapp, 1983). This procedure was repeated several times in different environments under conditions that do not interfere with the removal of SDS. Gel filtration using Sephacryl S-400 served to analyze the pattern of reassociation, and the cellulolytic activities were also determined. Reassociation of subunits occurred under all the conditions tried (presence of nonionic detergent, presence of 8 *M* urea, or in the absence of these additives). As can be seen in Fig. 11, in the presence of urea the "reconstitution" product was significantly larger than the average subunit but was smaller than that of the untreated cellulosome. Reconstitution

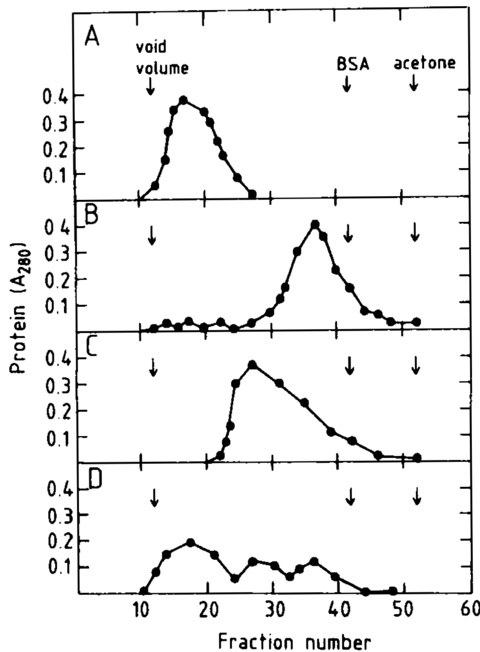


Fig. 11. Attempts to reconstitute intact cellulosome after dissociation by boiling in SDS. The figure represents the gel filtration pattern of the various fractions run on a Sephacryl S-400 column. (A) Intact cellulosome; (B) cellulosome after SDS treatment and boiling; (C) reaggregation in 8 *M* urea; (D) reaggregation in water.

attempts in water also resulted in a more heterogeneous mixture of aggregates. Interestingly, in this case the ability to bind to cellulose was also regained and about 25% of the original endoglucanase (measured using carboxymethylcellulose as substrate) was also restored. As a matter of fact, the complete removal of SDS was not necessary for regaining endoglucanase activity. Unfortunately, two very important distinguishing characteristics of the intact cellulosome were not recovered in the reassembled complex, namely "true" cellulase activity and dependence of the endoglucanase activity on the combination of thiol and Ca^{2+}. We are presently considering additional strategies for reconstituting the "true" cellulase activity in the cellulosome.

G. Cellulolytic Components Not Included in the Cellulosome

Early in our studies we noticed that only about 70–80% of the extracellular endoglucanase activity consistently adsorbed to cellulose and was eventually determined to be part of the cellulosome (Lamed *et al.*, 1983a, 1985). The unadsorbed fraction constituted the major part of the extracellular protein. These components were further purified by chromatography on DEAE-cellulose (eluting at 0.3 *M* KCl) and by gel filtration using agarose columns. The average molecular weight was estimated at 250,000. Since SDS–PAGE revealed numerous low molecular weight bands (30,000–45,000) the existence of protein complexes in this protein fraction was also apparent.

The most interesting activity exhibited by the DEAE-cellulose-adsorbed fraction (DE fraction) was its synergistic action with the cellulosome in the hydrolysis of microcrystalline cellulose. The synergistic action was only present in the absence of externally added thiol and Ca^{2+}. In addition, enhancement of defibrillating activity of the cellulosome was also observed (measured as an increase in turbidity of a suspension of microcrystalline cellulose). This activity apparently coincided with low levels of endoglucanase activity in the DE fraction. Our attempts to ascribe the observed activity to one or more of the major bands observed in this fraction or to an immunochemically defined entity were unsuccessful. Unlike the purified cellulosome, no cellobiohydrolase activity (using *p*-nitrophenylcellobioside as substrate) could be detected, whereas the cellulosome clearly showed such activity. As previously mentioned by Ljungdahl *et al.* (1983), we also observed the presence of a major viscous protein (approximately 130 kDa) with an unknown role in the cell growth fluid.

An additional low molecular weight component has been suggested to be associated in some manner with binding of the cellulase system to cellulose (Ljungdahl *et al.*, 1983). This "yellow affinity substance" (YAS) appeared to increase the adsorption of the cellulase complex to cellulose.

As suggested by our recent results (Lamed et *al.*, 1985), however, the YAS may constitute a secondary effect resulting from altered physicochemical characteristics of the cellulose surface (perhaps an increased hydrophobicity for example) rather than a direct effect of this material on the specific affinity of the cellulosome for its substrate. Indeed, the YAS may have an alternative cellular role unrelated to affinity.

V. Characterization of Adherence-Defective Mutant AD2

As mentioned in Section IV,A, the CBF (later termed the cellulosome) was absent from the cell surface of a nonadherent mutant when grown on cellobiose (Bayer et *al.*, 1983). In order to gain more information on the relationship between the cell-associated cellulases of *C. thermocellum*, mutant AD2 was further characterized (Bayer et *al.*, 1985).

Various properties of mutant AD2 and wild-type YS were compared, including (1) the effect of carbon source on adherence to cellulose, (2) endoglucanase activity, (3) cell-associated and cell-free antigenic activity, (4) the molecular weight distribution of complexes containing endoglucanase, and (5) the distribution of the respective polypeptide patterns.

Interesting differences were revealed with respect to these parameters when the cells were grown on cellobiose. Under these conditions, the major observed difference was that mutant AD2 apparently lacks the capacity to produce sufficient levels of the S1 subunit; notably, the cell surface of the mutant is virtually free of the cellulosome (see Fig. 5). At the same time, relatively low levels of extracellular S1 are secreted in the mutant compared to that of the parent strain (Fig. 12). However, in

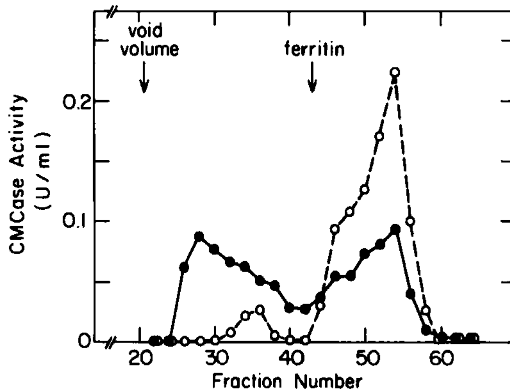


FIG. 12. Molecular weight distribution of extracellular endoglucanases derived from cellobiose-grown wild-type (•) and mutant cells (○). Gel chromatography of the respective cell-free growth concentrates was performed on a Sepharose 4B column.

contrast to the wild type, very high levels of free uncomplexed endoglucanases are present in the growth supernatant of mutant AD2. No antigenic activity could be found in the low molecular weight cellulase fractions.

By SDS–PAGE, it was found that the polypeptide pattern of the high molecular weight material derived from both mutant and wild-type supernatant fluids was identical to that of the purified cellulosome, but that the majority of the low molecular weight cellulases appear to consist of proteins that are not intrinsic to the cellulosome. It thus appears that the adherence-defective mutant, when grown on cellobiose, compensates for its inability to produce large quantities of cell-associated cellulosome by synthesizing very high levels of relatively uncomplexed cellulases.

It is also interesting to note that mutant AD2 is a conditional mutant. When grown on cellulose, the mutant regained most of the wild-type properties including adherence to cellulose, cell-associated endoglucanase, and its predominant presence in large cellulosome complexes which can be detached from the cell surface. Although the polypeptide composition of the cellulosome was similar for both wild-type YS and mutant AD2 cells grown on the same substrate, altering the carbon source (specifically from cellobiose to cellulose) caused concomitant qualitative and quantitative differences in the distribution pattern of the respective subunits within the cellulosome.

VI. Ultrastructural Studies

As shown in previous sections, the cell-associated form of the cellulosome appears to be organized in some manner on the cell surface. The very function of the cellulosome (cell adherence to, and hydrolysis of, an insoluble substrate) would in itself suggest that the responsible agent is positioned on the cell surface. This contention is supported experimentally by the inhibition of adherence by the purified cellulosome (Table IV) and by the agglutination of cells by anti-cellulosome antibodies. Moreover, we have shown (Lamed *et al.*, 1983b) that a portion of the cell-derived cellulosome (from whole-cell sonic extracts) is associated with a particulate or vesicular fraction.

More direct evidence in this direction is provided by ultrastructural analysis (Bayer *et al.*, 1985). The electron microscopic evidence presented in Figs. 13 and 14 delineates the precise position of the cellulosome on the surface of *C. thermocellum* strain YS. Remarkably, the cell-surface cellulosome is not distributed in a uniform fashion but appears to be centralized on protuberant structures which decorate the cell surface at almost periodic intervals. These protuberances were present only on wild-type cells and not on the adherence-defective mutant. Virtually the same structures were visible by both transmission and scanning electron

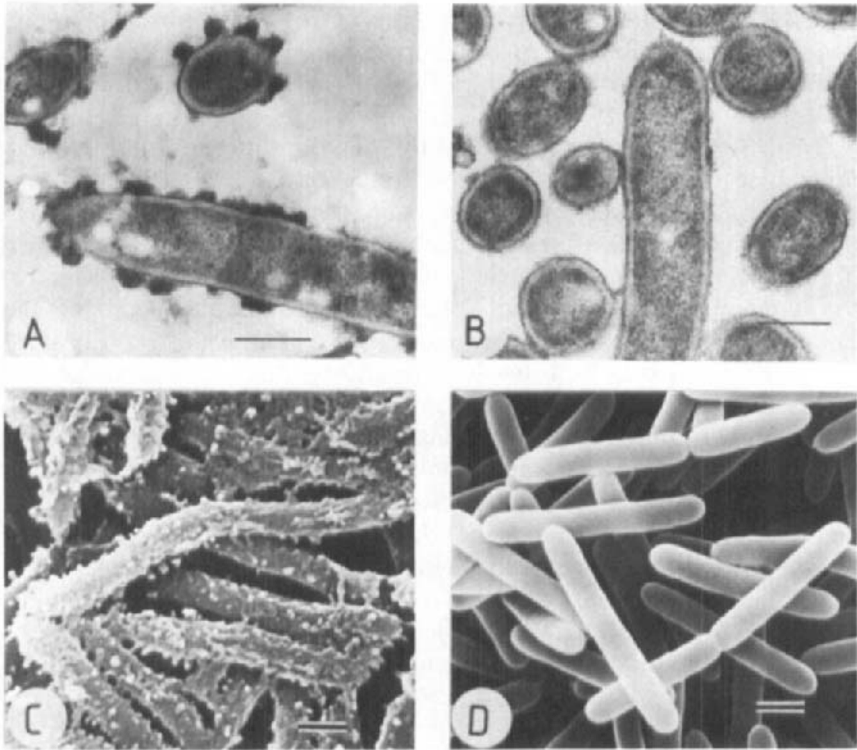


FIG. 13. Ultrastructural comparison of cellobiose-grown wild-type YS and mutant AD2 cells of *C. thermocellum*. Wild-type (A and C) and mutant (B and D) cells were stained with cationized ferritin before processing for transmission (A and B) or scanning (C and D) electron microscopy. Note the ferritin-stained nodulous protuberances which ornament the wild-type cells but not the mutant. Bars represent 500 nm.

microscopy, either by immunochemical staining or by using a general cell surface stain (cationized ferritin) which is specific for anionic sites. Selective immunochemical labeling of the cells demonstrated that cell-bound cellulosomes are directly associated with the cationized ferritin-stained protuberances on the cell surface. In the wild-type cell (Fig. 14A), only the protuberances appeared to be heavily stained, indicating that the cellulosome is associated with these exocellular structures.

These polycellulosomal protuberances apparently represent the major cellular loci of *C. thermocellum* attachment to cellulose. This is supported by the fact that the adherence-defective mutant lacks these exocellular structures. Moreover, direct ultrastructural evidence in this regard has been obtained (Lamed and Bayer, 1986; Bayer and Lamed, 1986). Growth of the

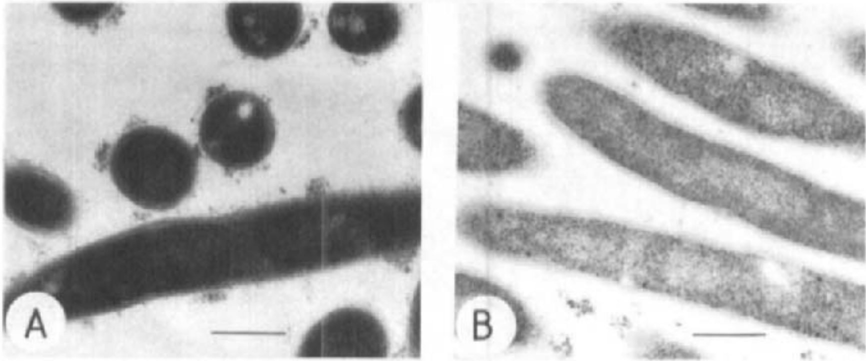


FIG. 14. Immunocytochemical staining of the cell surface cellulosome of *C. thermocellum*. Wild-type YS (A) and mutant AD2 (B) cells were treated with biotinylated anti-cellulosome antibody followed by incubation with ferritin--avidin conjugates. Cells were then processed for transmission electron microscopy. Compare staining pattern with that in Fig. 13A and B. Bars represent 500 nm. (Reproduced by permission from Bayer *et al.*, 1985.)

wild-type cell directly on cellulose as a substrate conferred a dramatic change in the constitution of the exocellular protuberances (Fig. 15A). Upon contact of the cellulosome with cellulose, some of the polycellulosomal protuberances appear to protract, yielding an amorphous or fibrous network. A distance of up to 400–500 nm often separates the cell proper from the surface of the cellulose, and the fibrous matrix appears to connect the two.

Similar fibers on cells of *C. thermocellum* had been described previously by Wiegel and Dykstra (1984) and by ourselves (Samsonoff *et al.*, 1982) which were then assumed to be directly involved in the binding of the cell to cellulose (Fig. 15B,C). However, the cellulosome appears to represent the major site of attachment to the cellulose surface, since labeling with anti-cellulosome antibodies revealed that most of the ferritin marker particles within the fibrous network are arranged in clusters which are intimately associated with the cellulose surface (Fig. 15A, arrows). This is in line with our previously published data (Bayer *et al.*, 1983) which connect the cellulosome directly with adherence. The fibrous structures, on the other hand, form a "contact corridor" which appears to connect the cell to the cellulosome, but is not directly responsible for the binding phenomenon *per se*. These contact zones may serve to direct cellulose degradation products systematically toward the cell surface. It would therefore appear that the hydrolysis is mediated by cellulosome clusters at the surface of the insoluble substrate. The transfer of the products toward the cell may also be a highly ordered efficient process mediated by the fibrous components in the contact zone. These products (in particular

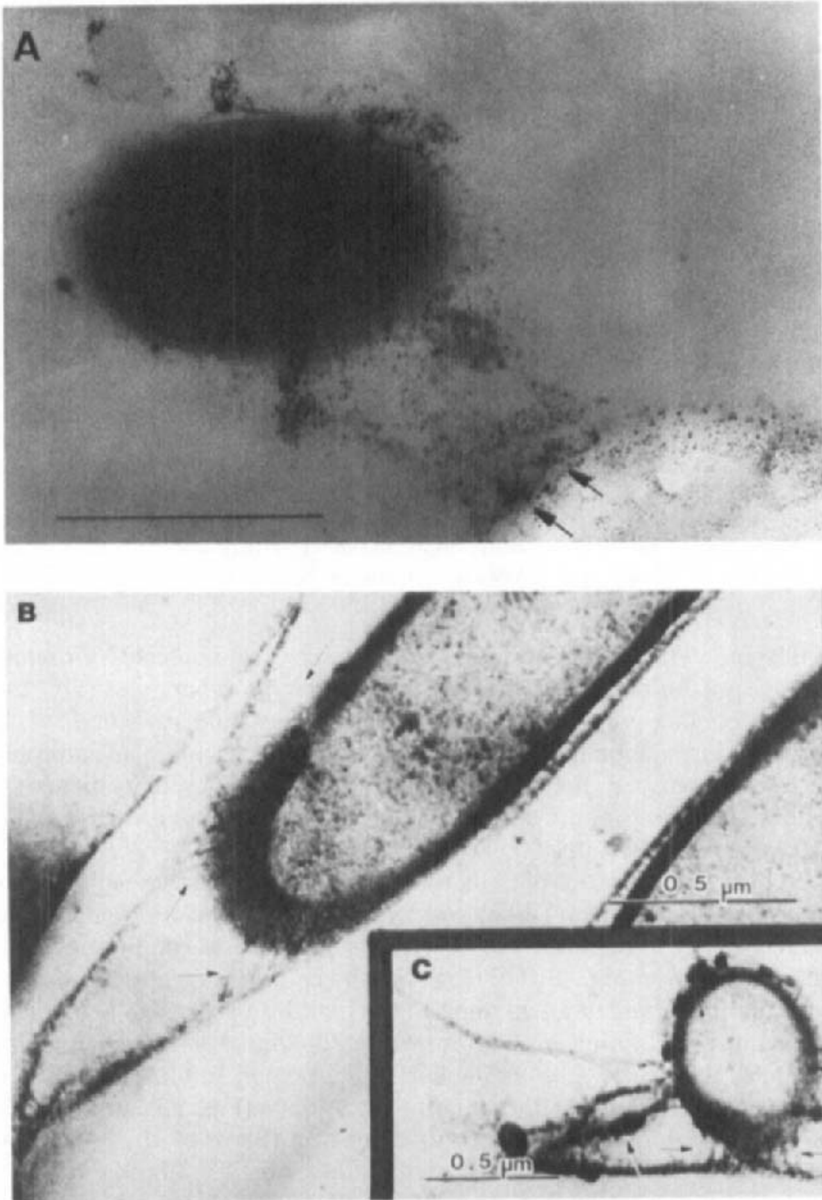


FIG. 15. Interaction of wild-type cells with cellulose. *Clostridium thermocellum* YS cells were grown on cellulose-containing medium and harvested. The cellulose–cell mixture was washed by sedimentation and then subjected either to immunochemical treatment (A) as described in Fig. 14 or to treatment with ruthenium red (B and C). The cells were then processed for transmission electron microscopy. Note the ferritin clusters (arrows in A) which indicate the presence of polycellulosome fragments attached to the surface of the cellulosic substrate. In B and C, the thin arrows designate fibrous material which appears to link the cell with the surface of the cellulose. Note also the nodular ruthenium red-stained structures on the cell surface in C which resemble in shape and size the protuberances shown in Fig. 13 and 15A. Bars represent 500 nm.

cellobiose) would then be taken up by the cell, presumably by an appropriate transport system (as discussed in Section II,A).

VII. Extension of the Cellulosome Concept

A. The Cellulosome in Other Strains of *C. thermocellum*

It was found in our initial studies (Bayer *et al.*, 1983; Lamed *et al.*, 1983a) that the cell-associated CBF (cellulosome) antigen was common to at least three different strains of *C. thermocellum*, along with a variety of other surface antigens. The antigenic similarity of these strains was evident despite the fact that each was isolated from a very different clime. Since other distinctive characteristics (adherence to cellulose, cellulolytic activity, agglutinating activity, etc.) are also shared by these strains, the cell-surface features described here may comprise a more general phenomenon common to this species. Moreover, comparative gel filtration analyses of the supernatant fluids derived from cellulose-grown cells of these strains were strikingly similar. In each case, the predominant fraction (>70%) of the endoglucanase activity was eluted at a position equivalent to that of large proteins (>1 MDa) and was accompanied by the cellulosome antigen.

It is also noteworthy in this context that the cellulosome purified from yet another cellulose-grown strain, *C. thermocellum* NCIB 10682, contained essentially the same components as strain YS, except that the S1 subunit in the former strain corresponded to a single polypeptide chain with molecular weight of about 250,000 rather than 210,000 as in the YS strain. Like the YS variety, the 250-kDa S1 interacted with the anti-cellulosome antibody and contained large amounts of carbohydrate. In addition to the difference in the size of the S1 subunit, the ratio of the polypeptide components was not the same in the two strains. The NCIB strain contained higher levels of subunits S13 and S14. The specific endoglucanase activity was also higher, thus reflecting some "flexibility" in the cellulosome structure.

Wu and Demain (1985) have also demonstrated the presence of Avicelase activity in a very high molecular weight ($\sim 6 \times 10^6$) fraction obtained from the growth supernatant of *C. thermocellum* ATCC 27405. (This strain is identical to NCIB 10682.) The SDS-treated complex could be resolved into several carboxymethylcellulose-hydrolyzing fractions.

Coughlan *et al.* (1985) studied yet another strain (ATCC 31449). Electron micrographic evidence revealed three major globular complexes: one with a diameter of 21 nm, similar perhaps to the 18-nm cellulosome in *C. thermocellum* YS (Lamed *et al.*, 1983a,b), and larger forms of 41 and 61 nm, respectively. These larger forms may resemble the cellulosome type(s) in peak I (Fig. 7B). As with the YS strain, each of these complexes was composed of the same 15–20 polypeptides. The globular complexes

remained attached to the cellulose after most of the cells were removed by washing with a buffer solution.

In recent studies, we have compared the surface features of several *C. thermocellum* strains by scanning electron microscopy using cationized ferritin to stabilize these structures. All of the additional strains (J1, LQRI, and NCIB 10682) possessed protuberant structures similar to those found in the YS strain (see Fig. 13C). It was noticed, however, that the size and distribution of the protuberances varied among the strains, and these qualities may therefore be strain specific.

These studies, which collectively employed five different strains of *C. thermocellum*, indicate that the cellulosome is an integral part of this organism. There may be various strain-specific features related to the relative size, the disposition of the subunits within the complex, and arrangement of the cellulosome on the cell surface.

B. Are Cellulosomes Present in Other Cellulolytic Bacteria?

Although the cellulosome has been fairly well characterized in *C. thermocellum*, there are an increasing number of reports in the literature indicating that the discovery of high molecular weight cellulolytic complexes associated somehow with exocellular structures (such as capsular material) may be a general phenomenon. This appears to be true, at least for many cellulolytic anaerobic bacteria.

In *Ruminococcus albus*, another gram-positive, cellulolytic anaerobe, the presence of a soluble affinity factor has been suggested by Leatherwood (1969, 1973). This claim was based on the observation that areas of solubilized cellulose occurred in the diffusion zones between variant colonies grown on cellulose-containing agar. The author proposed that the putative affinity factor combines with a hydrolytic factor to form a complete cellulase complex.

The occurrence of high molecular weight cellulase complexes has also been shown in *R. albus*. Wood *et al.* (1982) reported that most of the cellulase produced by the mesophilic rumen bacterium, *R. albus* SY-3, is cell bound. The enzyme could be released readily by washing with phosphate buffer or water. The cell-bound enzyme was of very high molecular weight ($>1.5 \times 10^6$) whereas the molecular weight of the extracellular enzyme was variable and depended on growth conditions. The high molecular weight cellulase bound very tightly to cellulose. The authors suggested that *R. albus* cellulase may exist as an aggregate of lower molecular weight components on the bacterial cell wall and in solution under specified conditions.

In another study, Stack and Hungate (1984) reported that both the morphology of the cell surface and the cellulase content of *R. albus* were markedly affected by 3-phenylpropanoic acid (PPA). In the presence of

this growth factor, substantial quantities of cell-bound cellulases were found. In addition, the extracellular enzymes were of very high molecular weight. In the absence of PPA, the total amount of cellulase produced was greater, but essentially all of the enzyme existed in a soluble low molecular weight form. Examination of thin sections by transmission electron microscopy of PPA-grown *R. albus* showed a lobed ruthenium red-stained capsule surrounding the cell wall, as well as small vesicular structures. In contrast, thin sections of PPA-deprived cells were devoid of vesicles and showed little or no capsule surrounding the cell. The behavior of these latter cells is somewhat reminiscent of the situation described for the adherence-defective mutant AD2 in *C. thermocellum*, whereas in the presence of PPA, *R. albus* cells are similar to the wild-type *C. thermocellum*.

The organization of cellulases into high molecular weight multienzyme complexes and their association with specialized cell surface structures have been noted in several gram-negative anaerobes as well. For example, vesicular structures associated with cellulase activity were reported for the rumen bacterium *Bacteroides succinogenes* (Groleau and Forsberg, 1981). Cellulose-grown cells of this organism released 90% of the endoglucanase into the supernatant, of which 50–60% was associated with sedimentable membraneous fragments.

In another example (MacKenzie *et al.*, 1985), the gram-negative bacterium *Acetivibrio cellulolyticus* was shown to be a highly efficient cellulolytic anaerobe which produces several electrophoretically distinct endoglucanases. As in *C. thermocellum*, Ca^{2+} is required for efficient cellulolysis of crystalline cellulose. The polypeptide pattern of the cellulose-adsorbed cellulase fraction bears considerable resemblance to that of *C. thermocellum* cellulosomes, including a large polypeptide of about 200 kDa.

The last example is a newly isolated gram-negative cellulolytic anaerobe, *Bacteroides cellulosolvens*, which has the ability to produce cellulase and to degrade cellulose to cellobiose and glucose (Giuliano and Khan, 1984). The presence of the cell-bound form of the cellulases appears to be necessary for formation of glucose. Significant cellulolytic activity was not found in cell-free cellulase preparations.

VII. Epilogue and Perspectives

In spite of the recent volume of new findings on the cellulolytic system of *C. thermocellum* and the potential extension of the "cellulosome" concept to include other anaerobic bacteria, we can only speculate at present on the contribution of each component of the complicated system to the observed efficiency of the complex as a whole. It is clear that the organism tends to conserve cellulase by preventing its random secretion into the medium. The binding of the bacterium itself to the insoluble

substrate takes place via its cellulosome system, which in *C. thermocellum* YS appears to be linked by an extensile connection to the cells. It is not clear, however, whether the "contact corridor" thus formed imposes diffusion boundaries on oligodextrin degradation products and in particular on cellobiose formed by the multicellulase complex, or whether the critical factor is simply the proximity of the site of cellulolysis to the cell membrane which actively pumps the hydrolyzed sugars into the cell.

The major organizational role of this complex might therefore be designed both for effective delivery to the substrate as well as to bring into proximity the various complementary enzymes. In addition, the complex may be structured in such a way as to enable the protection of various product intermediates and to facilitate their transfer to other cellulase components for further hydrolysis.

Simultaneous multiple-point cleavage may also be a key factor for effective disruption of the crystalline structure of the substrate. Such a process would take place within individual cellulosome particles, within polycellulosome clusters, and along the entire length of the bacterium. Under these conditions, extensive restoration of the newly severed glycosidic bonds would be highly unlikely.

The recognition that the intact system may be a requirement for effective cellulolysis has already led to a novel approach in cellulose research. As mentioned, the cellulase system in *B. cellulosolvens* was almost entirely cell bound (Giuliano and Khan, 1984). Nevertheless, a successful attempt to use intact cells of this organism was made, yielding significant production of sugars from crystalline cellulose. A resting cell suspension was thus used to effect hydrolysis of crystalline cellulose, leading to an accumulation of 34 g sugar/liter (Giuliano and Khan, 1985). This approach may be a logical alternative to the classical strategy of using extracellular enzyme preparations in industrial settings.

In a further step, one may consider immobilized cellulase systems for industrial application. Immobilization of the various cellulolytic enzymes may lead to enhanced activity over soluble cellulases due to improvved synergism and elimination of abortive, nonproductive adsorption considered to block the available surface area of cellulose. The coimmobilization of cellulase components or cellulosomes on microcarriers may also lead in the future to improved cellulolysis, despite the apparent illogic in using an immobilized enzyme to degrade an insoluble polymer. Indeed, to the surprise of classical enzymologists, several reports on immobilized cellulases acting rather avidly with crystalline cellulose have appeared in the literature (Woodward and Zachry, 1982; Fadda *et al.*, 1984). Contrary to the generally held opinion (Reese and Mandels, 1980), cellulases do not necessarily lose activity toward insoluble substrates after immobilization but may even become more efficient in their degradation of natural celluloses. It should be noted that in cell-associated systems,

which are the most efficient systems, nature has in fact chosen to degrade the insoluble substrate with an immobilized form of the cellulosome. If purified cellulases could be bound to an inexpensive support, the possibility of reusing the cellulases would also reduce the cost of enzymatic hydrolysis. It is not unlikely that immobilized cellulolytic organisms may eventually serve in bioreactors for conversion of cellulosic biomass (Horne and Hsu, 1983; Asther and Khan, 1984). This approach may prove to be the method of choice for industry.

For genetic engineering purposes, the structural complexity of the cellulosome in *C. thermocellum* may be somewhat discouraging. Nevertheless, the ease with which genetic expression of many of its components could be carried out in *E. coli* (Millet *et al.*, 1985) may be very helpful in further analysis of cellulosome structure. Use of the components expressed in *E. coli* and their respective antibodies should allow novel studies in this direction. Thus, the process of secretion and assembly of the complex may one day be elucidated. In addition, the unlimited availability of separate components may enable the reconstruction of at least a simplified version of the cellulosome which might prove useful in practical applications. Further genetic engineering studies could lead to the construction of a strain of bacterium or yeast with the necessary cellulolytic apparatus for direct conversion of cellulosic materials to ethanol and other useful products.

In summary, the cellulosome concept can be considered to encompass the general organization of various complementary cellulases and related components into defined complexes which may form larger polycellulosomal structures. These structures may be localized at some point in the cell cycle on the cell surface in the form of capsulelike material or other surface structure (e.g., the protuberances in *C. thermocellum* YS). In some cases, the cellulosome may be multifunctional; for example, in addition to the hydrolytic role, it may mediate the property of cellular adherence. The organization of cellulolytic enzymes into the cellulosome imparts synergy among its components, resulting in highly efficient solubilization of the insoluble, paracrystalline, cellulosic substrate. The cellulosome concept may bear particular relevance to other microbial systems wherein other structurally complex insoluble polymers (e.g., starch, chitin, and various insoluble proteinaceous matrices) are degraded enzymatically.

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Clonal Populations with Special Reference to *Bacillus sphaericus*

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I. Introduction

In recent studies of the genetic structure of bacterial populations (Orskov and Orskov, 1983), the word *clone* (and the clonal population concept) is used to denote a group of bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times but showing so many identical phenotypic and genotypic traits that the most likely explanation for this identity is that the cultures were derived essentially from the virtually complete asexual reproduction of a common ancestor. According to Ochman and Selander (1984), the analysis of the genetic structure of natural populations of bacteria should be founded on an assessment of the chromosomal genotype over a large number of genes. According to these authors the only practical way of determining chromosomal genotypes in the large number

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of isolates required for these studies of genetic variation in natural populations is by multilocus electrophoresis of enzymes. Multilocus enzyme electrophoresis has long been a standing method in eukarotic population genetics (Lewontin, 1974; Nei, 1975; Rattazzi *et al.*, 1983; Selander and Whittam, 1983; Oxford and Rollinson, 1983). Most recently the clonal population concept has been applied successfully in examining epidemiological, systematics, taxonomic, and evolutionary problems, primarily using the techniques of multilocus enzyme electrophoresis.

The value of biotyping and the more classical approaches is still seen in the work of Miller and Hartl (1986), but it should be of interest to those of us who work with large numbers of cultures of important taxa that serotyping and related techniques, so long an important practical approach to relating bacterial members of our collections, has come into question. Serotypes have been considered uniform entities and therefore meaningful, if not fundamental, units of population structure. This has been recently questioned (Ochman and Selander, 1984; Musser *et al.*, 1986). Musser *et al.* (1986) found that during the cultivation of the genus *Bordetella* the organism frequently expressed changes in many of its surface components, while the electrophoretic mobilities of metabolic enzymes were stable characteristics and thus could be used for epidemiological as well as genetic studies. The intensity of natural selection for cell surface antigens must surely be substantially greater than that for electromorph variants of enzymes (Caugant *et al.*, 1985).

Recent work in my laboratory dealing with the systematics of larvicidal and nonlarvicidal strains of *Bacillus sphaericus* has shown that the structure of the larvicidal *B. sphaericus* populations is clonal. In this article, results from this study (Cole *et al.*, 1987; Hessler *et al.*, 1987) are used to illustrate the utility of the clonal population concept for applied microbiology problems. In addition, the clonal population concept is discussed as recently applied to the areas of epidemiology, systematics, and evolution. But before we do this, we need to examine the methods used for measuring culture differences.

II. Measuring Culture Differences

Even before the advent of microbiology as a formal discipline, microbiologists had the need to differentiate among their cultures of interest. During the course of time, a large library of information has accumulated to aid the practioners of this art as well as the theoreticians. The nine editions of *Bergey's Manual*, whether designed as the classical determinative bacteriology of the first eight editions or the "systematics" of the ninth edition, are examples of this library of information. Until the advent of macromolecular biology, the primary approach for examining

diversity among strains used information from whole cells and their products to delineate differences between cultures. With improvement in techniques and technology, the emphasis more recently has been on the utilization of information at the macromolecular level. These techniques deal primarily with the various DNA and RNA components as well as at the epigenetic level with a direct product of the informational gene, the protein/enzyme.

A. Information from Whole Cells and Cell Products

1. *Auxanography (Biotyping)*

At the most elementary teaching level, the beginning microbiology student determines the reactions of a series of "biochemical" tests (e.g., does the unknown utilize lactose with the production of "acid and gas" in 24 hours?). At present, hundreds of carbon and nitrogen sources have been used for differentiation purposes for examining both clinical and nonclinical cultures. The task has been made easier by the availability of commercially prepared panels of such carbon and nitrogen sources (Logan and Berkeley, 1981). The use of panels has also been extended to testing for the presence of many of the constitutive enzymes as well (Logan and Berkeley, 1984). There is no paucity of availability of such aides for the auxanotyping of bacterial strains or their use in differentiating strain differences. The popularity of this technique lies in the ease and rapidity of identification, particularly when these results are coupled to variously designed diagnostic schemes using numerical taxonomy computer methods (Colwell and Austin, 1981).

2. *Serotyping and Bacteriophage Typing*

Strains of a bacterial species can be divided into serovars based on the antigenic complexity of their cell surface antigens (cell wall lipopolysaccharide, flagella, and capsular constituents). More than 1000 serovars have been detected within the genus *Salmonella* alone (Jones and Krieg, 1984). Jones and Krieg (1984) also stated that it is generally accepted that these techniques are of little value in classification but are valuable in epidemiological studies, in view of the fact that the volume of data is now so large and so many cross reactions occur that computer or similar programs are required for analyzing the material in an objective fashion. The same argument could be made for bacteriophage typing. Examples of the use of both methods are given below (Sections III and IV,C).

3. *Plasmid Profiles*

Plasmids are small circular pieces of DNA located outside the bacterial chromosome that have long been known to carry genes for properties

such as resistance to antibiotics, degradation of hydrocarbons, utilization of carbon and nitrogen sources, resistance to mutagenic agents, and production of antibiotics (Broda, 1979). Although these properties are usually considered to be optional (not absolutely essential for the survival of the host), under certain environmental conditions they may be essential for the survival of the organism (e.g., resistance to antibiotics). Plasmid profiles determined by agarose gel electrophoresis have been used as aides in classification (Orskov and Orskov, 1983). Although there is disagreement among taxonomists on whether known plasmid-determined characteristics should be used for taxonomic purposes, in practice this can be useful. For example, the species *Bacillus thuringiensis* is distinguished from *Bacillus cereus* solely on the basis of its toxicity for insects (Gordon *et al.*, 1973). This classification may not be taxonomically sound, yet it is very convenient for those interested in biological control agents. Also, because plasmid-determined characters may be somewhat variable and thus confuse the identification of bacterial taxa, it is useful to determine if plasmids are present in a particular strain and, if so, what characters are determined by the plasmid DNA.

Most plasmids found in natural populations are cryptic (Hartl and Dykhuizen, 1984). This does not mean that they do not carry valuable genetic information but rather that the particular property(s) has not been determined yet. Since plasmid DNA accounts for a small portion of the cellular DNA, perhaps less than 3% (Broda, 1979), it is not sufficiently large to interfere with the results of multilocus enzyme electrophoresis determinations (Selander *et al.*, 1986). Plasmid profiles, however, were essential in differentiating the epidemic clone from the nonepidemic clone (Orskov and Orskov, 1983). Identification of a specific plasmid (through restriction endonuclease digestion studies) is often more important than the plasmid profile. In studies of pathogenic clones, investigation of the evolution of the plasmids (gene sequencing) may be as important as study of the evolution of phenotypically identical groups of organisms. For example, it leads to very useful markers (Orskov and Orskov, 1983).

4. *Other Metabolic Processes*

What also needs to be mentioned here, in addition to the methods listed above, are several new techniques that have recently achieved prominence in attempts to measure culture difference, particularly using information from whole cells and their products (Goodfellow and Minnikin, 1985). One of these is pyrolysis mass spectrophotometry (PY-MS). Pyrolysis is the breakdown of complex materials (e.g., bacteria) in an inert atmosphere to produce a series of volatile lower weight substances using heat alone (Shute *et al.*, 1985). In PY-MS, these fragments are subsequently detected

and quantified using mass spectrophotometry. A similar technique, pyrolysis gas chromatography (PY-GC), uses gas chromatography as the detection system. Using PY-MS, Shute *et al.* (1985) studied four groups of closely related *Bacillus* strains. A study on the same group of strains was carried out by O'Donnell *et al.* (1980) using pyrolysis gas–liquid chromatography. The results were examined by principal coordinates analysis for strains of the same group and canonical variates analysis for strains from different groups.

If working with end products rather than whole cells, the volatile end products of gram-positive bacteria can be detected by gas chromatography analysis, thin-layer chromatography, or head space gas analysis (Drucker and Tavakkol, 1985). Similarly, one can detect acid end products of anaerobic gram-negative bacteria using gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC), or isotachophoresis (Shah *et al.*, 1985).

B. Information at the Macromolecular Level

DNA is the source of all taxonomic information since the contained genetic code ultimately spells out all of the potential characteristics of the culture. The richest source of information in the DNA macromolecule lies in the nucleotide sequences of complete bacterial genomes, but sequencing the latter is not practical at present. Bacterial genomes can be compared on the basis of genome size, by DNA homology, via comparison of nucleotide sequences of DNA–DNA pairing, by % G + C, or by DNA mapping following restriction endonuclease digestion (Goodfellow and Minnikin, 1985). Nucleotide sequencing of rRNA preparations, particularly the 16 S rRNA component (Stackebrandt and Woese, 1981), can provide a means of determining relationships among representatives of diverse taxa. In addition, the examination of extrachromosomal DNA (plasmid profiling) can be useful for taxonomic purposes (Orskov and Orskov, 1983).

C. Information at the Epigenetic Level

Although DNA is the source of all taxonomic information, proteins, the secondary macromolecular product of DNA, are usually easier to work with (Goodfellow and Minnikin, 1985). At this secondary or epigenetic level, comparative analysis of specific homologous proteins can provide an accurate means of establishing relationships among bacteria. More complex epigenetic analyses involve the comparison of groups of proteins by gel electrophoresis and of regulatory mechanisms governing the synthesis of enzymes operating in given pathways. Changes in amino acid

sequences of specific proteins can reflect evolutionary divergence between organisms (Goodfellow and Minnikin, 1985). One can measure the amino acid sequence of specific homologous protein from representative strains or one can use serological techniques (Schleifer and Stackebrandt, 1983). But the most powerful, relatively simple, yet cost-effective method is that of protein electrophoresis. The two general approaches using protein electrophoresis are (1) determination of specific protein patterns using the SDS–PAGE method (Dent and Williams, 1985), and (2) examination of specific enzyme patterns (Williams and Shah, 1980); both methods are of great taxonomic value.

D. Multilocus Enzyme Electrophoresis

In the past 20 years, the multilocus enzyme technique has been used to study variation and structure in hundreds of eukaryote species (Nevo *et al.*, 1980) but only recently have serious attempts been made to apply this technique to bacteria (Selander *et al.*, 1986).

Chemical profiling or fingerprinting methods [auxanotyping (biotyping), serotyping, phage typing, fibrimiation typing, etc.] which classically detect phenotypic variation do not easily relate, if at all, to allelic variation at specific gene loci. They have not provided the information on frequencies of alleles and multilocus genotypes that is required for analysis of the genetic structure of populations (Selander *et al.*, 1986). Selander *et al.* (1986) also noted that DNA hybridization studies, widely used to define species limits and relationships, have made little contribution to the study of genetic variation within species, particularly since variance is broad enough to make resolution of closely related strains difficult. Multilocus enzyme electrophoresis studies, however, have established basic population genetic frameworks for the analysis of variation in serotypes and other phenotypic characters, and such studies have provided extensive data for systematics and useful marker systems for epidemiology (Selander *et al.*, 1986).

The net electrostatic charge (as well as three-dimensional shape) and, therefore, the rate of migration of a protein during electrophoresis are determined by its amino acid sequence. As a consequence, mobility variants (electromorphs or allozymes) of an enzyme can be directly equated with alleles at the corresponding structural gene locus. Although electromorph profiles over loci can be equated with multilocus genotypes and electromorph frequencies yield an estimate of allelic frequencies, it should be understood that the alleles recognized may actually be groups of isoalleles. This is because some amino acid substitutions may not affect electrophoretic mobility (Selander *et al.*, 1986). One potential source of error in the application of multilocus enzyme techniques to population

studies noted by Selander *et al.* (1986), that of posttranslational modifications, may not occur frequently enough in any microorganism to bias seriously estimates of genetic variation derived from the electrophoresis of proteins. On a positive note, Selander *et al.* (1986) have demonstrated that estimates of genetic distance based on multilocus electrophoresis are strongly correlated with estimates of divergence in nucleotide sequences obtained from hybridization experiments. Similarly, Miller and Hartl (1986) using biotyping experiments have confirmed the clonal population structure in *Escherichia coli* that was previously based on multilocus enzyme electrophoresis analysis (Ochman and Selander, 1984; Whittam *et al.*, 1983).

E. METHODS OF GENETIC AND NUMERICAL TAXONOMIC ANALYSIS OF POPULATIONS

Systematics is concerned with the scientific study of the diversity of organisms and the relationships among them. Systematics includes taxonomy, which is the science of classification, its principles, procedures, and purposes (Goodfellow and Minnikin, 1985). Systematics includes elements of evolution and as a consequence is also concerned with the interaction of genes and their products. Classification of organisms can be either phenetic, based on shared properties, or phylogenetic, according to their degree of common ancestry (Goodfellow and Minnikin, 1985). There have been recent attempts to bring the two together (Schleifer and Stackebrandt, 1983).

In order to utilize rationally the information generated by modern techniques, computer-based numerical taxonomy approaches have been developed to handle the ever-growing data base. For purposes of discussion of clonal population concepts, the approaches of Selander and coworkers (Selander *et al.*, 1986) are most easily understood. Genetic distance (d) between pairs of isolates or groups of isolates (electromorph types or ETs) is calculated as the proportion of loci at which dissimilar alleles occur, i.e., the proportion of mismatches. In contrast, in my laboratory results are expressed as percentage similarity. A genetic distance of 99% would be equivalent to 1% similarity. Selander *et al.* (1986) noted that various multivariate statistical methods may be used to represent overall genetic relatedness among isolates. In my laboratory the analysis technique used is a simple match coefficient survey. This is derived through matching similar results between pairs of bacilli tested until each strain's results is compared with every other strain's results in the study. Similarity values are assessed as the total number of matching characters, whether these are both positive or both negative. From this information, $t \times n$ similarity matrices are formed, where the strains

(t) involved, operational taxonomic units (OTUs), are considered vs. the number (n) of the tests involved. As pairs are formed, those with the highest similarity indices are sorted out in succeeding order until a hierarchy of similarity between each of the strains involved is assessed. From this hierarchy of similarity it is possible to generate phenograms (dendrograms) showing the general relatedness of the cultures involved. Basically the genetic relationship among the strains is determined by using an unweighted pair group cluster analysis with arithmetic averages (UPGMA) to construct a dendrogram (Sneath and Sokal, 1973).

The genetic diversity for each locus can be calculated as

$$h = (1 - \Sigma x_i^2)(n/n-1)$$

where x is the frequency of the allele at the locus, n is the number of isolates in the sample, and ($n/n-1$) is a correction for bias in small samples (Nei, 1978). Mean diversity per locus can be calculated by the arithmetic average of h over all loci assayed (Selander *et al.*, 1986).

III. Use of the Clonal Population Concept in Epidemiology, Systematics, and Evolution

The use of the clonal population concept perhaps is best illustrated by the summary report of Orskov and Orskov (1983) of a workshop on the clone concept in epidemiology, taxonomy, and evolution of Enterobacteriaciae and other bacteria. The workshop considered epidemiological efforts based on examination of large numbers of enteric pathogen populations from around the world and in some instances back to 1943. The methods used in these examinations included auxanotyping; serotyping of somatic (O), casular (K), and flagellar (H) antigens; the analysis of outer membrane proteins (OMP) by SDS–PAGE patterns; examination of plasmid profiles per se as well as restriction endonuclease digestion studies of both plasmids and chromosomal DNA; peptide finger printing; and electrophoretic analysis of enzymes. The summary report concluded that with the increased ability to differentiate useful and stable phenetic characteristics, it is increasingly possible to delineate clonal associations among bacterial isolates. For example, the defining characters of *Salmonella typhi*, those that make it especially able both to infect human intestinal and reticuloendothelial tissues and to spread among humans, unlike its phage sensitivity and other variable characters, are found together in almost all isolates recovered from typhoid patients. The globally disseminated *E. coli* serotypes and biotypes recovered from diarrheal disease provide another example of the stability of widespread clones over the years. Although variations occur in biotype and serotype of an enteric pathogen over the years, one can still identify the basic few

clonal types although it may take some effort. It was evident from the authors' summary of the workshop that many, if not most, populations of epidemiological importance are the result of a limited number of clonal populations which according to Whittam *et al.* (1983) are mixtures of more or less independently evolving lines.

Multilocus enzyme electrophoresis has recently been successfully used in the assessment of the genetic structure of populations of *Yersinia ruckeri* (Schill *et al.*, 1984), *Legionella pneumophilia* (Selander *et al.*, 1985), *Bordetella* spp. (Musser *et al.*, 1986), and *Neisseria meningitidis* (Caugant *et al.*, 1986).

Forty-seven field isolates of *Yersinia ruckeri* were screened for electrophoretic variation at 15 enzyme loci (Schill *et al.*, 1984). Only four electrophoretic types were observed, a result indicating that the genetic structure of these populations was clonal. The low genetic diversity in this species was demonstrated by the fact that 42 isolates examined represented only one electrophoretic type. Earlier attempts to establish intraspecies classification criteria to be used in epidemiology and vaccine studies in which biotypes, serotypes, and plasmid profiles were defined did not give suitable information for defining the genetic structure of the species. Four major clonal groups or lineages of electromorph types (ETs) were also uncovered when 292 isolates of *Legionella pneumophilia* from clinical and environmental sources were screened for electrophoretic variation in 22 enzymes (Selander *et al.*, 1985).

In a screen of 60 strains of three nominal *Bordetella* spp., Musser *et al.* (1986) demonstrated by electrophoresis that the allelic variation of structural genes encoding 15 enzymes is clonal. They found that the genetic diversity of *Bordetella* is relatively limited compared with most other pathogenic bacteria and is insufficient to justify recognition of three species. The isolates of *B. pertussis* represented only two closely related clones. All isolates of *B. pertussis* from North America (except strain 18–323) were genotypically identical. All isolates of *B. parapertussis* were of one electrophoretic type. These isolates were closely similar to the electrophoretic types of isolates representative of *B. bronchiseptica*. The strain of *B. pertussis* (18–323) used in mouse potency tests of vaccines was apparently more similar genetically to *B. parapertussis* and *B. bronchiseptica* than to the other isolates of *B. pertussis*, while the Dejong strain of *B. bronchiseptica* was strongly differentiated from all other *Bordetella* isolates examined. Two well-defined clusters of ETs were observed when 152 isolates of *Neisseria meningitidis* were analyzed for variation in nine enzymes by multilocus enzyme electrophoresis (Caugant *et al.*, 1986). Each of the two clusters of ETs included one of the two most common ETs identified among isolates (ET-5 and ET-35). The distribution of alleles

in genotypes identified among the isolates indicated that genetic recombination may occur in natural populations of *N. meningitidis*.

Undoubtedly the natural bacterial population most completely studied in terms of population genetics is *Escherichia coli* (Hartl and Dykhiuzen, 1984). Single-locus diversity estimates of 1705 clones of *E. coli* surveyed for allozyme variation of 12 enzyme loci showed that the combination of alleles in electrophoretic types is highly nonrandom. These allele linkages reflect genetic differentiation of *E. coli* into three groups of strains. "Because of the restricted recombination, both the stochastic extinction of lines and selective differences between particular genetic combinations may have contributed to the evolution of subspecific structure in *E. coli*" (Whittam *et al.*, 1983). This subspecific substructure was supported by Ochman *et al.* (1983), who examined electrophoretic variation in 12 enzymes in 1600 isolates of *E. coli* from human and animal sources as well as 123 strains of four species of *Shigella*. Ochman and Selander (1984) examined 142 K1 isolates of four O serogroups of *E. coli* by electrophoretic analysis of allozyme variation in 12 chromosomally encoded enzymes and found that the distribution of O serogroups among the isolates did not consistently correspond to clonal structure. Miller and Hartl (1986) confirmed the nearly clonal population structure in *E. coli* using biotyping studies.

IV. Studies on the Larvicidal Populations of *Bacillus sphaericus*

A. Bacterial Insecticides

Prior to the 1930s cases of malaria and related mosquito-borne diseases in the United States were counted in the millions (Nielsen, 1979; NAS, 1976). Since then, the problems of mosquito control have been reduced to that of recreational concern. Occasionally there is a major outbreak of encephalitis, such as the one that occurred in 1975. Although there were only hundreds of cases of encephalitis, this belies the severity of the illness. Outside of the United States and most other developed countries, we have a different situation. Vector-borne tropical disease is measured in hundreds of millions of cases of malaria, yellow fever, dengue fever, and filariasis (Nielsen, 1979; NAS, 1976). Since World War II the major defense against vector-borne diseases has been chemical insecticides (Johnson, 1969). Although major emphasis has been placed on perturbation of the environment, the major cause of decline in effectiveness of chemical insecticides, from a practical viewpoint, has been the rise of resistance to the chemicals by the vector populations in endemic areas. In response to this situation there have been efforts to search out biological alternatives, new weapons, to supplement the use

of the chemicals (NAS, 1976; Cantwell and Laird, 1966). The most promising of these are a group of spore-forming bacteria (Singer, 1973, 1980, 1986; Krieg and Miltenburger, 1984; Yousten, 1984).

When insecticidal activity against mosquito larvae of isolates of *B. sphaericus* was first reported (Singer, 1973), these isolates stood unique in their potential for field use against mosquito larvae (Singer, 1974). Subsequently, isolation of new strains of *B. thuringiensis* [especially strain *israelensis* (*B.t.i.*)] effective against both mosquito and black fly larvae (Goldberg and Margolit, 1977; deBarjac, 1978) pushed to the sidelines interest in *B. sphaericus* in spite of the isolation of more stable varieties of *B. sphaericus* available for development. Industry appeared to be reluctant to invest the development dollars (or marks, or francs, or yen) in what was seemingly a narrow public health market. The isolation of *B.t.i.*, however, rekindled interest since its biotechnology fit more easily in corporate research and development plans, especially of those companies who were already marketing the agricultural varieties of *B.t.* Recently *B.t.i.* has seen full commercial development as a microbial insecticide against vectors of tropical disease, e.g., mosquitoes, black flies (*Simulium*), and is available commercially for field use (Singer, 1986), while *B. sphaericus* is still under development by the World Health Organization (WHO).

An informal consultation on the development of *B. sphaericus* as a microbial larvicide was recently held in Geneva under the auspices of WHO (WHO, 1985). The most noteworthy conclusion was that "the most toxic *B. sphaericus* isolates are more effective than *B.t.i.* against species of *Culex*, *Mansonia*, and some species of anopheline mosquitoes and on this basis alone warrant further development and evaluation" (WHO, 1985).

In contrast to *B.t.i.*, existing isolates of *B. sphaericus* are virtually nontoxic to black flies (*Simulium*). They are quite toxic to most of the important mosquito groups with the exception of some *Aedes* such as *Aedes aegypti* (Singer, 1980; Yousten, 1984). The three prime candidate *B. sphaericus* strains are 2362, 1593, and 2297 (discussed below). New primary powders and formulations have recently become available for laboratory and field testing (WHO, 1985). In general, strain 2362 was the most effective against all species and instars, followed by 1593, which in turn was more effective than 2297 when tested in the laboratory. Strains 1593, 2297, and 2362 all proved toxic to species of *Culex*, *Anopheles*, *Mansonia*, and *Psorophora*. None of the strains were significantly toxic to *Ae. aegypti*, although *Ae. melanimon*, *Ae. triseratus*, and *Ae. nigromaculis* were susceptible, indicating that *B. sphaericus* may prove useful as a larvicide against aedine mosquitoes (WHO, 1985). Of interest

is the effectiveness of primary powders of 1593 and 2362 against *Mansonia* with strain 2297 coming in a relatively poor third.

According to the report (WHO, 1985) some of the most impressive results achieved in the field to date with experimental formulation of *B. sphaericus* have been obtained against *Culex quinquefasciatus* in highly polluted waters in the Ivory Coast and in the United Republic of Tanzania. In the latter country, for example, the formulated 2362 provided effective control of *Cx. quinquefasciatus* in cesspits and latrines for as long as 6–10 weeks when applied at a rate of 10 g/m^2. In addition to efficacy against *Cx. quinquefasciatus*, the formulated preparation of 2362 and 1593 has been shown to be relatively effective against *Mansonia uniformis*, an important vector of Brugian filariasis in South East Asia. At a rate of 1 kg/ha, both these formulations provided 80% reduction of larval populations for as long as 14 days after application (WHO, 1985).

B. Origin of *Bacillus sphaericus* Strains

Forty-five out of 186 strains of *B. sphaericus* isolated and examined to date show some toxicity to mosquito larvae and provide 100% mortality in 48 hours at a concentration of 10^7 cells/ml (WHO, 1985). Most of these larvicidal strains carry an accession number from a collaborating center for the biological control of vectors of human disease (WHO/CCBC) at Ohio State University (Columbus) under the direction of Dr. J. D. Briggs. Much of the early development work of many of the more active strains, particularly the earlier ones, was done in the author's laboratory (Singer, 1980, 1986). Table I lists these strains according to country of origin and clonal grouping (of those done). Strain K was isolated by Kellen (Kellen and Meyers, 1964; Kellen *et al.*, 1965) and it, along with strain Q which was derived from it, can be said to be the first reported active *B. sphaericus* isolate. Strain SSII-1 (WHO/CCBC 1321), one of the earlier isolates from India (Singer, 1973), represents the first generally available active *B. sphaericus* strain, with which much of the earlier development work was done (Singer, 1986). In 1975 strain 1593, the first fermentation- and population-stable strain, was derived from field material sent to WHO/CCBC (Columbus). This strain still remains one of the three key field candidates. Wickremesinge and Mendes (1980) isolated strain 2297 (which they call MR-4), the second of the present-day key field candidates. Weisser (1984) isolated strain 2362 from *Simulium* adults in Nigeria. Strain 2362 is the third of the three key field candidates. The remaining strains listed in Table I (as well as the larvicidal strains listed in Table II) are of more than historical interest. They represent a genetic pool from which material for future genetic manipulation may be derived. Although a majority of research and development effort concentrated on the few

TABLE I

COUNTRY OF ORIGIN OF SEVERAL INSECTICIDAL STRAINS OF *Bacillus sphaericus* ACCORDING TO CLONAL GROUPS

Clonal group	Strain	Country of origin
1	Q	United States
	2317-3	Thailand
	1881	El Salvador
	2173	India
	2314-2	Thailand
	2297	Sri Lanka
	1894	Israel
	SSII-1	India
2	2013-6	Rumania
	2362	Nigeria
	2500	Thailand
	2117-2	Philippines
	1691	El Salvador
	2377	India
3	1404	Philippines
	1593	Indonesia
4	2115	Philippines
	K	United States
5	2315	Thailand

strains listed above, many of these "other" strains are as active as the ones just mentioned. They and similar strains yet to be isolated represent the potential to satisfy the local national need for "endogenously derived" strains.

C. PRESENT CLASSIFICATION OF *Bacillus sphaericus*

The members of the genus *Bacillus* in general and *B. sphaericus* in particular are ubiquitous saprophytes occurring universally in nature (Gibson and Gordon, 1974). *Bacillus sphaericus* is an aerobic rod-shaped endospore-forming bacterium with the endospores in a swollen terminal position. Until recently it was thought that *B. sphaericus* did not form a parasporal crystal like that of *B. thuringiensis*. However, Davidson and Myers (1981) found that some insecticidal strains, notably those that are highly toxic in the spore stage, produce parasporal inclusions that

resemble crystals of *B. thuringiensis*. *Bacillus sphaericus* does not ferment glucose, other sugars, or starch. Rather, it uses amino acids as its carbon and nitrogen sources (Singer *et al.*, 1966). Using the classical biochemical identification methods (biotyping), it is impossible to distinguish differences between the insecticidal and noninsecticidal varieties of *B. sphaericus*. There is no simple distinguishing test that one can utilize for the initial examination of populations of these bacteria when they are freshly isolated from dead larvae from the wild or that one can use to judge potential differences in biological activity among these strains.

Several studies have been conducted in an attempt to differentiate clearly the strains of *B. sphaericus*. Krych *et al.* (1980) examined 62 strains of *B. sphaericus* for certain phenotypic characteristics, including G + C content of DNA and DNA homology. They found little differences in mol% G + C content among the strains. However, the DNA homology study provided clear evidence that the strains of this species had little genetic relationship to one another. They found five homology groups with homology group II being subdivided into groups IIA and IIB. Group IIA was composed of seven *B. sphaericus* strains that were all pathogenic to the mosquito larvae of *Cx. quinquefasciatus*. Ordinarily these results would present the basis for establishment of several new species of bacteria. However biotyping did not reveal any properties aside from pathogenicity that could be used for routine separation of new species (Yousten, 1984).

deBarjac also attempted to distinguish between insecticidal and noninsecticidal strains using biochemical and auxanographic techniques (deBarjac *et al.*, 1980). Differentiation of the strains could not be made on the basis of 78 phenotypic characters. Yousten (1984) developed a bacterophage typing protocol which allows grouping of the insecticidal strains into seven phage types, with strain 1593 and 2362 classified in group 3 and strain 2297 into phage group 4. Phage group 3 reportedly contains strains showing the most insecticidal activity. Using H antigens, deBarjac *et al.* (1980) developed a serotyping protocol (similar to the one deBarjac developed earlier for *B. thuringiensis*) to distinguish the *B. sphaericus* strains. Their serotype groups coincide almost exactly with the phage groupings of Yousten *et al.* (1980).

I have arranged the strains of *B. sphaericus* (Table II) in light of available information on DNA homology (Krych *et al.*, 1980) and phage type for the insecticidal strains (Yousten *et al.*, 1980).

D. Plasmid Profiles of *Bacillus sphaericus* Strains

Delta endotoxin of *B. thuringiensis* is generally associated with large plasmids, which are reputed to be present in low copy numbers (Gonzales *et al.*, 1981). At present it is not known whether large plasmids are

TABLE II

ARRANGEMENT OF STRAINS OF *Bacillus sphaericus* IN LIGHT OF AVAILABLE INFORMATION

DNA homology groups[a]

I	IIA[c]	IIB	III	IV	V
ATCC 14577[b]	*1593*	*ATCC 7055*	*NRS 592*	*NRS 400*	*NRS 1198*
ATCC 10208	SSII-1	ATCC 7054	ATCC 12123	NRS 717	NRS 1199
NRS 967	1404	ATCC 12300	P1	NRS 1529	NRS 1184
NCTC 9602	Kellen K	NRS 718	NRS 800	ATCC 13805	
	Kellen Q	NRS 1191	NRS 1692	ATCC 245	
	1881	NRS 1196	NRS 593	NRS 1090	
	1691	ATCC 7063	NRS 1195	NRS 1693	
		NRS 1194	NRS 1193	NRS 1307	
		NRS 1200	NRS 1197		
		NRS 1201	NRS 1187		
		NRS 1192	NRS 1023		
		NRS 156	NRRL B4197		
			NRS 810		
			NRS 719		
			ATCC 4978		
			NRS 1223		

Phage groups[d]

1	2	3	4	5	6	7
Kellen K	SSII-1	1593	2297	1894	2115	2315
Kellen Q	1404	1691	2173			
	1883	1881	2377			
	1885	2013-4	2314-2			
	1886	2013-6	2317-3			
	1887	2117-2				
	1888	2362				
	1889	2500				
	1890	2501				
	1891	2537-2				
	1892	2533-1 (K1)				
	1893	2533-1 (K2)				
	1895	2601				
	1896	2602				

[a]From Krych *et al.* (1980).
[b]Italicized strains denote the type strain of the DNA homology group.
[c]Members of this group are all insecticidal.
[d]From Yousten (1984).

associated with insecticidal activity in *B. sphaericus*. The search for plasmids present in *B. sphaericus* has been incomplete and somewhat conflicting (Yousten, 1984). The presence of small plasmids has been reported (Davidson *et al.*, 1982; Abe *et al.*, 1983; Yoshimura *et al.*, 1983). Davidson *et al.* (1982) reported the presence of a single large plasmid

in strains 1593 and 1881 but not in 1691 or 2362; Abe *et al.* (1983) found a large plasmid in 1881 but not in 1593 or 1691. Abe *et al.* (1983) found five plasmids in strain K; Davidson found none. These differences are probably due to differences in techniques, since plasmid isolation techniques undoubtedly result in the loss of large plasmids whereas direct lysis of the whole protoplast in the wells of the agarose gel (Eckhardt, 1978) preserves the large plasmids. Recent work in my laboratory (Shelley and Singer, 1986) using the Eckhardt technique (Eckhardt, 1978) indicates that most of the highly active *B. sphaericus* strains contain one or more large plasmids (Table III; Fig. 1), but so do several of the noninsecticidal strains. Table III illustrates the plasmid pattern of at least one insecticidal strain from each of the DNA homology groups, with strain 1593 (bacteriophage group 3) representing DNA homology group IIA.

Although no plasmids were detected for the strains from DNA homology groups I, III, and V (chosen for illustration), other strains of the same groups (not shown here) do possess plasmids (Shelley and Singer, 1987). Note also that no plasmids were detected in strain 1691. This did not surprise us. We could not detect plasmids in strain SSII-1

TABLE III

INSECTICIDAL AND NONINSECTICIDAL STRAINS OF *Bacillus sphaericus* AND THEIR PLASMIDS

Strain	Homology or Bacteriophage group	Number of plasmids	Plasmid mass (MDa)
Insecticidal	Bacteriophage group		
K	1	1	29
SSII-1	2	1	75
1593	3[a]	1	75
2362	3	1	75
2297	4	3	75, 3.4, 3.2
1894	5	1	5.7
2115	6	1	4.2
2315	7	7	75, 40, 15, 7.6, 5.7, 3.7, 2.9
Noninsecticidal	Homology group		
14577	I	–[b]	–
7054	IIB	2	5.2, 3.4
1191	IIB	3	75, 5.2, 3.4
4978	III	–	–
1090	IV	3	35, 9.2, 2.7
1198	V	–	–

[a]Homology group IIA.
[b]None detected.

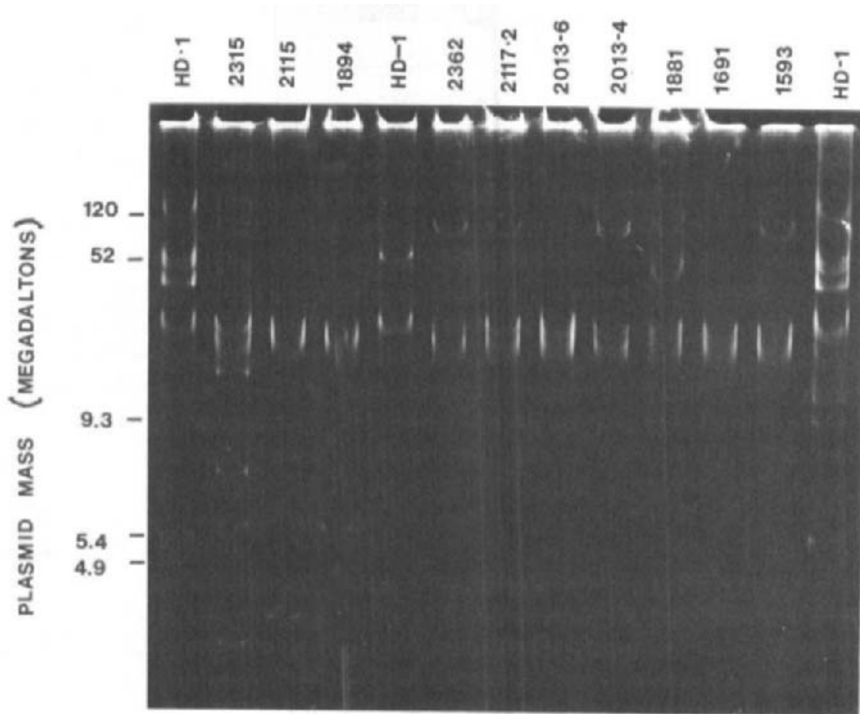

FIG. 1. Agarose gel (0.5%) showing plasmid profiles of insecticidal *Bacillus sphaericus* strains. Marker strain was *B. thuringiensis* HD-1 (lanes 1, 5, and 13).

the first time we examined it, yet when we examined SSII-1 from Yousten (who originally obtained the strain from us) we found it possessed the 75-be Da plasmid. Obviously plasmids were lost (or the plasmid copy number was reduced) during slant-to-slant passage over the long period that we had been working with it. Media variation appears to affect not only the growth and fermentation of these strains but also plasmid replication (Ditto, 1985; Ditto and Singer, 1985). In general we have had less plasmid loss with the *B. sphaericus* strains than we have had with the *B. thuringiensis* strains. We used B.t. strain HD-1 as our marker strain in lanes 1, 5, and 13 (Fig. 1). The cultures for all three lanes were derived from the same initial slant in our inoculum buildup (but different subsequent shake flasks), yet HD-1 in lane 5 shows a loss of plasmids. An apparent loss of plasmids in one out of three cultures is not uncommon in our work with *B.t.*

Single and multiple antibiotic-resistant strains were isolated from four insecticidal and two noninsecticidal parent strains of *B. sphaericus* (Yurks and Singer, 1986). Several of the single resistant strains isolated were also

cross-resistant to several antibiotics of different modes of action. All of the parent strains were naturally susceptible to 11 and naturally resistant to two out of 22 antimicrobics examined. The majority, 17 out of 23, of antibiotic-resistant strains, that were isolated from the insecticidal parent strains retained their insecticidal activity while the noninsecticidal strains remained noninsecticidal.

Of the 25 single antibiotic-resistant strains isolated, five strains displayed multiresistance to an array of antibiotics in addition to the target antibiotic. This would imply the presence of multiresistant plasmids. The plasmid DNA complement of *B. sphaericus* has been shown to be quite simple compared to the complex arrays observed in *B. thuringiensis* (Singer, 1986). Work in the author's laboratory (Shelley and Singer, 1987) has demonstrated through the examination of plasmid profiles that no new plasmids appeared when antibiotic-resistant isolates were obtained from the insecticidal populations. Unless the multiresistance somehow relates to the cryptic plasmids present, it would appear that these multiresistant events involved mutational resistance in the chromosomal DNA.

The most unusual finding, however, was the distinctive pattern of seven plasmids in strain 2315. This insecticidal strain would make an excellent marker culture for both studies and genetic work. We have also selected antibiotic-resistant variants of this strain in a manner similar to that done with several of the most active *B. sphaericus* (Yurks and Singer, 1986).

The results of the plasmid profile work in my laboratory would indicate that the plasmid profiles per se cannot differentiate among the insecticidal or noninsecticidal strains of *B. sphaericus*.

E. Multilocus Enzyme Electrophoresis of *Bacillus sphaericus* Populations

1. *Background*

Bacillus sphaericus is considered to be a loosely clustered species (Logan and Berkeley, 1981; Priest, 1981; Priest *et al.*, 1981). Although *B. sphaericus* has been studied by a number of workers since its original description by Neide (1904), the boundary around the species continues to be "diffuse and arbitrary" (Gordon *et al.*, 1973). The means presently used to identify strains of *B. sphaericus* is the examination of the spore (terminal and swollen) and several biochemical tests concerned with the inability to use most sugars. Approximately half of the strains now classified as *B. sphaericus* differ with respect to urease production, the utilization of citrate, and toxin production for the larvicidal group. As indicated earlier (Table II), phage typing (as well as serotyping) is a

successful approach when initially examining fresh strains isolated from dead larvae. None of these approaches, however, yield information with regard to the genetic diversity of this group. A good beginning to a molecular approach has been the DNA homology studies of Krych *et al.* (1980). Some of the pertinent findings from this study are that (1) each strain within each homology group has 80–100% homology to the group type strain; (2) strains from homology group 2, divided into two subgroups, show about 60% homology to type strains of the other subgroup; (3) all of the larvicidal strains tested fall within subgroup 2A; (4) group 1 which contains the species type strain shows less than 20% homology to the remaining 4 groups; (5) similarly, group 5 shows only 20% homology to the remaining 4 groups; (6) while groups 2, 3, and 4 show approximately 40% homology to each other.

The use of enzyme electrophoresis offers some advantages over DNA hybridization. The former is easy and quick and does not require special expensive equipment. Baptist *et al.* (1978) have used electrophoresis of limited numbers of enzymes to differentiate *Bacillus* species. According to these workers if a sufficient number of enzymes are compared (more than five), then the results will show that two individuals from different species will differ in electrophoretic mobility by about 50% or more, whereas two members of the same species will usually differ 20% or less. Preliminary experiments in my laboratory using aminopeptidase panels (Kohl and Singer, 1985) and later using polyacrylamide gel electrophoresis of amino peptidases from nine strains of *B. sphaericus* demonstrated differences in electromorph patterns between insecticidal and noninsecticidal strains [Williston (Kohl) and Singer, 1987]. This initiated a multilocus electrophoretic study of further members of *B. sphaericus* using 20 strains from the five homology groups and 12 enzymes (29 loci) (Hessler and Singer, 1986) and using 19 strains from the seven groups of the insecticidal DNA homology group IIA with a similar number of enzymes (Cole and Singer, 1986).

2. *Experimental Methods*

Either horizontal starch gel electrophoresis or vertical polyacrylamide gel electrophoresis (PAGE) may be used to examine the electromorph mobility of the specific enzyme (Selander *et al.*, 1986). While both methods were used in the following experiments to be discussed, either permits examination of the enzymes. In my laboratory PAGE is preferred because in our hands clearer bands are obtained on PAGE using *B. sphaericus* strains. The use of starch does, however, permit a more rapid examination of the starch gels. This is because many "slices" can be obtained per starch gel and stained for the appropriate enzymes, while only one stain per gel can be accomplished with PAGE unless one uses

transfer techniques (McLellan and Ramshaw, 1981), which in our hands is not particularly satisfactory. Starch gels also have the advantage of allowing one to note cathodal movement of some enzymes. No such movement, however, was observed with the enzymes used. Starch gel electrophoresis was used to basically scan or quickly identify the presence of the enzyme allozymes. For the enzyme systems where the resolution was not good on starch, they were done on PAGE. The enzyme systems that were to be examined were selected either from the results of preliminary use of commercially available enzyme panels or from previous experience with enzyme systems used by colleagues for examining other taxa and available for our use. It is important that the enzymes systems to be used are randomly selected in order not to introduce bias in the protocol (Selander *et al.*, 1986). This was carefully practiced during the selection of the enzymes.

The enzymes used for the following studies were, on starch, alanine dehydrogenase, fumarase, isocitrate dehydrogenase, malate dehydrogenase, and shikimate dehydrogenase; on PAGE, alanine aminopeptidase, diaminopimilate dehydrogenase, diaphorase, esterase (α-acetate substrate), glutamate dehydrogenase, threonine dehydrogenase, and superoxide dismutase.

The protocol used was growth of the strain in a standardized inoculum buildup procedure (Singer, 1986); a 20-hour broth culture was then used to prepare cell-free material by passage through a French pressure cell, followed by adjustment of the protein content based on a Lowry determination. The cell-free extract was then used either in a horizontal starch gel electrophoresis unit according to the methods of Smithies (1955) or in a vertical PAGE until using a modification of the alternative method of Davis (1967). Staining of the enzymes in both electrophoresis systems was according to Harris and Hopkinson (1978). Genetic relationships among the bacilli used was established as previously described (see Section II,E).

3. *The Species* Bacillus sphaericus *and Its Clonal Components*

Figure 2 (taken from Hessler *et al.*, 1987) and Fig. 3 (taken from Cole *et al.*, 1987) relate the percentage similarities of the strains from each of the two experimental series based on electrophoretic mobilities of the allozymes of 12 separate enzymes (29 loci). In Fig. 2 we see that all of the strains examined cluster according to their DNA homology group with variation within and between homology groups but with greater similarity within groups than between groups.

Homology group I is a tightly clustered group quite distinct from the other groups. This is interesting in light of the fact that group I contains strain ATCC 14577 which is the *B. sphaericus* type strain. This group

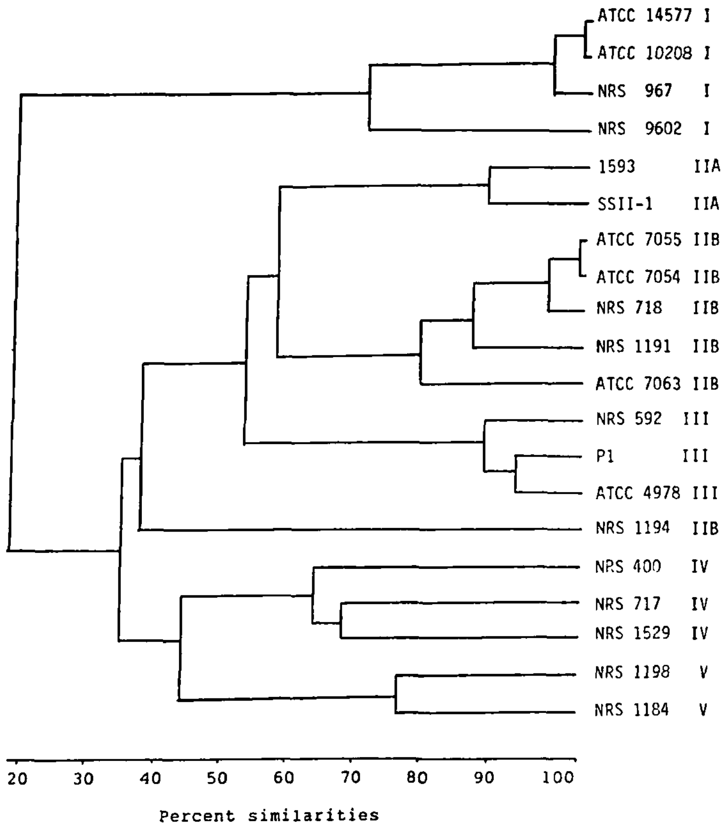

FIG. 2. Phenogram of percentage similarities among 20 *Bacillus sphaericus* strains, representative of DNA homology groups. Roman numerals represent DNA homology groups according to Krych *et al.* (1980).

also contains strain NRS 9602 which has been the source of much of the physiological information on *B. sphaericus* (White and Lotay, 1980; White, 1983). DNA homology group I would therefore be a good candidate for separate species status. Group IIA is also a good candidate for separate species status. This group has many unique alleles (not shown here) which set it apart from the noninsecticidal strains. Each of the strains in this group produces toxin(s) which cause them to be larvicidal to certain mosquito larvae. Krych *et al.* (1980) showed this group to be related to the noninsecticidal group IIB, and so classified them in the same homology group although in different subgroups. According to our electrophoretic data, the strains of DNA homology group IIB cluster tightly (except for strain 1194) and are distinctly different from group IIA. Most

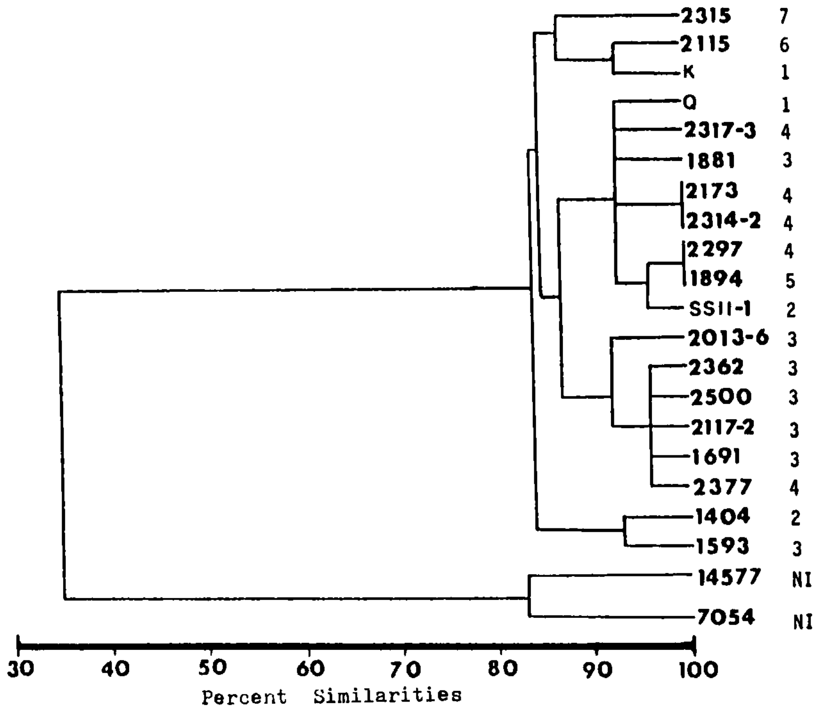

FIG. 3. Phenogram of percentage similarities among 21 *Bacillus sphaericus* strains, representative of insecticidal bacteriophage groups. The numbers in the right-hand column are according to Yousten (1984). NI, Noninsecticidal.

group IIB alleles, however, are shared with at least one other homology group but the group does have some unique alleles (not shown here). Strain 1194 shows some unique alleles as well as alleles in common with groups IIB and V (not shown here). Group V, according to Krych *et al.* (1980), is a small group with very little relation to any of the other groups. The two strains tested in this study support this. Although groups III and IV appear to be more diffusely clustered than the other groups, members of each of these two groups are closer to each other than to the other groups in this study. Members of both of these groups also share many electromorphs with other groups and each other (not shown here). Group IV is the most heterogeneous of the groups, much more so than anticipated from the DNA homology study (Krych *et al.*, 1980). Other members of groups III and IV (Table II) will have to be examined before more can be said of the relation of these two groups of strains to each other and to the other homology groups.

DNA homology group IIA is particularly interesting since it contains all of the insecticidal strains. The dendrogram shown in Fig. 3, based on electrophoretic examination of representatives of the strains from the seven phage groups (Table II), shows the populations of 19 members of this group to be essentially clonal. The noninsecticidal and insecticidal strains examined here were only 35% similar while the insecticidal strains cluster closely between 82 and 100% similarity. Within the insecticidal cluster there are five distinctive subclusters or clonal groups. Clonal group 1 consists of eight strains including strain 2297 (see Table I). Clonal group 2 consists of six strains including strain 2362. Both clonal groups 3 and 4 consist of two strains: strains 1593 and 1404 in group 3, and 2115 and K in group 4. Clonal group 5 only contains one strain (2315), but it should be remembered that this strain is the unusual one that showed seven plasmids in the plasmid profile experiment (see Section IV,D).

The mean genetic diversity of all of the 19 strains was substantially higher than that of each individual clonal group (not shown here). While each of the five clonal groups of strains showed genetic diversity within each group, there was greater genetic similarity within a group than between groups.

The three key field candidates strains (1593, 2297, 2362) separated into three different clonal groups. Each of the clonal groups contain strains of various insecticidal activity with none of the clonal groups having a monopoly on this important biological activity.

Strains 1404 and 1593 were 90% similar (Fig. 3) and belong to the same clonal group. Strain 1593 was isolated from material from Indonesia and strain 1404 was isolated from material from the Philippines (Singer, 1977). This is in agreement with the clone concept of bacterial cultures isolated from different sources in different locations, and often at different times, that are so similar to one another that they must have derived essentially from virtually asexual reproduction of a common ancestor (Orskov and Orskov, 1983).

There was no relationship between the bacteriophage groups (or serogroups) and the clonal groups. *Bacillus sphaericus* strains 2297 and 1894 were 100% similar, but strain 2297 belongs to phage group 4, serogroup H25, and strain 1894 belongs to phage group 5, serogroup H2 (Yousten, 1984). Genetically distinctive strains can evidently converge to serotypes that are indistinguishable by conventional criteria and genetically identical or nearly identical strains can have different serotypes (Hartl and Dykhuizen, 1984). The problem is that serotypes reflect variation in an unknown but probably small number of genetic loci involved in the synthesis of antigenic molecules, the chemical

structure of which are not well understood (Selander *et al.*, 1985). Furthermore, electrophoretic data provide estimates of the degree of genetic relatedness or distance among isolates, whereas serological data indicate only the existence of differences (Caugant *et al.*, 1985).

Our electrophoretic data are in close agreement with the DNA homology studies of Krych *et al.* (1980) and are in disagreement with the serogrouping and bacteriophage grouping of deBarjac *et al.* (1980) and Yousten et *al.* (1980), particularly for the insecticidal strains of *B. sphaericus*. Whether *B. sphaericus* will be divided into several species according to the DNA homology and the electrophoretic data (which is probable) or remains a single large species containing many clonal groups (which is unlikely) requires further examination of all of the strains (Table II). There is no question, however, that the classification of the insecticidal DNA homology group IIA would be best served by the clonal groupings indicated earlier. This would be particularly true in the future examination of newly derived culture candidates for field use. There is little value in having 25 or more genetically unrelated serogroups when 4 or 5 electrophoretically derived, genetically related clonal groups are so easily matched and examined. It should also be emphasized that it is from the examination of genetic diversity of the strains rather than from their cell surface changes that the greatest economic and public health benefits will be derived.

V. The Importance of the Clonal Concept in Problems in Applied Microbiology

The one area that unites most microbiologists, whether applied or nonapplied, is the integrity of the identity of one's own important taxon and its relationship to other taxa. To maintain this integrity or even to establish it in the first place requires a constant effort, especially for those of us who are not taxonomy specialists. This is as true for the establishment (assignment) of proprietary rights (as in a patent) as it is for understanding the genetic baseline of the microorganism upon which one is about to perform some recombinant DNA feat. It is as important in examining a large number of isolates (for whatever reason) as it is in constructing a plasmid for vectoring into a crucial strain. What the population geneticists have given us in the form of the population clone is a means of maintaining the strain's integrity. The concept also gives us the opportunity to consolidate in an era where the flood of information tends to divide and separate the strains in our culture collections, one from the other, more and more. It, above all, confirms and reinforces the conservatism of the taxonomists who insist on using only stable characteristics in their work.

There are many examples where one can cite the importance of the clonal concept. For example, one can refer to the importance of population clones in epidemiology (Orskov and Orskov, 1983), in vaccine production (Schill *et al.*, 1984), and in my own specialty, the isolation and construction of newer and better bacterial insecticides. All of us upon reflection should be able to relate the importance of the concept to our own areas. What is of particular importance to my laboratory is that I am now able to examine the cultures in my collection for a stable set of characteristics quickly, inexpensively, with a minimum of new math and come out with a set of relationships that can presumably lead me to new unsuspected activity. Let me cite two examples of this: (1) how to handle new field isolates and (2) how to get a new view of old isolates.

In screening for new isolates a technique is needed to identify whether an isolate is a new strain, or rather a duplicate of a strain or a group of strains already isolated. Through the use of serotyping or bacteriophage typing, this would not be possible since two genetically identical clones (isolates) could have different serotypes or phage types. Also I could not tell if the isolates are insecticidal or not without going through a lengthy inoculum buildup and bioassay protocol. Since I am now aware that in *B. sphaericus* we are dealing with only four or five clones (to date) among the insecticidal strains, all I need do is use the appropriate combination of a few enzymes in a multilocus electrophoretic study and the appropriate type clonal strains, and I can not only determine which clone it belongs to (how they are related to existing strains) but at the same time determine if it is presumptively insecticidal. The procedures are such that it would be more efficient to test many strains than only a few strains. Also according to Selander *et al.* (1986) I could just as easily start with scrapings from a plate (which of course is anathema to a fermentation person).

In the second instance we have available in my own collection or through the courtesy of USDA locally a vast number of *B. thuringiensis* (*B.t.*) strains. The HD (for Howard Dulmage) collection alone has over 800 cultures. The *B.t.* strains are a good example of the use of serotyping in identifying strains. The last time I looked we were up to serotype H-26 and undoubtedly by the time you read this we will be past serotype H-30. The literature (Dulmage *et al.*, 1981), folklore, and personal experience tell me that within the various specific serotypes there are strains that differ (perhaps slightly) in their larvicidal activity against specific target lepidoptera. It would be nice to apply multilocus electrophoretic testing of the type serotypes (as well as select members within a few of the serotypes) and determine if the *B.t.* populations are clonal. This is already being attempted in my laboratory. There are two applications that I can immediately apply this forthcoming information to. The first one is to

aid in selecting candidates for genetic manipulation (or at the very least protoplast fusion). The second is to see if any relationships develop that gives me insight into which clone(s) are better for particular target agricultural pests or vectors of tropical disease. Is *B.t. israelensis* (serotype H-14) related to any of the other serotypes? In the aforementioned effort with the *B.t.* serotypes, we are also examining *B. cereus* strains to see if there are any population genetic associations between the two "species."

As mentioned in the Introduction, the clonal population concept is not new. It has only just been applied to bacteria. At the worse it will only supplement existing information, while at the best it may lead to new insights into the bacterial populations that we use.

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Molecular Mechanisms of Viral Inactivation by Water Disinfectants

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I. Introduction

Hepatitis, gastroenteritis, and other diseases (Chambers, 1971; Harakeh and Butler, 1984) may be transmitted when viruses evade filtration and pass through water treatment plants (Poduska and Hershey, 1972; Shuval, 1970). Coagulation and sedimentation enhance virus removal (O'Connor *et al.*, 1982); however, further treatment is required to ensure that those viruses which successfully pass through the filtration process become inactivated. The addition of disinfectants to prevent the spread of waterborne disease (Varma *et al.*, 1974) dates back to early times. Disinfectants are defined as agents, usually chemical, which destroy disease-causing or other harmful microorganisms when applied to inanimate objects (Block, 1983).

Inactivation of bacteria is usually more easily accomplished than is inactivation of viruses. This may be due in part to the complexity of bacteria and the interaction between a bacterium and its environment. Viruses, on the other hand, are very simple. They are composed of a protein coat (capsid) which surrounds either RNA or DNA (Poduska and

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Hershey, 1972). No metabolic processes occur outside a host cell. Disinfectants must remove or destroy viruses to such an extent that successful reproduction in a susceptible cell is prevented. This may be accomplished by permanently immobilizing viruses on a surface, blocking or destroying host cell receptors on the virus, or inactivating the nucleic acid within the viral capsid.

The complex interaction of viruses and disinfectants varies from one type of virus to another (Harakeh and Butler, 1984), making the mode of inactivation unclear (Butler *et al.*, 1985). Therefore, chemical analyses of disinfectants is not enough in order to evaluate how they may work. Most existing knowledge of the action of disinfectants on viruses has been obtained under a variety of experimental conditions, and thus apparently conflicting data may be reported (Chen and Koski, 1983). What is clear is that many factors affect disinfection (Boardman and Sproul, 1977; Culp, 1974) and that no single disinfectant is equally effective under all conditions for all viruses (Kawata *et al.*, 1979).

II. Factors Affecting Disinfection Efficiency

Disinfecting ability is dependent on the virus type (Jensen *et al.*, 1980) and the concentration of disinfectant species available (Engelbrecht *et al.*, 1980; Kelly and Sanderson, 1960; Taylor and Butler, 1982; Varma *et al.*, 1974), as well as the pH, reaction temperature, contact time, ionic strength of the suspending medium. state of virus clumping and aggregation, and the presence of interfering substances (Payment *et al.*, 1985; Grabow *et al.*, 1983; Keswick *et al.*, 1985; Akey and Walton, 1985; Kruse *et al.*, 1970; Roy *et al.*, 1982; Engelbrecht *et al.*, 1978; Sharp *et al.*, 1980; Snead et *al.*, 1980). Hoff and Geldreich (1981) have claimed that ranking biocidal efficiences of disinfectants is not possible because inactivation efficiences vary with different microorganisms and experimental conditions. Redox potentials also do not correlate well with germicidal activity (Morris, 1970).

Popular types of disinfectants for water treatment include soluble agents, insoluble (contact) disinfectants, and light-induced disinfection (Chang, 1970). A major shortcoming of all soluble disinfectants is their short half-life. Soluble disinfectants need to be replenished periodically. This in turn requires monitoring of the disinfectant concentration and determination of optimal quantities to add to the water system. This disadvantage may be overshadowed by the ability to alter significantly the concentration of the disinfectant to meet the varying demands of water treatment. Soluble disinfectants can be applied in doses well above normal operating concentrations for special treatment conditions, and then easily returned to normal levels as diffusion and dilution occur. In

contrast, the concentration of contact disinfectants cannot be easily adjusted.

Insoluble (contact) or surface-active disinfectants are those disinfectants requiring physical contact between the virus and the surface of the disinfectant. A distinction should be made between time-released disinfectants and insoluble contact disinfectants. Time-released disinfectants such as trichloroisocyanuric acid (Scott and Bloomfield, 1985) are really soluble disinfectants, as direct contact is not required even though a residual substance may be present for some time within a treatment system. True contact disinfectants are insoluble and have unique properties. While constant monitoring of disinfectant concentration is not a concern, fouling of the active surface and flexibility of treatment become important considerations.

Another major concern of insoluble contact disinfectants is the inflexibility of treatment. Once added to a water treatment system, contact disinfectants may be difficult to remove. Immediate alteration of disinfection capability may not be as readily achieved as with soluble disinfectants.

III. Disinfectant Kinetics

A. Soluble Disinfectants

Chick (1913) described disinfection kinetics as being analogous to a chemical reaction. For disinfectants to inactivate viruses efficiently, first-order kinetics should occur. Ideally, this will occur if (1) viruses act as discrete units equally susceptible to a single species of disinfectant; (2) both viruses and disinfectant are uniformly dispersed within the water being treated; (3) the disinfectant remains unchanged in chemical composition and substantially constant in concentration; and (4) the water being treated contains no interfering nitrogenous substances (Fair *et al.*, 1971; Hoff, 1986).

Unfortunately, different types of viruses have varying susceptibilities to disinfectants and even the susceptibility of individual virus particles within a population may vary. In addition, the susceptibility of a given virus will vary with the type of disinfectant. This may be dependent on the ionic species of the disinfectant present in the water. The distribution of viruses in water may not be uniform due to clumping or aggregation with particles present in the water, which can protect viruses from disinfection (Galasso and Sharp, 1962; Young and Sharp, 1985; Emerson *et al.*, 1982; Keswick and Gerba, 1980; Hejkal *et al.*, 1979). The distribution of soluble disinfectants or the uniform flow past a contact disinfectant is dependent on thorough mixing of the water during disinfection.

The disinfectants themselves may form different species having different inactivation properties dependent on the pH, temperature, and ionic nature of the water (Fig. 1) (Cramer *et al.*, 1976; Sharp *et al.*, 1980: Taylor and Butler, 1982; Wiedenkopf, 1958). This may, in part, be due to variations in ion pairing (Jensen *et al.*, 1980). The concentration of available disinfectant may vary as the presence of competing or interfering substances which may combine with it vary.

Any condition of the virus, disinfectant, or water being treated which alters the kinetics of disinfection will cause a deviation from ideal linear inactivation (Chang, 1966; Churn *et al.*, 1984). Curves illustrating deviations from ideal conditions are noted in Fig. 2. The "shoulder" effect may perhaps be due to improper mixing or a delay due to diffusion of a disinfectant across the viral capsid protein coat to its target. Gard (1959) suggested that substances acting on nucleic acids must modify the protein coat in order to reach the nucleic acid. Conversely, a "shoulder" could be caused by a multihit requirement for inactivation due either to clumping or to one site on the viral protein coat requiring numerous interactions with the disinfectant or to many sites requiring a single interaction before inactivation is complete. Rapid, initial inactivation may be due to the extreme sensitivity of a portion of the microbial population. "Tailing off" may be the result of disinfectant depletion or the presence of a more resistant subpopulation due to viral protection by clumping or aggregation, conformational change of the viral capsid, or innate genetic variation in some members of the population (Hoff, 1986).

Genetic variation, or drift, caused by point mutations in the virus genome may result in decreased susceptibility of a subpopulation of viruses to disinfection by a particular species of disinfectant. This could occur due to altered hydrophobicity caused by variations in protein

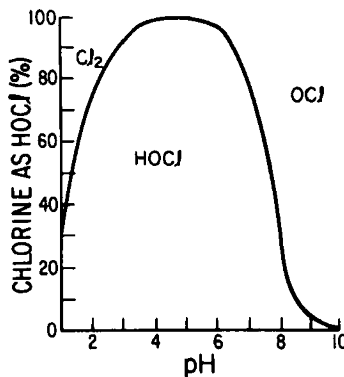

FIG. 1. Effect of pH on the concentration of the ionic species of hypochlorous acid.

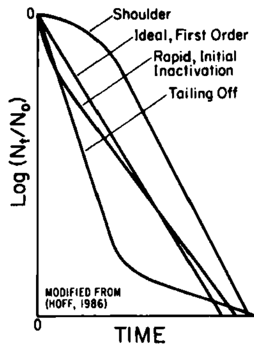

FIG. 2. Typical survival curves for disinfection experiments.

side-chain exposure to the disinfectant, and thus decreased interaction between viral target sites and the disinfectant. More directly, if the site of action of the disinfectant is the RNA itself, then point mutations in the genome may affect RNA folding or in some other manner lead to less opportunity for the disinfectant to act on target sites. Any change in the target site of a virus that does not impair its ability to engage in productive infections will be expected to alter the efficiencies of water disinfection processes.

Poliovirus evolves at a rate approximating two bases per week (Kew *et al.*, 1985). Effectively, this means that its 7441 bases could become completely changed every 71 years. While this is perhaps highly unlikely it stresses the probability that a population of viruses will in fact contain subpopulations having genetic variations that could potentially affect inactivation kinetics (Charney *et al.*, 1960).

Viral clumps are generated in large, tightly packed cytoplasmic crystals in cells and are not completely dispersed upon release from an infected cell (Berman and Hoff, 1984: Floyd *et al.*, 1976; Sharp *et al.*, 1975). Clumping may also occur as single virions aggregate while in water due to shifts in the pH or salt concentration, and can be reversible (Floyd and Sharp, 1977; Vrijsen *et al.*, 1983). Although inactivation of single-particle virus suspensions should reflect first-order kinetics, large clumps of viruses or aggregates in the suspension will lead to a deviation from linearity (Emerson *et al.*, 1982; Sharp *et al.*, 1976). Unfortunately one cannot be assured that complete dispersion of viruses at the time of disinfection has occurred.

Small clumps of 2–10 viruses have no detectable effect on plaque-forming titers, whereas large clumps or aggregates seem to be involved (Sharp *et al.*, 1975; Stagg *et al.*, 1977) (Fig. 3). The clumping of virus

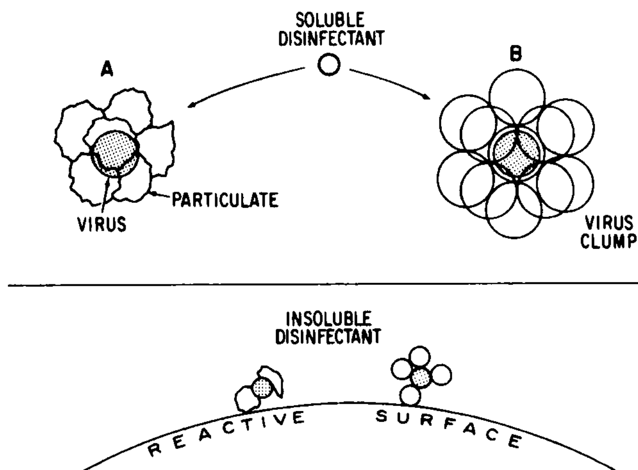

FIG. 3. Protective action of particulate matter (A) and virus clump (B). Soluble disinfectants and the reactive surface of insoluble disinfectants may be prevented from acting on a virus particle surrounded by particulate matter or a clump of viruses.

particles or the aggregation of organic material around a virus may protect a central virion from the action of a disinfectant even at high concentrations (Hejkal *et al.*, 1979). Clumps of 16 identically sized spheres (virions) are required to form a protective coat one sphere (virion) thick around a sphere (virion) of equal diameter. Hence clumps involving 16 or more viruses will probably exhibit resistance to soluble disinfectants. Small clumps of viruses may not completely envelop a central virus, allowing a soluble disinfectant to reach its target site. Contact disinfectants will be expected to show a greater loss of activity due to clumping or aggregation. Clumps smaller than 16 may prevent contact between the central virus and the disinfectant surface. Thus, any degree of clumping or aggregation of organic materials around a central virus may alter inactivation kinetics, depending on the nature of the disinfectant being used.

Virus sensitivity to disinfectants may alter due to pH changes in the water in which they are suspended. Viruses have isoelectric points (IEP) at which the electric charge on the virus is changed. Shifts of pH from below the IEP (virus has an overall net positive charge) to above the IEP (virus has an overall net negative charge) can be expected to alter the sensitivity of target sites in or on the virus. Negatively charged amino acids in the viral capsid may become more exposed to the environment, while others (positively charged) may become internalized and unavailable for

interaction with the disinfectant. This change in conformation may affect the susceptibility of viruses to disinfection by changing the spatial arrangement of target sites or inducing clumping or aggregation, perhaps by increasing virion hydrophobicity (Sharp and Leong, 1980; Vrijsen *et al.*, 1983). Poliovirus is most sensitive to disinfection near its IEP (Butler *et al.*, 1985). Some viruses have more than one IEP (Vrijsen *et al.*, 1983) and there is evidence for a correlation between one of these IEPs and sensitivity to disinfection (Butler *et al.*, 1985).

Changes in the hydration state of the virus at low concentrations of disinfectant may also cause irregularities in the disinfection rate of viruses (Fig. 4) (Floyd *et al.*, 1976). As shown in Fig. 4, low bromine concentrations and temperature virions become hydrated and become much more susceptible to disinfection.

B. Insoluble Disinfectants

Murray (1980) described virus adsorption and degradation on insoluble metal surfaces as being caused by the ionization of prototropic groups (Park, 1976) and the formation of a double layer as ions present in the liquid phase collect at the surface. At the pH of most natural waters, viruses will have an overall net negative charge. Therefore, positively charged surfaces will allow virus adsorption. Other negatively charged particulates in the water will also be electrostatically attracted and compete with virions for adsorption sites or neutralize the charge on the disinfectant surface. Likewise, positive ions may adsorb onto the viral surface and block the action of the disinfectant. The pH of the water also

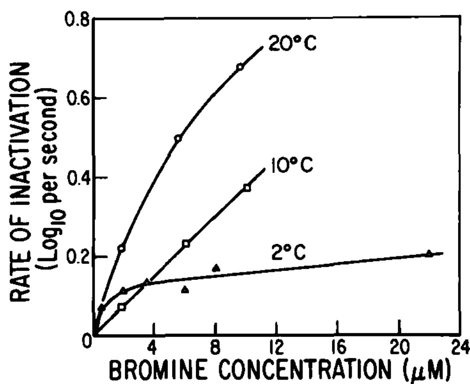

FIG. 4. Effect of hydration on survival of virus.

influences virus conformation (Fig. 5), which in turn influences exposed charge groups on the capsid protein and sensitivity to disinfection (Grimmel *et al.*, 1983; Mandel, 1971; Murray, 1980; Young and Sharp, 1985; Young *et al.*, 1977).

Water at a pH less than the IEP of the virus will result in a more positively charged virus, perhaps affecting repulsion rather than attraction between the virus and the charged disinfectant unless salt bridging occurs (Woodard *et al.*, 1968). However, electrostatic attraction is a relative condition. Two objects having the same type of charge (both positive or both negative) may still be attracted to one another if the difference in their charges is of a large enough magnitude.

The double layer has two regions, the compact inner Stern layer and the diffuse outer Gouy layer. The free energy of adsorption which results in repulsion or attraction between a virus and a surface depends on whether the double layers from different particles are of like or opposite electrical charge. The Shultz–Hardy rule as described by Atkins (1982) explains that the thickness of the double layer decreases as the ionic strength of the surrounding medium increases (Bitton *et al.*, 1976). Therefore, as the double layer becomes thinner, it allows similarly charged particles to approach one another until salt bridging occurs.

After initial electrostatic attraction (physisorption) which draws two oppositely charged particles together, van der Waal interactions which operate over distances of 5 nm or less become important. These forces are due to electromagnetic fields generated from dipole oscillations (Murray and Laband, 1979; Stumm and Morgan, 1981). The initial energy drop associated with physisorption (reversible) is supplanted by a rise in energy as bonds stretch prior to chemisorption (irreversible), in which chemical bonding occurs with the surface. Upon chemisorption the

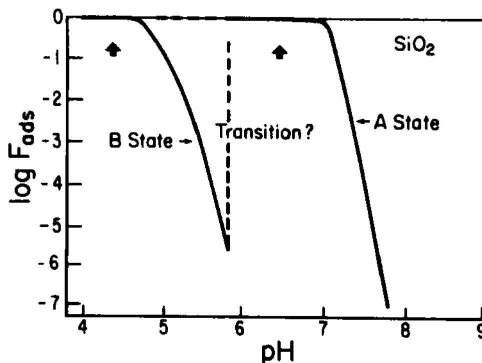

FIG. 5. Effect of pH on the conformation of poliovirus.

energy level drops as surface-associated bonds reach their full strength (Uhlig, 1952). Although no evidence for chemisorption of viruses has been reported, chemisorption may in fact play a significant role in the degradation of viruses adsorbed onto a surface. This may present a problem for contact disinfectants unless bonds between virions and active sites on the surface of the contact disinfectant can be regenerated by sloughing of chemisorbed particles. Otherwise periodic "cleaning" or replacement of the contact disinfectant will need to be undertaken.

Viral removal may occur due to physisorption without chemisorption occurring. In the case of permanent adsorption of viruses periodic replacement of the disinfectant would be required, whereas viruses temporarily immobilized on a surface may be eluted at a later time while maintaining their infectivity (Lance *et al.*, 1976). Surface-bound viruses may also be influenced by the pH at the surface of a contact disinfectant. El-Amamy and Mill (1984) noted that the pH at a surface may be 2 to 3 units lower than that of the bulk solution, and thus enhance inactivation of acid-sensitive functional groups of viral capsid proteins.

According to Murray (1980), the double layer theory and the Lifshitz theory (general theory of van der Waal interactions) when combined may be used to predict adsorption characteristics of viruses in aquatic systems. If all other conditions are constant the combined theory predicts that large particles will, in general, adsorb onto a surface more strongly than will small particles. Exceptions to this prediction are adenoviruses and reoviruses, which are larger than enteroviruses, but which adsorb less strongly to aluminum hydroxide and phosphate precipitates, perhaps due to differences in interaction configuration and electrokinetic properties.

Further predictions state that if the virus and the solid have similar charge, increasing the ionic strength of the suspending solution will increase the tendency of the virus to adsorb. Conversely, if the virus and the solid are of opposite charge, an increase in ionic strength will decrease the tendency to adsorb. Materials with high dielectric susceptibilities such as metals and metal sulfides should develop higher van der Waal potentials and adsorb viruses more strongly than minerals or humic substances: metals > sulfides > transition metal oxides > silicon dioxide > organic substances (Gerba, 1984; Murray, 1980).

IV. Mechanisms of Inactivation

A. General Concerns

Viruses can potentially interact with disinfectants in a variety of ways (Fig. 6). The infectivity of the virus may remain undisturbed as nontarget sites are attacked. Inactivation may occur temporarily due to a reversible

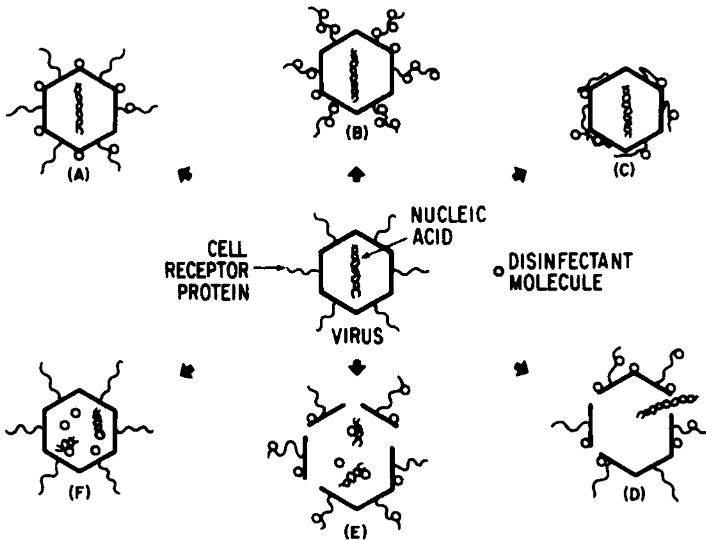

FIG. 6. Possible disinfectant-virus interactions: (A) nontarget sites attacked; (B) reversible adsorption of disinfectant to capsid receptors or nonreversible adsorption resulting in inactivation of the virus; (C) reversible conformation change of virus due to reversible adsorption of disinfectant; (D) capsid destroyed, releasing infectious nucleic acid; (E) capsid destroyed and nucleic acid rendered noninfectious; (F) nucleic acid rendered noninfectious while capsid remains intact.

change in the virus conformation, or permanent damage to either the capsid proteins and/or the nucleic acid may result. Virus clumping or aggregation may prevent inactivation of all viruses as some may be protected. Turbidity is important in this regard. Any property of the water such as incomplete mixing, which reduces the likelihood of allowing sufficient contact time between the virus and the surface of the disinfectant pH, or ionic strength, which may enhance aggregation or change conformation of proteins in the virus capsid, may reduce the efficiency of that disinfectant.

The surface area of the disinfectant available to viruses is important. A contact disinfectant has only a small percentage of reactive sites on its surface (Murray, 1980). Ideally, contact time for inactivation will be brief, with immediate and complete release of the inactivated virus and thus regeneration of the virus attachment site on the surface of the disinfectant. Should release of the inactivated virus leave a portion of the viral capsid proteins attached to the contact surface, then, as mentioned above, a limited lifespan of the disinfectant is assured. Means to

ensure that the contact disinfectant presents its maximal surface area to the water being treated is also important. To achieve this direct flow through a column of resin beads or inorganic substance may be more efficient than lining a container with the same substance.

B. SOLUBLE DISINFECTANTS

1. *Halogens*

Chlorine is used in many water treatment facilities, because it is an effective oxidizing agent (Dugan, 1978; Kruse *et al.*, 1970). Figure 7 shows the reactions chlorine can typically undergo in an aqueous environment. The presence of nitrogenous substances can reduce the efficiency of viral inactivation as chlorine forms less effective chloramines. For example, Berman and Hoff (1984) noted that monochloramine requires 6 hours to inactivate the same amount of rotavirus as chlorine will inactivate in 15 seconds.

Chlorine dioxide is used extensively in Europe as a disinfectant for treating water because it does not form carcinogenic trihalomethanes (Murray, 1980; Noss and Olivieri, 1985), bad-tasting chlorophenols, or chloramines, as does chlorine (Faust and Hunter, 1967; Kinman *et al.*, 1970; Llabres and Ahearn, 1985; Longley *et al.*, 1980; Taylor and Butler, 1982). Chlorine dioxide has been found superior to combined chlorine in a number of investigations (Aieta *et al.*, 1980).

The concentration of the different species of chlorine is influenced by pH (Vaughn *et al.*, 1986). Hajenian and Butler (1980a) found that chlorination has a reduced effect at a pH near the IEP of the virus. Some confusion

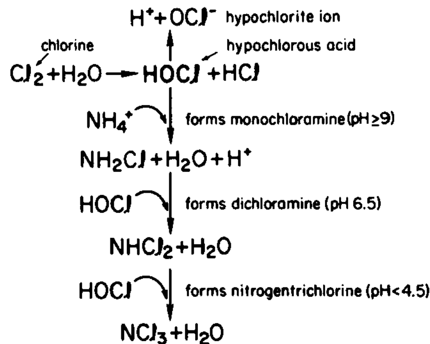

FIG. 7. Possible reactions of chlorine in water.

appears to exist concerning the effectiveness of the different species of chlorine. Scarpino *et al.* (1972) concluded that the hypochlorite ion (OCl^-) is more effective than hypochlorous acid (HOCl), refuting earlier work by Wiedenkopf (1958). Koh *et al.* (1975), on the other hand, reported that HOCl is more effective than OCl^-. The mechanism of viral inactivation due to chlorine seems to be unresolved. Two schools of thought revolve around the action of chlorine: one assumes that viral capsid proteins are attacked, the other that viral nucleic acids are attacked.

Chang (1970) stated that chemical agents denature the protein shell of viruses. leaving the nucleic acid unaffected. Oxidation of sulfhydryl groups is not enough to inactivate viruses, which explains why higher residuals are required for viruses. Young and Sharp (1985) found that chlorine may alter poliovirus conformation, making it less infective. Even though these altered viruses are incapable of causing an infection by themselves, they may collectively produce an infection (see Section V, Multiplicity Reactivation).

The conformation of a virus may be reversible if the virus has not become stabilized in one conformation state by ultraviolet light or heat (Mandel, 1971). One may hypothesize that as a change in pH occurs, a resulting change in conformation may occur that alters the hydrophobicity of the virus and affects its ability to recognize and adsorb onto cell receptors. Hence, if chlorine does not inactivate a virus outright, but instead temporarily renders the virus noninfectious, then under appropriate conditions the virus may revert to an infective conformation. This may help account for the recovery of infective viruses from finished drinking water having a free chlorine residual (Payment *et al..*, 1985; Rose *et al.*, 1986; Snead *et al.*, 1980) greater than 1 mg/liter (Shaffer *et al.*, 1980).

Venkobachar *et al.* (1977) determined that chlorine changes the membrane permeability of *Escherichia coli*, allowing macromolecules such as proteins and RNA to leak out. They also showed that DNA is released at high concentrations of chlorine. One may speculate whether the capsid proteins of viruses react similarly, allowing RNA to leak out. Dennis *et al.* (1979) noted that chlorine has an affinity for RNA at a pH of 5.6–9.9, which is greater than its affinity for protein. Cytosine monophosphate and adenosine monophosphate consume HOCl at low pH more actively than does guanosine monophosphate. Uridine monophosphate appears quite unreactive (Dennis, 1979). Hauchman *et al.* (1986) found that chlorine dioxide can react with nucleotides, but inactivation of intact f2 virus may be due to discrete reactions with cysteine, tyrosine, and tryptophan moieties in the viral protein (Noss *et al.*, 1986).

Tenno *et al.* (1979) concluded that HOCl inactivates poliovirus by reacting with the protein component of the virus, but that it does not result

in any detectable change in the structure of the virus and does not affect the infectivity of the viral RNA. The work of Roller *et al.* (1980) supported this statement by showing that bacterial inactivation is primarily through irreversible sulfhydryl oxidation. Thus, while chlorine may damage bacteria and viruses, their nucleic acids may remain infectious (Chang, 1970; Yeager and O'Brien, 1979). Taylor and Butler (1982) and O'Brien and Newman (1979) showed that cleaved viral RNA is released from the poliovirus capsid following treatment with chlorine. The viral sedimentation coefficient changes from 156 S for native poliovirus to 80 S after treatment with chlorine, due to the release of its RNA (Alvarez and O'Brien, 1982a). The IEP and cell attachment capabilities of the virus, however, remain unchanged (Olivieri *et al.*, 1971). This seems to indicate that no major capsid conformational changes occurred during chlorine inactivation. O'Brien and Newman (1979) maintained that chlorine inactivation occurs due to RNA degradation before release of the degraded RNA and that the RNA release is a secondary event (Alvarez and O'Brien, 1982b).

If this scenario is correct, then chlorine must diffuse across the virus capsid in order to reach its target site—the viral RNA. This mode of inactivation may be dependent on the availability of a chlorine species that is electrically neutral. Sletten (1974) reported that HOCl is more effective than OCl^- in inactivating bacteria; this may be because it has a neutral charge and can penetrate the bacterial cell wall (Lippy, 1986). Inactivation of poliovirus due to HOCl would be expected to occur most readily at a pH between 3 and 6.5 where 90% of chlorine is present as HOCl.

Alvarez and O'Brien (1982b) attempted to settle the discrepancy in reports on the action of chlorine on viruses by stating that the end result is dose related. Chlorine concentrations less than 0.8 mg/liter result in inactivation without major structural changes, whereas chlorine concentrations in excess of 0.8 mg/liter result in 156 S to 80 S conversion with RNA loss from the viral capsid. Hence, damage may occur to either viral proteins and/or RNA (Harakeh and Butler, 1984; Leong, 1983; O'Brien and Newman, 1979: Tenno *et al.*, 1979).

Bromine undergoes reactions similar to chlorine in an aqueous medium. However, its disinfecting capacity and mode of action differ from that of chlorine. Keswick *et al.* (1981) reported that while viral proteins are affected by bromine, the RNA is not. Floyd *et al.* (1978) found that hypobromite ions inactivate poliovirus more rapidly than OCl^- ions (see Sharp, 1982), suggesting they operate at different sites on the virus. Bromine concentrations of 0.3–0.5 mg/liter bromine chloride (BrCl) cause virus inactivation with no apparent structural damage, while stronger concentrations of 10–20 mg/liter BrCl cause structural damage to the virions. Olivieri *et al.* (1971) indicated that bromine (15 mg/liter) is capable

of inactivating nucleic acids, yet does not appear to penetrate the protein coat of the virus. This action must probably occurs at a pH where bromine is present as neutral hypobromous acid (HOBr) and may pass through the viral capsid to attack the nucleic acid.

Bromine chloride is two to three times more effective than HOCl in the disinfection of sewage (Hajenian and Butler, 1980b) seeded with poliovirus 1, coxsackie B4, echovirus 7, and reovirus 2 (Keswick *et al.*, 1978, 1979). Damage to the protein capsid of reovirus after exposure to HOBr (2–3 μM) can be very rapid and has been detected by electron microscopy in as little as 1 minute (Sharp *et al.*, 1975).

Iodine has been reported to have greater stability and general utility when compared to chlorine (Hsu, 1964). While elemental iodine can inactivate viruses, the more effective form is the hydrated form of elemental iodine. Iodine decomposition products iodate and iodide are formed at a pH above 8 and are nonvirucidal (Cramer *et al.*, 1976).

Iodine displays first-order inactivation kinetics, indicating single-site inactivation unless clumping occurs. Iodine oxidizes sulfhydryl groups and tryptophan (Cramer *et al.*, 1976) and perhaps more importantly substitutes tyrosyl on histidyl moieties at neutral pH and room temperature (Hsu, 1964). Structural changes in viral integrity may be noted by electron microscopy after treatment with iodine (Taylor and Butler, 1982) and thus infectious RNA could be released into the environment (Berg *et al.*, 1964; Olivieri *et al.*, 1971). Hsu *et al.* (1966) noted that the virucidal properties of iodine in waters of low pH and high organic content could be inadequate regardless of the free iodine concentration when viruses require iodination of tyrosine for inactivation. Viruses resistant to iodine may have buried sulfhydryl groups or their exposed sulfhydryl groups might not be essential for productive viral infections, as is the case for tobacco mosaic virus (Hsu, 1964).

2. *Ozone*

There are many difficulties associated with the utilization of ozone as a disinfectant. Ozone produces mutagens (Burleson and Chambers, 1982; Murray, 1980), is difficult to handle, does not disinfect well at low pH, is adversely affected by organics, and has poor penetrating ability into solutions. Walsh *et al.* (1980) found that ozone can diffuse into flocs and inactivate viruses. They determined that at turbidities from 1 to 5 NTU viruses are afforded no protection at ozone concentrations of < 0.25 mg/liter. While ozone does not impart taste or color to water (Majumdar *et al.*, 1973), its ability to inactivate viruses is dependent on both the initial ozone concentration and the quantity of organic matter in the water (Katzenelson *et al.*, 1974). An acidic pH helps stabilize ozone and increases its half-life. Ozone bubbles with an ozone residual are more

effective a disinfectant than an ozone residual alone. According to the film theory, the ozone concentration at the gas–liquid interface of the bubble exceeds the ozone concentration in the bulk liquid; thus, viruses in direct contact with ozone bubbles will be inactivated more readily than those viruses in the bulk liquid (Farooq *et al.*, 1977).

Enterovirus inactivation due to ozone in order of decreasing resistance is poliovirus 1 > echovirus 1 > poliovirus 2 > coxsackie B5 > echovirus 5 > coxsackie A9 (Roy *et al.*, 1982). Farooq and Akhlaque (1983) stated that viruses are more resistant to ozone than bacteria. Controversy still exists regarding the mechanism by which ozone inactivates viruses. Although it is agreed that ozone can react with both RNA and viral capsid proteins, there is disagreement over the degree of interaction of these viral components.

Roy *et al.* (1981a) found that while polypeptide chains Vp1 and Vp2 present in the viral protein coat of poliovirus 1 become altered in the presence of ozone, virus adsorption and the integrity of the virus are not significantly impaired. Damage to viral RNA was determined by ozonating poliovirus virions and extracting the RNA. Sucrose gradient analyses revealed two broad peaks instead of a single, well-defined peak representing native poliovirus RNA. It was then suggested that mass transfer of ozone into the virus particle occurs where it breaks down the RNA, inactivating the virus (Roy *et al.*, 1981b,c). This process is adversely affected by low pH, and resistance to inactivation increases approximately four times as the pH drops from 7.2 to 4.3.

DeMik and DeGroot (1977), in contradiction to Roy *et al.* (1981a), concluded that ozone attacks sulfhydryl groups and can directly inactivate cysteine, methionine, and tryptophan. They further stated that ozone modifies pyrimidine and purine bases, as well as alters the adsorption spectra of nucleic acids, nucleotides, and nucleosides. Shinriki *et al.* (1981) and Ishizaki *et al.* (1981) reported that the degradation rate due to ozone of the base portion of nucleotides and nucleosides is in the order of decreasing sensitivity to degradation: guanine > thymine > uracil > cytosine > adenine. The ribose moiety is also degraded, but more slowly than the base portion of the nucleic acid.

Kim *et al.* (1980) detected structural changes in the protein coat of bacteriophage f2 using electron microscopy after ozonation. It is suggested that ozone breaks the capsid proteins into subunits and releases the RNA which then may become damaged afterward. As infectious DNA has been recovered from ϕX174 after treatment of ozone, it is believed that while DNA–RNA destruction can occur, it is the proteins which first are affected and which inactivate the viruses. The nucleic acid may be secondarily affected if access to them occurs during contact with ozone (DeMik and DeGroot, 1977).

3. *Metal Ions*

Metal ions may inactivate viruses in a number of ways by binding electron donor groups on proteins or nucleic acids. Overall charge, size, coordination number, and lability help determine the nature of the interaction (DeLeers, 1985; Dixon, 1984; Hutchinson, 1985; Plastourgou and Hoffman, 1984; Puck *et al.*, 1951). Adsorption of metal ions to viruses is pH dependent and competition for adsorption sites may occur if more than one kind of cation is present (Netzer and Hughes, 1984; Wallis and Melnick, 1962).

Inactivation of biological macromolecules due to transition metals is thought to involve a modified site-specific Fenton mechanism (Amram *et al.*, 1983; Samuni *et al.*, 1984). It is assumed that the metal ion binds to a biological target and is reduced by superoxide radicals or other reductants and subsequently reoxidized by H_2O_2 generating hydroxide radicals. Repeated cyclic redox reactions may result in multihit damage as radical formation occurs near the target site.

The interaction of copper, zinc, lead, cadmium, nickel, silver, cobalt, and manganese ions with biologic molecules has been reported (Chang, 1970; Martin, 1967; Plastourgou and Hoffman, 1984; Sundberg and Martin. 1974) as mediating hydrolysis (Mingelgrin and Saltzman, 1979). This occurs by nucleophilic displacement either by polarization allowing subsequent external attack by a nucleophile or by generation of a reactive basic reagent in which a hydroxyl ion coordinates itself with the metal in forming a reactive nucleophile that attacks amides and peptides (Hay and Morris, 1976).

Decarboxylation reactions (Plastourgou and Hoffman, 1984) may alter the function of molecules necessary for virus infectivity, resulting in inactivation. Chelation prior to hydrolysis or decarboxylation, involving short-range interactions, polarizes the carbonyl group and assists the transfer of electrons from the carbon–carbon bond undergoing cleavage (Hay, 1976). Indirect chelation may occur if a coordinated water molecule hydrogen bonds to a ring nitrogen or to a phosphate oxygen.

Should metal ions come in contact with nucleic acids due to viral capsid protein degradation or by diffusion through the capsid, it is possible for degradation of the backbone, disruption of base pairing, or protection from inactivation to occur (Nishikawa and Kuriyama, 1968). Metal ions may attack the nucleic acid backbone to induce cleavage as a cyclic intermediate connecting the 2′ and 3′ oxygen atoms of the ribose with the phosphorus atom of the phosphodiester bond forms. Ring opening may then occur secondary to backbone cleavage as the cyclic configuration degenerates.

Metal ion intercalation may neutralize electrostatic repulsion between neighboring-strand phosphate groups, thus decreasing the overall free

energy and stabilizing the double helix. Intercalation of metal ions can be detected by determining if the migration rate of closed, relaxed nucleic acids has increased, as intercalation increases superhelicity, resulting in faster electrophoretic migration. Preference for specific base-rich regions has been demonstrated for Cu(II), Hg(II), and Pt which prefer G-C-rich regions, while Ag prefers A-T-rich regions, and Zn(II) prefers C-U (Eichhorn *et al.*, 1973).

Metals binding exocyclic oxygen atoms of purine and pyrimidine bases usually disrupt base pair hydrogen bonding and destabilize the helix by lowering the melting temperature T_m, but particular reactions depend on the tertiary structure of the nucleic acid (Barton and Lippard, 1980). At low concentrations metal ions bind to phosphates, resulting in either backbone cleavage or increased T_m. At high metal ion concentrations base binding becomes more significant, reducing the T_m and causing helix destabilization. Increasing or decreasing the T_m will alter the normal function of nucleic acids and may result in virus inactivation or an increased rate of mutagenicity as mispairing occurs (Eichhorn *et al.*, 1973). Barton and Lippard (1980) prepared a list of metals showing their decreasing order of preference for binding to phosphate groups over bases: Mg(II) > Co(II) > Ni(II) > Mn(II) > Zn(II) > Cd(II) > Cu(II).

Silver has been used for years as a protein coagulant in treatment for burn patients. Silver has also found use as a disinfectant in water treatment. Its great affinity for electron donor groups containing sulfur, oxygen, or nitrogen inhibits bacterial enzymes and may interfere with respiration at the cell membrane. Colloidal silver is, however, not an effective enteric virus disinfectant in water at permissable concentrations (PHS Drinking Water Standards, 1962; Chang, 1970; Thurman and Gerba, unpublished data). Silver ions may form complexes with nucleic acid bases (Richards, 1981) without causing clumping or disruption of the double helix (Grier, 1983; Tilton and Rosenberg, 1978). This mode of inactivation, however, seems unlikely for intact viruses unless diffusion across the viral capsid is achieved. Chambers *et al.* (1962) noted that it is the concentration of silver, not the nature of the ion, which is vital in bacterial inactivation.

C. Insoluble Disinfectants

1. *Metal Oxides*

Oxides of silicon, iron, copper, manganese, magnesium, and aluminum have all been reported as adsorbing viruses (Atherton and Bell, 1983; Murray and Laband, 1979; Thurman and Gerba, unpublished data). Silicon appears to bind viruses loosely and no significant loss of virus infectivity is noted upon elution of viruses from the oxide surface. Iron

oxide and aluminum oxide produced slight virus inactivation, while manganese dioxide and copper oxide showed significant inactivation. Murray (1980) showed that as the incubation time of virus and metal oxide increases, so does the amount of virus inactivated increase.

Magnesium oxide and magnesium peroxide cause significant virus inactivation which may in part be due to their effect on the pH of the bulk solution, which is greatly increased (Thurman and Gerba, unpublished data). Hydrolysis of viral proteins may result, destroying cell receptors. These substances, while efficient in viral inactivation, may cause problems due to the significant increase in pH of the treated waters.

2. *Aluminum*

Metal surfaces, such as aluminum, act as reducing agents and, while not well characterized, it is believed that hydroxylation (Barna *et al.*, 1984; Schindler, 1984) and superoxide radical formation can occur at the surface of the metal. The reactions are the results of chemisorption of water molecules or dissolved oxygen. An oxide coating several nanometers thick forms on aluminum. Thin spots in this coating are believed to be the reactive sites of virus adsorption and subsequent degradation. Poliovirus capsid proteins are destroyed (Thurman and Gerba, unpublished data) as well as the RNA (Murray, 1980), resulting in a significant decrease in virus titer (Thurman and Gerba, 1985).

Strong van der Waal forces generated by the aluminum have been suggested as the cause of the degradation (Murray, 1980). The formation of superoxide radicals or peroxides at the metal surface may play a role in viral inactivation (Michelson, 1980; Yamamoto *et al.*, 1964). Poliovirus adsorbs to metallic aluminum and desorbs within 3 days. Viral fragments and clumped viruses, which prove to be noninfectious upon dispersion with freon 113 (Sobsey *et al.*, 1980), can be seen by electron microscopy after exposure to metallic aluminum (Thurman and Gerba, unpublished data). Canter and Knox (1985) found that phosphates become chemisorbed on the surfaces of iron and aluminum minerals in strongly acid to neutral systems. Because aluminum does not significantly alter the pH of neutral water contacting it, this might occur once the viral capsid is destroyed. If phosphate groups or other pieces of destroyed viruses remain chemisorbed onto the metal surface, then a decreased efficiency of virus removal over time should occur.

D. Light-Induced Disinfection

1. *Ultraviolet Light*

Ultraviolet light has been used to disinfect public water supplies since 1909 (Ellis *et al.*, 1941). The biocidal effectiveness of ultraviolet radiation

varies with the wavelength of the radiation (Clarke and Berman, 1983). The most effective wavelengths range from 253.7 to 265 nm (Chang, 1970). Ultraviolet light-disinfecting devices used to treat water are usually either immersed in the water or positioned just above the water. The effective depth of penetration of ultraviolet light in water is dependent on the nature of the water. Ultraviolet light of wavelength 253.7 nm retains 90% of its intensity at 5 in. in distilled water, while in Ringer's solution ultraviolet light retains 90% of its intensity at only 0.5 in. (Luckiesch *et al.*, 1944). This variation is apparently due to dissolved salts. Water in contact with iron pipes and iron storage tanks may acquire sufficient iron for such variation in effective penetration and special techniques to enhance disinfection may be required. Film-spreading devices may be used to increase the surface area and reduce the depth of waters having a high ultraviolet adsorption, thus allowing disinfection even in the presence of absorbing salts.

In order for photochemical reactions to mutate or inactivate a virus, the light energy must be absorbed by a target molecule that possesses a bond of importance to the function of the virus. A sufficient amount of excitation energy of the absorbed photon must alter this vulnerable bond such that the normal function of the molecule is permanently altered (Jagger, 1967). Ultraviolet irradiation may act in this way on viral capsid proteins or the viral nucleic acids.

The aromatic amino acids tyrosine, tryptophan, and phenylalanine are particularly sensitive to ultraviolet light and may undergo decarboxylation, deamination, or ring rupture. Cystine may convert to cysteine, altering the tertiary structure of the viral capsid proteins and affecting adsorption to host cell receptors. This may not be significant if a virus has a sufficient excess of cellular receptors to negate the loss of some of them. Peptide bonds also may be an important target site due to their frequency. Energy absorbed by amino acids may be transferred short distances to other sites (resonance transfer). Such chromophores may, themselves, not be altered by the radiation (Jagger, 1967). Resonance transfer may be involved in ultraviolet-induced cross-linking between double-strand DNA and the major core protein VII of adenovirus (Sato and Keiichi, 1984). Cross-linking also has been reported between single-strand DNA and the gene 5 protein of bacteriophage fd.

The effects of ultraviolet light on nucleic acids may be more significant than on viral capsid proteins. Any alteration in the genetic information may cause an inability to replicate or alter the genetic information and thus effectively inactivate the organism or cause a mutation. Mutations may occur through misreplication or pyrimidine dimers or pyrimidine(6-4) pyrimidine lesions, which would indicate that targeted mutagenesis is the most probable event (Bourre and Sarasin, 1983), while

pyrimidine dimers may directly obstruct transcription (Eglin *et al.*, 1980; Ross *et al.*, 1972).

Luria (1955) showed that most absorption takes place in the nucleic acid component of the virus. This is in part due to the fact that all nucleic acid bases are good absorbers, whereas only about 1 in 10 amino acid residues are good ultraviolet absorbers. Pyrimidines are 10 times more sensitive to ultraviolet irradiation than are purines and may form cyclobutane dimers with adjacent pyrimidines. Base stacking allows overlapping electron orbitals and may enhance resonance transfer. This may be dependent on the constraint of volume within the viral capsid, which may limit the ability for dimers to form.

The effects of ultraviolet irradiation seem to be dose related. Low doses may form reversible hydration products whereas large doses may inhibit virus uncoating (Miyamoto and Morgan, 1971) or cause disintegration of the particle. Luria (1955) reported that inactivated phage can still adsorb and kill a host cell by suppressing host cell syntheses, yet no new phages are produced.

Katagiri *et al.* (1967) studied in detail the changes that occur to poliovirus virions after exposure to ultraviolet light. Analysis of the exposed virions showed that there were four stages in the morphological appearance and biological properties of the virus. In the first stage, virus particles lose their infectivity. In the second stage, the viral RNA is rendered RNase sensitive. The alteration of viruses by ultraviolet light is a prerequisite for development of sensitivity of virus RNA to RNase since RNase is ineffective on the intact virion. Compared with the loss of infectivity, the development by virus RNA of RNase sensitivity requires a relatively high dose of ultraviolet light. In the third stage, the viral RNA is no longer enclosed, but is still adherent to the capsid. In the fourth stage, the viral capsid, and eventually viral particles, become empty shells.

The disadvantages of using ultraviolet light for the disinfection of water are that no residual disinfectant is maintained, it is generally more expensive than other means of disinfection, and variations in the quality of the water being treated may be such that film-spreading devices must be used, which may increase the time and cost required to disinfect a given volume of water (Buttolph, 1955).

Photoreactivation of RNA and DNA may be a concern if irradiated viruses are exposed to cells or cell extracts (Jagger, 1967). Eglin *et al.* (1980) reported that the survival of herpes simplex virus (type 1) shows a multicomponent curve which is greatly influenced by host–cell reactivation and to some extent by postreplication repair.

2. *Photodynamic Inactivation*

The usefulness of photoreactive dyes to inactivate microorganisms and to oxidize toxic compounds and organic matter in wastewater has been

demonstrated (Gerba *et al.*, 1977; Seely and Hart, 1977; Acher and Rosenthal, 1977).

Photodynamic action may be defined as the sensitization of microorganisms to inactivation by visible light through the action of certain dyes. It is generally held that viral inactivation results from alteration of the nucleic acid (Spikes and Livingston, 1969). According to Hiatt (1960), the dye combines with one or more critical sites on the virus particle under the proper conditions, to yield a dye–virus complex. Upon irradiation, this complex absorbs light energy and attains an excited energy state. The excited complex then combines with oxygen as the energy is released in a reaction that results in loss of infectivity.

Poliovirus is inactivated in tapwater, sewage, or seawater by methylene blue in the presence of visible light. Typically, almost 2.5 logarithmic units of virus can be inactivated by a 5-minute exposure to 670-nm light in solutions containing 1 mg/liter of methylene blue at pH 10.0. Various factors such as temperature, pH, oxygen concentration, and light energy affect the rate and sensitivity of the virus inactivation (Wallis and Melnick, 1965; Spikes and Livingston, 1969). The effectiveness of methylene blue as a photosensitizing dye has been shown to increase with increasing pH, probably because of an increase in the nonionized form of the dye (Gerba *et al.*, 1977). This allows for a greater penetration rate of the dye into a virus particle as compared to the ionized forms.

A variety of inactivation curves has been encountered in studying the kinetics of the inactivation of viruses by dyes (Hiatt, 1960). Linear, first-order inactivation curves have been observed for T-even coliphages and adenoviruses (Hiatt, 1960), while shouldered curves are produced during the inactivation of T-odd coliphages, vaccinia virus, and enteroviruses (Wallis and Melnick, 1963, 1965; Witmer and Fraser, 1971). Gerba *et al.* (1977) found that poliovirus sensitized with methylene blue undergoes a distinct induction period upon exposure to visible light and thereafter follows an exponential decline. This type of curve is produced independent of sensitization time, pH, light intensity, temperature, or dye concentration, although the length of the shoulder may be influenced by these factors. It was concluded that a true multihit kinetics occurred in the inactivation of the virus.

V. Multiplicity Reactivation

Multiplicity reactivation is a process in which damaged, noninfectious virions pool their resources in order to generate an infectious unit. Sharp (1982) noted that the plaque titer of well-dispersed echoviruses was reduced by several factors of 10 upon exposure to HOCl. By lowering the pH to 4.5 and inducing clumping, the titer was increased by a factor ranging from 10 to 3000. This phenomenon (Fig. 8) is not the result of

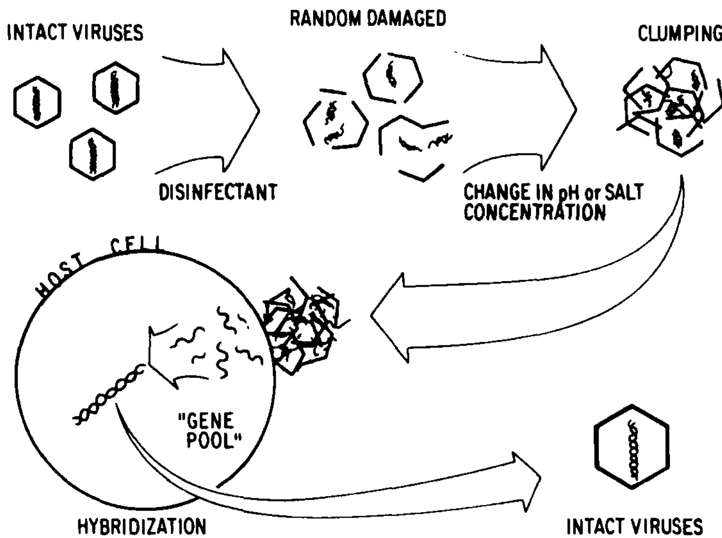

FIG. 8. Multiplicity reactivation.

virion repair, but complementation in which two or more damaged virions, each incapable by itself of forming a plaque, can produce a plaque (Young and Sharp, 1979, 1985; Sharp *et al.*, 1975). Luria (1947) first postulated multiplicity reactivation as a means of explaining an increase in titer of f2 bacteriophage upon clumping afrer a previous reduction in titer due to treatment with a disinfectant. The degree and nature of damage to the virion may be such that complementation is highly unlikely or that larger clumps are required before a productive infection can occur.

Luria assumed that random damage to either viral proteins or nucleic acids occurred due to a disinfectant. Resulting viral particles were inactivated and unable to cause a productive infection, hence the drop in titer after disinfection. Inducing these inactivated viral particles to clump together by changing the pH or salt concentration of the suspending medium mysteriously caused an increase in plaque-forming units. The reactivation process is thought to have a topographical role. Clumping of inactivated viruses ensures that the partially damaged viruses reach the cell surface at the same time and place. Each clump of damaged viruses is thought to contain at least one functional copy of each damaged unit (discrete, independent hereditary locus). Reconstitution of a plaque-forming unit is dependent on intracellular reincorporation of the active units derived directly or indirectly in a kind of "hybridization" of a gene pool from the inactive particles (Luria and Dulbecco, 1949).

Recombination due to crossing-over between poliovirus genomes has been described both *in vitro* and *in vivo* (Agut *et al.*, 1984; Kew and Nottay, 1983). This evidence adds support to Luria's hypothesis that fragmented viral nucleic acids can combine to form functional units sufficient to induce a productive infection. For effective disinfection of viruses to occur, viral capsid proteins need to be destroyed, reducing the ability of the virus to adsorb to a susceptible cell and releasing RNA to the environment where it may be degraded. The RNA itself needs to be nonspecifically attacked and fragmented followed by dispersion of the fragments such that the likelihood of multiplicity reactivation is severely diminished.

VI. Final Remarks

A suggestion made by Tifft *et al.* (1977) of using two-stage disinfection may offer an efficient means of inactivation of viruses. Initial treatment using chlorine followed by chlorine dioxide is vastly superior to single-stage disinfection. Taylor (1982) stated that iodine increases viral permeability by reacting with the capsid proteins, while chlorine dioxide attacks an internal target. In the two-stage approach to disinfection an initial attack of capsid proteins alters the structural integrity of the virus. Second-stage disinfectants, which normally would be unable to diffuse across the intact viral capsid, are then able to reach their target sites easily and inactivate the RNA, reducing the likelihood of multiplicity reactivation. Greater rates of inactivation of viruses with lower concentrations of chemical disinfectants have been achieved.

The literature to date seems contradictory in describing the action of various disinfectants on different virus types. It is, however, important to remember that the experimental conditions are not uniform from one investigation to the next and that many factors influence the outcome and interpretation of data. As Kawata *et al.* (1979) stated, no single disinfectant is equally effective under all conditions for all viruses.

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Microbial Ecology of the Terrestrial Subsurface

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I. Introduction

A. Out of Sight, Out of Mind?

Vast regions beneath the surface of the Earth may be inhabited by microoorganisms. For the most part, terrestrial subsurface environments are invisible, inaccesible, and remote. Until recently most people, including microbial ecologists, had never thought about them seriously.[1]

[1]Except as the fabled residence of gnomes and as literary and poetic allusions (e.g., F. M. Dostoevsky, "Notes from Underground"; Bob Dylan, "Subterranean Homesick Blues").

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Indeed, the aphorism "out of sight, out of mind" aptly describes a traditional view of the terrestrial subsurface held by generations of human beings. Unfortunately, the lack of interest implicit in this view has created serious environmental problems which ultimately may cost billions of dollars to rectify.

Toxic waste dumps and municipal landfills exemplify the problems. From a gnomic point of view, microbial activities in the soils beneath terrestrial dump sites were thought to promote the gradual conversion of the waste material into harmless products. Despite the potential of soil microorganisms for transforming many components of the wastes, toxic leachates are generated. But it was believed that the residual chemicals in the leachate would be removed as the leachate percolated through the upper soil mantle and entered unsaturated subsurface zones, eventually reaching groundwater in a purified state. Thus, terrestrial dump sites were not perceived as a threat to groundwater supplies until very recently, when improved analytical capability revealed widespread presence of toxic wate chemicals in groundwater. Similar examples can be cited for land applications of pesticides, fertilizers, and industrial chemicals which are now polluting many groundwater supplies around the world. (For comprehensive reviews of the sources and types of groundwater pollution, see Keswick, 1984; Carun, 1984; Zoetman, 1985.)

Until a little more than 15 years ago, we believed that groundwater supplies were safe from pollution. Sadly, we (and the gnomes) were wrong. In many areas of the world today aquifers containing vital groundwater supplies are polluted with toxic chemicals with little hope of reclaiming them in the near future. Polluted aquifers are still out of sight, but they are no longer out of mind.

B. Emergence of Subsurface Microbiology Research Programs

At approximately the same time as the magnitude of our groundwater pollution problems was dawning on environmental scientists, it was recognized by some that subsurface biological activity provided hope for cleaning up polluted aquifers (McNabb and Dunlap, 1975; Freeze and Cherry, 1979; Matthess, 1982). It was also realized that knowledge of biological activity in shallow aquifers was almost completely lacking. Indeed, a comprehensive search of the literature conducted by United States Environmental Protection Agency (USEPA) scientists in the early 1970s (Dunlap and McNabb, 1973; McNabb and Dunlap, 1975) found reports on bacteria in deep formations associated with petroleum and sulfur deposits (Kuznetsov *et al.*, 1963; Davis, 1967; Zobell, 1957, 1958) and reports on the transport of pathogenic bacteria and viruses in aquifers (see reviews in Gerba and Bitton, 1984; Matthess, 1982). In addition, there

was a small body of literature on the fauna and microflora of underground environments directly connected to the surface, such as caves, cavernous aquifers, and aquifers directly connected to stream beds (Caumartin, 1963; Gounot, 1973; Longley, 1981; Hynes, 1983; Sinton, 1984). Virtually no information was found on the indigenous microflora of common drinking-water aquifers a few meters to several hundred meters deep. Dunlap and McNabb (1973) questioned the results of many of the earlier studies because they were done on samples that were not obtained aseptically and because identification of the microorganisms often relied on enrichment cultures or other cultural techniques that were subject to contamination. Furthermore, with very few exceptions (for examples of the exceptions, see Kuznetsov *et al.*, 1963; Zobell, 1957, 1958) earlier studies were conducted on water samples taken from long-standing wells, rather than on sediment core samples. Wells are open to the surface and, thus, are subject to colonization by surface microorganisms.

Stimulated by the lack of reliable information in the literature and by an ever increasing public urgency concerning groundwater pollution (for a discussion of these concerns, see Keely, 1985; Lehr, 1985), studies of subsurface biological processes were initiated as part of an EPA groundwater protection research program (Lobel, 1986). One goal was to study microbial activity in shallow aquifer sediments, particularly the type used commonly as sources of drinking water in North America and Europe. The development of a successful microbiological research program relied first on the availability of aseptic sampling techniques and second on the adaptation of existing microbiological methods for the study of microbial abundance and activities in subsurface material. An important feature of the EPA program was its limited scope. Because of high drilling costs, the number of sampling sites and size and depths of core samples were limited. Also, sharing of samples between laboratories fostered collaboration between investigators. Without a collaborative approach, progress would have been much slower.

Other subsurface microbiology programs currently under way in the United States and Europe are beginning to show results. The United States Geological Survey carries out a broad range of groundwater studies with biological components (Chappelle *et al.*, 1987; Ehrlich *et al.*, 1982, 1983; Harvey *et al.*, 1984; Harvey and George, 1987) and the United States Department of Energy recently has initiated a deep subsurface microbiology program which has alreaded yielded significant results (Fredrickson and Hicks, 1987). In Germany, groundwater microbiology is being studied under sponsorship of the Federal Ministry of the Interior (Umweltbundesamt) (Hirsch and Rades-Rohkohl, 1983; Rades-Rohkohl and Hirsch, 1983; Matthess, 1985; Matthess and Pekdeger, 1985), and in England microbiological aspects of groundwater pollution by nitrate and

landfill leachate are being investigated at the Institute for Geological Sciences, Wallingford (Foster *et al.*, 1985) and at the Water Research Center, Medmanham (Towler *et al.*, 1985), respectively. Several other European nations with serious groundwater problems also have incorporated subsurface microbiology in their groundwater pollution programs.

C. Purpose

The main purpose of this article is to review past and present research on the microbial ecology of terrestrial subsurface environments. To achieve this we first examine the physical and chemical properties of subsurface sediments and groundwater that will affect their ability to support microbial life. We also examine the growing body of recent work on microbial abundance and activities in shallow subsurface zones, focusing on biotransformations of organic compounds in water table aquifers. We hope that this article will provide background information and guidance for those interested in pursuing further research in this underdeveloped area of microbial ecology.

II. The Terrrestrial Subsurface as a Microbial Habitat

A. General Description

To describe the environmental properties of the entire terrestrial subsurface would require description of the multitude of geologically different subterranean environments that comprise the earth's crust to a depth where life can no longer exist. Obviously, description of such vast and variable environments would require far more space and information than is available to us now. Therefore, for the purpose of this article, we shall focus on the important environmental factors that may affect life in the upper few hundred meters of the earth's crust. These regions contain the subsurface zones that are most important for maintaining the quality of groundwater aquifers that serve as sources of drinking water for a large portion of the world population.

Despite immense geological variability from one geographic region to another, one striking feature of the shallow, subsurface zones of interest is the similarity of the types of strata in which drinking water aquifers are found (Freeze and Cherry, 1979; Bouwer, 1984). For example, most aquifers used for water supplies are found in sand and gravel layers or in mixes of alluvial or unconsolidated material where groundwater flow is greatest. In some localities consolidated sandstone and porous limestone layers are used. Other less productive strata usually are avoided.

Thus, on a large scale the terrestrial subsurface can be viewed as an endless variety of different types of sedimentary environments encompassing a wide range of geological and geochemical conditions, but on a smaller scale at each location each stratum will have some relatively constant physical and chemical properties that will determine whether or not they can be used as sources of drinking water; these properties also may determine the types, abundance, and activities of microorganisms.

B. Environmental Factors

McNabb and Dunlap (1975), Matthess (1982), and Bouwer (1984) have reviewed the principal hydrogeochemical factors that may influence the presence and activities of microorganisms in shallow aquifers. Kuznetsov *et al.* (1963) and Davis (1967) have also discussed factors affecting microbial activity in deeper subsurface zones with regard to mineral and petroleum deposits. In this section we review the principal factors that may control microbial abundance and activity in vadose zones and water table aquifers.

1. *Spatial Limitation*

Texture, porosity, and permeability of the subsurface material will affect water availability and determine pore space which, in turn, will limit the types and sizes of organisms present. The size of the interstices is critical, and may vary widely in subsurface sediments. The interstices can range from minute pores to cracks, fissures, and large solution cavities. Gravelly aquifers associated with river terraces and cavernous limestone aquifers can contain spaces large enough to allow a variety of subterranean animals to live in them (Longley, 1981; Matthess, 1982). Because of the smaller size and nutritional diversity of microorganisms, they will be far more widely distributed in the subsurface. McNabb and Dunlap (1975) concluded that, except for massive crystalline rock containing no fractures and clay and rock layers with very low permeability, sufficient space exists in most unconsolidated and porous subsurface strata to allow microorganisms to live. The limiting factor will be the sizes of individual interstices which must be large enough to accommodate the dimensions of microbial cells.

Most laboratory-grown bacterial cells are on the order of 1 μm in size, but the smallest survival forms in soil may be only a few tenths of a micrometer in diameter (Bae *et al.*, 1972; Balkwill and Casida, 1973). Small bacterial cells can be expected to increase in size when more nutrients are available (Bae and Casida, 1973). Most eukaryotic microorganisms and their survival forms are between 1 and 10 μm in size. Thus, as a rough gauge, openings between 0.1 and 1.0 μm will be required to accommodate

bacteria; openings between 1 and 10 μm will be required for eukaryotic microorganisms. The sizes of openings in subsurface material can be assumed to be variable and generally they are not measured directly, but porosity and permeability measurements on aquifer sediments indicate that adequate spaces for bacteria exist in many sediment types, even in some rather dense porous rocks (McNabb and Dunlap, 1975). The interstices of the shallow aquifer sediments of interest can easily accommodate bacteria and probably algae, protozoa, and fungi as well. Larger organisms will be excluded from most subsurface formations, except for gravelly and cavernous aquifers. Microorganisms certainly will be the dominant forms of life and, in most cases, they will be the only forms of life present in aquifers.

2. *Nutrient Availability and Redox Conditions*

a. Carbon, Nitrogen, Phosporous, Sulfur, and Trace Elements. In most regions not associated with petroleum, peat, or coal deposits the concentration of organic carbon in subsurface water and sediments is very low. Most groundwaters contain less than 1 mg dissolved organic carbon (DOC)/liter and aquifer solids contain only trace amounts of organic carbon that are water soluble (Thurman, 1985). It can be assumed that most pristine subsurface environments are oligotrophic. The population density of heterotrophic microorganisms will be limited by the low concentrations of metabolizable organic carbon (Atlas, 1986; Poindexter, 1981). However, oil-bearing stratal waters and waters associated with peat or coal deposits may contain 2–10 mg DOC/liter or more with very high levels near the deposits (Matthess, 1982; Davis, 1967; Thurman, 1985). Such deposits may support quite large populations of hydrocarbon-utilizing bacteria (Kuznetsov *et al.*, 1963; Davis, 1967). Carbon dioxide usually is abundant in soil and ground air (Alexander, 1977; Matthess, 1982). Therefore, this inorganic carbon source, which is required by all autotrophic and heterotrophic bacteria, will be plentiful in most near-surface groundwaters. Older and deeper groundwaters may be depleted in CO_2 (Freeze and Cherry, 1979). However, recent evidence shows that heterotrophic bacterial activity may be a source of CO_2 in some deep aquifers (Chappelle *et al.*, 1987).

The types of organic compounds in subsurface zones will depend to a large extent on the parent material of the formation, the time recharge water takes to reach the zone, and the biological activity in the recharge zones (See Section IV for further discussion). Most readily metabolized compounds will be consumed by the surface microflora before they reach unsaturated subsurface horizons above a water table aquifer (Alexander, 1977; Matthess, 1982; Thurman, 1985). The identity of soluble organic compounds dissolved in groundwater is still a matter for speculation,

but generally it is thought that subsurface organic matter consists mostly of humic substances, naphthenic acids, and phenolic compounds derived from either the organic matter in sedimentary rocks or lignin degradation products derived from plant residues in surface soil (Davis, 1967; McNabb and Dunlap, 1975; Matthess, 1982).

Nitrogen and phosphorus limitations are more likely to occur in subsurface environments than is limitation by sulfur. Sulfate is plentiful in groundwater of near-surface zones (Matthess, 1982; Freeze and Cherry, 1979), but ammonium, or nitrate, and phosphate may be extremely low in specific locations (McNabb and Dunlap, 1975). The frequent observation that dissolved phosphate is very low or absent in groundwaters (Matthess, 1982) may be explained by the low solubility of the phosphate salts of magnesium, calcium, and iron. However, as argued previously by McNabb and Dunlap (1975), the absence of soluble phosphate and, in some locations, inorganic nitrogen in groundwater does not mean that lack of these nutrients limits growth or normal activity of the microflora. Indeed, phosphate can be readily mobilized from mineral phases by microorganisms. Furthermore, assuming that the organic matter of shallow subsurface sediments is similar in composition to humus of soil (Alexander, 1977), the concentrations of organic nitrogen and phosphorous may be high enough to support a small standing crop of oligocarbophilic microorganisms. Only when utilizable carbon sources are present in excess, such as in a pollution episode, would lack of nitrogen or phosphorus be expected to limit the size and activity of the microbial population.

b. Oxygen and Other Electron Acceptors. Oxygen concentration and redox potential will greatly affect microbial activity and the geochemistry of various elements in the subsurface; however, except for studies on redox potential in flooded soil profiles (for summary, see Bouwer, 1984), reports on direct measurements of redox conditions in subsurface pore waters are rare. This lack of direct measurements apparently results from methodological problems involved in measuring redox potential and O_2 concentrations in subsurface sediments (McNabb and Dunlap, 1975). Because of these problems, it has been assumed, perhaps wrongly (Winograd and Robertson, 1982), that most deep groundwaters are depleted in O_2. This assumption is based on the idea that O_2 dissolved in recharge water is consumed as organic matter is oxidized in soil, subsoil, and vadose zones, or as inorganic species (e.g., Fe^{2+}, Mn^{2+}, S^{2-}) are oxidized in groundwater zones. These processes do consume O_2 and shallow aquifers in some regions are anaerobic (for discussion of conditions that lead to anaerobiasis see Section IV); however, most pristine drinking-water aquifers do contain significant amounts of O_2.

Unless there is pollution from agricultural fertilizers, NO_3^- concentrations usually are low in shallow groundwater zones. But the ambient NO_3^- concentrations probably are adequate to supply nitrogen to the bacterial population. The fact that denitrifying bacteria occur in these zones (McNabb and Dunlap, 1975; Matthess, 1982; Foster *et al.*, 1985) shows that the potential for denitrification exists. The other common alternate electron acceptors, SO_4^{2-} and HCO_3^-, are plentiful in shallow subsurface zones (Matthess, 1982). Manganese oxides and iron oxyhydroxides, which may act as electron acceptors in bacterial respiration (Ghiorse, 1988), are abundant in many subsurface zones. Microbial reduction of these solid-phase minerals may account for the oxidation of organic matter and mobilization of Fe^{2+} and Mn^{2+} in some anaerobic groundwaters (Matthess, 1982).

It is generally agreed that the redox state of aquifer sediments will be a very important factor controlling microbial transformations of many inorganic and organic compounts (McNabb and Dunlap, 1975; Freeze and Cherry, 1979; Matthess, 1982; McCarty *et al.*, 1984). Indeed, laboratory column studies that simulated subsurface conditions (Bouwer and McCarty, 1984; Bouwer and Cobb, 1987) showed that a thermodynamic biofilm model was applicable to trace organic biotransformations in subsurface environments , provided that biodegradable organics, O_2, NH_4^+, NO_3^-, and SO_4^{2-}, were present. In this model, O_2-terminated heterotrophic respiration and nitrification lead a succession of anaerobic redox processes, principally denitrification, sulfate reduction, and methanogenesis in that order, as water moves away from the source of contamination. The model predicts that different electron acceptors and redox conditions will prevail as organic substrates move downstream; and each different redox environment created in the succession may favor the secondary degradation of a particular set of organic micropollutants. From their studies of idealized column systems, Bouwer and McCarty (1984) concluded that prediction of organic micropollutant transformations in the subsurface will require the ability to predict the presence of particular electron acceptors and populations of microorganisms that can use them. Bouwer and Cobb (1987) have confirmed and extended these concepts by employing a subsurface biofilm model to predict the degradation of trace halogenated organic pollutants when acetate was the primary substrate.

The application of a sequential electron acceptor model to predict redox conditions in aquatic environments including groundwater systems has been discussed previously (Stumm and Morgan, 1981; Matthess, 1982). For a contaminated groundwater system Matthess (1982) assumed that a confined aquifer contained excess DOC and solid-phase Mn(IV) oxides and Fe(III) oxyhydroxides and other minerals. In such a system, the reduction sequence should be O_2, NO_3^-, MnO_2, $Fe(OH)_3$, SO_4^{2-}, HCO_3^-, and N_2.

Adjacent uncontaminated zones or transitional zones should contain free O_2. Although supportive field measurements are not abundant, there is no apparent reason why a natural confined aquifer polluted by excess DOC should not behave as predicted by the thermodynamic models. It should be emphasized that the models are based on thermodynamic considerations for chemical reactions which operate in nature only at very slow rates unless microorganisms are present (Stumm and Morgan, 1981). It should also be emphasized that the oxidation—reduction zones predicted by these models may greatly affect the behavior of minor and trace constituents in the system, including trace metals associated with iron and manganese oxides (Matthess, 1982; Ghiorse, 1984) as well as trace organics.

c. *Nutritional Ecology of Aquifers.* Microbial abundance in aquifers will be limited by availability of nutrients, especially organic carbon sources. Nitrogen and phosphorus limitations are also possible, especially when DOC levels are high, but sulfur and other mineral nutrient limitations are unlikely. Redox conditions will be very important in determining microbial activity, especially for predicting biotransformations in groundwater zones. These transformations may be controlled by the presence of O_2-consuming biological and abiological oxidation reactions in soil, subsoil, and subsurface zones. Although re-aeration of O_2-depleted groundwater is unlikely to occur to any great extent, because of their low organic matter content, most pristine aquifer sediments probably will contain sufficient O_2 or other electron acceptors to support a minimum level of microbial respirative activity. However, aquifiers contaminated with high levels of utilizable organic carbon may quickly be depleted of suitable electron acceptors which will be replenished only very slowly under natural conditions. Indeed, along with nitrogen and phosphorous sources, the presence of sufficient electron acceptors will be a principal factor in predicting the degradation of organic contaminants in contaminated aquifers (see Section V).

Some questions on the nutritional ecology of subsurface ecosystems can be raised at this point. Assuming that the nutrients and redox conditions of a subsurface will select for specific physiological groups of microorganisms, can we not extrapolate from our present knowledge of microbial physiology and ecology and assume that subsurface ecosystems will function in the same way as ecosystems at the surface? If subsurface ecosystems are indeed comparable to surface systems, will microbial maintenance and survival processes be emphasized to the same extent in the oligotrophic subsurface as they are in other oligotrophic environments? What is the relative significance of reproductive growth versus dormancy for such environments? If, as we suspect, growth rates

of bacteria in subsurface populations normally are very low, then maintenance strategies may be the rule. If that is the case, then how do the physical properties of sediments and the chemical properties of groundwater affect microbial activity and survival processes? How might perturbation of subsurface environment affect these processes?

The fact that we can ask such naive questions, even about the relatively shallow subsurface zones of interest in this article, shows the importance of studying these microbial realms. It also shows the importance of investigating life in deeper subsurface zones which now appear to contain a greater abundance and diversity of microbial life than was previously expected (Fredrickson and Hicks, 1987).

3. *Activity on Surfaces*

The high specific surface area of aquifer sediments deserves special consideration with regard to its potential effects on microbial activity (for a concise discussion of the surface area available in subsurface sediments, see Bouwer, 1984). McNabb and Dunlap (1975) argued that the solid surface area characteristic of highly structured subsurface zones may influence microbial activity through sorptive effects on nutrients, extracellular enzymes, and the microbes themselves. These authors implied, but did not state directly, that most of the effects would tend to increase microbial activity. More recently McCarty and co-workers (McCarty *et al.*, 1984; Bouwer and McCarty, 1984) have argued that the high specific surface area of subsurface sediments would favor predominance of attached bacteria in the form of biofilms, particularly in surface zones containing organic pollutants. Presumably, attached bacteria would have an advantage over bacteria suspended in groundwater because the attached bacteria would be exposed to a constant supply of nutrients in the water that flows over them (Bouwer and McCarty, 1984). These arguments certainly justify the application of biofilm kinetic models on aquifer-simulating laboratory systems and for the modeling of polluted aquifers themselves (McCarty *et al.*, 1984). However, because very little is known of underlying physiological mechanisms, the effects of surfaces on microbial activity are difficult to generalize. The modeling of surface effects on bacterial activities in natural aquifer sediments may be far more complex than the present models indicate.

Some of the physiological complexities of surface effects were discussed at a recent Dahlem Conference (Marshall, 1984) which focused, in part, on the physiological consequences of microbial adhesion and aggregation. One important consensus of the conference participants was that no predictable pattern of surface effects on bacterial physiology can be discerned from past investigations. Physiological processes such as uptake and utilization of carbon substrates, respiration, heat generation, survival,

and growth all have been compared for attached versus unattached cells in a variety of laboratory investigations; however, no consistent trends have emerged. Attachment can result in either enhancement or depression of activity or no effect at all, depending on the specific microorganisms, the surface to which they are attached, or the activity being measured. Indeed if other factors are equal, the rates of nutrient intake by bacterial cells would likely be slower for attached bacteria than for freely dispersed cells (Breznak *et al.*, 1984). In contrast, other ecologically significant processes such as cross-feeding, extracellular degradation of isoluble polymers, growth in anaerobic microenvironments, and protection from abiotic (e.g., toxic substances, suboptimal pH) and biotic (e.g., grazing and attack by bacteriophage) stresses would be enhanced by attachment.

Of particular interest for our discussion of oligotrophic subsurface environments are the effects of surfaces on nutrient availability. Surfaces should confer a significant survival advantage to attached bacteria in such environments. For example, if surfaces adsorb macromolecules which can be exploited by attached cells via extracellular hydrolytic enzymes, the molecules will provide a source of nutrients (Hoppe, 1984). If low molecular weight nutrients are absorbed, the degree of availability will depend on whether or not the nutrients and substratum are charged. Bacteria attached to charged surfaces may have more NH^{+}_{4} or NO_3^- and PO_3^{2-} available to them than bulk-phase bacteria do. However, the charged nutrient species must not be so tightly bound to the substratum that they cannot be assimilated by the bacteria (Fletcher, 1984). Low molecular weight uncharged nutrients are likely to be equally distributed on surfaces and in the bulk phase unless other forces like hydrophobic interactions come into play (Filip and Hattori, 1984; Kjelleberg, 1984). Nevertheless, attached bacteria that take up uncharged nutrients via a high-affinity transport system (Poindexter, 1982) may still have an advantage over unattached bacteria simply by virtue of their ability to create steep diffusion gradients in their microenvironments (Breznack *et al.*, 1984). Finally, some bacterial activities such as CO_2 production and substrate uptake can be decreased by attachment to surfaces (Fletcher, 1984), suggesting that, in some instances, surfaces may reduce overall activity and perhaps induce dormancy.

Other possible surface effects may be important in contaminated subsurface environments containing high levels of organic compounds: (1) Modifications of the surface chemistry of cells and substratum by cellular contact with substratum. For example, the formation by bacteria of specific negatively charged extracellular polymers when they contact surfaces (Fletcher, 1984) may provide a means for nutrient capture. However, the formation of a thick biofilm will not necessarily promote nutrient

assimilation because the hydrated matrix may act as a gel diffusion barrier, retarding diffusion of nutrients and waste products (Fletcher, 1984). (2) The formation of mutualistic consortia which, in the subsurface, may be important for anaerobic decomposition of organic compounds especially when tightly coupled redox reactions are required for decomposition to occur (Fletcher, 1984). The formation of consortia also may facilitate extracellular enzyme activity (Hoppe, 1984) which can promote cross-feeding and other cooperative activities. (3) Protection from environmental stresses. Although experimental evidence is scarce, surfaces may also provide protection for attached cells against the effects of toxic substances and predation (Breznack *et al.*, 1984).

High specific surface area of subsurface sediments may greatly influence subsurface microbial activity by affecting nutrient availability; but other possible effects of surfaces such as stimulation or depression of overall metabolic activity, enhancement of dormancy and survival, and protection from environmental stresses must also be considered. There do not appear to be any universal surface effects that apply to subsurface sediments in general. To predict the effects of surfaces, each case may have to be assessed individually with regard to the microbial process of interest and the particular sediment, groundwater, and microorganisms involved.

4. *pH, Temperature, Hydrostatic Pressure, and Dissolved Salts*

Possible effects of environmental factors such as pH, temperature, hydrostatic pressure, and dissolved salts have been reviewed in detail by Kuznetsov *et al.*, (1963), McNabb and Dunlap (1975), and Matthess (1982). Because their biological effects are most influential at extremes, these factors are not likely to limit microbial activity appreciably in most shallow aquifer zones in which variations of pH, temperature, pressure, and salts usually are minimal. Indeed, only the top 10 m of the subsurface profile is affected by seasonable variations in temperature; at 10–20 m depth the temperature is constant and corresponds roughly to the mean annual air temperature of the locality (Kuznetsov *et al.*, 19163; Matthess, 1982). In the United States temperatures in this zone range from 23 to 25°C (McNabb and Dunlap, 1975). Below 20 m and outside of areas of high tectonic and geothermal activity, temperatures increase at a rate of approximately 3°C per 100 m. Thus, the temperatures in shallow aquifer sediments down to several hundred meters typically are ideal for mesophilic microorganisms. The temperture at 1000 m may approach 50–55°C, the lower range of temperatures for thermophilic microorganisms.

Hydrostatic pressure, like temperature, increases with depth. With few exceptions, the pressure within subsurface rocks can be approximated

from the hydrostatic pressure produced by a column of water extending from the surface to the depth of the measurement (McNabb and Dunlap, 1975). Thus, assuming an average rate of increase of approximately 1 atm per 10 m, increases of pressure experienced by microorganisms in the shallow subsurface zones of interest will be on the order of tens of atmospheres reaching 100 atm at 1000 m. Because surface microorganisms have been shown to tolerate hundreds of atmospheres before their growth and activity are inhibited, hydrostatic pressure is unlikely to have any great influence on the activities of microorganisms in the shallow aquifer zones of interest.

Variations in groundwater pH will be governed to a large extent by the pH of recharge water and by other geological and geochemical factors (Matthess, 1982). However, because of the buffering capacity of HCO_3^- in shallow groundwater aquifers, extremes of pH are rare. Even though extremes of pH may limit the diversity of microorganisms and alter the effects of other environmental factors on microbial activity (e.g., low pH may alter an organism's sensitivity to temperature or to heavy metal toxicity), extremes of pH are not likely to preclude microbial activity in any shallow aquifer sediments; however, low pH and high metal content may be influential in some aquitard clays (McNabb and Dunlap, 1975). It should be noted that the bulk pH of the groundwater may be entirely different from the pH in the microenvironment in the vicinity of particles where attached microorganisms live (Filip and Hattori, 1984); thus pH effects at surfaces may greatly affect the activities of subsurface microorganisms even though bulk pH does not change markedly.

The concentration of salts dissolved in groundwater may vary considerably from site to site depending on the geology of the formation. As a rule, total salts increase with increasing depth and age of the groundwater (Freeze and Cherry, 1979; Bouwer, 1984); however, drinking water aquifers usually contain water with salt concentrations on the order of hundreds of milligrams per liter which will not greatly affect microbial activity. Older water in deeper zones of alluvial aquifers may contain higher salt and toxic metal concentrations that may limit the types of microorganisms in them. Some very deep hydrogeologically isolated salaquifers may contain more than 20% total dissolved salts (Kuznetsov et *al.*, 1963; McNabb and Dunlap, 1975; Bouwer, 1984).

C. How Deep Is the Terrestrial Biosphere?

In this section we consider the possibility of microbial life at much greater depths than a few hundred meters, in subsurface zones thousands of meters deep where temperatures may approach or exceed the limits of life on the surface. This question has been considered previously by

others who were concerned with the existence of sulfate-reducing and hydrocarbon-oxidizing bacteria in deep formations containing oil and sulfur deposits (e.g., Zobell, 1957, 1958). Also the role of microorganisms in the genesis, degradation, and recovery of petroleum has been of considerable interest in the past (for reviews, see Davis, 1967; Kuznetsov *et al.*, 1963; Yi-gang, 1981). Microbial activity in the deep subsurface has also been of interest to researchers concerned with the injection of liquid waste into deep subsurface strata (e.g., Willis *et al.*, 1975), the use of deep aquifers as industrial water supplies (Olson *et al.*, 1981), and the use of iron- and sulfur-oxidizing bacteria in deep solution mining (Davidson *et al.*, 1981).

The information on microbial life at great depths is fragmentary and incomplete. During the 1940s and 1950s, Claude E. Zobell in collaboration with Shell Oil Company demonstrated the existence of viable sulfate-reducing bacteria in waters and core samples taken from strata down to 3700 m near oil and sulfur deposits (estimated *in situ* $T = 95°C$, $P = 400$ atm). Zobell (1957, 1958) reported that sulfate-reducing bacteria grew in enrichment cultures derived from these samples which were incubated at 104°C under 1000 atm pressure. Several Soviet workers also have reported thermophilic sulfate-reducing and hydrocarbon-oxidizing bacteria in deep stratal waters and sediment core samples down to 2200 m (Kuznetsov *et al.*, 1963). Olson *et al.*, (1981) found sulfate-reducing and methanogenic bacteria in stratal waters from wells 1800 m deep.

Most of the earlier results are equivocal because of possible contamination problems in the cultural methods employed to identify bacteria. However, in agreement with the conclusions of Kuznetsov *et al.* (1963), the results suggest that microbial life may exist in subsurface strata down to about 4000 m, provided that enough water, pore space, and nutrients are available. Because there is no evidence to the contrary, the possibility exists that microbial life could exist even at greater depths, in zones where temperatures exceed the currently accepted upper temperature limit for life (~120°C) (Trent *et al.*, 1984, Bernhardt *et al.*, 1984; White 1984). Deep subterranean environments, like deep sea vents, are characterized by constant elevated temperature and pressure. Thus, they may harbor extremely thermophilic bacteria, comparable to those in the deep sea thermal vents and other caldoactive environments (Belkin and Hannasch, 1985; Stetter, 1985). Although the deep terrestrial subsurface is equally as remote as the sea floor, the technology for drilling deep wells and retrieving deep core samples under pressure is only now being developed (Kozlovsky, 1984; J. Oliver, personal communication). Thus, it may be feasible in the future to obtain intact core samples for microbiological analysis from depths of 10,000 m or more.

III. Characterization of Microorganisms and Their Activities in Subsurface Environments

A. SAMPLING

Sampling of subsurface material is not a trivial matter. To obtain representative samples for microbiological investigations is difficult, tedious, and expensive. Sampling must be done by digging or drilling a hole through the A and B horizons of soil to reach the subsurface strata below. For water samples, a well must be constructed. These operations are often technologically complex and expensive. They require special equipment and highly trained personnel. The expense of sampling increases drastically with depth and with special sampling requirements. Thus, the microbiologist's request for "uncontaminated" samples, even from shallow depths in the subsurface, further complicates the difficulties. Needless to say, the possibility of contamination by soil microorganisms in surface soil, water, and drilling muds is a constant problem. Another problem is sample variability. It is often difficult to obtain enough core material from one stratum or location to satisfy the usual field sampling statistical requirements. Despite the problems, sampling methods have been developed (McNabb and Mallard, 1984) and numerous microbiological studies discussed in this Section were completed during the last decade.

B. METHODS FOR DETECTION, ENUMERATION, AND METABOLIC ACTIVITY

There is no lack of methods that might be applied to detect microorganisms and estimate their biomass and metabolic activities in subsurface samples (for recent reviews of methods applicable to bacterial ecology, see Poindexter and Leadbetter, 1986a). The range of available methods extends from high-resolution transmission electron microscopy and ultrasensitivie enzyme-linked and gas chromatographic—mass spectrometric assays to enrichment culture procedures, microcosm studies, and radioisotopic measurements of heterotrophic activity and growth. The applications of these methods to shallow subsurface samples have been reviewed (Bengtsson, 1985; Ghiorse and Balkwill, 1985; Hirsch and Rades-Rohkohl, 1983; Leach *et al.*, 1985; White *et al.*, 1985; Wilson and Noonan, 1984; Wilson *et al.*, 1985b). Most of the methods have been adopted from soil and aquatic microbiology; however, usually it has been necessary to extend their sensitivity because of low population densities and low levels of activity in subsurface samples. In the following paragraphs, we describe briefly some methods that have been successfully applied to subsurface samples.

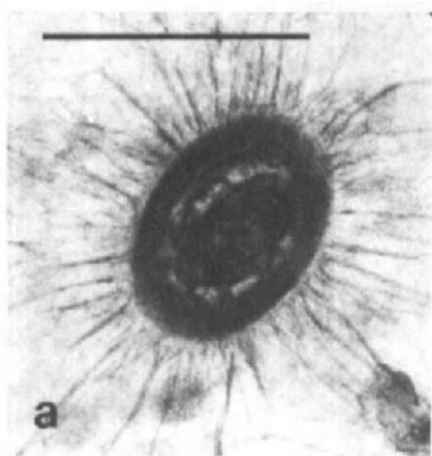
1. *Microscopic Methods*

The presence of microorganisms in environmental samples and some of their distinguishing morphological features (size, shape, eukaryotic or prokaryotic cell organization, cell wall type, internal storage bodies, external polymer structures, etc.) can be established most convincingly by direct light and electron microscopy. Because subsurface samples may contain very low microbial population densities, conventional microscopic procedures may not always be effective. Indeed, the lower limit of detection of cells in unconcentrated liquid medium by conventional light microscopy is approximately 10^6 to 10^7 bacteria/ml (Brock, 1984) which is close to the average population densities observed in subsurface samples (Tables I and II).

Because the presence of microorganisms in subsurface environments was questioned in the past, it has been worthwhile to improve the sensitivity of the microscopic counting procedures to demonstrate their presence directly. For water samples, this is done by concentrating cells on membrane filters and staining with a fluorescent dye to improve their visibility in the light microscope. Filtration procedures have been successful for examining groundwater samples (Ehrlich *et al.*, 1982, 1983; Harvey *et al.*, 1984; Olson *et al.*, 1981; Marxsen 1981a,b; Dockins *et al.*, 1980; Larson and Ventullo, 1983; Ladd *et al.*, 1982). However, they have been less successful for sediment and rock core samples because of particle interference and because of the difficulty of desorbing cells from surfaces. These problems have been partly overcome by Towler *et al.* (1985) who recently reported a direct microscopic-filtration counting procedure in which solid chalk was dissolved by acid treatment in the presence of phenol aniline blue to reveal bacteria in the chalk.

Direct observation and counting of bacteria in subsurface sediment samples has been achieved by staining smears with acridine orange (AO) (Balkwill and Ghiorse, 1985a,b; Bone and Balkwill, 1986; Ghiorse and Balkwill, 1983, 1984, 1985; Wilson *et al.*, 1983a). Bacteria have also been released and concentrated from sediment particles and then viewed by transmission electron microscopy (Figs. 1 and 2). The release and concentration methods are not suitable for quantitative cell counts because only a small portion of the cells are released (Bone and Balkwill, 1986).

FIG. 1. Transmission electron micrographs showing thin sections of sediment bacteria from various depths at the pristine subsurface site at Lula, Oklahoma. (a) Bacterial cell with exopolymers projecting from surface, subsoil. (b) Gram-negative cell surrounded by exopolymers coated with clay particles, unsaturated zone. (c) Gram-positive cell, interface zone. (d) Gram-negative cell containing large poly-β-hydroxybutyrate storage body, saturated zone. (e) Gram-positive cell, saturated zone. Note particles (probably clay) attached to surfaces of cells in (c), (d), and (e). Bars, 0.5 μm. (Courtesy, D. L. Balkwill.)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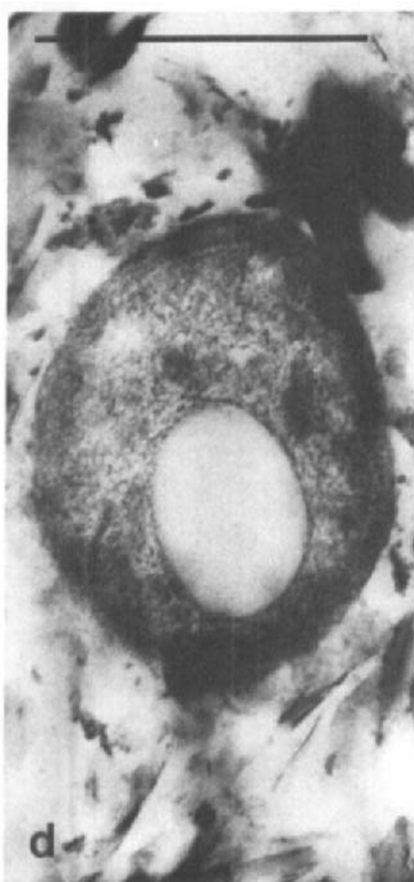
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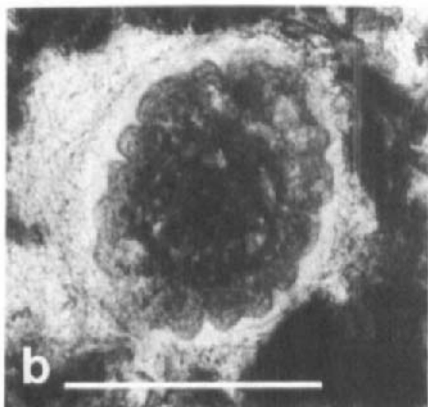
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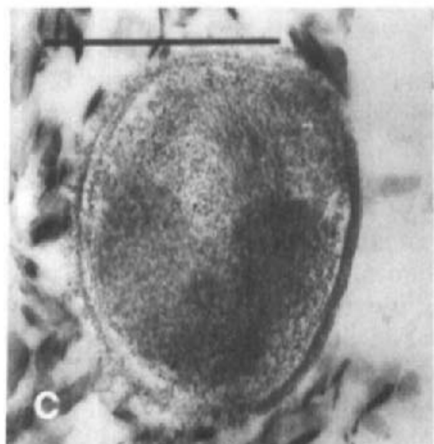
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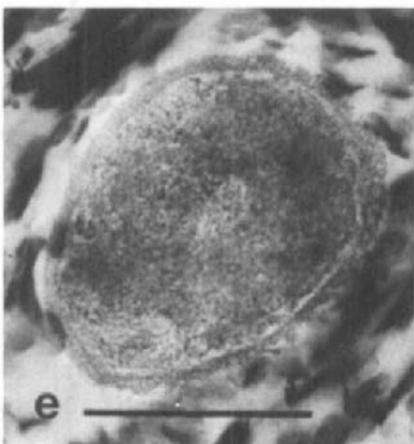
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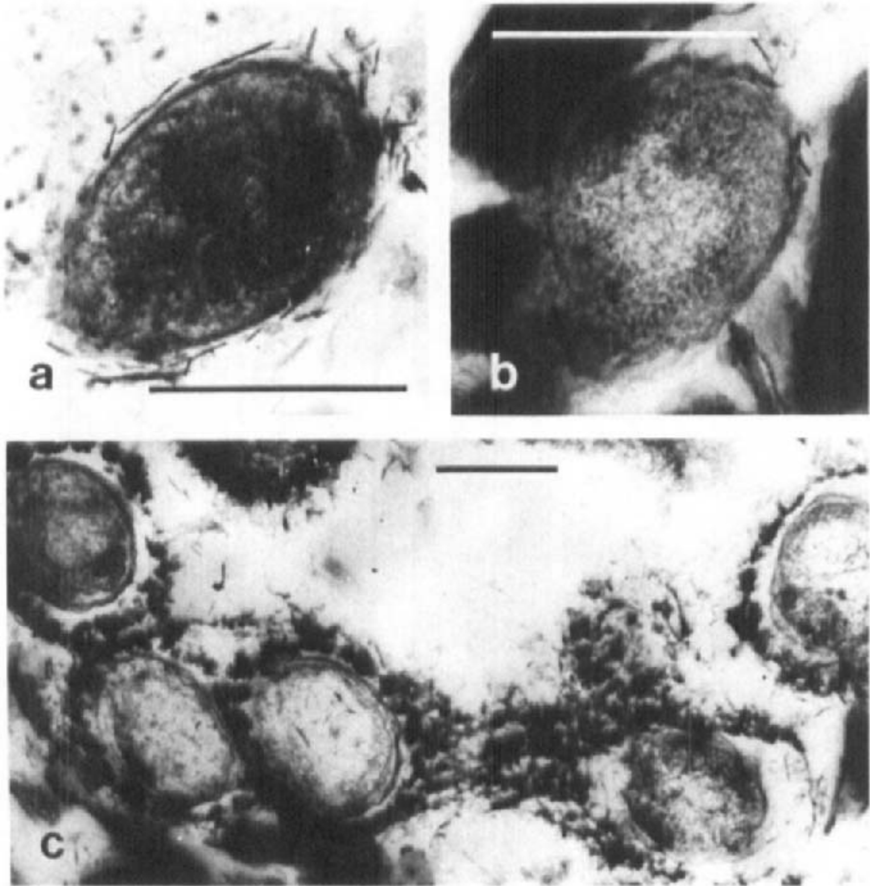

FIG. 2. Transmission electron micrographs showing thin sections of sediment bacteria from saturated zone obtained just outside a plume of contamination at a buried creosote waste site in Conroe, Texas. All cells are gram-negative. Note the claylike particles attached to cells in (a) and (b) and electron-dense material (probably exopolymer) surrounding cells of a microcolony in (c). Bars, 0.5 μm. (Courtesy, D. L. Balkwill.)

However, the smear technique has been employed successfully for direct counts (Balkwill and Ghiorse, 1985; Ghiorse and Balkwill, 1983; Wilson *et al.*, 1983a). In addition, a tetrazolium dye reduction variation of the direct counting procedure has been developed to estimate the proportion of respiring bacteria in subsurface sediments (Webster *et al.*, 1985; Beloin *et al.*, 1988). Application of many other methods for estimating cell viability and growth in subsurface samples certainly is possible and should be useful for future work (for examples, see Karl, 1986; Newell

et al., 1986; White, 1986). Indeed, Harvey and George (1987) showed that a frequency of dividing cells method was better than a tritiated thymidine uptake method for assessing differences in growth rate along the containment plume of a sewage polluted aquifer.

2. *Cultural Methods*

Standard cultural procedures, e.g., plate counts, most probable number counts, and enrichment culture procedures, have been employed in many subsurface studies (Tables I and II). Usually they are more sensitive than microscopic methods of detection, especially for diverse microbial types that may be present at very low population density, or for those whose properties are not well known (Poindexter and Leadbetter, 1986b). Because such procedures are sensitive to contamination, samples should be taken and handled with great care and interpretation of results should be cautious. Hirsch and Rades-Rohkohl (1983) have successfully applied enrichment techniques to studies of microbial diversity of groundwater and subsurface sediments. Their techniques reveal the enormous diversity of microbial life that can exist in subsurface aquifer zones and groundwater.

3. *Biochemical Indicators*

Biochemical indicators of metabolic activity and detection of specific molecular markers in natural samples (e.g., ATP, GTP, phospholipid, muramic acid) provide very sensitive estimates for biomass and activity. These methods often employ very sensitive biochemical assays, or large samples to improve sensitivity (Karl, 1986; White, 1986). White *et al.* (1985) and Leach *et al.* (1985) have reviewed the application of such methods to subsurface sediments. Because these methods cannot show directly the presence or activity of cells, results should be interpreted cautiously. Ideally, these methods should be supported by direct microscopic and cultural methods applied to the same samples (Tables I and II).

4. *Radioisotope Methods*

Radioisotope-labeled substrate transformation and uptake methods provide another sensitive means of measuring metabolic activity and growth of microorganisms in environmental samples. The advantages and problems associated with their use have been reviewed (Karl, 1986). Ideally, the rate of labeled substrate transformation should be compared to total direct counts, plate counts, or another biomass indicator to normalize the rates to population density, but this is not always done. Rates of heterotrophic mineralization activity (Ladd, *et al.*, 1982; Larson and Ventullo, 1983; Ventullo and Larson, 1985; Ventullo *et al.*, 1983) and tritiated thymidine incorporation growth rates have been measured in

subsurface samples (Thorn and Ventullo, 1988; Harvey and George, 1987) (Tables I and II).

5. *Microcosms*

The application of microcosm methods to studies of biotransformation in subsurface samples and the advantages and problems associated with their use for studies of subsurface microbial activity have been reviewed previously (Bengtsson, 1985; Wilson and Noonan, 1984; Wilson *et al.*, 1985b). Despite the major criticism of all microcosm studies, i.e., the difficulty of extrapolating results from a simplified system to a more complex natural system, simple static microcosms have been very useful in estimating potential for biotransformations of synthetic organic pollutants in subsurface sediments and groundwater (see Tables I–V).

C. Types, Abundance, and Activities of Subsurface Microorganisms

During the past decade, the number of reports concerned with characterization of subsurface microorganisms and their activities has increased steadily from less than 1 per year in the period 1977 to 1980 to more than 10 per year in the period from 1984 to 1987 (Tables I and II). McNabb and Dunlap (1975) noted that most of the work published before 1980 either was directly related to petroleum microbiology or it was focused on special problems such as iron-depositing bacteria in water wells (Cullimore and McMann, 1977) or microbial activity in injection wells (e.g., Godsy and Ehrlich, 1978). No concerted effort was made to study the indigenous microflora of common water table aquifers until 1980.

Tables I and II summarize work reported between 1977 and 1987 showing types, abundance, and activities of microorganisms in samples from subsurface environments. For these tables, we created two groups based on whether material from pristine (Table I) or contaminated (i.e., chemically polluted or otherwise perturbed) sites (Table II) were studied.

Some patterns emerge from these two tables that might be predicted from our considerations of the subsurface as a microbial habitat (Section II). First, it is apparent that significant numbers of microorganisms, primarily bacteria but also protozoa and fungi, are present in most shallow subsurface zones. Not surprisingly, the high numbers of active microorganisms are detected in groundwater and sandy aquifer sediments, but most vadose zone samples, and even clayey aquitard sediments, also harbor microorganisms. The population densities of pristine subsurface sediments and groundwater, as determined by total cell counts, are remarkably consistent with depth. Generally they fall in a range between 10^5 and 10^7 cells per gram of sediment or per milliliter

TABLE I

TYPES, ABUNDANCE, AND ACTIVITIES OF MICROORGANISMS IN SUBSURFACE MATERIAL FROM PRISTINE SITES

Location and site description	Type of sample	Depth	Findings[a]	Reference
Saskatchewan, Canada	Groundwater from iron-bearing formations	Various depths	Diverse types of iron-depositing bacteria present in wells and and cultures	Cullimore and McCann (1977)
Montana, USA	Groundwater from Madison limestone	10–263 and 1264–1752 m	Total counts ~10^3/ml. Sulfate-reducing bacteria present	Dockins *et al.* (1980); Olson *et al.* (1981)
Fulda Valley, Germany	Groundwater from sandy and gravelly sediments	3–6 m	Total counts 10^6–10^7/ml. [^{14}C]glucose uptake correlated with C.O.D. and D.O.	Marxsen (1981a)
West Neidersachsen, Germany	Unsaturated and satured zone sediments	10–90 m	Viable counts 10^3–10^6/g. 11 physiological groups of bacteria detected	Hoos and Schweisfurth (1982)
Marmot Basin, Alberta, Canada	Groundwater	1.5 m	Total counts 10^5–10^6/ml. Viable counts 10^3–10^7/ml. [^{14}C]lignocellulose degraded[b]	Ladd *et al.* (1982)
Southern Arizona, USA	Groundwater	Various depths	Viable counts ~10^3/ml. Aerobic bacteria isolated	Stetzenbach and Sinclair (1983)
Segeberger Forest, northern Germany	Groundwater and sandy aquifer sediments	5–31.5 m	Enrichment of diverse bacteria, fungi, protozoa. Total counts 10^6–10^7/g. Viable counts 10^3–10^4/g	Hirsch and Rades-Rohkohl (1983); Rades-Rohkohl and Hirsch (1985)
Fort Polk, Louisiana, USA	Unsaturated and saturated sandy and clayey sediments	1.2–6.7 m	Total counts 10^6–10^7/ml. Viable counts <10^2–10^5/g. Bacteria observed by TEM. Phospholipid 0.90–5.5 nmol/g. Muramic acid 2.0–11.3 nmol/g. Toluene, styrene degraded in microcosms[b]	Ghiorse and Balkwill (1983); Wilson *et al.* (1983b); White *et al.* (1983a,b); Balkwill and Ghiorse (1985)

(continued)

TABLE I *(continued)*

Location and site description	Type of sample	Depth	Findings[a]	Reference
Bucatunna aquifer near Pensacola, Florida, USA	Clayey sediment	410 m	Phospholipid 0.52 nmol/g. Muramic acid 0.62 nmol/g. Ratio higher in PHB and uronic acid/muramic acid than surface sediments	White *et al.* (1983a,b)
Williamsburg and Sault Ste. Marie, Ontario, Canada	Groundwater	3–3.5 m	Total counts $\sim 10^6$/ml. Viable counts 10^1–10^5/ml. ^{14}C-glucose, -amino acids, -benzoate mineralized[b]	Larson and Ventullo (1983); Ventullo and Larson (1985)
Butler and Dayton, Ohio, USA	Groundwater	10 and 12 m	Total counts 10^4–10^5/ml. Viable counts 10^2–10^4/ml. ^{14}C-glucose, -amino acids mineralized[b]	Ventullo and Larson (1985)
Pickett and Lula, Oklahoma, USA	Unsaturated and saturated sandy, clayey sediments	1.2–8.0 m	Total counts 10^6–10^7/g. Viable counts 10^3–10^6/g. ATP and other biomass and activity estimates decreased with depth in unsaturated zone, but varied in saturated zone.[c] Protozoa and fungi detected in some samples. Bacterial colony diversity decreased with depth. PHB-containing cells observed in saturated zone by TEM.[d] Toluene degraded rapidly, chlorobenzene slowly in microcosms.[b] Plasmids found in 2–8% of isolates.	Wilson *et al.* (1983a); Bengtsson (1984); Ghiorse and Balkwill (1984, 1985); Balkwill and Ghiorse (1985a,b); Webster *et al.* (1985); Sinclair and Ghiorse (1987); Bone and Balkwill (1986, 1988); Wilson, J. T. *et al.* (1986); Beloin *et al.* (1988); Ogunseitan *et al.* (1987); Thorn and Ventullo (1988)

Kaiserslautern, Germany	Unsaturated sandstone rock over coal deposit	2–405 m	Viable counts decreased with depth. No viable counts at 24–343 m, 10^3/g at 405 m coal layer	Weirich and Schweisfurth (1983)
Maryland, USA	Coastal plain sediments, interbedded clay, silt, sand	14–182 m	Total counts 10^4–10^8/g. Viable counts 10^3–10^6/g. Methanogenic and sulfate-reducing bacteria found in <40% of samples. Heterotrophic bacteria may be a source of CO_2.	Chapelle *et al.* (1987)
Nemaha County, Kansas, USA	Clayey, sandy, and gravelly sediments in glacial buried-valley aquifers	26–86 m	Total counts 10^6–10^7/g. Viable counts <10^2–10^7/g. Protozoa found in very permeable layers.	Sinclair *et al.* (1987)

[a]Abbreviations: C.O.D., chemical oxygen demand; D.O., dissolved oxygen; TEM, transmission electron microscopy; PHB, poly-β-hydroxybutyrate.
[b]See Table IV.
[c]See Table III.
[d]See Figure 1d.

TABLE II

TYPES, ABUNDANCE, AND ACTIVITIES OF MICROORGANISMS IN SUBSURFACE MATERIAL FROM CONTAMINATED SITES

Location and site description	Type of sample	Depth	Findings[a]	Reference
Sewage injection well, Magothy aquifer, Bay Park, New York, USA	Groundwater	127–146 m	Viable counts 10^2–10/ml. Numbers of anaerobic and facultative bacteria declined with distance from injection well.	Godsy and Ehrlich (1978)
Organically polluted and unpolluted sandy, gravelly acquifer, Fulda Valley, Germany	Groundwater	3 m	Total counts 10^6–10^7/ml. [^{14}C]glucose uptake highest in oxygenated part of contaminated zones. >90% bacteria attached to particles.	Marxsen (1981b)
Hydrocarbon-polluted well, Shilo, Manitoba, Canada	Groundwater	27–32 m	Viable count 10^1–10^5/ml. Seasonal variation in microbial numbers depending on D.O. and hydrocarbon concentration	Cullimore (1983)
Reclaimed coal strip mine, Canmore, Alberta, Canada	Groundwater	2–12 m	Total counts ~10^5/ml. Viable counts 10^2–10^4/ml. Seasonal variations in microbial population depended on flow	Wallis and Ladd (1983)
Creosote waste site, St. Louis Park, Minnesota, USA	Groundwater from contaminated and uncontaminated zone	0–30 m	Total counts 10^6–10^7/ml. Viable counts 0–10^3/ml. Diverse anaerobic bacteria detected. Methanogens only in contaminated zone[a]	Ehrlich *et al.* (1982, 1983)

Creosote waste site,[b] Conroe, Texas, USA	Unsaturated and saturated sediments from contaminated and uncontaminated zones	2.0–9.0 m	Total counts 10^6–10^7/g. Viable counts $<10^2$–10^5/g. INT reducers 10^5–10^6/g. ATP 0.01–0.17 ng/g. Lower ratios of glycerol teichoic acid/phospholipid and PHB/phospholipid in contaminated zone. Bacteria observed by TEM (Fig. 3). Rapid degradation of hydrocarbons at margin of waste plume. 19% of isolates contained plasmids	Wilson, J. T. *et al.* (1985a, 1986); Smith *et al.* (1985); Webster *et al.* (1985); Ogunseitan *et al.* (1987)
Sewage-contaminated aquifer, Cape Cod, Massachusetts, USA	Groundwater and sand and gravel sediments	5–40 m	Total counts declined from 10^7 to 10^5/ml and [^{14}C]glucose uptake rate declined with distance from infiltration beds. ~60% attached bacteria in contaminated zone; ~95% attached in uncontaminated zone. Estimated generation times 16–139 hours	Harvey *et al.* (1984); Harvey and George (1986, 1987)
Sewage polluted aquifer, Templeton, New Zealand	Groundwater from glacial outwash gravel formation	12–24 m	10% of phreatic crustaceans contained sewage-derived bacteria in guts	Sinton (1984)
Confined aquifers, Lincoln and Norfolk Counties, England	Limestone, glacial sand, and chalk cores, from unsaturated and saturated zones	3–50 m	Viable counts of aerobic and anaerobic bacteria 10^3–10^5/ml. Dentrifying bacteria found in enrichment cultures of most samples. Presence of bacteria in chalk related to occurrence of fissures	Foster *et al.* (1985)

(continued)

TABLE II *(continued)*

Location and site description	Type of sample	Depth	Findings[a]	Reference
Chalk aquifer affected by landfill contamination, England	Core samples from unsaturated and saturated zones in uncontaminated regions and beneath landfills	2–35 m	Total counts 10^5–10^8/g. Viable counts <10^2–10^7/g. Bacteria unevenly distributed in depth profile. Highest densities at interfaces	Towler *et al.* (1985)
Groundwater treatment system, southwest Germany	Hydrocarbon-polluted groundwater		Total counts 10^5–10^7/g. Viable counts 10^4–10^5/ml. Diverse bacteria detected at low population density. Differences in metabolic activity of bacteria in polluted and nonpolluted zones	Dott *et al.* (1984); Frank and Dott (1985)
Septic tank tile field, Williamsburg and Sault Ste Marie, Ontario, Canada	Surface and subsurface soil	1.2–3.0 m	Aerobic and anerobic ^{14}C mineralization rates lower in subsurface than in surface soil. Denitrification observed.	Ward (1985)
Underground iron and manganese removal system, Germany	Loose rock aquifer material from aerated and untreated zones	0.5–1.8 m	Viable counts 10^0–10^6/g. Diverse bacterial populations in both aerated and untreated zones	Gottfreund *et al.* (1985a–c)

[a]Abbreviations: D.O., dissolved oxygen; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; PHB, poly-β-hydroxybutyrate; TEM, transmission electron microscopy.

[b]See Table V.

of groundwater. In deeper groundwaters total cell counts may be lower (see Olson *et al.*, 1981; Dockins *et al.*, 1980, in Table I); however, not enough deep subsurface samples have been examined to generalize these findings. Considerably more variability is encountered in viable cell numbers. Viable cell counts can range from essentially zero to values that are nearly as high as the total microscopic counts. The variability may reflect the trophic status of the particular subsurface zones under investigation, i.e., aquifer regions polluted with high levels of assimilable carbon sources may show higher plate counts than unpolluted regions (see Harvey *et al.*, 1984; Marxsen, 1981, in Table II). However, the inability of microoorganisms to grow on the media provided probably explains much of the observed variability.

The work cited in Tables I and II also shows that diverse physiological groups of bacteria are present in subsurface zones depending on environmental conditions. Several types of protozoa have been detected in groundwater (Hirsch and Rades-Rohkohl, 1983) and in very permeable aquifer layers (Sinclair and Ghiorse, 1987; Sinclair *et al.*, 1987). Fungi also have been reported. When biochemical and radioisotope estimates of biomass and activity are compared with direct microscopic or cultural estimates positive correlations have been obtained (e.g., Webster *et al.*, 1985; Smith *et al.*, 1984). In pristine zones the metabolic activity of subsurface microflora may be very low compared to surface soil zones (Ventullo and Larson, 1985; Thorn and Ventullo, 1988), but in polluted zones, the activity may be higher (Harvey *et al.*, 1984; Marxsen, 1981a,b; Dott *et al.*, 1984; Frank and Dott, 1985). Interestingly, recent evidence shows that fewer isloates from a pristine aquifer contained plasmids than from a contaminated aquifer (Ogunseitan *et al.*, 1987). The significance of these findings with respect to biodegradation potential of bacteria in the aquifers remains to be established.

A general conclusion concerning pristine sites (Table I) is that the low population densities reflect the oligotrophic conditions of the pristine aquifers. But, few studies have attempted to compare directly the organic composition of subsurface water with the presence of microorganisms. One exception is the work of Wallis and Ladd (1983) (Table II) who noted a seasonal correlation of viable cell counts with dissolved organic matter (DOM) in the groundwater of a reclaimed coal strip mine in Alberta, Canada. Total cell counts in groundwater were near 10^5/ml year around, and viable counts varied between 10^2 and 10^4/ml while the average amount of DOM varied froom 1.15 to 1.98 mg/liter over a 12-month period. The DOM consisted of 20% humic acids, 6% carbohydrate, 4% tannins and lignins, 2% phenols, amino acids, and protein. The remaining 68% was designated as fulvic acids. More studies of this type are needed. Such studies could help to elucidate effects of DOM fluxes on microbial

populations in unperturbed aquifers and groundwater-stream ecosystems, especially if they can be combined with parallel studies of streamwater biogeochemistry (Hynes, 1983; Bott *et al.*, 1984; Kaplan and Bott, 1985; Ladd *et al.*, 1982).

Work on contaminated sites (Table II) shows the general stimulatory or selective effects of organic pollutants on subsurface microbial population densities, which normally may be limited by the amount of organic carbon and the availability of electron acceptors (see Section II,B,2,b). The consequences of organic pollution on the function of pristine and contaminated aquifers are discussed more fully in Sections IV and V. It should be reemphasized that the introduction of organic matter into an oligotrophic subsurface environment may result in stimulation of microbial activity provided that sufficient nitrogen, phospohorus, and electron acceptors are available (Section II,B,2). Also, the toxicity of the organic pollutants must not be too great. Toxic effects of leachates beneath a landfill in England have been reported (Towler *et al.*, 1985). At this site the total bacterial numbers in subsurface zones beneath the landfill dropped markedly in coincidence with a front of leachate organic acids percolating into the aquifer. A similar situation has been observed at a site in Georgia polluted by a high level of trichloroethylene (T. Phelps and C. Fliermans, personal communication).

Another effect of high levels of organic pollution, or higher than normal levels of natural organic matter, may be a shift from predominantly attached populations to a higher proportion of free-swimming bacteria. Marxsen (1981a,b) and Harvey *et al.*, (1984) both observed this effect in sandy and gravelly aquifers. A high proportion of bacterial cells (>90%) were attached to particles in zones where organic pollutant concentrations were low. Fewer cells (<60%) were attached in polluted zones where higher growth rates occurred (Harvey *et al.*, 1984; Harvey and George, 1987). Such findings suggest that bacteria in uncontaminated subsurface sediments live primarily attached to surfaces. Only when the environment is perturbed by introduction of larger than normal doses of matabolizable organic matter does the proportion of unattached bacteria increase.

D. Vertical Distribution of Microorganisms in Shallow Aquifer Systems

In view of the known geological and stratigraphic variability of subsurface environments (Freeze and Cherry, 1979), vertical variation of microbial abundance and activity may be expected to occur. On the other hand, the similarities of physical and chemical properties of shallow aquifers (see Section II,A) suggest that microbial populations in a given

type of drinking water aquifer may be predictable. These predictions have been tested only recently by systematic vertical profile studies of the distribution of microbial abundance and activity (Beloin *et al.*, 1988).

The early work of soil microbiologists showed clearly that microbial abundance as measured by viable cell counting procedures declined drastically with depth in subsoil horizons. In most cases little, if any, evidence was found for microbial life below the B horizon at depths greater than 2 m (Alexander, 1977). Recently, more sensitive biochemical assays for microbial biomass and activity have been employed to confirm these results (Federle *et al.*, 1986). However, conventional direct counting and cultural methods also have been used recently to show that microbial populations extend below the B horizon into saturated aquifer zones (Hoos and Schweisfurth, 1982; Foster *et al.*, 1985; Towler *et al.*, 1985; Gottfreund *et al.*, 1985a–c). These studies all show that variations in microbial abundance may occur in subsurface profiles. Also, although the microbiological data are not always easily interpreted in relation to geochemical data, these studies show that subsurface microorganisms can profoundly affect groundwater geochemistry. Interpretations often are complicated by the complexities involved in relating microbiological data to hydrogeological and geochemical data, which frequently are obtained from different types of samples (i.e., water samples versus core samples) or from cores of different boreholes at the same site. Efforts to study the role of microorganisms in the biogeochemistry of subsurface sediments and groundwater should receive more emphasis in the future.

Detailed population profile studies have been carried out at a shallow aquifer study site at Lula, Oklahoma (Sinclair and Ghiorse, 1987; Beloin *et al.*, 1988; Bone and Balkwill, 1988; see also Table II and Part IV). This site is located on the margin of the floodplain of a small river. It contains an aquitard layer just below the water table at 3 m. Sandy and gravelly aquifer layers between 5 and 8 m are underlain by massive plastic clay bedrock layer. An expected decline in microbial abundance and activity occurred in the subsoil and unsaturated zones, but in the saturated zone further declines did not occur. Instead, an increase occurred at the interface between the unsaturated and saturated zones. Also, bacterial populations in the saturated zone gave rise to different types of colonies than were found in samples from the soil and in the unsaturated zones (Bone and Balkwill, 1988). Abundance and activity were highly variable in stratigraphically complex layers; depending on sediment texture, sandy layers generally showed higher and clayey layers lower values for biomass and activity. The most biologically active layer in the subsurface profile was a gravelly sand layer at 7.5 m (Beloin *et al.*, 1988; Sinclair and Ghiorse, 1987). This very permeable layer was sampled

on different dates from different boreholes. It contained a higher proportion of viable to total bacteria and significantly higher ATP content than the layers above and below it. Also surface-type protozoa and cyanobacteria were detected in this layer, indicating that it may be connected hydrologically to a stream approximately 60 m from the boreholes. The stratigraphy and hydrology of this alluvial site were very complex, but the microbiological data suggest that lateral underground connections to the streambed may have influenced microbial activity in this layer.

It is of interest that in profile studies such as those of Beloin *et al.* (1988) and Towler *et al.* (1985) some layers show very low numbers of viable bacteria relative to total cell counts. While the usual arguments concerning the unsuitability of growth media, etc., may explain these results, such arguments are not totally satisfying. In the work of Beloin *et al.* (1988), the disparity between viable and total counts was related to sediment texture (i.e., clayey texture = low viable/total count; sandy texture = high viable/total count), but the correlation did not always hold, indicating that other environmental factors are also important in determining microbial activity in these sediments. Most clayey sediments did have low viable/total count ratios, however. Viable cells were rarely detected by any cultural techniques in the bedrock clay layer, but significant total numbers of bacteria ($\sim 10^6$/g dry wt) and measurable amounts of ATP were detected (Beloin *et al.*, 1988). Furthermore, intact bacterial cells were observed in samples from this layer by transmission electron microscopy (Bone and Balkwill, 1988). These observations raise questions on the survival of subsurface microorganisms in high-clay layers which are relatively dehydrated compared to sandy aquifer sediments. Study of the bacteria in these layers may help to answer questions on long-term survival of microorganisms which were raised many years ago by petroleum microbiologists (Davis, 1967; Kuznetsov *et al.*, 1963).

E. Lateral Distribution and the Origin of Microorganisms in Aquifers

Most of the microbiological studies listed in Tables I and II were done on superficial aquifers that were not confined beneath aquitard layers. In this type of geological setting the lateral distribution of microorganisms in the aquifer may be controlled by infiltration from the unconfined zones above. Also, it has been shown that lateral transport of microorganisms over considerable distances (2–3 km) can occur in unconfined sandy aquifers (Harvey *et al.*, 1984). New populations of microorganisms may colonize an aquifer by filtration from above or by lateral migration. However, there is no *a priori* reason to believe that some microorganisms

in shallow aquifer formations could not have colonized the formation at the time the sediments were originally deposited.

Presumably, bacteria can be transported by water movement through aquifer sediments, yet biological phenomena such as motility and chemotaxis and cell surface adhesion phenomena may also be important. At this time, the mechanisms by which microorganisms may migrate in aquifer sediment are poorly understood.

The mechanisms of colonization of deeper confined aquifers may be the same as for unconfined aquifers. However, in the confined aquifers new populations might have to migrate considerably longer distances from recharge areas in order to colonize the aquifer. In most cases, the deeper a confined aquifer is at a particular location, the farther away it is from its recharge area (Freeze and Cherry, 1979). Thus colonization by surface microorganisms cannot occur very rapidly unless the microorganisms are introduced artificially. Artificial colonization of deep aquifers by injection of surface waters has been found in secondary oil recovery situations. For example, Gorlatov and Belyaev (1985) reported on a 1500–1700 m deep saline aquifer of an oil field in which fresh water was injected. The majority of hydrocarbon-oxidizing bacteria in the mixed stratal waters could only grow at low or moderate salinity, indicating that they had originated in the injection water.

Lateral distribution of hydrocarbon-oxidizing and sulfate-reducing and sulfur-oxidizing bacteria in oil-bearing aquifers has been investigated in the past by petroleum microbiologists (Kuznetsov *et al.*, 1963; Davis, 1967). The rudimentary microbiological methods employed in these studies and the possibility of contamination by surface microorganisms in well water give rise to doubts concerning their validity (McNabb and Dunlap, 1975). Nevertheless, in many instances convincingly large differences in total abundance of different bacterial types between wells were observed, indicating that the different types were present in deep formation waters (Kuznetsov *et al.*, 1963; Davis, 1967).

One interesting study of this type was conducted by the Socony Mobil Oil Company in the oil-bearing Carrizo sands formation, a very permeable freshwater artesian aquifer in south Texas (Davis, 1967). Formation water samples were obtained from seven wells along a 56-km transect across the oil field. The samples were analyzed for their organic and mineral contents and numbers of viable sulfate-reducing and facultative bacteria were determined. The highest numbers of bacteria were found in the waters of the oil-producing well, but waters from two up-dip wells near the recharge area (66 and 350 m deep, respectively) and the well furthest down-dip (1292 m deep) also contained high numbers of bacteria. Intermediate wells contained at least 10-fold fewer bacteria than the

production well, and one well contained no detectable bacteria. The distribution of sulfate-reducing bacteria in the wells corresponded to an apparent bacterial influence on the geochemistry of sulfur in the aquifer. Despite the obvious possibility that colonization of the wells by surface bacteria could have influenced the results, this and similar studies conducted by Soviet microbiologists during the 1940s and 1950s (Kuznetsov *et al.*, 1963) should not be ignored. The older literature can provide valuable background information for future investigators of the microbial ecology of deep subsurface strata.

IV. Function of Pristine Aquifers

Aquifers are unique ecosystems in that there is little if any primary production. There is a chance for chemoautotrophic production in locations where reduced and oxygenated groundwaters mix together, but in general the energy to sustain microbial communities in aquifers is provided by secondary organic materials that are produced in the surface environment and then are transported into the aquifer by infiltrating water. As a result, the nature and properties of the microbial population at a particular point in the subsurface may depend on the composition and nutritional quality of the dissolved organic matter in the water that perfuses that particular subsurface material. The nutritional quality of the dissolved organic materials is controlled by their sources (see Section II,B,2,a) and by any alterations they experience as the water moves along the flow path from the point of entry to the subsurface to the point of discharge or extraction.

The quantity and quality of the organic matter leached from soil by infiltrating rain water or snowmelt can be very high. Thurman (1985) referred to the work of Cronan and Aiken, who measured the concentration of dissolved carbon in podsols. The surface layers contained waters with 10–30 mg DOC per liter while the pore water of deeper layers only contained 2–5 mg/liter. Wallis (1979) reported that groundwater under a subalpine forest in Alberta contained (per liter) 21 μg carbohydrates, 14 μg combined amino acids, 3 μg free amino acids, 1 ng phenols, and 1800 μg humic and fulvic acids. Whitelaw and Edwards (1980) found carbohydrates in the Chalk Aquifer of England at concentrations of 10–100 mg/kg aquifer material. Spitzy (1982) found 20–100 μg total disolved amino acids, 105–125 μg carbohydrates, and 3 mg dissolved organic matter per liter of water from shallow wells in the Hamburg area, and 121–367 μg total amino acids, 65–121 μg carbohydrates, and 0.2–1.2 mg dissolved organic carbon per liter in deeper wells in the same area.

Although pore water in surface soils may contain 5–50 g dissolved organic carbon/liter, aquifers underlying well-drained soils usually

contain less than 1 mg dissolved organic carbon/liter (Leenheer *et al.*, 1974; Thurman, 1985), probably because of degradation of the dissolved organic matter during passage to the aquifer. Because the oxygen demand of the remaining organic matter does not exceed the ambient levels of dissolved oxygen, these aquifers tend to remain aerobic (see Section II,B,2,b). Aquifers that receive most of their recharge after snowmelt, particularly under podsols, are often anaerobic. There is a wide belt of anaerobic water table aquifers in the northern United States and Canada. It is possible that recharge in these aquifers is too rapid, or the water is too cold for significant biodegradation in the unsaturated zone of the organic matter leached from the surface soils. Thus, the biological oxygen demand of the dissolved organic matter reaching the aquifer exceeds the oxygen available in the water. Aquifers that do not have an aerobic unsaturated zone between the surface soil and the water table are usually anaerobic, particularly if the soil is flooded most of the time and the water table is actually in the surface soil. Many anaerobic aquifers in the southeastern United States fit into this description.

The nutritional quality of leached organic mater is controlled by conditions in the soil. The quality of dissolved organics should be high if the soil is productive, has a neutral to alkaline pH and is high in exchangeable bases, has a high organic matter content, has been tilled, was frozen and then thawed, was very dry and then rewetted, or was puddled and anaerobic for a long period of time. In fact, the high nutritional value of leachable soil organic matter is exploited to make soil extract culture media. As the infiltrating water perfuses through the subsurface material, dissolved organic material is exposed to alteration by the indigenous population situated along the flow path. Organisms at any one place in the flow path must accept the dissolved organic matter passed to them by the organisms above and extract whatever they can for their own nutrition. The remaining dissolved organic constituents then pass on down the flow path to populations below. Certainly the quantity, and probably the quality of the dissolved organic matter, is reduced as the water moves through the various subsurface zones. Below the water table there is limited opportunity for reaeration, and there is also a corresponding reduction in the concentration of oxygen as it is used to metabolize the dissolved organic matter (Matthess, 1982; Jackson and Patterson, 1982). In theory, then, a point will be reached when the readily degradable components of the dissolved organic are consumed to such an extent that microbial life can no longer be supported. At this point no further biological alteration will occur. Such an end point has not been established for any subsurface ecosystems yet examined.

It can be assumed from the foregoing discussion that the nutritional ecology of a particular aquifer will depend to a large extent on the

residence time of the water in the system, and on the alterations experienced by the dissolved organic carbon as it moved along the flow path to the point of interest. Once water leaves the surface soil it percolates down through deeper unsaturated material until it reaches the water table; then it turns and moves laterally with the flow in the aquifer (Freeze and Cherry, 1979). In sandy subsurface sediments, or in subsurface materials with contents of silt and clay but whose structure includes cracks and fissures, the passage of recharge water to the water table can be rapid—on the order of days to weeks. Once below the water table vertical penetration of the aquifer is much slower, and most of the movement is lateral. Recently recharged water tends to pool above water already in the aquifer.

The pooling effect is well illustrated by the distribution of tritium in shallow water table aquifers from 1953 to present (Munnich *et al.*, 1967). Tritium from atmospheric testing of hydrogen bombs was restricted to the top few meters of water table aquifers; the penetration of tritium was directly related to the amount of recharge since atmospheric testing began. Such observations indicate that the age of groundwater can be estimated roughly from its depth below thhe permanent water table. The porosity of the aquifer and the rate of groundwater recharge must also be considered. For example, if mean annual recharge in an area is 10 cm and the porosity of an aquifer is 0.2, then for every 50-cm increase in depth, the water in the aquifer is roughly 1 year older. A shallow domestic well screened at 6 m below the water table would be producing water that was recharged more than a decade ago. As a result, the dissolved organic matter in pore water at the very top of the water table may not be very different from that in pore water of the surface soil, but just a few meters into the aquifer there will have been adequate time for extensive microbial alteration of the dissolved organic matter.

This simplistic view of the relationship between age and depth is complicated by variations in the transmissivity of different layers in an aquifer. The rate at which water moves laterally through an aquifer depends on the slope of the water table and the permeability of the geological material (Freeze and Cherry, 1979). In the same aquifer, the water moves much more quickly through sands and gravels than it does through silts and clays. As a result, water in a gravel layer might be younger and, therefore, of higher nutritional value than water in a layer of fine sand lying above it. Such stratification may greatly influence microbial abundance and activities in a different sedimentary strata of an aquifer (see Section III,D; Beloin *et al.*, 1988; Sinclair and Ghiorse, 1987). On a larger scale, however, the residence time of water in an aquifer, and the associated opportunity for reduction in its nutritional quality, increases with depth below the water table and with decreasing permeability of the geological matrix.

These relations are illustrated by the subsurface microbiology of the extensively studied pristine site at Lula, Oklahoma (Table III) (see also Tables I and IV, and Section III,D). The site is unconsolidated alluvial material that is free of industrial pollution. The surface soil is fertile and presently is in permanent pasture. The permanent water table is within a confining layer approximately 3.0 m below land surface. The water in the aquifer has 2.6 mg oxygen/liter, 197 mg sulfate/liter, less than 5 mg total organic carbon/liter; the pH is 7.1; and the temperature is 17°C.

There was little change in the total number of bacterial cells in material just below the root zone, material from the confining layer, and material from below the water table (Table II). However, viable cell counts varied from zone to zone (Table I), and the ATP content of materials in the aquifer was 10–15 times lower than that of materials from the unsaturated zone and the interface region near the water table (Table III). The rate of biotransformation of organic materials was slower in the aquifer than in the unsaturated zone above. At time scales relevant to the residence time of water, amino acids were turned over very rapidly. A gravelly sand layer just above the bedrock clay contained less ATP on the average and turned over amino acids at about one-tenth the rate of organisms in the unsaturated zone. Microbial degradation of toluene was rapid but chlorobenzene was degraded very slowly in the unsaturated zone and an interface zone at the top of the water table. The microbial population of the aquifer did not discriminate against the generally more refractory xenobiotic compounds; they degraded chlorobenzene as rapidly as they degraded the natural analog. There is reassuring uniformity in the rates of chlorobenzene and toluene degradation in samples of the layer of uniform fine sand. These samples were acquired at different times and examined with different techniques. There are dramatic differences in the rate of toluene and chlorobenzene biodegradation in samples of the uniform fine sand and of the gravel layer just below it which were separated in the aquifer by only 1 or 2 m. The kinetics of biodegradation of amino acids, toluene, and chlorobenzene were first-order within a range of concentrations that could be entirely removed under conditions that existed in the aquifer. The half-saturation concentrations of (K_S) for all the organics were all much greater than the quantity of organic compounds that could be removed by oxygen-supported metabolism.

Rates or extent of biotransformations of naturally occurring and xenobiotic compounds also have been determined at a number of other pristine subsurface sites. Table IV summarizes some of the information from these studies. A surprisingly large data base has accumulated during the last 5 years, probably reflecting the recent interest of many environmental scientists in groundwater pollution. Many of the rates of

TABLE III

BIOMASS AND BIOTRANSFORMATION RATES IN PRISTINE, UNCONSOLIDATED SUBSURFACE MATERIAL FROM LULA, OKLAHOMA

Subsurface material	AODC[a] (10^6/g)	ATP[b] content (ng/g)	Rate of biotransformation[c] ($week^{-1}$): Amino acids	*m*-Cresol	Toluene	Chlorobenzene	Fraction of total material biotransformed that was mineralized to CO_2 (%): Amino acids	*m*-Cresol	Toluene	Chlorobenzene
Fine silty clay with iron stains (has cracks and fissures)	7	1.2	146	17	>2.5	0.05	56	64		
Fawn-colored sand with minor clay content (not transmissive, confining layer for artesian layers below)	5	1.9 1.1	11	2.4	>2.4	0.05	37	63		
Mixture of coarse sands and gravels (very transmissive)			336	5.1	>3.5	<0.01	51	51		
Uniform fine sand (moderately transmissive)	4	0.16	332	8.5	0.25 0.89	0.20 0.15	57	2.1	11	36
Gravel with sand matrix (very transmissive)	9	0.03[d]	9.1	8.2	<0.007	0.006	49	5.4		

[a]Number of bacteria determined by acridine orange direct count (Balkwill and Ghiorse, 1985b).
[b]ATP data are from Wilson, J. T. *et al.* (1986).
[c]Amino acid and *m*-Cresol rates derived from Dobbins and Pfaender (1987). Toluene and chlorobenzene rates derived from Aelion *et al.* (1987) and Wilson, J. T. *et al.* (1986).
[d]Other samples obtained in January and June, 1985, contained 0.09 and 0.17 ng ATP/g, respectively, suggesting that seasonal variations in biomass and activity may occur in this layer (Beloin *et al.*, 1988).

TABLE IV

RATE OR EXTENT OF BIODEGRADATION OF VARIOUS ORGANIC COMPOUNDS IN SUBSURFACE MATERIAL FROM PRISTINE SITES

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
Common metabolites				
Arginine	Oxygen	Groundwater from 10–12 m in monitoring wells in gravel aquifers, Hamilton and Dayton, Ohio, USA	Normalized to 10^7 cells/ml groundwater, 27–130/week	Ventullo and Larson (1985)
Glucose			36–45/week	
Glutamate			120–300/week	
Glutamic acid	Oxygen	Groundwater from shallow soils under a subalpine forest in Marmot Basin, Alberta, Canada	Normalized to 10^7/ml groundwater, 61–200/week	Ladd *et al.* (1982)
Glycolate			3.8/week	
Phenylalanine			115/week	
Volatile fatty acids acetic, propionic, butyric	Oxygen	Chalk from 10 m below water table, Ingram, Suffolk, England	0.3/week, no evidence of adaptation after 50 days	Kiene and Capone (1986)
Halogenated hydrocarbons				
Bromodichloromethane	Oxygen	Sandy clay just above the water table at Lula, Oklahoma, USA	Not detected, <0.03–0.02/week	Wilson *et al.* (1983a)
Bromoform	Oxygen	Sand water table aquifer on Borden Air Base, Ontario, Canada	0.02/week, field experiment	Roberts *et al.* (1986)
Carbon tetrachloride	Oxygen	Sand water table aquifer on Borden Air Base, Ontario	Not detected in 2 years, <0.002/week, field experiment	Barker *et al.* (1983a,b)

(continued)

TABLE IV *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
Chlorobenzene	Oxygen	Sandy clay just above and within a water table aquifer at Fort Polk, Louisiana, and Pickett, Oklahoma, USA	<0.01/week, not greater than abiotic control	Wilson *et al.* (1983a,b)
		Sandy clay within a shallow water table aquifer at Lula, Oklahoma	Not detected, <0.01–0.05/week	
		Sand in a shallow confined aquifer at Lula, Oklahoma; 3–5 m deep	Not detected in 7 months, <0.009/week	
		Sand and gravel in a shallow confined aquifer at Lula, Oklahoma, 5.5–6.7 m deep	0.06–0.02/week	Wilson *et al.* (1986)
1,2-Dibromoethane (ethylene dibromide)	Oxygen	Sand and sandy clay in and just above a shallow confined aquifer at Lula, Oklahoma	Not detected, <0.03/week	Wilson *et al.* (1983a)
		Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	7/week	Aelion *et al.* (1987)
		Sandy clay just above and within a water table aquifer at Fort Polk, Louisiana	Not detected, <0.012/week	Wilson *et al.* (1983a,b)
		Sandy clay just above and within a water table aquifer at Pickett, Oklahoma	Not detected, <0.012/week	

		Sand water table aquifer on Borden Air Base, Ontario	0.03/week in field experiment, locally degradation was rapid and extensive	Roberts *et al.* (1986)
1,1-Dichloroethylene	Carbonate	Muck from Florida Everglades, USA	0.03–0.06/week, $K_m = 4$ mg/liter	Barrio-Lage *et al.* (1986)
cis-1,2-Cichloroethylene	Carbonate	Muck from Florida Everglades	0.01–0.06/week, $K_m = 5$ mg/liter	
trans-1,2-Dichloro-ethylene	Carbonate	Muck from Florida Everglades	0.03–0.04/week, $K_m = 3$ mg/liter	
Hexachloroethane	Oxygen	Sand water table aquifer on Borden Air Base, Ontario	0.1/week, field scale	Criddle *et al.* (1986)
Tetrachloroethylene (perchloroethylene)	Oxygen	Sand water table aquifer on Borden Air Base, Ontario	Not detected in 2 years, <0.0009/week, field experiment	Roberts *et al.* (1986)
		Sandy clay just above the water table at Fort Polk, Louisiana	0.009–0.002/week, not greater than abiotic control	Wilson *et al.* (1983a,b)
		Sand just above and within a water table aquifer at Pickett, Oklahoma	0.01/week, not greater than abiotic control	
		Sandy clay just within a shallow water table at Lula, Oklahoma	Not detected, <0.03/week	
1,2,4-Trichlorobenzene	Oxygen	Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	No adaption in microcosms after 7 months, <0.01/week	Aelion *et al.* (1987)

(continued)

TABLE IV *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
1,1,1-Trichloroethane	Oxygen	Sandy clay just above and within a water table aquifer at Fort Polk, Louisiana and Pickett, Oklahoma	Not detected, <0.01/week	Wilson *et al.* (1983b)
		Sandy clay and sand just above the water table and in a shallow confined aquifer at Lula, Oklahoma	Not detected, <0.05/week	
Trichloroethylene	Oxygen	Sandy clay and sand just above and within a water table aquifer at Fort Polk, Louisiana and Pickett, Oklahoma	Not detected, <0.01/week	Wilson *et al.* (1983a,b)
Phenols				
m-Aminophenol	Oxygen	Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	0.3–0.5/week	Aelion *et al.* (1987)
2-Chlorophenol	Oxygen	Shallow water table aquifer near Pickett, Oklahoma	50–100 μg/liter disappeared in effluent of column microcosm in 2 weeks	Suflita and Miller (1985)
p-Chlorophenol	Oxygen	Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	3.3/week	Aelion *et al.* (1987)

m-Cresol	Oxygen	Sandy clay, sand, and gravel just above and within a water table aquifer at Lula, Oklahoma	2.4–17/week	Dobbins and Pfaender (1987)
		Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	0.5/week	Aelion *et al.* (1987)
2,4-Dichlorophenol	Oxygen	Shallow water table aquifer near Pickett, Oklahoma	50–100 μg/liter disappeared in effluent of column microcosm in 2 weeks	Suflita and Miller (1985)
p-Nitrophenol	Oxygen	Sand in a shallow confined aquifer at Lula, Oklahoma	<0.005/week. At 0.5 μg/liter, no adaptation in 90 days; <1.6 μg/liter, adaptation in 7–42 days	Aelion *et al.* (1987)
Phenol	Oxygen	Shallow water table aquifer near Pickett, Oklahoma	50–100 μg/liter disappeared in effluent of column microcosm in 2 weeks	Suflita and Miller (1985)
		Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	18/week	Aelion *et al.* (1987)
2,4,6-Trichlorophenol	Oxygen	Shallow water table aquifer near Pickett, Oklahoma	50–100 μg/liter disappeared in effluent of column microcosm in 2 weeks	Suflita and Miller (1985)

(continued)

TABLE IV *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
Petroleum-derived hydrocarbons				
Acenaphthene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas, USA	Not detected, <0.03/week	Wilson *et al.* (1985a)
Benzene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.03/week	Wilson, J. T. *et al.* (1986)
	Oxygen	Sand in water table aquifer in Borden Air Base, Ontario	0.1/week in laboratory, 0.2 mg/liter-week in field	Barker and Patrick (1986)
		Sand just below the water table in a terrace of the South Canadian River in Norman, Oklahoma, USA	0.2/week	Mahadevaiah and Miller (1986)
Dibenzofuran	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.1/week	Wilson *et al.* (1985a)
Ethylbenzene	Oxygen	Just below the water table in a terrace of the South Canadian River at Norman, Oklahoma	0.2/week	Mahadevaiah and Miller (1986)
Fluorene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.1/week	Wilson *et al.* (1985a)
2-Methylnaphthalene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.1/week	
Naphthalene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.01/week	
Styrene	Oxygen	Sand and sandy clay just above and within the water table aquifer at Pickett, Oklahoma	0.02–0.04/week	

Styrene	Oxygen	Sandy clay just within a shallow water table aquifer at Fort Polk, Louisiana	0.1/week	Wilson *et al.* (1983a,b)
Toluene	Oxygen	Sand just above the water table at Pickett, Oklahoma	0.009 (abiotic) - 0.52/week	
		Sand and sandy clay just above and within a water table aquifer at Lula, Oklahoma	>2.5/week	
		Gravel in a shallow confined aquifer at Lula, Oklahoma	Not detected, <0.01/week	Wilson, J. T. *et al.* (1986)
		Sand in a shallow confined aquifer at Lula, Oklahoma, 5.5 m deep	3/week	
		Sand in water table aquifer on Borden Air Base, Ontario	0.1/week in laboratory, 0.3 mg/liter-week in field	Barker and Patrick (1985)
		Sandy clay just above and within a shallow water table aquifer at Fort Polk, Louisiana	0.03/week, not greater than abiotic control	Wilson *et al.* (1983b)
		Sand in shallow water table aquifer in Conroe, Texas	0.04/week, not greater than abiotic control	Wilson, J. T. *et al.* (1986)
		Sand just below the water table in a terrace of the South Canadian River at Norman, Oklahoma	0.2/week, adaptation occurred in 2–4 weeks	Mahadevaiah and Miller (1986)

(continued)

TABLE IV *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
o-Xylene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.03/week	Wilson, J. T. *et al.* (1986)
		Sand just below the water table in a terrace of the South Canadian River at Norman, Oklahoma	0.2/week	Mahadevaiah and Miller (1986)
		Sand in water table aquifer on Borden Air Base, Ontario	0.2/week in laboratory, 0.4 mg/liter-week in field	Barker and Patrick (1986)
m-Xylene	Oxygen	Sand in shallow water table aquifer in Conroe, Texas	Not detected, <0.05/week	Wilson, J. T. *et al.* (1986)
		Sand just below the water table in a terrace on the South Canadian River at Norman, Oklahoma	0.2/week	Mahadevaiah and Miller (1986)
		Sand in water table aquifer on Borden Air Base, Ontario	0.3 mg/liter-week in field	Barker and Patrick (1986)
p-Xylene	Oxygen	Sand just below the water table in a terrace of the South Canadian River at Norman, Oklahoma	0.2/week	Mahadevaiah and Miller (1986)
		Sand in water table on Borden Air Base, Ontario	0.2/week in laboratory, 0.4 mg/liter-week in field	Barker and Patrick (1986)

Miscellaneous				
Aniline	Oxygen	Sand in a shallow confined aquifer at Lula, Oklahoma, 5.0 m deep	0.4–1.3/week	Aelion *et al.* (1987)
Methanol	Oxygen	Sandy unsaturated material in Pennsylvania, USA	40–46 mg/liter-week	Novak *et al.* (1985)
	Sulfate	Very shallow water table aquifer, black to gray marl soil in New York, USA	Lag for 30 days, then 20 mg/liter-week	
	No oxygen, no nitrate, little sulfate	Saturated sand and silty clay in Virginia, USA	6–16 mg/liter-week	
Nitriloacetic acid	Carbonate	Anaerobic sands from the floodplain of the South Canadian River in Byers, Oklahoma, USA	4 mg/liter-week	Dunlap *et al.* (1972)
Tertiary butyl alcohol	Sulfate	Very shallow water table aquifer, black to gray marl soil in New York	<0.02/week	Novak *et al.* (1985)
	Oxygen	Sandy unsaturated material in Pennsylvania	4–12 mg/liter-week	
	Oxygen-depleted	Sandy unsaturated material in Pennsylvania	4 mg/liter-week	

degradation shown in Table IV are derived from our best estimates of the published data. In many cases we reinterpreted data from published figures, or used a different description of the kinetics of degradation to derive first-order rates from the published data. The original authors referenced in Table IV should not be held accountable for any error we may have made in interpreting their work.

Generally, the studies listed in Table IV show that the rates of degradation of common metabolites is almost instantaneous in the context of the residence time of water in the subsurface environment. Degradation rates of aromatic compounds such as toluene that are not important components of humic materials are variable. Degradation of phenols, which are common humic components, is rapid; even chlorophenols are degraded with no appreciable lag. Degradation of benzoic acid is also rapid. These compounds may be structural analogs to phenolic components of dissolved organic matter that probably provide carbon and energy for indigenous microorganisms. Biodegradation of halogenated aliphatic hydrocarbons is generally undetectable in oxygenated subsurface environments, although they can undergo reductive dehalogenation in anaerobic subsurface materials.

Relatively small proportions of the dissolved organic matter consumed by the microflora of the Lula site were completely mineralized. Major portions of these materials may have been diverted into storage products such as poly-β-hydroxybutyrate (PHB) and extracellular polymers (see Figs. 1d and 2c). Compared to organisms just below the root zone, the microbial population in the confined aquifer at Lula appear to contain almost 10 times as much uronic acid-containing exopolysaccharide and polyhydroxyalkanoic acid storage products (Smith *et al.*, 1986). At first glance the diversion of carbon resources to exopolysaccharide synthesis is puzzling. Surely aquifers are among the most oligotrophic sedimentary habitats in the biosphere. The water in the Lula aquifer sediments contain 3.5 mg nitrate/liter and 0.12 mg orthophosphate/liter. These concentrations are low compared to culture media, but they are more than adequate to support growth of the resident bacteria. The phosphate concentration is above the half-saturation constant for uptake by many bacteria (Rosen, 1978). One might presume that the microorganisms in the aquifer are carbon limited (see Section II,2,a), but the observations of Smith *et al.* (1986) indicated that the bacteria divert a large portion of their carbon to storage products and exopolysaccharide rather than growth. The diversion of carbon to storage products makes sense in any oligotrophic environment (Poindexter, 1981); however, diversion of scarce carbon resources to exopolysaccharide synthesis at first appears to be wasteful. In the following paragraphs we offer some observations and a hypothesis that may explain this paradox.

Individual organic compounds are frequently not degraded in natural systems unless they are present at or above some critical concentration.

This critical concentration may be related to the concentration required to support growth (Alexander, 1985). Compounds present below this critical concentration are only degraded when other substrates are available to maintain the microbial population. If the concentration of a foreign organic compound is great enough and if organisms capable of degrading the compound are available, the organisms in the region of the aquifer that first encountered the foreign compound will adapt and start to reduce the concentration of the contaminant. Ultimately the concentration will fall below the level required for adaptation, and the contaminant will enter regions of the aquifer where its biodegradation occurs much slower, if at all. Groundwaters resulting from the land disposal of municipal wastewater or from bank infiltration of polluted industrial rivers contain a plethora of organic compounds at concentrations of 10–100 ng/liter (Tomson *et al.*, 1981; Piet and Smeenk, 1985; Piet and Zoeteman, 1980). This may represent the concentration below which adaptation in aquifer cannot occur.

Dissolved organic materials in pristine aquifers are likely to be depleted of individual organic compounds able to elicit adaptation. How can this material maintain the apparently healthy populations of microorganisms found in aquifers? The uniform fine sand at the Lula site containing 0.3 mg or organic material per gram provides an example. Dissolved organic carbon was not detected in the pore water from the sand (detection limit 5 mg/liter). At 5 mg/liter, the pore water in a gram of the sand would contain 0.0005 mg dissolved organic matter. Therefore, by far the greater part of organic matter in the sand is associated with solids, rather than the aqueous phase. Furthermore, we can hypothesize that surface-associated organic matter, which is bathed in a stream of dissolved organic matter, may absorb and concentrate metabolizable organic compounds, bringing their concentration up to the critical level required to support a standing crop of attached microorganisms.

To complete our explanation of the exopolysaccharide paradox, it also may be assumed that when a solid surface is colonized in an oligotrophic aquifer sediment, a local area supports the growth of the colonizing microflora until the metabolizable compounds in that area are depleted. The cells in that area would then either die or disperse in a dormant form, and surface-associated organic matter would begin again to absorb and concentrate organic compounds. This sequence of events would result in a continuous cycle of colonization, exploitation, abandonment, and regeneration of concentrated carbon source on the surface. Such cycles are common in other ecosystems. For example, the familiar fairy ring of mushrooms results from such a cycle. Therefore, if the exopolysaccharide helps cells attach to surfaces and to absorb metabolizable organic materials from the bulk water phase (see, Part II,B,3), then the investment of carbon would make sense. Such a role for exopolysaccharides in aquifer

bacteria is supported by the observation that surface-associated bacteria turned over glucose, glutamate, and arginine more rapidly than planktonic bacteria (Ventullo *et al.*, 1983).

V. Function of Contaminated Aquifers

The long residence time of contaminants in aquifers allows ample opportunity for microbial adaptation. As observed at field scale, organic contaminants seem to persist for various time periods; then, over a very short interval, they disappear. This effect is well illustrated in a field study conducted in a shallow sandy aquifer at Canadian Forces Air Base Borden, in Ontario, Canada (Roberts *et al.*, 1986). An artificial plume of pollution was created in an oxygenated aquifer, and allowed to move with the natural flow of groundwater through a dense nest of monitoring wells. The plume contained chloride as a tracer for water, tetrachloroethylene which was not degraded biologically, and 1,2-dichlorobenzene. Figure 3 presents a schematic diagram of the experiment and breakthrough curves for the solutes at one point in the aquifer. Sorption of tetrachloroethylene and 1,2-dichlorobenzene chromatographically separated them from the chloride. Chloride reached the sampling point first. After the chloride plume moved past the sampling point, the concentration of chloride dropped sharply. Tetrachloroethylene reached the sampling point next. After the tetrachloroethylene plume moved past the sampling point, its concentration dropped slowly, probably resulting from a process similar to chromatographic tailing (Roberts *et al.*, 1986). Finally, 1,2-dichlorobenzene reached the sampling point. A month later, the concentration of 1,2-dichlorobenzene dropped precipitously. No significant concentrations of 1,2-dichlorobenzene were measured for the remainder of the field experiment even though the water still contained appreciable concentrations of tetrachloroethylene. The best explanation for these observations is that microbiological adaptation had occurred and subsequent metabolic activity had selectively removed 1,2-dichlorobenzene from the ground water.

Because the concentration of 1,2-dichlorobenzene was low, there was adequate oxygen to allow for its complete degradation to CO_2. In this case, the contaminant completely disappeared at the first evidence of adaptation. At higher concentrations a contaminant may persist after adaptation, because the extent of biodegradation may be limited by the supply of an appropriate electron acceptor such as oxygen or nitrate (see Section II,B,2,b). In such circumstances, the rate of further biodegradation may not be limited by the kinetics of microbial metabolism; rather the transport processes in the aquifer that supply appropriate electron acceptors can limit the rate of biodegradation.

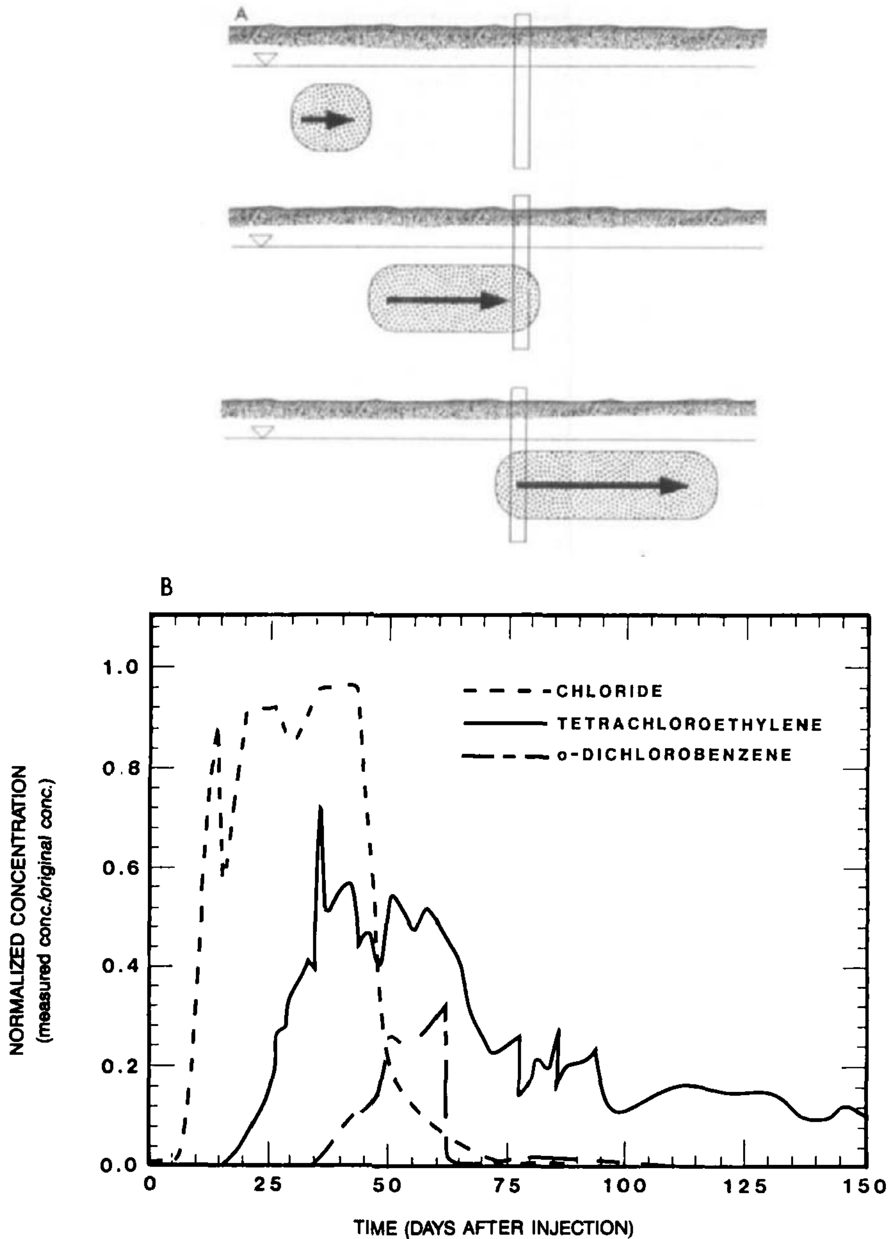

FIG. 3. A field experiment performed at Canadian Forces Air Base Borden, Ontario. (A) Diagram showing movement of an artificial plume of pollution that was allowed to move with the natural flow of groundwater through a series of monitoring wells. (B) Breakthrough of three components of the plume at one position in the aquifer. Redrawn from Roberts *et al.* (1986).

Electron acceptor limitation has been documented at the site of a buried disposal lagoon for wood creosoting wastes in Conroe, Texas (Borden *et al.*, 1986; Borden and Bedient, 1986; Wilson *et al.*, 1985a). At this site a shallow water table aquifer was contaminated with alkylbenzenes and polynuclear aromatic hydrocarbons of the waste. Water that contained hydrocarbons did not contain oxygen, and water that contained oxygen did not contain hydrocarbons. The rate of biodegradation of the hydrocarbons in the contaminated portion of the aquifer, as determined by applying a solute-transport model to the disposition of the plume, was 10% per year (Borden and Bedient, 1986). The rate of degradation of the same hydrocarbons in subsurface material collected from a remediated zone just outside of the plume was 10% per day. These observations strongly suggest that an adapted subsurface microbial population was present. This population was able to remove the organic contaminants very rapidly when sufficient oxygen was available.

A similar situation existed at another site in Traverse City, Michigan (Wilson, B.H. *et al.*, 1986) where a spill of aviation gasoline had contaminated a water table aquifer with petroleum hydrocarbons, primarily alkylbenzenes. In this case, the rate of aerobic biodegradation in the plume, determined by modeling the disposition of the contaminants in the field, was 1% per day (personal communication, Philip Bedient, Rice University, Houston, Texas). The rate of degradation in subsurface material collected just outside of the plume was >30% per day.

Both of these sites were contaminated at least a decade ago, and the spread of the plumes was in a steady state. Apparently, the limiting transport mechanism was dispersion and diffusion of oxygen from the water table down into the plume. As the plumes moved away from their source, they appeared to sink, which increased the distance over which oxygen transport was required and weakened the diffusion of dispersion gradient. As a result, the rate of aerobic biodegradation slowed as the plumes moved away from their sources. At field scale the rate of biodegradation was adequately described by the semiempirical first-order rate constants (Borden *et al.*, 1986). This description may be the most appropriate model for plumes that originate at the water table and are reaerated by oxygen transport through the unsaturated zone.

In the field study illustrated in Fig. 3, the artificial plume was injected over an interval of 1.4–2.0 m below the water table in the oxygenated aquifer. In this case, degradation of the hydrocarbons at field scale was zero-order. The fractional loss of hydrocarbons was directly proportional to the distance the plume had moved. There was very little vertical mixing through dispersion, and not enough time for significant diffusion to occur. Oxygen could not enter the plume from waters above or below. As a result, oxygen required for biodegradation of the alkylbenzenes was

supplied principally through longitudinal dispersion of oxygenated water to the plume, a process that should be first-order with respect to the distance the plume has moved (Freeze and Cherry, 1979).

These field studies clearly show that contaminant biodegradation processes in aquifers can be controlled largely by site-specific geochemical and hydrogeologic factors. Because oxygen transport limitations may retard the aerobic biodegradation of contamination in groundwater, anaerobic processes may often determine the fate of pollutants. This appears to be the case at many contaminated sites (Table V). When the biodegradation rates of various organic compounds have been measured employing various alternate electron acceptors (nitrate, sulfate, and carbonate), the rates of transformation were relatively rapid compared to aerobic rates. Perhaps more significant is the diversity of organic compounds that were degraded anaerobically (Table V). The studies of contaminated sites also show that alkylbenzenes were rapidly degraded anaerobically in methanogenic landfill leachate and in methanogenic plumes from gasoline spills, and that anaerobic degradation of xylenes can be supported by denitrification. It should also be noted that in anaerobic environments contaminant compounds themselves may also serve as electron acceptors. Indeed, reductive dechlorination of alkyl or aryl chlorines has been demonstrated in contaminated subsurface environments (Gibson and Suflita, 1986). After dechlorination organic compounds such as phenols and benzoates may enter anaerobic degradation pathways, and be mineralized.

This discussion of the function of contaminated aquifers is brief, but it serves to emphasize the importance of anaerobic processes in aquifer restoration (see Section II,2,B,b). Now that the rudiments of subsurface ecology are partly understood, the microbiological bases of these important processes undoubtedly will receive more attention in the future.

VI. Summary and Conclusions

We have presented a current view of the microbial ecology of the terrestrial subsurface by considering primarily the ecology of shallow aquifer sediments. The properties of the aquifer sediments and groundwater determine their ability to support microbial life and control the abundance and activities of microorganisms. Pore size, nutrient limitations, availability of electron acceptors, and large surface area for attachment all may have major effects on microbial abundance and activities in aquifer material. Microorganisms are the predominant forms of life in the subsurface. They will be found wherever enough space, nutrients, and water are available for them to live. Environmental factors such as pH, temperature, hydrostatic pressure, and dissolved salts also may

TABLE V

RATE OR EXTENT OF BIODEGRADATION OF VARIOUS ORGANIC COMPOUNDS IN SUBSURFACE MATERIAL FROM CONTAMINATED SITES

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
Common metabolites				
Acetate	Oxygen	Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario, Canada	14/week	Ward (1985)
Acetate	Nitrate	Shallow water table aquifer near domestic septic tile field and garden soil, Williamsburg, Ontario, Canada	8/week 84/week	
Benzoic acid (ring and carboxyl)	Oxygen	Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	6–30/week	
Glucose	Nitrate	Shallow water table aquifer near domestic septic tile field and garden soil, Williamsburg, Ontario	4.5/week 94/week	
Glucose	Oxygen	Garden soil, Williamsburg, Ontario	25/week	Ward (1985)
		Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	8.6/week	
Glutamic acid	Oxygen	Shallow water table aquifer near domestic septic tile field and garden soil, Williamsburg, Ontario	6–7/week 47/week	

		Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	16/week	
Glutamic acid	Nitrate	Garden soil, Williamsburg, Ontario	69/week	
		Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	69/week	
Stearic acid	Oxygen	Shallow water table aquifer near domestic septic tile field and garden soil, Williamsburg, Ontario	0.34/week	
Stearic acid	Nitrate	Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	1.3/week	
volatile fatty acids (acetic, propionic, butyric)	Oxygen	Chalk from 10 m below the water table, Ingram, Suffolk, England	1.0/week, no evidence of adaptation after 30 days	Kiene and Capone (1986)
Halogenated hydrocarbons				
bromoform, chloroform, chlorodibromo-methane	Carbonate	Confined aquifer injected with treated municipal wastewater, Palo Alto Baylands, California, USA	0.2/week	Roberts *et al.* (1982)
1,2-Dibromoethane (EDB)	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	99% removed in 16 weeks	Wilson, B. H. *et al.* (1986)

(continued)

TABLE V *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
1,2-Dichlorobenzene	Oxygen	Field site on banks of Glatt River in Switzerland	1/week, exposed to 0.3 μg/liter; 270/week, after acclimation to 30 μg/liter	Kuhn *et al.* (1985)
Dichlorobromo-methane	Carbonate	Confined aquifer injected with treated wastewater, Palo Alto Baylands, California	0.2/week	Roberts *et al.* (1982)
1,1,1-Dichloroethane	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	77–99% removed in 16–40 weeks	Wilson, B. H. *et al.* (1986)
trans-1,2-Dichloroethane	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	87% removed in 40 weeks	
2,4-Dichlorophenoxy-acetic acid	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	99% removed in 3 months	Gibson and Suflita (1986)
Tetrachloroethylene	Oxygen	Field site on banks of Glatt River in Switzerland	Not detected, <0.01/week	Kuhn *et al.* (1985)
Tetrachloroethylene	Carbonate	Confined aquifer injected with treated municipal wastewater, Palo Alto Baylands, California	0.02/week	Roberts *et al.* (1982)
1,1,1-Trichloroethane	Carbonate	Sand influenced by leachate from North Bay municipal landfill, Ontario, Canada	Field data showed disappearance	Barker *et al.* (1986)

Trichloroethylene	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	66–99% removed in 40 weeks; 90% removed in 8 weeks	Wilson, B. H. *et al.* (1986)
Phenols				
2-Chlorophenol, 3-chlorophenol, 4-chlorophenol	Sulfate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	100% removed in 3 months	Gibson and Suflita (1986)
2-Chlorophenol	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	Not detected, <0.02/week	Gibson and Suflita (1986)
o-Cresol	Sulfate or carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	No acclimation within 90–100 days	Smolenski and Suflita (1987)
m-Cresol	Sulfate or carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	43–90 days lag time for acclimation	
p-Cresol	Sulfate	River alluvium contaminated by landfill leachate, Norman, Oklahoma	<10–46 days lag time; 180–3300 mg/liter-week after acclimation; 3 mg sulfate required per mg *p*-Cresol consumed	
o-, *m*-, *p*-Cresol	Carbonate	Sand contaminated with creosote waste	Extensive degradation	Goerlitz *et al.* (1985)
2,4-Dichlorophenol	Sulfate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	Not detected, <0.04/week	Gibson and Suflita (1986)

(continued)

TABLE V *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
2,5-Dichlorophenol	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	83% removed in 3 months	
3,4-Dichlorophenol	Sulfate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	Not detected, <0.01/week	
Phenol	Sulfate or carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	99–100% removed in 3 months	
Phenol	Carbonate	Water table aquifer contaminated with creosote, St. Louis Park, Minnesota, USA	Extensive degradation to methane	Ehrlich *et al.* (1982)
		Water table aquifer contaminated with creosote, Pensacola, Florida, USA	>0.1 mg/liter pentachlorophenol exhibited degradation	Godsy *et al.* (1983)
2,4,5-Trichlorophenol	Sulfate	River alluvium contaminated by landfill leachate, Norman, Oklahoma	Not detected, <0.04/week	Gibson and Suflita (1986)
Petroleum-derived hydrocarbons				
Acenaphthene	Oxygen	Shallow water table aquifer on Texas coast	>1.3/week	Wilson *et al.* (1985)
Benzene	Oxygen	Sand 5 m below the water table in a shallow unconfined aquifer contaminated by a gasoline spill	2.3–2.7/week, degradation slowed at 4–5 μg/liter	Wilson, B. H. *et al.* (1987)

Benzene	Carbonate	Sand 3 m below the water table in a shallow unconfined aquifer contaminated by a gasoline spill	0.9/week, degradation slowed at 12 μg/liter	
Benzene, ethylbenzene	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	70–74% removed in 40 weeks, 99% in 120 weeks	
Dibenzofuran	Oxygen	Shallow water table aquifer on Texas coast	>1.8/week	Wilson *et al.* (1985a)
Fluorene	Oxygen	Shallow water table aquifer on Texas coast	>0.9/week	
1-Methylnaphthene, 2-methylnaphthene, naphthalene	Oxygen	Shallow water table aquifer on Texas coast	>1.6/week	
Naphthalene	Carbonate	Water table aquifer contaminated with creosote, St. Louis Park, Minnesota	No degradation detected	Ehrlich *et al.* (1982)
Styrene	Carbonate	Alluvium contaminated by landfill leachate, Norman, Oklahoma	90% removed in 8 weeks, 99% in 16 weeks	Wilson, B. H. *et al.* (1986)
Toluene	Oxygen	Sand in shallow water table aquifer exposed to wood creosoting waste	0.2/week	Wilson, J. T. *et al.* (1986)
		Sand 3–5 m below the water table in shallow unconfined aquifer contaminated by a gasoline spill	Degradation ceased at 3–5 μg-liter	

(continued)

TABLE V *(continued)*

Compound	Dominant electron acceptor	Type of sample and location	Biodegradation rate or extent	Reference
		Sand 3 m below the water table in plume of contamination	0.5/week, slowed at 56 μg/liter	
		Alluvium contaminated by landfill leachate, Norman, Oklahoma	99% removed in 40 weeks	
1,2,4-Trimethylbenzene	Carbonate	Sand influenced by leachate from North Bay Municipal landfill, Ontario	No degradation	Barker *et al.* (1986)
o-Xylene	Oxygen or carbonate	Sand 3–5 meters below the water table in shallow unconfined aquifer contaminated by a gasoline spill	2.6–4.8/week, degradation ceased at 1–3 μg/liter	Wilson, B. H. *et al.* (1987)
		Sand 3 m below the water table in plume of contamination	0.6/week	
o-Xylene	Nitrate	Sediments from banks of Glatt River in Switzerland	160/week, after acclimation to 400 μg/liter	Kuhn *et al.* (1985)
o-Xylene	Carbonate	Sand influenced by leachate from North Bay Municipal landfill, Ontario	0.01/week, estimated from field data	Barker *et al.* (1986)
		Alluvium contaminated by landfill leachate, Norman, Oklahoma	78% removed in in 40 weeks, 99% in 120 weeks	Wilson, B. H. *et al.* (1986)

m-Xylene	Oxygen	Field site on banks of Glatt River in Switzerland	>16/week, exposed to 0.2 μg/liter	Kuhn *et al.* (1985)
	Nitrate	Sediments from banks of Glatt River in Switzerland	65/week, after acclimation to 400 μg/liter	
p-Xylene	Oxygen	Field site on banks of Glatt River in Switzerland	160/week, after acclimation to 400 μg/liter	
m- and *p*-Xylene	Oxygen	Sand 3–5 m below the water table in a shallow unconfined aquifer contaminated by a gasoline spill	3.0–4.8/week, degradation slowed at 2–3 μg/liter	Wilson, B. H. *et al.* (1987)
	Carbonate	Sand 3 m below the water table in the plume of contamination	0.4/week	
Miscellaneous				
Nitrilotriacetic acid	Oxygen or nitrate	Unsaturated zone below septic tile field, Sault Ste. Marie, Ontario	0.7–0.8/week	Ward (1985)
		Shallow water table aquifer near domestic septic tile field, Williamsburg, Ontario	1.3–2.3/week	
2,4-Dichlorophenoxy-acetic acid	Sulfate or carbonate	River alluvium contaminated by landfill leachate, Norman, Oklahoma	<0.01/week, 99% removed in 3 months	Gibson and Suflita (1986)
Quinoline and isoquinoline	Carbonate	Sand contaminated with creosote wastes, Pensacola, Florida	Extensive degradation	Pereira *et al.* (1987)

influence subsurface microbial populations, but these factors do not exhibit great extremes in shallow water table aquifers, and thus only in very deep formations might they limit diversity or preclude the existence of microorganisms.

Although the presence and activity of microorganisms in most subsurface environments are predictable, only recently have subsurface microbial populations in shallow subsurface zones been characterized. Aseptic sampling methods have been employed and microbiological and biochemical methods have been adapted to determine the types, abundance, and metabolic activities of microorganisms in subsurface material. Bacteria dominate, but eukaryotic microorganisms also are present. Vertical profile studies of a shallow aquifer in Oklahoma showed that active microbial biomass declined with depth to the unsaturated zone, but was variable in saturated sediments. Such a distribution of active biomass may be common in shallow aquifers. Studies on the lateral distribution of microorganisms in shallow and deep aquifers suggest that microorganisms are transported or migrate over fairly long distances in aquifer sediments. Surficial aquifers may be colonized by vertical or lateral transport and migration of surface microorganisms from recharge areas, but microorganisms could also have colonized when sediments were originally deposited. The biological and physical mechanisms controlling the migration of microorganisms in aquifers are not well understood.

The function of shallow aquifers was considered with regard to nutritional ecology. Most pristine aquifers are oligotrophic. Heterotrophic life in these unique ecosystems is supported by secondary organic compounds that filter down from the soil above. The quantity and quality of organic nutrients depend on the age of water and rate of recharge of the aquifer. Metabolic activity may be regulated by the amount of metabolizable organic matter and availability of electron acceptors, especially oxygen. Anoxic conditions may prevail if excess organic matter enters the aquifer. Alternate electron acceptors such as nitrate, sulfate, and carbonate may be used for biodegradation of organic compounds in heavily polluted aquifers. According to thermodynamic models, sequential utilization of electron acceptors should govern the biodegradation processes.

The function of contaminated aquifers should be regulated by the same processes that occur in pristine aquifers. Microbial populations will be selected and regulated by the availability of space, surface area for attachment, nutrient supplies (especially nitrogen and phosphorus), and electron acceptors. Anaerobic processes will be more influential in contaminated aquifers than in pristine aquifers.

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NOTE ADDED IN PROOF. Since we completed this article in the middle of 1987, several relevant papers on microbial ecology of shallow aquifers have been published or are in press. Beeman and Suflita (1987) studied the ecology of an unconfined sandy riverbank aquifer beneath a municipal landfill in Norman, Oklahoma. Carbon and energy flow in this anoxic aquifer appeared to be controlled by the availability of electron acceptors, sulfate or carbonate. Distinct regions of the aquifer were found to be either sulfate-reducing or methanogenic. Suflita *et al.* (1988) reviewed biotransformation of pollutant chemicals in anaerobic aquifers. Pollutant chemicals were discussed with respect to their biodegradation potential under methanogenic or sulfate-reducing conditions. Alkylated aromatic compounds were found to be degraded under both conditions, but the rate of degradation was higher under sulfate reducing conditions. Halogenated aromatic compounds were found to be reductively dehalogenated under methanogenic, but not under sulfate-reducing conditions; however, reductive dehalogenation of nonaromatic compounds such as tetrachlorethylene were not inhibited by sulfate. Tetrachloroethylene was reductively dehalogenated to trichloroethylene at the same rate under both conditions. Biodegradation potential under aerobic conditions was studied by Swindoll *et al.* (1988). Experiments were designed to test the effects of added nutrients on mineralization of ethylene dibromide, p-nitrophenol, phenol, and toluene in samples from the Lula, Oklahoma aquifer (see Tables III and IV). Although the results of inorganic nutrient additions varied, in general, multiple inorganic nutrients enhanced activity more than single substances. Addition of glucose or amino acids inhibited mineralization of the xenobiotic substrates, possibly reflecting preferential utilization of the more easily degradable carbon sources. Finally, eight papers on microbial abundance and activities in pristine groundwater and aquifer sediments are scheduled to appear in a special groundwater microbiology issue of *Microbial Ecology* **16** (1988). We reviewed some of these papers in prepublication form (see in Table I, Beloin *et al.*, 1988; Bone and Balkwill, 1988; Thorn and Ventullo, 1988).

Foam Control in Submerged Fermentation: State of the Art

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I. Introduction

Foam, a dispersion of gas in liquid with bulk density approaching that of the gas (Evans and Hall, 1971), is an agglomeration of gas bubbles separated from each other by a thin liquid film (Bikerman *et al.*, 1953). Thus it is invariably formed in a system in which gas is a participant either as reactant or as product (Howe, 1978). Foam formation is a common phenomenon in many industrial processes including chemical and fermentation processes. Its formation is a desirable property in some

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processes such as in the production of beer. Similarly, the foaming property finds useful applications in fire fighting (Fry and French, 1951), froth floatation (Gaudin, 1953), foam separation (Perri and Hasel, 1946; Rubin and Everett, 1963), foam fractionation (Brady, 1949), and a variety of miscellaneous uses such as in immobilization of gas or liquid (Vana, 1949), faster absorption of liquids and gases (Bransky and Diwoky, 1940), manufacture of foam glass (Long, 1934), analytical work (American Public Health Association, 1925), and medicine (Bering, 1944).

In fermentations, the aeration and agitation of the medium generate foam. It is also formed in anaerobic fermentations due to the agglomeration of bubbles of the gases generated during fermentation (Solomons, 1967). In most fermentations, the foam is usually formed on the surface of the broth with a clear distinction between the foam layer and the rest of the broth. However, in fermentations involving thick mycelium, this distinction does not exist and the gas dispersion gradually increases from the bottom of the fermentor to the surface (Bryant, 1970).

Foam formation in submerged fermentation is not only a nuisance and health hazard but also adversely affects productivity and downstream processing. In spite of the importance of the problem, the attention paid to it in the literature and experimental investigations has been less significant than it deserves. During our investigations on scale-up of laboratory-scale processes, we have encountered serious problems with foams, leading to the necessity for high concentration of antifoam agents in the media. For effective prevention of foam formation and for foam control during fermentation, it is essential to understand thoroughly the mechanism of foam formation as well as foam breaking. This article is an attempt to review critically the present knowledge on aspects of foam formation and control. The aspects on mathematical considerations are not dealt with as these have been reviewed recently by Viesturs *et al.* (1982).

II. Adverse Effects of Foam

Foam formation in fermentations is undesirable because of its many adverse effects (Table I). Along with the foam, some of the cells are separated from the bulk of the culture broth and are deposited on the inner side of the head plate and on the upper walls of the fermentor vessel where they are no longer useful in the fermentation reaction. Such cells liberate proteins, due to autolysis, which act as catalysts for additional foam formation (Bryant, 1970). The loss of culture fluid and microorganisms due to foam leads to wetting of the outlet air filters and seepage in the stirrer bearings and other attachments on the fermentor head plate. The presence of foam, even in small amounts, also alters the

TABLE I

ADVERSE EFFECTS OF FOAM IN FERMENTATION

Effect	Reference
Reduction in working capacity	Bungay *et al.* (1960)
Loss of culture fluid and cells through air line	Solomons (1967)
Contamination of the atmosphere	Bryant (1970)
Increased chances of fermentor contamination	Solomons (1967)
Barrier to oxygen transfer	Solomons and Perkins (1958); Deindoerfer and Gaden (1955)
Promotion of lysis of cells	Bryant (1970)
Invalid fermentation data	Bungay *et al.* (1960)
Deposition of cells on upper part of fermentor	Bryant (1970)
Shutdown of the plant	Bungay *et al.* (1960)
Preferential removal of surface-active agents from medium	Bryant (1970)
Additional expenses for foam control	Bungay *et al.* (1960)
Creation of additional non-homogeneity in fermentation system	Viesturs *et al.* (1982)

conditions of aeration and agitation in foaming and nonfoaming regions (Bryant, 1970). The liquid from overflow or off-take lines in continuous fermentations may have an increased concentration of microorganisms due to foam formation, and thus the samples may not be representative of the bulk culture liquid if the overflow or off-take line is situated in a layer of foam (Bryant, 1970).

In the foam layer, the microorganisms are reported to adhere at the foam bubble surface and respire using gaseous oxygen (Bertholomew *et al.*, 1950). Due to such consumption of oxygen, the rigid bubble of foam thus will become depleted of oxygen and cause oxygen limitation in the foam layer (Sukan and Güray, 1985). In addition, the higher soluble protein concentration in the foam layers as compared to the rest of the culture broth (Sukan and Güray, 1985) causes problems. Further, foam stabilization by proteins of molecular weights of 3000–10,000 (Saruno and Ishida, 1963) and protein denaturation in foams (London *et al.*, 1954) lead to a more complicated situation.

Cellulolytic microorganisms are reported to form intense foam in pure cellulose media, especially during the active enzyme production phase (Mou, 1975; Mandels and Weber, 1969; Sukan *et al.*, 1984). The effect of such foaming during fermentation on cellulase synthesis and activities

in media based on crystalline cellulose and beet sugar cosett was studied by Sukan and Güray (1985) employing *Trichoderma reesei* and *Sporotrichum pulverulentum*. In the case of *T. reesei* growing on crystalline cellulose, the cellulase activity in an experimentally forced foam layer was significantly higher than in a naturally formed foam layer and in the nonfoaming portion of the broth. However, this was not observed for other experimental setups which, in contrast, showed decreased cellulase in both naturally formed foam and experimentally forced foam layers.

III. Classification of Foams

Based on their characteristics, foams are classified into various categories, listed in Table II (Bryant, 1970; Dawson, 1961). In general, fluid foams are encountered in submerged fermentations and these can be unstable, metastable, transient, or persistent.

Two important characteristics of foams are foaminess and foam stability. Foaminess is a measure of the foaming capacity and is independent of the geometry of the equipment as well as the technique employed. It is largely dependent on the nature of the ingredients incorporated in the fermentation medium, their relative concentrations, and a variety of physical factors (Currie, 1952). On the other hand, the stability of the foam is determined by the number of lamellae and the angles between them (Bikerman *et al.*, 1953). The presence of three lamellae at an angle of 120° between each lamella imparts stability to the foam. At all other angles and lamella numbers, the foam becomes unstable because the equilibrium state is continuously approached (but not achieved). The foam also becomes stable if the surface tension of the system is less than

TABLE II

CLASSIFICATION OF FOAMS

Type	Characteristics
True	Predominantly gaseous dispersion
Fluid	Predominantly liquid dispersion with enhanced holdup of gas in a large portion of the liquid
Unstable	Equilibrium state is continuously approached
Metastable	Progress to the equilibrium state is arrested
Transient	Lifetime of seconds
Persistent	Lifetime of hours or days if undisturbed

that of the pure solvent of the solution. The liquid film in a stable foam can endure rapid variations in the area of the liquid film and the surface tension.

IV. Factors Affecting Foam Formation

Even when aerated and agitated at high rates, liquids do not foam unless some impurities are present in them (Evans and Hall, 1971). The presence of another component, in either soluble or insoluble form to produce a different surface layer as compared to the bulk liquid, is essential for foam formation (Solomons, 1967). The physical reasoning for foam formation in an aerated liquid containing solid substrate particles was described by Howe (1978). According to this description, the gas phase attracts the water-resistant phase of the liquid–solid mixture while the hydrophilic phase of the mixture holds water due to abnormal surface tension. Consequently, the gas becomes emulsified with the liquid–solid system and is not allowed to evolve freely due to entrapment and the surrounding physical limitations. Changes in various factors such as pH, molecular surface electrical charge, surface tension, and temperature also influence the rate of evolution of entrapped gas. The cycle of the solid substrate particle, uncharged emulsoid formation, charging of emulsoid, charged suspended lyophobic solution based upon the theory of electrical charges, and foam formation described by Howe (1978) is shown in Fig. 1.

The chemical and biochemical factors (Evans and Hall, 1971; Pirt and Callow, 1958; Kouloheris, 1970; Szarka and Magyar, 1967; Lucke and

TABLE III

CHEMICAL AND BIOCHEMICAL FACTORS AFFECTING FOAM FORMATION

Concentration of soluble, insoluble, and colloidal components	Temperature and vapor pressure
	Geometrical restriction of the vessel
Cell concentration and density of solution	Aeration and agitation rates
Surface tension-reducing compounds	Formation of gaseous and foam-promoting metabolites
Salt and salt-forming compounds	Sudden changes in pH
	Foam life and foaming velocity
Polymers	Poor O_2 transfer and lack of O_2
Viscosity and elasticity	Dispersed growth of the strain
Acidity and alkalinity	Ratio of inoculum
Duration of sterilization	Proteinaceous compounds

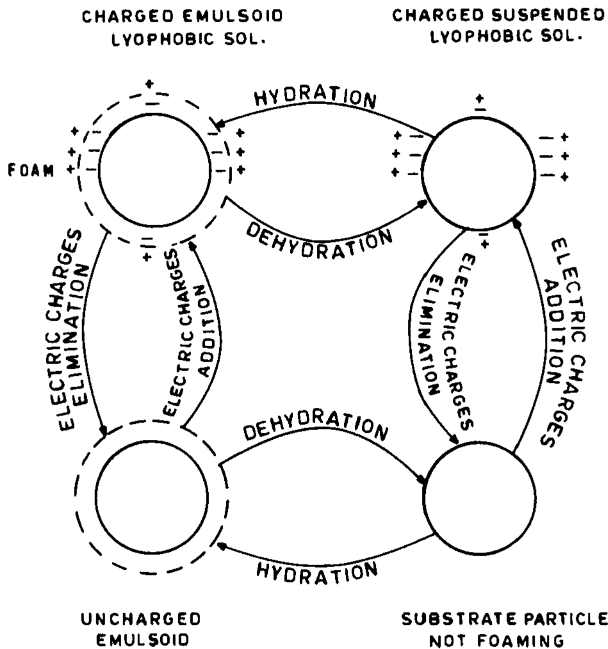

FIG. 1. Diagrammatic presentation of foam formation due to electric charges on solid substrate particles.

Schügerl, 1976; Gifford and Scriven, 1971; Howe, 1978) affecting foam formation are listed in Table III. They influence foam formation due to their ability to affect surface properties, permit discontinuities, promote drainage, form gelatinous surface layers, accelerate the life cycle of foams, promote a surface layer of a composition different from that of a bulk liquid, and cause damping of shock waves, double-layer repulsion as well as steric interactions. The manner of influence of these factors may be complex. For example, the surface viscosity influences the gas diffusion rate across the lamellae and, therefore, the presence of polymer at even very low concentrations in the biological medium affects foam structure with fermentation time (Bumbullis *et al.*, 1981). Salt-forming substances, in general, are known to increase foaming and surface viscosity as well as decrease the surface tension (Wolfer and Schügerl, 1983). An increase in temperature enhances foaminess but diminishes foam stability (Mokrushin and Zhidkova, 1959). Salts such as KCl and $MgSO_4$ are reported to behave similarly (Bumbullis and Schügerl, 1981). It is even possible to describe this effect quantitatively by means of turbidity–temperature shift due to the interactions between the salts and water

structure (Bumbullis and Schügerl, 1981). The protein denaturation and Maillard reaction during sterilization in potato–protein liquor and glucose medium is known to increase the foaminess by a factor as high as 2100 (Kotsaridu *et al.*, 1983).

The relationships between the foam stability and the drainage of a liquid from foam lamellae (Bikerman, 1973) as well as between foaminess and surface tension in the system (Sie and Schügerl, 1983) are well known. Considerable work on foam stability and syneresis was carried out by Kann and his group (Kann, 1979; Kann and Druzhinin, 1978, 1979a,b) and includes studies on criteria for foam stability, effect of foam structure on foam stability, hydrodynamic and structural stability of foams, and the criteria for estimating the foam structural stability. In addition, the total time of syneresis, the maximum syneresis rate, rate of hydrodynamic liquid flow through channels between foam bubbles, and rate of liquid flow due to the contraction of foam bubbles were studied. The relative velocity of liquid outflow as a criteria for foam stability and the dependence of foam stability on the dispersivity of the foams were stressed by these workers. The foams become thermodynamically unstable due to high specific surface area of the foams, resulting from the lower surface energy of the gas and liquid in separated states (Bryant, 1970).

On the whole, the ability to resist local thinning of the liquid film while allowing general thinning to proceed decides the vulnerability of the solution to form foams. The presence of foam as well as its extent represents a balance between the processes creating it and the forces causing its destruction (Bryant, 1970). Many theories postulated for unstable foams take into account the relationship between surface tension and solute concentration in a static or dynamic system or surface viscosity and liquid film as well as surface area and surface tension (Kouloheris, 1970; Gibbs, 1928). On the other hand, the thermodynamic theory for stable foam formation in a dilute solution of low viscosity is based on the correlation between the stability of the foam and transfer of excess solute from the surface to the bulk of the liquid (Nakagaki, 1957) or on the electrical double-layer repulsion (Derjaguin and Titijersbaya, 1957).

V. Foam Control in Fermentation

In view of its adverse effects including safety hazards, it is essential to control foam in fermentation. In most cases, chemical methods are preferred for foam control although a mechanical foam breaker is usually incorporated in modern sophisticated fermentors. In some cases, a combination of chemical and mechanical methods is employed for more effective control of the foam. Foam breaking by thermal means is also possible

but is not generally employed in fermentations. It is interesting to note that similar control methods are usually employed even with different types of foams formed. Foam formation in fermentors can also be prevented to varying degrees without employing any external means. Considering the advantages of the latter techniques, they deserve more attention but are usually neglected.

Reviews on foam formation and control in fermentors are available in Japanese (Abe, 1981; Takahara, 1978a,b) and in Russian (Viesturs *et al.*, 1973, 1980). Reviews in English are also available (Viesturs *et al.*, 1982; Schügerl, 1985; Evan and Hall, 1971; Howe, 1978; Solomons, 1967; Hall *et al.*, 1973) in addition to the treatment of the topic in books dealing with various aspects of fermentation (Bryant, 1970; Solomons, 1969). Most of these reviews deal with specific aspects and are more or less theory oriented. Methods for modeling and design of mechanical foam breakers or chemico-mechanical foam breakers as well as methods for comparison of these devices were developed and described in detail by Viesturs *et al.* (1982).

VI. Chemical Methods of Foam Control

Foam control by chemical methods relies on the use of a class of compounds known as antifoam agents or defoamers. These are extensively employed and constitute a method of choice in fermentation (Solomons, 1969; Kitchener and Cooper, 1959; Ross, 1967). There is no clear-cut demarcation between antifoam and defoamer compounds. However, when the agent is added to the medium before foaming occurs, it is sometimes referred to as antifoam, whereas it is called a defoamer when used to knock down the foam (Evans and Hall, 1971). The antifoams are usually surface-active agents which are unable to produce stable foam by themselves (Solomons, 1967). In some fermentations, the antifoams are used in excess quantities for the dual purpose of foam control and crude pH adjustment by the acids produced during the metabolism of the oil (Bungay *et al.*, 1960). These antifoams are sterilized before use by autoclaving at 15 psi for 20–30 minutes or by direct steam for 2–3 hours. Direct heat at 160°C for 2–3 hours or at 127° for 90 minutes is employed in some cases (Bungay *et al.*, 1960; Solomons, 1969). Antifoam agents may sometimes cause some adverse effects, and judicious selection of proper agents and their doses is essential.

A. Type and Nature of Antifoams

Chemical antifoam agents are classified as fats, oils, waxes, aliphatic acids or esters, alcohols, sulfates, sulfonates, fatty acid soaps, nitrogenous compounds, phosphates, sulfides, thio compounds, organosilicons,

organogermanian compounds, and halogenated and inorganic compounds (Bickerman *et al.*, 1953). Examples of some of the members of these groups which are recommended and employed in fermentations are listed in Table IV. In addition, Table V lists the chemical antifoams used in some specific fermentation processes in laboratory and industrial-scale operations. In general, most of the research papers on fermentations do not specify the antifoam agent used and this amply illustrates the apathy toward this aspect of fermentation.

Many of the commercial antifoams require the presence of carriers to assist in the suppression of the foam (Bryant, 1970; Solomons, 1969). A mixture of more than one antifoam is also necessary in some cases for

TABLE IV

CHEMICAL ANTIFOAMS EMPLOYED IN FERMENTATIONS

Chemical antifoam agent	Reference
Lard oil/soybean oil/linseed oil/tall oil/rapeseed oil/ polyglycol	Bungay *et al.* (1960)
Fat	Podgorski and Ichniowski (1982)
Molten lard	Meyer (1930)
Sulfonated olive oil	Barti (1935)
Sunflower oil/cod liver oil/hydrogenated cod liver oil/lanolin	Viesturs *et al.* (1982)
Oxazolines	Johnson (1948)
Degras	Auclair (1939)
Defoamer FTS	Bickerman *et al.* (1953)
Polydimethylsiloxanes	Evans and Hall (1971)
Sulfanol/awirol/PMS-154/silicon S/adekanol/aseol/ struktol/propynol B-400	Viesturs *et al.* (1982)
Alkaterge C in paraffin oil/grape oil/lard oil	Solomons (1967); Diendoerfer and Gaden (1955)
Silicon in castor oil/liquid paraffin	Solomons (1969); Solomons and Perkin (1958)
Polyethylene glycol(2–3.6%) + surfactant + 1–1.2% unsaturated vegetable oil/mineral oil	Cristian *et al.* (1981)
Emulsion containing stearic acid + glycol/polyglycol esters of fatty acids/an insoluble metal soap + an emulsifying agent	Fritz (1945)
Tri- and tetraalkylsilanes dissolved in a combination of water-soluble and water-insoluble solvents	Chiba Ltd. (1949)
Mixture of polydimethylsiloxane, esters of $C_{15\text{-}30}$ fatty acids with cyclopentanophenanthrene-type alcohol and partial esters of $C_{16\text{-}24}$ fatty acids with glycercol containing >90% monoglycerides, dispersed in 875 g oleic acid ester containing 6 mol ethylene oxide	Jaroslav *et al.* (1981)

TABLE V

CHEMICAL ANTIFOAMS USED IN SPECIFIC FERMENTATIONS

Antifoam agent	Product/process	Microorganism	Reference
Bacterial/actinomycete fermentation			
Decanol/propynol B-400/polypropyleneglycol R-2000	L-Lysine	*Micrococcus glutamicus*	Shchekotovo (1974)
Alkaterge C, 30% in paraffin oil/ 70% silicon antifoam A in silicon fluid MS-200-IOCS/30% antifoam 60 in water	2,3-Butanediol	*Aerobacter aerogenes*	Kouloheris (1970)
Stearyl 0.1–0.5%, polyoxyethylene (polyoxybutylene 18-lamate)	L-Glutamic acid	*Micrococcus* sp.	Asahi Denka Kogyo (1981)
Octadecylalcohol, 1.5% in ethanol	2-Ketogluconic acid	*Psudomonas fluorescens*	Dworschack *et al.* (1954)
Silicon oil	5-Exonuclease and phosphatase complex	*Actinomyces coelicolor*	Shakhova *et al.* (1982)
Siloxanes, 40 ppm (polymeric dimethylsiloxanediol/decamethylcyclopentasiloxane/octamethylcyclotetrasiloxane)	Streptomycin	*Actinomyces griseus*	Coppock (1950)
RD emulsion, 30%, aqueous suspension	Monamycin	*Streptomyces jamaicensis*	Hall and Hassall (1970)
Organic polyester, 0.1%, sperm whale fat/50% aqueous emulsion of polymethylsiloxane/adekanol	Dactinomycin	*Actinomyces parvullus*	Kartasheva *et al.* (1977)
Silicon defoamer 21	Glucose isomerase	*Streptomyces fradiae* ScF 5	Prapulla *et al.* (1987)
Yeast fermentations			
Amersperse 750 + surfactant/ antifoam AEL-900C + surfactant	Alcohol	*Saccharomyces cerevisiae*	Ademir (1982)

Turkey oil	Rum	*Saccharomyces cerevisae*	Arroyo (1948)
Lauric acid	Distillary products		Modiano (1933)
Polyoxypropylene glycol, 100 ppm	Hydrocarbon fermentation	*Candida novellus*	Kanegafuchi Chem. Industry (1982)
Desmophen 3600	Biomass	*Hansenula polymorpha*	Wolfet and Schügerl (1983)
Antifoam A(DCA), 0.1%, Hodag K-4/lard oil	Biomass	*Torulopsis utilis*	Phillips *et al.* (1960)
Span 85/Span 20/heptadecanol/ Monsanto AE-1	Biomass	Yeasts	Bickerman *et al.* (1953)
Octyl alcohol/liquid mixture of solid aliphatic alcohol and a liquid aliphatic alcohol	Biomass	Yeast	Steibert (1934, 1937)
Oleic acid + solar and mazut oils + petrov catalyst	Biomass	Yeast	Metyushev and Kharin (1938)
Soapstocks from plant oil refining/ fatty acids from tallow/oleic acid from hide scrapings	Nutrient yeast	Yeast	Edomina *et al.* (1978)
Fungal fermentations			
Soybean oil/lard oil/1% octadecanol in ethyl alcohol or lard oil	Sodium gluconate	*Aspergillus niger*	Blom *et al.* (1980)
Silicon defoamer 21	α-Amylase	*Aspergillus niger*	Ghildyal *et al.* (1980)
Octadecanol, 0.75%, in 95% ethanol + soybean oil	Itaconic acid	*Aspergillus terreus*	Pfeifer *et al.* (1952)
Propinol B-400, 0.1% + 0.5% palm or sunflower oil/0.7% silicate (AS-60) + 0.5% sunflower oil	Lipase	*Geotrichum candidum*	Fedorva *et al.* (1984)

(continued)

TABLE V *(continued)*

Antifoam agent	Product/process	Microorganism	Reference
Lard oil/silicon antifoam A/ polypropylene glycol 2000/lard oil containing 3 or 6% Alkaterge C	Penicillin	*Penicillium chrysogenum*	Rollinson and Lamb (1953); Goldschmidt and Koffler (1950); Shiro *et al.* (1972); Brown and Peterson (1950a,b); Deindoerfer and Gaden (1955); Anderson *et al.* (1953)
Octadecanol (stearyl alcohol)/3% octadecanol in lard oil	Penicillin	*Penicillium notatum*	Solomons (1967); Stefaniak *et al.* (1946)
Octadecanol 3% in paraffin oil/ lard oil + 3% octadecanol in mineral oil/2% Atlas G 5600 (glycerol alkyl oleate)	Citric acid	*Aspergillus niger*	Martin and Waters (1952); Steel *et al.* (1955); Betovič and Cimerman (1979)

effective foam control (Cristian *et al.*, 1981; Fritz, 1945; Ademir, 1982). Jaroslav *et al.* (1984) have reported threefold more effectiveness, compared to the use of oleic acid alone, when 20 g oleic acid was supplemented with 5 mol ethylene oxide and 10 g rapeseed fatty acids, or when 8 mol ethylene oxide and 5 mol propylene oxide were added to 70 g sunflower oil during molasses fermentation. Ricken Vitamin Co., Ltd. (1985) has patented a process for achieving better foam control in tofu production. It is based on a mixture of chemicals containing (1) one or more of polyricinoleic acid glycerol ester, polyricinoleic acid sorbitan ester, polyricinoleic acid propylene glycol ester, and polyricinoleic acid sucrose ester, (2) soybean phospholipid, (3) one or more of $CaCO_3$, $CaHPO_4$, Ca silicate, $MgCO_3$, Mg phosphate, and Mg silicate, and (4) animal or vegetable fats or oils. An example given was a mixture containing 10 parts tetraricinoleic acid glycerol ester, 5 parts soybean phospholipid, 55 parts edible tallow, and 30 parts $CaCO_3$, which was claimed to be 150% more effective than a conventional antifoam agent in tofu fermentation.

A novel antifoam agent for use in fermentations was developed by Leubbert *et al.* (1984). It is a lipophilic microbial metabolic product containing a mixture of lipids, phosphatides, $C_{12\text{-}30}$ fatty acids, fatty acid esters, vitamins, and their precursors such as ergosterol and ubiquinone. The treatment of the mixture with a nonoxidizing mineral acid yields the novel antifoam agent.

B. Foam Breaking Mechanism

It is essential to note that the mode of action of a chemical antifoam agent cannot be predicted by analogy and that misleading information may be obtained from any generalization of the mechanism (Bryant, 1970). The mode of action of a chemical antifoam agent in the destruction of foam varies with the nature of the compound, the type of the foam, and nature of the substances causing foam formation. The carrier that is used with commercial antifoam agents, in general, acts as a reservoir from which the antifoam agent is liberated. However, many carriers have defoaming ability and thus may also influence the mechanism of foam breakage by the antifoam agent which is dispersed in the carrier. Knowledge of the mode of action of antifoams is useful and also aids in selecting appropriate antifoam agents. Generalized mechanisms of foam breakage by chemical antifoam agents are presented in Table VI. It is evident from the table that these mechanisms are diverse in nature and are specific to the nature of the chemical antifoam agent.

It is interesting to study the mode by which antifoam agents are able to destroy foams. For example, antifoam agents with surface-active properties act by competitively replacing the surface-active compounds that

TABLE VI

MECHANISMS OF FOAM BREAKAGE BY CHEMICAL ANTIFOAM AGENTS

Reduction in surface elasticity
Changes in physical properties of foam
Displacement of foam-stabilizing surface active agents
Local reduction in surface tensions
Formation of mixed film
Discontinuities in surface tension
Promotion of drainage
Lowering of viscosity
Dissipation of double-layer repulsion forces
Increasing of surface viscosity
Improvement in the electrical conductance of the film

are causing the foam in the system (Solomons, 1967). The antifoam agent also enters the film between two bubbles and then spreads as a thick duplex film. The consequent setting up of a tension mechanically breaks the foam (Goldschmidt and Koffler, 1950; Ross, 1950; Robinson and Woods, 1948). In some cases, the antifoam agent spreads to a limited extent after entering the liquid film and thus produces a mixed monolayer with a foaming substance. The resulting poor coherence in the mixed monolayer breaks the foam (Bungay *et al.*, 1960). The above facts indicate that the positive spreading and/or penetration coefficient of the antifoam agents are involved in destruction of the foam. However, in spite of having these abilities, silicon antifoam agents are not able to break the foam unless hydrophobic, highly dispersed solid silica particles are present (Ross, 1950; Robinson and Woods, 1948). The mechanism in the case of silicon antifoams involves the transfer of the solid silica particles to the lamella from the silicon oil and the development of a contact between hydrophobic solids and the film of surface-active agents by spreading of the solids on the lamella (Kulkarni *et al.*, 1977; Ross and Nishioka, 1978). Consequently, the lamella bursts due to the resulting diminished concentration of the lamellae-stabilizing agents because of their adsorption on the surface of the hydrophobic solids. At the same time, the originally hydrophobic surface of the solid particles becomes hydrophilic and therefore the particles move from the oil phase into the aqueous phase and lose antifoam activity (Kulkarni *et al.*, 1977; Ross and Nishioka, 1978).

C. CRITERIA FOR SELECTION OF ANTIFOAMS

Often a great deal of expenditure is incurred in controlling foam in fermentors due to the use of improper defoaming agents and inaccurate

doses (Kouloheris, 1970). In spite of the availability of numerous commercial defoaming agents, no objective comparison of the performance of these agents is available except for some isolated reports (Sie and Schügerl, 1983). Laboratory tests for effective screening of defoaming agents and for zeroing in on proper dose have been developed by some workers (Kouloheris, 1970; Sie and Schügerl, 1983). The methodology developed by Sie and Schügerl (1983) gives a good evaluation, and is based upon the use of a bovine serum albumin system wherein the height of the foam is a function of time.

It is beneficial to evaluate various defoaming agents in laboratory and pilot-plant fermentors before selecting the most appropriate agent. But this is rarely followed and in most cases the selection is based on the availability of a particular antifoam agent in the laboratory or on earlier experience. In fact, evaluation studies need to be further continued, even after selection of the agent on preliminary screening, to select the dosage and the point of application in the foaming medium. It is also necessary to establish optimum environments such as pH, substrate concentration, microbial density, temperature, oxygen supply rate, oxidation rate, viscosity, and carbon–nitrogen ratio to delineate the effectiveness of the defoaming agent under evaluation (Howe, 1978).

The desired characteristics sought in antifoam agents are described by various workers (Evans and Hall, 1971; Solomons, 1967, 1969; Bikerman *et al.*, 1953) and are listed in Table VII. In addition to these major desirable

TABLE VII

DESIRED CHARACTERISTICS OF ANTIFOAMS

Fast foam breaking	Stability during sterilization
Long-lasting action	Insolubility in foaming medium
Nonexplosiveness	No chemical action with finished product
Nonvolatility	No imparting of color or odor to the product
Noncorrosiveness	Inability to affect O_2 transfer rates adversely
Low cost	Better ability to destroy surface elasticity/surface viscosity
Non- or low flammability	Nonresistance for relatively faster biooxidation/biodegradation
Nontoxicity to microorganisms, humans, and animals	High entering and spreading coefficients
Lower BOD and COD values[a]	Low intramolecular cohesive forces
Nonmetabolizability by microbial strain under use	Presence of some hydrophobic character
Inability to form corrosive products during sterilization/fermentation	
Low surface tension	
Low interfacial tension	
Effectiveness at low concentration	

[a]BOD, biological oxygen demand; COD, chemical oxygen demand.

characteristics, the degree of ability to form solid films or completely fluid films, ability to bring about changes in surface tension, hindrance to restoring processes of foams, local disruption of liquid films, and dispersion in the foaming system with a minimum of agitation determine the comparative effectiveness of the antifoam (Bickerman *et al.*, 1953; Bryant, 1970; Saito and Friberg, 1975). The overall cost of the defoaming operation plays an important role not only in the economics of the process but also in the selection of the defoamer. The cost factor is not generally analyzed properly and, as a result, cheaper antifoam agents with a low degree of effectiveness are employed with consequent higher expenditure. For example, antifoam agent X may be three times as expensive as antifoam Y. However, based on the requirement of a smaller dose for effective foam control, X may be seven times more effective than Y.

It must be understood that some of these desired characteristics are too rigid and demanding and therefore one may not be able to get the most ideal antifoam agent. For example, one of the desired characteristics is the nonmetabolism of the antifoam agent by the miocrobial strain employed in the fermentation. This is essential as otherwise higher doses at frequent intervals will be needed for effective foam control. Considering the voracious nature of microorganisms, it may be difficult to comply with this requirement. In such cases, the otherwise efficient antifoam agent is selected even if it is metabolized by the strain. However, care is taken to avoid those agents which are metabolized into substances that are antibiotic in nature or into fatty acids that cause larger foam buildup (Howe, 1978).

Sie and Schügerl (1983) have compared 17 different commercially available silicon emulsions in which the active compound varied between 16 and 100% and the concentration employed was between 0.00 and 0.01% of the active component. No simple relationship between foaminess and surface tension in the system was evident but the silicons with polyethers, silica, and modified polydimethylsiloxanes were found to be most effective. The other observations reported by them include higher efficiency of antifoam agents when foaminess is lower, diminished foaminess with increasing active component concentration as well as increasing aeration rates, diminished equilibrium surface tension with increasing antifoam concentration, and different comparative efficiency at low and higher concentrations of the antifoam agents in some cases.

Among the commercially available antifoams, polydimethylsiloxanes most nearly approach the ideal antifoam for use in fermentation. These silicon compounds are nontoxic and are permitted in foodstuffs up to 10 ppm. They are most suitable for bacterial fermentations at alkaline pH and for yeast fermentations. However, silicon defoamers are not satisfactory in mold fermentations because the mold itself inactivates the

silicons (Fink *et al.*, 1976). In addition, certain other disadvantages associated with them include breakup of the watery emulsion after sterilization, development of viscosity, difficult handling when used with carriers such as castor oil or liquid paraffin, and higher oxygen demand due to the metabolism of castor oil by microorganisms (Solomons, 1967; Fink *et al.*, 1976). Another promising antifoam for bacterial and yeast fermentation is Alkaterge C in paraffin or grape oils but it is quite expensive (Bryant, 1970). Texofor D 10, an alkonylated fatty base and alkylene glycol, is especially useful in media containing higher electrolyte concentrations (Solomons, 1967; Bryant, 1970).

For mold fermentations, octadecanol in cold-pressed lard oil is employed but has a moderate action and poor lasting effects (Deindoerfer and Gaden, 1955). A combination of glycerides, oils, and fatty acids is reported to be suitable for mold fermentations (Solomons, 1967) as it is nontoxic, heat stable, and moderately priced. Polypropylene glycol 2000 is, however, most suitable for mold fermentation. One reason is that it is inexpensive and very convenient in handling (Solomons, 1967; Shiro *et al.*, 1972). Moreover, polypropylene glycol is not metabolized significantly by microorganisms (Solomons, 1967). Other advantages include improved efficiency of microbial growth, no noticeable decrease in product yield, nontoxicity, effective long-lasting action, and control of oxygen disturbances in the medium (Asahi Denka Kogyo K. K., 1981; Kanegafuchi Chemical Ind., 1982).

D. Adverse Effects of Antifoams

It is well known that the use of chemical defoamers for control of foam during fermentation leads to many adverse effects. Changes in the physical properties of the culture broth, deterioration of the operational characteristics of the fermentor, and a marked decline in fermentor performance are reported to be associated with the use of chemical antifoam agents (Deindoerfer and Gaden, 1955; Dawson, 1961; Yagi and Yoshida, 1974). The presence of antifoam in the system also causes difficulty in extraction and purification of the product, formation of difficult emulsions in aqueous solvent systems, and the necessity for extra separation stages for the removal of defoamer from the product (Solomons, 1967; Evans and Hall, 1971; Hall *et al.*, 1973; Ebner *et al.*, 1967), thereby making the process more expensive. The effect of several biocompatible chemical antifoam agents on the performance of ultrafiltration membranes for yeast cell concentration was studied by Cabral *et al.* (1985). They reported decreased flux rates of water solution as well as the suspension of yeast cells and the cumulative fouling effect on the membrane. Although these studies were carried out in order to determine the efficiency of cleaning

procedures on cross-flow filtration of microbial suspensions in membrane ultrafiltration equipment, it amply illustrates the adverse effect of chemical antifoam agents in downstream processing operations involving ultrafiltration.

Enzyme systems of the fermenting microorganisms may be damaged by some of the oils used as defoamer or carrier, causing rates of sugar utilization to decrease and production of desired metabolites such as antibiotics to be inhibited (Bungay et *al.*, 1960). Also, the control valves and metering devices of the fermentor are reported to become clogged by the carbonaceous compounds or greasy sediments formed during microbial decomposition of the natural oils (Bungay *et al.*, 1960). Moreover, the diaphragms and gaskets of the fermentor system are softened by the oils and it is even necessary to keep the storage vessel and pipe lines warm in order to reduce viscosity and to aid the flow of the oil. The effect of soybean oil on mass transfer in bubble columns was investigated by Vanett Riet *et al.* (1984). They reported minor effects on mass transfer rate and bubble coalescence along with decreased foam formation in a sodium caseinate–water solution. Various other adverse effects of antifoams have been reported by other workers (Evans and Hall, 1971; Bungay *et al.*, 1960; Solomons, 1969; Blom *et al.*, 1952; Phillips *et al.*, 1960; Pfeifer *et al.*, 1952; Ishida and Isano, 1952; Rollinson and Lumb, 1953; Hall and Hassall, 1970; Beroviç and Cimerman, 1979; Fowler, 1982); these are summarized in Table VIII.

TABLE VIII

SOME ADVERSE EFFECTS OF ANTIFOAM AGENTS AND THEIR CAUSES

Effect	Cause
Changed pattern of dissolved gases	Rapid bubble collapse
Change in air bubble size	Effect on surface properties
Gradual drop in mixing power and consequent changed pattern of agitation	Buildup of air entrapment and consequent lower liquid density and partial flooding of impellers
Sharp increase in power requirement	Degassing
Changes in pH pattern	Release of fatty acids due to lipase action on the oils
Production of totally different metabolite (antibiotic)	Radical changes in metabolism
Preferential utilization of antifoams as carbon source	Characteristics of the strain under use
Decrease in product/biomass, reduced fermentation rate, delayed onset of metabolite production	Altered metabolism rates of microorganisms

The judicious use of the antifoam agent, in minimal possible quantities, is vital not only because it reduces the adverse effects but also because it can lead to improved economics in view of the high cost of defoamers. In addition, it prevents fine emulsion formation in the fermentation medium, which is highly desirable for maintaining a large surface area between the phases in many cases (Chemap A. G., 1977, 1978). Finally, the use of too low or too high a concentration of the antifoam agent can lead to stabilization of the existing foam (Sonntag and Strenge, 1970).

E. Effect on Oxygen Transfer Rate

Most antifoam agents have a profound effect on oxygen transfer rates (Yagi and Yoshida, 1974; Bull and Dempe, 1971; Sawada *et al.*, 1977), either lowering or increasing the rates. The behavioral properties of antifoams responsible for lowering (Solomons and Perkins, 1958; Deindoerfer and Gaden, 1955; Pirt and Callow, 1958; Ebner *et al.*, 1967; Phillips *et al.*, 1960; Phillips and Johnson, 1961) or increasing (Phillips *et al.*, 1960; Benedik and Heideger, 1971; Astarita, 1965; Zandi and Turner, 1970) oxygen transfer rates are presented in Table IX.

Several studies have indicated a marked drop in the uptake of oxygen and production of carbon dioxide soon after application of the antifoam dose (Deindoerfer and Gaden, 1955; Phillips *et al.*, 1960; Corman *et al.*, 1957). Such a waste of expensive sterile air and energy involving agitation efficiency is uneconomical. The lowering of oxygen transfer rates by some antifoam agents by as much as 12–50% (Evans and Hall, 1971;

TABLE IX

Behavioral Properties of Antifoams Having Effect on Oxygen Transfer Rate (OTR)

Increased OTR	Decreased OTR
Displacement of the barrier by a permeable silicon monolayer	Barrier for gas diffusion across the interface
Release of oxygen-depleted air from the foam and the consequent entry of fresh air in the vortex	Decreased effective contact between culture and air trapped in foam
Increased diffusibility of gases in liquids	Formation of large bubbles with a smaller surface-to-volume ratio
Increased interfacial area	Decreased contact time of gas bubbles with liquid medium
Progressive decrease in concentration of the antifoam in the medium due to its separation on the walls of the fermentor	Additional gas–liquid interfacial resistance due to surface-active nature of antifoams
	Gas diffusion across the interface to the extent determined by its monolayer

Solomons, 1967; Dawson, 1961) can have a serious effect on fermentations, such as in white vinegar production which is very sensitive to oxygen, and in bakers' yeast production, which requires high aeration rates (Ebner *et al.*, 1967). Hence it is essential to overcome or avoid the lowering of oxygen transfer rates by the antifoam agent. The strategy often used is to switch to another defoamer with a lesser effect on oxygen transfer rates (Solomons, 1967). It is also interesting to note that the addition of the antifoam agent in small increments depressed the oxygen absorption rates to a lesser extent (Phillips *et al.*, 1960).

In contrast, about a 10-fold increase in oxygen transfer rates was reported with some antifoam agents such as silicon compounds (Phillips *et al.*, 1960; Chain and Gulandi, 1954) and sodium dodecyl sulfate (Benedik and Heideger, 1971) as well as in certain fermentors such as the vortex type (Chain and Gulandi, 1954) or the Waldhof type (Brown and Peterson, 1950a) and in shake flasks (Starks and Koffler, 1949). Moreover, the values of K_La (volumetric mass transfer coefficient) in the presence of these antifoams were reported to be larger than the corresponding values in the absence of the antifoam at high driving forces (Astarita, 1965; Zandi and Turner, 1970). However, the K_La values were lower in the presence of antifoams than in its absence at low driving forces.

Phillips *et al.* (1960) reported interesting results linking the behavioral pattern of some of these defoamers, e.g., silicon-based compound, lard oil, and Hodag K-4, in enhancing the oxygen transfer rates. The silicon compound formed a thick gummy ring on the walls of the fermentor vessel and on the impeller shaft up to a height of about 4 in. above the liquid level in an unsparged fermentor. The lard oil formed a greasy layer in a similar manner, but not to the same extent. However, with Hodag K-4, the entire inside of the fermentor vessel became coated. The gradual increase in oxygen transfer rates as fermentation proceeded was therefore attributed to the progressive decrease in the concentration of the antifoam in the medium as it deposited on the walls of the fermentor (Phillips *et al.*, 1960).

F. Automatic Antifoam Addition Devices

The antifoam may be added in a single dose before sterilization or in necessary quantitites whenever foam forms during the course of the fermentation. Incremental or continuous addition in very low quantities is preferred to one large dose added arbitrarily at the beginning of the fermentation run (Evans and Hall, 1971). For continuous delivery, manual addition is too laborious, often leads to contamination, and dictates the presence of the operator at all times. Instead, the most popular method of delivery is the use of automatic antifoam addition devices which are

incorporated in modern laboratory fermentors and commercial plants (Kroll *et al.*, 1956; Bertholomew and Kozlow, 1957). All of these devices operate in a similar manner with minor variations or modifications, and consist of a foam sensor, an electronic controller, a timed delivery metering device, a pump, a reservoir for antifoam, delivery tubes, and a distribution device.

Various level-sensing devices such as conductance or capacitance probes, paddles, turning valves, or floats are used as foam sensors in automatic foam controllers for generating the signals. These activate, through the electrical circuit, the metering pumps which may be of various types such as a gear pump, reciprocating pump, centrifugal pump, diaphragm check-valve pump, flexible liner pump, peristaltic pump, clamps, or similar devices (Evans and Hall, 1971; Solomons, 1969). Next, the metering and addition of the antidfoam are effected by timed delivery through a valve. The latter may be any one of the various types such as a solenoid valve, a motor-driven hypodermic syringe pump, sequential timing of two solenoid valves with a diaphragm expansion chamber between the valves, a drip or sight feeder, a drum feeder placed under the fermentor, the blow pot system, or a pressurized oil system with needle valve and on–off delivery. In many cases, distribution devices such as a spray distributor, wicks, diverter bars fastened to the impeller shaft, a nozzle injector with discharge pointed upward or an air stream as carrier are used to improve the distribution efficiency of the antifoams over straight pipe entry (Bungay *et al.*, 1960; Redikul'tsev and Lit'vinenko, 1983).

Additional features of these devices include a delay mechanism to prevent oil shots due to flash or transient foam rise, devices to sense the change in capacitance as the foam becomes dielectric, and an alarm for manual attention (Solomons, 1967, 1969; Bryant, 1970). Two conductance probes, one at a higher level than the other, are also used to initiate addition of antifoam by the former and to turn off the system by the latter probe (Solomons, 1969). A trap and suction pump are also employed to collect any defoamer that is not caught by the foam and to return it to its container (Redikul'tsev and Lit'vinenko, 1983). In such a system, the air pressure in the defoamer container is kept higher than that in the fermentor for maintaining a constant recycling system. The use of a closed loop connected to the dosing system for continuous sterilization of antifoam and to eliminate delays in its discharge into the fermentor was also reported (Podgorski and Ichiniowski, 1982).

The automatic antifoam adding device developed by Anderson *et al.* (1953) uses a metering valve for regulating the flow of the antifoam agent. This valve is actuated by an electronic relay adjusted to prevent a false

response due to mycelial deposition on the probe. In another device, the actuated valve controls the air passage to the antifoam reservoir for controlled addition (Brown and Peterson, 1950b). Dworschack *et al.* (1954) have used an automotive spark plug with a 3-in. prong inserted through the cover as an electrode for making contact with the foam. The insulation of the electrode with porcelain was reported to be highly effective. The electrode actuates a solenoid valve which controls the addition of the antifoam agent. An internal timer was also provided to further regulate the time of addition to an interval of 3 seconds in a 3-minute period. In addition, a valve was used to throttle the antifoam agent. An electronic device was also developed by Pfeifer and Heger (1957).

A device for delivering definite increments of antifoam each time the foam comes in contact with an insulated electrode was developed by Stefaniak *et al.* (1946). In the system developed by Echevarria (1955), the electronic controller is actuated by two electrodes. The foam level in the reactor is maintained at or below the upper electrode tip. The use of an electronic interface controller working on the differences in electrical conductivity of the two liquids at the interface of the liquid and air was reported by Hersh *et al.* (1938).

Probeless automatic antifoam addition devices also have been developed and are reviewed in detail by Viesturs *et al.* (1982). Such controllers may be actuated based upon the set load on the fermentor shaft. Photoelements, fixed at the wall of the fermentor, are widely used and these actuate the controller when completely covered by the foam. The physical parameters of microbial growth may also be employed for actuating the controller. For example, two differentially actuated thermosensitive elements are placed in a heat-insulated sensor for generating the signal. The piezometric level meter is also employed for the above purpose (Viesturs *et al.*, 1982).

An electronic automatic foam controller, developed at the Central Food Technological Research Institute (CFTRI) in Mysore, India (Venkatakuppaiah *et al.*, 1978) is shown schematically in Fig. 2. It is based on conductance and includes a transistorized device with a solid-state time delay mechanism for eliminating the response to erroneous signals from sporadic splashing of fermentation broth on the sensor. A safety device which stops the air supply to the fermentor and simultaneously turns off the antifoam addition pump is also incorporated, and is automatically activated if the antifoam agent is added continuously for 2 minutes of addition and mixing time. In turn, this activates an audiovisual alarm to call the operator. The device is incorporated in an automated modular laboratory fermentor, the Digiferm, developed by CFTRI (CFTRI, Mysore, 1979–1980).

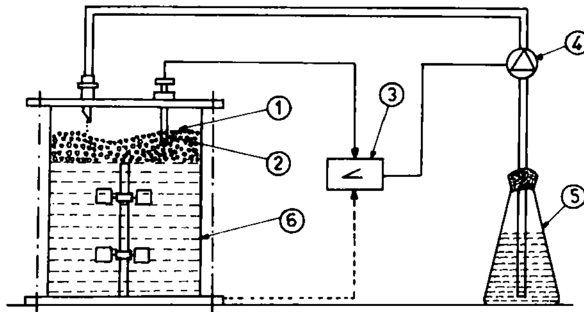

FIG. 2. Automatic electronic foam controller incorporated in the Digiferm laboratory ›rmentor developed by CFTRI, Mysore, India. 1, Foam; 2, foam probe; 3, electronic foam ontroller; 4, peristaltic pump; 5, chemical antifoam reservoir; and 6, fermentor.

The foam probe employed in automatic antifoam addition devices is ept inserted in the fermentor vessel through the head plate for the entire uration of the fermentation. It is essential that the fittings used for this urpose are airtight to prevent contamination. A commonly used fitting vhich does not allow contamination is shown schematically in Fig. 3. The conductance probes are reported to short-circuit due to collection f foam or liquid material across its insulation at the point of entry into he fermentor head plate or at the tip of the probe (Nakagaki, 1957). Thick nold growth deposited on the underside of the fermentor head plate can lso cause short circuiting, resulting in the addition of antifoam even

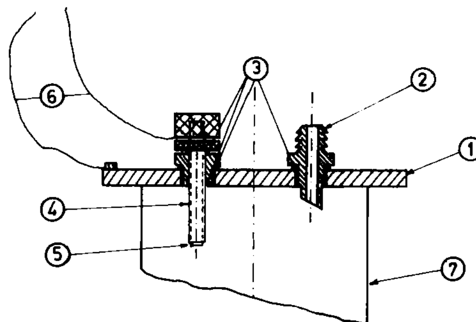

FIG. 3. Diagrammatic sketch of the airtight fitting for inserting a foam probe through the ead plate of the Digiferm fermentor and the antifoam addition port. 1, Head plate; 2, ntifoam addition port; 3, O rings; 4, Teflon cover of the foam probe; 5, metallic probe; , leads to foam controller; and 7, fermentor.

when it is not required. Such problems are eliminated in capacitance probes, since they are passive elements which cannot be fouled by such phenomena (Solomons, 1969). However, conductance probes are preferred due to their efficiency and lower cost.

The use of solenoid valves and conventional metering pumps is associated with various disadvantages such as valve leaks, sticking of oil in the devices, difficult absolute cutoff, maintenance of sterility, pronounced wear and tear, high cost, and deterioration of the delivery tube during sterilization as well as due to contact with oil. The drum feeder traps the moisture and even floats out the oil during sterilization and the drip or sight feeders are best suited for manual control. The on–off delivery by pressurized oil systems with needle valves is usually poor in performance. Motor-driven hypodermic syringe pumps are highly efficient for small fermentors due to their ability to add the antifoam dropwise at slow rate, but are expensive and of low capacity. Timed delivery by opening the valve for a desired interval is reported to be best even for larger fermentors (Evans and Hall, 1971; Solomons, 1967, 1969; Bryant, 1970).

VII. Physical Methods of Foam Breaking

Various physical devices to break foams rely on physical changes in the foaming region, and some of these methods are popular in the chemical industry. Physical methods based on ultrasound, thermal, or electrical treatments are employed in some fermentations; the ultrasonic defoamer is most studied. However, these methods are not popularly used in control of foam formation in fermentors because microorganisms are known to be highly sensitive to the influence of most physical factors (Viesturs *et al.*, 1982).

A. Ultrasonic Defoamers

The potential use of ultrasonic vibration for foam breaking was first suggested by Ross and MacBain (1944), and disintegration of foam by sufficiently intense ultrasonic vibration was reported by Gaden and Kovorkian (1956). Sonic defoamers are based on magnetic transmitters, aerodynamic as well as acoustic approaches and are of various types such as the Hartmann type (Hartmann, 1922), the Allen and Rudnick type (Allen and Rudnick, 1947), the cylindrical horn (Boucher, 1957), the Demister AB (Le Landers, 1960), and the nozzle–deflector type (Boucher and Weiner, 1963). Sonic defoamers transmit vibrations directly to the foam using a contact element consisting of a conical disk with radial holes and lateral rims or an acoustic transmitter (Viesturs *et al.*, 1982).

These are efficient even in destroying tough foams in flotation cells (Ramsey, 1948), in degassing of liquids (Hay and Shapland, 1974), in preventing dodecylbenzene sulfonate foaming (Hass and Johnson, 1965), and with highly frothable liquid (Shiou Chuan Sun, 1951). The destruction of foam by sonic defoamers is attributed to acoustic pressure, undirected radiation pressure, induced resonant vibrations in the bubble, high internal pressure in foam bubbles as compared to that in surrounding particles, vacuum caused by sonic energy, and turbulence produced by sonic waves (Boucher and Weiner, 1963; Steel and Miller, 1970). It was reported that radiation pressure forces are predominant at medium or high frequency and high intensity, whereas acoustic pressure and natural resonance frequency play the major role at low frequency and high intensity (Boucher and Weiner, 1963).

The device developed by Dorsey (1959) consists of an aluminum ultrasound generator fitted with a jet of 0.078-in. diameter. The depth of the jet is 0.078 in. It was mounted in a Plexiglas lid that fitted the top of the fermentor. The device is equipped with a vent for gas escape and filters for sterilization of incoming air. The device can also be installed inside the fermentor by fixing it at a distance of 8 in. from the surface of the liquid. In such cases, the vent for gas escape must be larger to allow the escape of gas from the foam as well as the effluent gas arising from the aeration of the medium. A line diagram of the assembly is shown in Fig. 4.

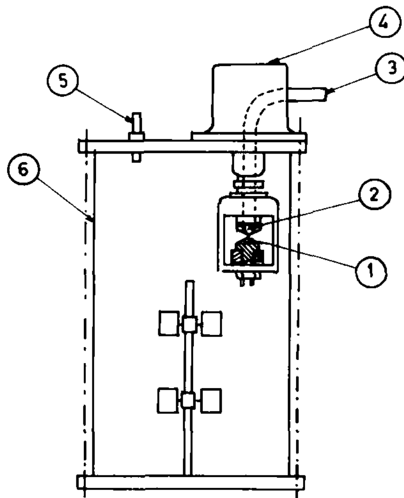

FIG. 4. Line diagram of a ultrasonic defoamer. 1, Primary resonance chamber; 2, nozzle; 3, air inlet port; 4, top body of defoamer; 5, air outlet; and 6, fermentor.

Sonic defoamers have been used successfully in fermentors and bioreactors by some workers (Dorsey, 1959; Steel and Miller, 1970; Dorothy, 1949; Miller, 1967; Hollfelder, 1967). Sound waves at 6–11 kc/second are reported to be able to control foam (Shiou Chuan Sun, 1951) and require 8–11.6 ft^3/minute air at 20–40 psig (Steel and Miller, 1970). The advantages (Miller, 1967; Boucher and Weiner, 1963; Solomons, 1969; Dorsey, 1959) and disadvantages (Dorothy, 1949; Roth *et al.*, 1956; Boucher and Weiner, 1962; Bryant, 1970; Schwan and Carstensen, 1952) of ultrasonic foam breakers are presented in Table X.

Recently, the effect of the exposure of brewers' yeast to 22-kHz ultrasound for 60–300 seconds was studied by Lyashenko *et al.* (1986), and enhancement of wort fermentation rate as well as the degree of alcohol production was reported. At an exposure rate of 120–180 seconds, the fermentation rate increased by 33–40% and alcohol formation was greater by 4.1%. The period of fermentation was also decreased by 1–2 days. Improvements in the quality of yeast and minimization of contamination by bacteria and molds were also stressed (Lyashenko *et al.*, 1986). Similarly, no harmful effect on *Serratia marcescens* was reported by Dorsey (1959). However, control of only 50–60% of foam in a 150-liter fermentor by a 10-kg sonifier was stressed (Dorsey, 1959).

B. Thermal Foam Breakers

Thermal foam breakers, as the name suggests, depend on the effect of temperature on the foam. The mechanism of collapse of the foam by the thermal method is based on the intensive expansion of the bubbles,

TABLE X

Advantages and Disadvantages of Ultrasonic Defoamers

Advantages	Disadvantages
Efficient control of medium viscosity foam	Detrimental effect on bacterial cells
Suitability for larger fermentors	Damage to spore coats
No moving parts	Poor acoustic coupling between air and water
Readily sterilizable	Noise problem
No effect on downstream processing	Need for exceptionally high air generation capacity
Ability to control tough foam	Too high power consumption
	Reflection of the majority of the ultrasonic energy
	Uneconomical

evaporation of moisture and solvent causing foam, decrease in surface viscosity, thermal degradation of the foam-producing material, freezing, and reduction in surface tension (Goldberg and Rubin, 1967). Commonly used thermal foam breakers include a hot wire over the surface of the solution or heating tape wrapped around part of the foaming region of the bioreactor (Karger *et al.*, 1966; Karger and Rogers, 1961; Gastrock and Reid, 1938; Poncha and Karger, 1965; Kishimoto, 1963; Shinoda and Mashio, 1960). Alternatively, a condensor through which steam or hot water is passed is placed near the top of the foam layer. In some cases, the heat is oriented directly toward the foam through either a nozzle or hollow shaft for better efficiency (Shkinder, 1975). Foam is controlled in some cases by lowering the temperature of the foam region of the reactor. A method involving heating and cooling cycles was reviewed by Viesturs *et al.* (1982).

Thermal foam breakers are rarely used in the fermentation industry, probably due to the sensitivity of microorganisms to temperatures. However, these methods are popular in the chemical industry. In fermentations, this method is used by M/s Distillers Co., Ltd., United Kingdom, and is based on the use of a heating coil to heat the upper part of the fermentor (Meyer, 1930). Molten tar or other suitable material is employed to heat the coil. These methods are cost intensive and are unsuitable with heat-sensitive materials (Goldberg and Rubin, 1967).

C. Electrical Foam Breakers

These devices are based on passing an electric current through the foamy region to break up the foam. Though the exact mechanism of foam breakage is not known, the activity is probably based on the creation of forces that act differently on liquid and gas (Viesturs *et al.*, 1982). The methodology employs grounding of the bioreactor and incorporates an electrode and a DC or AC source (Hirakawa, 1976). These techniques are rarely employed in fermentations. Moreover, information on the efficiency of these methods or the influence of the electric field on microorganisms is lacking (Viesturs *et al.*, 1982).

VIII. Mechanical Foam Breakers

Mechanical foam breakers are becoming more popular in modern laboratory fermentors mainly to overcome the disadvantages associated with the use of chemical antifoam agents. In many cases, their action is enhanced by simultaneously using chemical antifoam agents at the lowest possible concentration, as the mechanical devices alone were not found to control the foam effectively. The mechanical foam breakers are

especially useful when chemical antifoam agents cause undesirable contamination, or when downstream processing operations such as centrifugation, filtration, and drying are affected by antifoams, or when refoaming is desired for foam separation of the product (Goldberg and Rubin, 1967; Dorsey, 1959). The action of these devices is based on mechanisms such as rapid pressure change, shear force, compressive force, and impact force, either alone or in combination (Goldberg and Rubin, 1967), which lead to the collapse of the bubbles.

Mechanical foam breakers are indispensable in some fermentations wherein even the most efficient chemical antifoam agents are not able to cope with the copious foam formation. For example, cultivation of *Pseudomonas* sp. strain Rsan ver for the production of biosurfactant is impossible to carry out in a flat-blade turbine bioreactor equipped for automatic control of foam by chemical antifoam agent, due to excessive foam formation (Guerra-Santos *et al.*, 1983). However, a completely filled bioreactor with a standard mechanical foam breaker proved successful for growth and biosurfactant production by this strain. Another example is the process for white vinegar production, which is very sensitive to an interruption of oxygen supply. A large percentage of cells are affected by an interruption of oxygen supply even for a short period of time (Ebner, 1966; Ebner *et al.*, 1967). The use of a chemical antifoam agent increases the size of air bubbles and thereby lowers the rate of diffusion of oxygen into the fermenting medium, resulting in lower productivity. This example explains the nonsuitability of chemical antifoam agents in such fermentations and the consequent dependence on mechanical foam breakers. It is also claimed by Chemap AG (1977, 1978) that the whey-based yeast process is not economically feasible without a Fundafom, a mechanical foam breaker.

The drawbacks of mechanical foam breakers include high running cost, complicated as well as largely diverse designs, possible damage to the product or the microorganism, risk of disturbances to the unit operations of the process, and their effectiveness with only frail foam or under limited foam formation (Kotsaridu *et al.*, 1983; Hall *et al.*, 1973). In fact, these have failed with large-scale foam mass or with the foam formed by high aeration and mixing rates. Moreover, these are not usually preferred by the fermentation industry due to their expense (Kotsaridu *et al.*, 1983). For this reason, the need for more detailed investigations on this aspect was advocated by many workers (Hall *et al.*, 1973; Takahara, 1978a; Schügerl *et al.*, 1978). The difficulties in comparative estimation and scaling up of these devices are well known. However, a simple technique for determination of foam stability was developed, as it is an important factor in control of foam by mechanical means (Bumbullis and Schügerl, 1981). Recently, energy consumption and distribution in

bioreactors were studied by Viesturs *et al.* (1984) with particular reference to energy requirements of foam breakers.

A. Foam Breakers with Rotating Parts

Among the mechanical foam breakers, those with rotating parts are most popular and considerable work is reported on them. They can be of various types ranging from simple to complex designs. The simplest devices consist of either a bent, rotating stirring rod (Goldberg and Rubin, 1967), rotating paddle, or rotating vanes (Bryant, 1970) which are attached to the stirrer shaft. These devices, described in detail by Steel *et al.* (1955), are reported to destroy the foam by a combination of impact and shear forces (Goldberg and Rubin, 1967). Commonly referred to as whirling paddle foam breakers, these devices were used in fermentations by some workers (Burnner and Lemlich, 1963; Steel *et al.*, 1955; Walling *et al.*, 1952), and a diagrammatic sketch of the system is presented in Fig. 5. It is reported that these devices are not effective in collapsing wet and stable foams (Goldberg and Rubin, 1967). However, they may prove to be most effective when the foam is self-drained and has lost its self-healing properties (Bryant, 1970). They may also be of significant importance when used to supplement the action of a chemical antifoam agent (Steel *et al.*, 1955).

Another widely used foam breaker with rotating parts is the centrifugal or basket foam breaker, which brings about sudden pressure changes and the shear of bubbles in the spinning bowl (Goldberg and Rubin, 1967). Mounted on top of the fermentor tank, it can handle foams of different

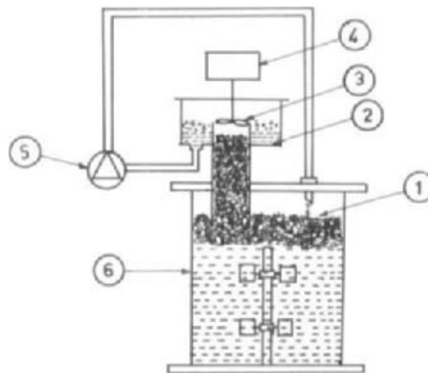

Fig. 5. Diagrammatic sketch of the whirling paddle foam breaker. 1, Foam; 2, condensed foam; 3, whirling paddle; 4, motor; 5, pump; and 6, fermentor.

composition and quantity (Ebner, 1966; Ebner *et al.*, 1967). In its simplest form, it consists of a device rotating inside an appropriate housing. In addition, a reflector is generally included with or without a flow pattern generator for flow of foam, liquid, and gases. The device breaks the foam bubbles by centrifugal forces into gas and liquid. The liquid is returned to the fermentor while the gas is allowed to escape into the atmosphere. The foams that are difficult to destroy are pumped back into the lower part of the vessel (Ebner *et al.*, 1967). However, a part of the foam bubble is reduced in size and thus the recycled foam is in the form of a more condensed emulsion. A capacity of up to 24 liters of foam collapsing per minute in a 4-in diameter and 4-in.-high basket device rotating at 3800 rpm (Hass, 1965) and operating up to a pressure of 28 psi has been reported, with an average consumption of 0.30–1.20 kW-hour power per 1000 ft^3 gas in the foam (Ebner *et al.*, 1967).

These devices employ either disks (Gurkin and Iosseliani, 1974; Chechura, 1976; Kalosha, 1979; Ibragimov, 1974) or conical dishes (Smirnov and Zuev, 1976; Verzhbitsky and Liplyagin, 1978; Zolotov and Filinnov, 1977; Kuzmichev, 1978; Shesterenko and Shesterenko, 1978). The latter consists of a disk with blades and a packet of conical dishes. Conical dishes are popular and are employed in laboratory, pilot plant, and industrial fermentors (Viesturs *et al.*, 1982). These conical dish-based devices can be added on separately and can be connected with the fermentor either permanently or temporarily (Anthon, 1977). Alternatively, they can be placed in a dome, open from below, and fixed at the upper part of the fermentor (Müller, 1978a,b). In addition, they can be separately connected to the fermentor by pipes for foam supply and feedback of liquid (Schering AG, 1977). They can also be fixed at a hollow shaft. In the other type of centrifugal foam breaker, rotating disks are fixed at various heights and the device is placed below the liquid level not more than half the disk diameter. In some cases, such devices mounted on the fermentor tank (Ebner *et al.*, 1967) or connected to a motor on the head space (Ohkawa *et al.*, 1978a,b, 1979).

The centrifugal foam breaker designed by Ebner and his group (Ebner, 1966; Ebner *et al.*, 1967) consists of a spiral housing, a rotor with radial blades, and inlet as well as outlet pipes. The spiral housing is sealed against the gas outlet by a peripheral ring and the rotor is placed parallel to its shaft. The rotor is belt-driven and turns at 1000–1450 rpm. The foam enters through the foam inlet pipe into the rotor assembly under a small positive pressure. After breaking the foam, the liquid as well as the undestroyed foam are thrown toward the circumference and pumped back into the lower part of the tank through a return pipe while the gas is allowed to escape through the gas outlet. It is claimed that the defoamer automatically adapts itself to the various kinds of dense or light foam

(Ebner *et al.*, 1967). A device based on shearing force of a high-speed rotating disk for complete collapsing of foam at a disk speed of 2500 rpm was developed by Goldberg and Rubin (1967). It was claimed that the device is simpler to build as well as to operate than the conventional centrifugal basket. The use of Teflon as wall material was also shown to be beneficial due to its highly water-repellent nature. It was, however, noticed that the device forms a small amount of foam at the container wall due to the liquid impact on wet surfaces resulting from the high disk speed.

Foam breakers with rotating parts are covered by various patents (Edwards, 1946; Gordon and Veldhuis, 1953; Humfeld *et al.*, 1952; Naucler, 1948; Yamaguchi *et al.*, 1978) and involve modifications for efficient function. Humfeld *et al.* (1952) used rotating vanes for effective foam control in fermentations for the production of bakers' yeast, torula yeast, subtilin, penicillin, and other antibiotics. The device developed by Naucler (1948) uses a stationary funnel-like casing, tapering downward, which encloses a concave plate rotating on its axis. The foam reaches the center of the plate through a pipe and is confined by a screen. The friction between the plate and the foam mass imparts rotation to the foam and thus the foam is thrown into the ambient atmosphere for transformation into liquid. The liquid is caught by the screen, from which it drops down into a collecting vessel (Naucler, 1948). A system of communicating pipes and targets is employed in the device patented by Gordon and Veldhuis (1953). A rotary device equipped with a gas-separating vessel placed at an equal or lower level of the foam ejector has been developed by Yamaguchi *et al.* (1978).

Considerable work on these devices was carried out in the USSR, culminating in various modifications for improved efficiency. These aspects are reviewed in detail by Viesturs *et al.* (1982), and some of the modifications are listed in Table XI. The efficiency of these devices is judged by rotational characteristics, restricting surfaces of the devices, and flow behavior patterns. The modifications suggested by various workers for optimum restricting surfaces (Plyehov, 1977; Bortnykov, 1977; Afanasiev and Krasnopyerova, 1977; Fadyeev and Cretkov, 1977) and flow patterns of foam, liquids and gases (Cameron, 1978; Hodgson and Gravis, 1977; Richard and Reese, 1977) are also dealt with in detail by Viesturs *et al.* (1982) and are summarized in Table XII.

In addition to these modifications, many other specific improvements or devices were shown to improve the foam breaking capacity of these units. These include (1) perpendicularly mounting the rotor with the lower part immersed in foam (Foch, 1976); (2) using a screw-type rotor for sucking and lifting foams; (3) using a pump for foam breaking along the whole fermentor volume; (4) having a propeller perform the role of a pump; (5) using blades with sharp edges and fixed at an angle; (6) using

TABLE XI

MODIFICATIONS/IMPROVEMENTS IN CENTRIFUGAL FOAM BREAKERS FOR EFFICIENT PERFORMANCE

Disk-based units	Conical dish-based units
Combining two disks with a hole on lower disk for foam sucking	Placing blades and mobile surface above the packet of plates
Providing supports for scattering destroyed foam	Using a separation chamber in the form of truncated cone
Mounting radial blades under the disk and placing them in mutually perpendicular planes	Placing a wall with holes in the lower part of the foam outlet
Locating radial blades at the larger diameter from the rotational axis	Fixing the packet of dishes inside the wall with the large base upward
Using an additional plate disk with perforated blades in a combined disks device	Providing a nozzle on the periphery for a liquid outlet
Providing gas distribution head in a combined disks device	Using a closed body unit with holes for foam drainage
Using a cone-shaped float	Making the lower dish hollow
Using a large number of vertical blades to form a squirrel cage	Using a funnel and bushing or restricting cone
Making support of the disk to adjust itself at the liquid–gas interface level	Designing working parts in the form of truncated cones with uneven inner surfaces and connected with the axial wheel
Providing controlled opening of blades depending on rotational speed and properties of foam	Fitting blades on the outside surfaces of the cone
Using a hollow rotor filled with bodies of irregular forms	Using an additional disk at shaft
Using conical vibrating disks	Placing radial plates opposite the rotor blades
	Using separate drives in opposite rotational directions for each cone
	Fitting with longer base facing downward
	Keeping tapering point in liquid layer
	Using a motionless conical dish fixed at top and at bottom

rotating wheels driven by gas or liquid and partially mounted in the upper part of the liquid; and (7) throttling the foam with a nozzle (Viesturs *et al.*, 1982).

A special device mounted in the apparatus for elimination of splashings from the fermentor, the use of a packet of disks mounted on a hollow shaft to enhance the phase rupture, and placement of the packet of smooth disks in a stationary drum for further enhancing the phase rupture as well as for lowering the energy consumption were also suggested by Soviet workers (Viesturs *et al.*, 1982).

TABLE XII

MODIFICATIONS FOR OPTIMUM RESTRICTING SURFACES AND FLOW PATTERNS OF FOAMS, LIQUIDS, AND GASES IN CENTRIFUGAL FOAM BREAKERS

For optimum restricting surfaces	For optimum flow patterns
Using a conical reflector screen	Directing the gas flow along the spiral but directing foam along the axis
Perforating the screen	Using a ring-shaped static device with blades along perimeter
Employing a reduction chamber	Imparting a different density to the gas during circulation
Covering a perforated disk with screen	Using a perforated cone-shaped disk with a hole in the center
Using guide blades	Scattering the flow with glass, plastic, or metal packing
Using a reflector composed of a number of truncated cones	Using conical sieves with a partition or sieve plate column
Using a horizontal reflector	Employing a motionless centrifugal deaerator
Partitioning the horizontal reflector into two chambers	Using a vertical partitioned device
	Employing a nozzle and ejector for throttling

Foam rupture is due to sudden changes that occur when the foam leaves the device as well as the shear of the bubbles in the spinning device (Goldberg and Rubin, 1967; Burnner and Lemlich, 1967). Consequently, various factors such as disk diameter, disk rotational speed, liquid feed rate, air sparge rate, and position of the disk govern the foam breaking capacity (Ohkawa *et al.*, 1972). Empirical equations are available for predicting the performance of the foam breakers (Ohkawa *et al.*, 1978b, 1979).

An improved device referred to as mechanical foam breaking rotating disk (MFRD) was developed by Ohkawa *et al.* (1978a, 1984a) to give effective foam breaking action in a stirred draft tube reactor. Various factors were investigated for reliable foam breaking operation of the MFRD (Ohkawa *et al.*, 1984a). Recently, the effect of impeller design on foam breaking and power characteristics with the MFRD was investigated using five different impeller designs (Ohkawa *et al.*, 1985). A six-blade vaned disk was reported to be superior to a six-blade turbine, six-blade paddle, or other designs. It was also stressed that this superiority is not just confined to the foam breaking performance but is also applicable to power requirements. Another device called a foam breaking apparatus with a rotating disk (FARD) was also developed by Ohkawa *et al.* (1984b) and was evaluated in a bubble column. In comparison studies of effectiveness, the highest ranking by FARD and lowest by spray nozzle (Sonada *et al.*, 1973) was reported; and it has been noted that the spray nozzle and

liquid-impact spray mechanism (Fuutai *et al.*, 1976) consume more noneffective power than effective power for foam breaking (Ohkawa *et al.*, 1984b). In addition, FARD offered operational flexibility (Ohkawa *et al.*, 1981a). The relationship between foam breaking performance and hydraulic behavior of liquid dispersed from a mechanical foam breaking apparatus was evaluated, and FARD was claimed to meet all these requirements (Ohkawa *et al.*, 1981b). The critical disk rotational speed required for foam breaking was found to be lower for smaller air sparging rate and working liquid volume as well as the larger feed rate and the disk diameter. In the case of FARD, the upper and lower limits of the disk diameter are about 80 and 50% of the column diameter, respectively (Ohkawa *et al.*, 1981b, 1983a,b).

Fundafom foam separators in various sizes have been developed and patented by Chemap AG (1977, 1978) for mechanical foam separation based on separating the used-up air phase from the liquid phase without allowing any droplets into the effluent system. The device may be centrally mounted on a flange on the fermentor cover within the fermentor vessel. It is also possible to mount the device off-center and at a 0–15° angle in a shoulder mounting. It consists of a rotating perforated hollow shaft, conical disks with radial vanes, a mechanical seal, and an electric motor and is equipped for pressure overlay, cooling water circulation, and steam condensation. It operates independently of the rotation speed of the fermentor stirrer and was reported to result in increased oxygen transfer rate values by 50% in the large fermentations over those observed in fermentors without the Fundafom. In addition, the device is claimed to have solved the most difficult foam problems in a simple and elegant manner (Chemap AG, 1977, 1978). The other advantages claimed include steady and noiseless operation, absolute sterility, reduction of aerosol load on the effluent air filter, the possibility of using a large working volume in a given fermentor, elimination of the need for use of chemical antifoam agent, and about 50% increase in production values for antibiotic, citric acid, and rennet fermentations. A diagrammatic presentation of a Fundafom-incorporated fermentor assembly is shown in Fig. 6.

Foam breakers with rotating parts have been used in fermentations by various workers, for example, in the production of vinegar as well as bakers' yeast (Ebner, 1966) and citric acid (Martin and Waters, 1952) and in the biological treatment of wastewater (Ebner *et al.*, 1967). Centrifugal foam breakers with rotating disks were also extensively evaluated by Ohkawa *et al.* (1978a,b, 1979). These are available commercially (Solomons, 1969) and are also patented (National Research Development Corporation, 1949; Denhard, 1949; Sharples, 1949; Schering AG, 1975; Vogelbusch, 1942; Jantzner, 1933). The use of centrifugal foam breakers offers various advantages such as providing the possibility of breaking

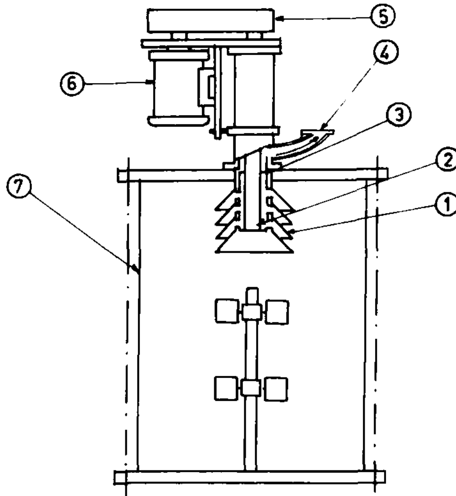

FIG. 6. Diagrammatic presentation of the Fundafom foam breaker developed by Chemap AG. 1, Conical disks; 2, perforated hollow shaft; 3, double mechanical seal; 4, air outlet; 5, transmission assembly for drive system; 6, electric motor; and 7, fermentor.

wet and stable foams of different composition and quality (Ohkawa *et al.*, 1978a), the direct feeding of substrate on the rotating disk, and the combination with a rotating shaft having wings for gas–liquid mixing (Ohkawa *et al.*, 1972). These are reported to be reliable and efficient (Ohkawa *et al.*, 1978a) and are suitable for cultures having low foaming potentials as well as low aeration requirements (Fiechter, 1962). Among the disadvantages, the complicated design of the device and the high cost of operation are critical. In addition, the need for increasing the rotating disk speed with the increase in the air sparging rate for reliable foam breaking action of MFRD has been stressed (Ohkawa *et al.*, 1984a). Moreover, rotating disk foam breakers of high speed on the order of 25,000 rpm were found to be unsuitable by Goldberg and Rubin (1967).

B. Cyclone-Type Defoamers

A cyclone-type defoamer usually consists of an entry pipe for flow of the foam, a cyclone separator for liquid and gases, a reverse circulation pipe for return of the liquid to the fermentor, and an outlet pipe for discharge of gases (Hass, 1965). Various modifications for better efficiency of foam breakage have been reported, including the use of twin cyclones, an additional perforated truncated cone inside the cyclone cone, baffles for forming a space for liquid flow, and variable cross section of channels

in the foam inlet pipe (Nieden, 1977). Foam breakage in these devices is reported to be due to the flow of the foam through the inlet pipe into the cyclone, forcing of gas to the cyclone center due to centrifugal forces for discharge through the outlet pipe, and the return of the liquid to the fermentor through the return pipe.

The cyclone-type foam breakers are rarely employed in the fermentation industry. However, considerable work on design, improvement, and evaluation of these devices has been carried out in the USSR (Viesturs *et al.*, 1982). These devices have been used in the production of biomass from starch industry by-products or molasses (Stakheev, 1976). The efficiency of cyclone-type foam breakers, in general, is not satisfactory in fermentations and failure to achieve foam breaking in molasses medium even with devices incorporating various modifications is reported (Viesturs et *al.*, 1982). It is often necessary to use these in combination with other mechanical foam breakers or chemical antifoam agents. Other negative aspects include difficulty in cleaning the cyclone and maintaining sterility. Moreover, mounting the cyclones in close proximity to the disk rotor leads to secondary foam formation (Viesturs *et al.*, 1982).

C. Impact Spray Foam Breakers

In its simplest form, the impact spray foam breaker consists of a nozzle and a sprayer. Its use involves jet spraying of the liquid or air on the foaming region of the medium (Sonada *et al.*, 1973). The distance between the top of the nozzle and the impact plate is a significant parameter and optimum results were achieved at a 0.5 mm distance (Fuutai *et al.*, 1976). The breakage of foam is reported to be due to the kinetic energy of the jets which act on the liquid film of the foamy liquid (Viesturs *et al.*, 1982). The strong jets cause impact, compression, and shear forces and their combined action is responsible for the breaking of the foam (Goldberg and Rubin, 1967). The nozzle is placed over a liquid layer (Fuutai *et al.*, 1976) while either simple or special sprays are employed. The fermenting medium, fresh medium, air, or nitrogen is used for impact spraying (Fuutai *et al.*, 1976). When part of the fermenting medium is used for spraying, it is drawn from the fermentor by pumps for feeding into the nozzle. The fermentor manufactured by M/s Vogelbusch, an Austrian firm, even combines the aeration and impact spray foam control systems by using deep jet aeration in the unit (Viesturs *et al.*, 1982).

Various modifications in the design and operation have been suggested for improved action and these include a combination of several nozzles at an angle of 80° with respect to foam level and spraying on the rotating disks of various designs. In addition, a fluid impact dispersion apparatus and a liquid impact spray mechanism have been developed by Fuutai *et al.*, (1976). In fermentation processes, the impact spray nozzle has been

successfully employed by Sonada *et al.* (1973) as a foam control device in cultivation of yeasts on n-paraffins. For the same fermentation system, improved foam control was achieved by Fuutai *et al.* (1976) using a fluid impact dispersion apparatus or liquid impact spray mechanism. A combination of liquid spray and chemical antifoam agent increases the efficiency of foam breaking to a considerable extent (Goldberg and Rubin, 1967).

Recently, interesting results have emanated from the evaluation studies conducted by Ohkawa *et al.* (1984a,b) on the impact spray nozzle developed by Sonada *et al.* (1983), the liquid impact spray mechanisms developed by Fuutai *et al.* (1976), and the foam breaking apparatus with a rotating disk developed by Ohkawa *et al.* (1984a,b) in a bubble column. The foam breaking apparatus with a rotating disk was given the highest ranking; the impact spray nozzle received the lowest. It was also noted that the impact spray nozzle and liquid spray mechanism consumes more noneffective power than effective power for foam breaking (Ohkawa *et al.*, 1984a,b). The high specific energy consumption is largely responsible for nonuse of these devices in fermentation processes (Viesturs *et al.*, 1982). The use of these devices is mainly confined to large-scale foaming in sewage effluent treatment plants and in experimental studies (Metzner and Brown, 1956).

D. Nozzle Foam Breakers

The nozzle foam breaker was first described by Hass and Johnson (1965), and works on the principle of rapid acceleration of foam through a nozzle (Phillips *et al.*, 1960). The foam is destroyed by sharp pressure changes or by pressure discontinuities (Hass, 1965) resulting from the throttling of the foam through a narrow hole such as a nozzle. The effluent air and foam are drawn through a nozzle by vacuum (Goldberg and Rubin, 1967) or by a pressure built up in the vessel at velocities in the range of 100–300 ft/second (Solomons, 1969). The sudden acceleration results in almost complete destruction of foam (Phillips *et al.*, 1960). After foam is broken into gas and liquid, the latter is returned to the fermentor by a positive displacement pump and the air is released to the atmosphere through an outlet pipe originating from the top of the device (Fig. 7). The pump operates only when foam formation is active as its operation is based on the completion of an electronic circuit.

A combination of a nozzle and an ejector was suggested for better action (Viesturs *et al.*, 1982). Interchangeable nozzles with varying diameters are also used to impart the required velocity (Solomons, 1969; Goldberg and Rubin, 1967) while the pump is controlled to operate only when the foam is forming actively. In experiments by Phillips *et al.* (1960), the head pressure in the fermentor usually did not exceed 5 psi. A pressure drop

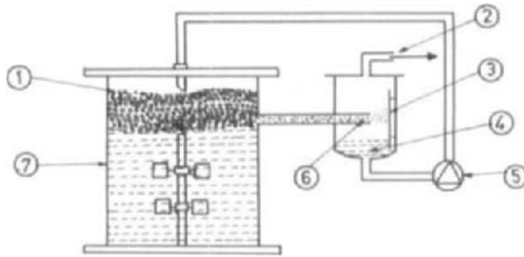

FIG. 7. Nozzle foam breaker assembly. 1, Foam; 2, vacuum-cum-gas exhaust line; 3, deflector; 4, condensed foam; 5, pump; 6, orifice; and 7, fermentor.

from the atmosphere for the inlet foam to 0.5 atm in vacuum is adequate and the device is effective with residual foam volumes of about 0.001 of the inlet volume for well-drained foam (Goldberg and Rubin, 1967). Nozzles of diameter 0.15, 1.0, and 2.5 mm were found to be equally adequate. Foam destruction occurs in the nozzle itself and the subsequent impingement may not be necessary (Bryant, 1970).

Nozzle foam breakers were used in fermentation by some workers (Phillips *et al.*, 1960; Phillips and Sallans, 1967; Loginov, 1947; Boiko *et al.*, 1962). Though the apparatus is very simple and the foam destruction is almost complete, it involves relatively higher power costs for pumping gas (Goldberg and Rubin, 1967) and thus has not found much use in fermentation. Moreover, the nozzle foam breakers are reported to be unsuitable for mold fermentations or for culture broths with particulate materials (Solomons, 1969).

E. SYNERESIS-BASED FOAM BREAKERS

Syneresis, a phenomenon involving natural breakdown of foams due to liquid escape (Bikerman, 1973), is also employed in some fermentors. It involves the natural running down of the liquid from the foam layer to the lower region, leading to gas escape. These devices are usually employed in the form of vertical cylinder vessels (Konstantinov and Prihodchenko, 1973; Graf, 1974) and are equipped with a central pipe for removing treated emulsion, a fan for sucking of gases, a multistep conical element, and partitions in the upper part with a plate fixed beneath it. Combinations of the device with chemical antifoam agents have been reported (Severov, 1974).

The typical device employed in the production of fodder yeast from molasses involves separation, settling, and mechanical destruction of foam. It consists of a diffuser, sprayer, hydroshutter, suction fan, dosator for chemical foam suppression, control windows, slop lever sensor, foam

level sensor, inlet pipe for slop, pipe for treated slop, circulation pump, and regulating valves (Viesturs *et al.*, 1982).

These devices are characterized by lower efficiency and larger space requirements. Moreover, syneresis is a complex multistep process and involves five simultaneous operations (Kann, 1979; Kann and Druzhinin, 1978, 1979a,b) which considerably affect the rate of syneresis. These were used in some cases in the 1960s, for example in the production of fodder yeast using molasses medium (Viesturs *et al.*, 1982). Compared to other mechanical foam breakers, they offer the advantage of low energy consumption. However, they are not employed currently because the disadvantages outweigh the advantages.

F. Vacuum-Based Foam Breakers

The ability of a vacuum to create sharp pressure drops is gainfully utilized in vacuum-based foam breakers. The application of vacuum results in a sharp pressure drop in the fermentor vessel and also in foam bubbles and, therefore, ruptures the interbubble film in a gas-liquid emulsion (Viesturs *et al.*, 1982). Various techniques have been suggested, such as contacting the foam with a porous plate beyond which a vacuum is maintained, occasional placing of the whole fermentor vessel into a vacuum chamber, collection of foam in a conical vessel connected to a vacuum source, and creation of the vacuum in a hollow whirl formed during whirling liquid flow at high speed (Anderson, 1977; Viesturs *et al.*, 1982). The vacuum was applied in impulses in the device developed by Nesmelov and Gulyaev (1968) and the medium was supplied with compressed gas during the intervals between the impulses.

These devices are not popular in fermentations and bioreactors despite extensive studies and good efficiency, probably due to the need for costly equipment and insufficient economic feasibility data (Viesturs *et al.*, 1982).

IX. Combined-Action Foam Control

Although the use of chemical antifoam agents offers advantages such as simplicity, ease of operation, and acceptable economics in most cases, its disadvantages are sometimes serious. Most of the efficient mechanical foam breakers are too complicated, consume a lot of power, and do not provide ease of operation. On the other hand, the simpler mechanical devices are not efficient enough to be used alone even though they are capable of overcoming the disadvantages associated with complicated

devices. Among the physical methods, successful foam control is possible with the use of ultrasonic foam breakers in some cases, but in-depth studies are required before it is considered safe for the strain under study. Thus, a single method may not be effective enough to eliminate the foam problem, and so the combined action of more than one type of defoamer has been practiced. These devices can be operated simultaneously or in a predetermined sequence. Alternatively, the least damaging device could be allowed to work alone, with another device to be pressed into service for supplementing the action of the former whenever it is not able to cope with the job.

Various combinations are reported in the literature. These include chemical and mechanical defoamers (Kallenbach, 1977), mechanical and thermal foam breakers (Bull and Solomons, 1977; Karger *et al.*, 1966; Sotirianos, 1979; Viesturs *et al.*, 1973), whirling paddle and chemical defoamers (Steel *et al.*, 1955), cyclone-type and mechanical or chemical defoamers (Viesturs *et al.*, 1982), liquid impact spray and chemical foam controllers (Goldberg and Rubin, 1967), fluid impact dispersion and liquid impact spray foam breakers (Fuutai *et al.*, 1976), and syneresis-based and chemical defoamers (Severov, 1974). Other combinations as reported by various workers (Smirnov and Zuev, 1974; Müller, 1978a,b; Petushinsky and Baskakov, 1975; Afanasiev and Krasnopyerova, 1978) are reviewed by Viesturs *et al.* (1982) and include combining disks with a blade and a packet of conical dishes, centrifugal and perpendicular forces, a cone and a chemical antifoam agent, centrifugal forces and filtration, and a static foam breaker with a moving centrifugal foam breaker. In addition to the above, a three-stage apparatus involving nozzle throttling, impact action by air, and striking against a blade has been described (Viesturs *et al.*, 1982).

Automatic devices for combined action have been developed. The device developed by Kallenbach (1977) for a combined system based on chemical and mechanical defoamers involves amplification of the signals from a sensor which alternatively actuates the mechanical and chemical foam breakers at set time intervals. An interesting and efficient automatic control system consisting of a rotating foam breaker and two automatic chemical antifoam addition devices was developed by Viesturs *et al.* (1982). The other components of the system include a foam level sensor A, final control elements B and C, delay lines, and the electrical/electronic circuit. Sensor A, when in contact with foam, actuates the mechanical foam breaker while the further actuation of the control loop is stopped if the foam is effectively collapsed by the mechanical foam breaker. However, if the foam is not broken effectively and reaches to control element B, it actuates a chemical addition device B, resulting in an impulse supply of the chemical agent through a solenoid valve.

The persistence of foam even after this leads to a signal via control element C to another chemical antifoam addition device C, employing an antifoam agent of greater efficiency. The unit is designed for effective economics in the process in that the antifoam agent employed in device B could be much cheaper, though relatively less efficient, compared to that employed in device C. The automatic resort to the use of a more efficient and costly antifoam agent employed in device C is made only when the foam is not controlled by the other two means.

X. Prevention of Foams by Appropriate Strategies

Although foam control systems are used extensively in practice, the inhibition of foam formation in the fermentor itself is preferable if it can be achieved without any adverse effect on the fermentation. In fact, foam formation can be minimized by the use of lower rates of aeration and agitation, but this usually affects the productivity adversely. In general, the control of foam in fermentors without external means is considered more or less impossible by most workers. However, it is stressed that the use of preventive measures would be far more advantageous and could be achieved by adopting appropriate strategies. For example, foam formation in proteinaceous media is mainly caused by protein denaturation and the Maillard reaction. Therefore, it may be possible to reduce foam formation in such media by employing shorter sterilization times or by reducing the sterilization temperature to the extent possible during sterilization of the medium (Kotsaridu *et al.*, 1983).

The foaming activity of a system might also be decreased without lowering the productivity by selecting non-foam-forming compounds as medium constituents and also by eliminating those which cause foam formation. For example, adjusting the mash composition to 2.0% amino acid, the fermentable-to-nonfermentable sugar ratio to 0.6, and external components to 5% was claimed to prevent foam effectively in a brewery (Q.P. Corp. *et al.*, 1984). Similarly, treatment of molasses with lime milk (Tuli, 1966) is recommended for overcoming the problems such as foam formation, coloration of yeast cells, and a lower growth rate due to the presence of colloidal particles in molasses.

The Waldof fermentor, which involves mechanical dispersion of air and simultaneous forced circulation through a central draft tube, is reported to combine the functions of fermentor vessel and foam controller (Saeman, 1947). The rapid passage of the medium through a central draft tube prevents accumulation of foam on the top of the liquid. The use of Waldof fermentors for the production of food yeast from waste substrates such as sulfite waste liquor, wood sugars, and wastes from dairy, cannery, and fruit processing industries was therefore advocated. In conventional

fermentors, these substrates pose difficulty due to excessive foam formation. In fact, foaming is one of the main obstacles to successful food yeast production from these substrates. In addition, the large quantities of chemical antifoam agents required for conventional fermentations based on these wastes constitutes a major expense. In Germany, centrifugal foam breakers were employed in Waldof fermentors, but it was shown by Saeman (1947) that the use of mechanical defoamers in Waldof fermentors was not necessary.

The comparative foaming capability of industrially used yeast strains was studied extensively by Ouchi and Akiyama (1971). About 3.5% of all industrially used yeast strains were found not to form any foam. It may even be possible to obtain mutants of the industrial strains which do not foam during growth and product formation stages. An interesting technique for prevention of foam formation in fermentors is based on manipulation of cultural parameters. It was reported by Schevchenko *et al.* (1978) that higher chitin concentration enhances the hydrophobic character of cells and makes them float better. This hydrophobic character reduces foam formation as well as consumption of chemical antifoam agents. Thus the manipulation of cultural conditions to impart hydrophobic character to the cells can be beneficial.

The use of mixed cultures of microorganisms was also proposed as a promising alternative to chemical foam control (Maiers and Veinir, 1979; British Petroleum Co. Ltd., 1976). It consists of a system in which the foam-forming substances produced by one culture are utilized by another. For example, in a methane fermentation based on *Methylococcus capsulatus*, the foam-forming compounds were found to be consumed by other bacteria belonging to genera *Moraxella*, *Alcaligenes*, *Acinetobacter*, *Agrobacterium*, and *Psuedomonas*. The use of such mixed culture fermentations for prevention of foam formation could be beneficial in other cases, provided it does not affect the productivity of the fermentation. Foam suppression by aerodynamic control was investigated by Chagin *et al.* (1985) in the production of riboxin in glucose—salt medium using *Bacillus subtilis* GEN 265. Intensive foaming between 15 and 40 hours of culture was observed and it was reported to coincide with intensive evolution of CO_2 in the fermentor. The foaming could be reduced by controlling aeration rates and CO_2 pressure and also by lowering the incubation temperature from 32 to 28°C. It was reported that foam suppression by this aerodynamic control was more effective than with chemical antifoam agents, and resulted in increased yield of riboxin (Chagin *et al.*, 1985).

XI. Research and Development Needs

For quick, efficient, and economic control of foams without significant adverse effects on fermentation, the following aspects require further study.

1. Better mechanical foam breakers should be developed, in view of the fact that no extraneous agents are needed by such devices to destroy foams. Alternatively, a simple mechanical device such as whirling paddles used in complement with very small doses of antifoam needs further evaluation. Research on the foam breaking capacity and effectiveness of whirling paddles is also lacking.

2. Careful investigations to ensure that no damage is done to microorganisms by sonic vibrations are needed before sonic defoamers are employed in fermentations.

3. The chemical and mechanical properties of the foams and their dynamic behavior should be evaluated systematically. Studies are also needed on factors stabilizing the foam, the relation between changes in foaming tendency and ingredients of the fermentation medium, the relation between foaming propensity and surface parameters of the medium, and the correlation between foaming and surface viscosity as well as surface tension.

4. Simpler devices to distribute the antifoam uniformly in the fermentor medium in the shortest possible time.

5. Studies on the synergistic effects of carrier on antifoam and any modification of the surface characteristics of either antifoam or the carrier would be interesting.

6. The effect on foaming of individual components of commonly used fermentation media needs to be determined so as to be able to replace them by nonfoaming components.

7. Studies are needed on the permeability of silicon antifoam monolayers to oxygen, carbon dioxide, and other gases, the reasons for low effectiveness of silicons in mold fermentations, and the effectiveness of various concentrations of silicons and other antifoams on oxygen transfer rates.

8. Systematic studies need to be done to select the correct antifoam for a particular foaming problem, to evaluate commonly used antifoams at various pH values employed in fermentations, and to search for oils with more specific properties.

9. The codification of chemical antifoams regarding their properties, suitability for various fermentation, rapidity of action, concentrations required, as well as periods for which further foam formation is prevented is essential to replace the current trial-and-error method for their selection.

10. Attention to the application of microbial mutants which do not foam during growth and product formation may prove very useful.

11. The use of mixed culture of microorganisms needs special attention as a promising alternative to chemical foam control.

12. Intensive research is needed for successful prediction of the foaming activity of fermentation media baseed on their composition.

13. The imparting of hydrophobic character to cells for reducing consumption of antifoam agent is worth investigating.
14. The relationship between surface phenomena and the behavior of cell populations within foam films needs to be studied (Atkinson, 1974).
15. Efforts should be made to develop energy-efficient systems for foam suppression/control with provision for reliable discharge of the used aerating gas without loss of culture medium, guaranteed sterility, as well as electronically programmed injection of antifoam agents.
16. Automatic control loops for the synchronous action of chemical and mechanical foam breakers operating in combination needs improvement for optimum efficiency.

XII. Summary

Foam, an agglomeration of gas bubbles separated from each other by thin liquid films, is a common phenomenon in aerated and agitated systems. Many theories have been put forth to describe the mechanisms of foam formation but few reports are available on the properties of the foam and the factors affecting its formation. In general, foam is controlled by employing, mechanical, physical, or chemical methods either individually or in combination.

Foam in submberged fermentations poses a serious problem and results in the loss of material through the exhaust lines as well as the danger of contamination of the fermentation batch and the environment. Its presence also acts as a barrier to oxygen transfer when surface aeration is used, as in the Waldof fermentor, and it reduces the operating capacity of the fermentor. The preferred method of foam control in fermentation is by the use of antifoam agents, although in a few instances, a mechanical foam breaker is used along with the chemical antifoam. In fermentations, the control of foam is a costly and nonproductive operation and therefore an effective foam control with reduced costs will greatly influence the economics of the process.

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Applications and Mode of Action of Formaldehyde Condensate Biocides

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I. Overview of Formaldehyde

A. HISTORY OF USE

The earliest published use of formaldehyde (FA) as an antimicrobial dates to 1886 by Loew and Fisher (Walker, 1964). Subsequently it was promoted as a vapor phase fumigant for infected enclosures. Its later use has been extensive in a variety of applications, including agriculture (seed fungicide, soil), egg incubators, cold sterilization, toxin and vaccine inactivation, and urinary tract infections. These areas have all been documented in a number of reviews (Nordgren, 1939; Walker, 1964; Tulis 1973; Hoffman, 1971).

There appears to be agreement on some properties—advantages and shortcomings—as well as lack of conformity. There are differences of opinion regarding the effect of organic matter and susceptibility differences between bacteria and fungi and the potential for resistance development. The use of FA in tanning and embalming attests to its

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reactivity, and it is no doubt because of its pungency the most recognizable of antimicrobial agents.

B. Chemistry and Pharmacology

1. *Chemistry*

Formaldehyde is available commercially as a 37–50% by weight aqueous solution. The one-carbon aldehyde is unique in its reactivity among carbonyls; all save FA have a C-C′ bond adjacent to the carbonyl group. This increases its polarity and its nucleophilic reactivity. Although it is structurally written CH_2O, it is only in that configuration in the gaseous or anhydrous form. In water it becomes hydrated to methylene glycol, $CH_2(OH)_2$, greater than 99% (Le Hénaff, 1963) although it is in equilibrium with the active carbonyl form. However, FA efficacy is directly related to atmospheric humidity levels in vapor phase. Under alkaline conditions, it readily polymerizes, another indication of its reactivity.

Formaldehyde is a nucleophile reactor (e.g., amines, amides, sulfides, purines, pyrimidines) (Feldman, 1973; Kallen and Jencks, 1966; Farrar, 1968). It also reacts readily with nitroalkanes, the electronegative nitro group directing the C-CH_2OH adduct (Dewey and Bollmeier, 1981). The reaction in all the above is C- or N-methylol which may be temporary or transient. The subsequent reaction being methylene bridge formation certainly has greater biological significance. These factors will be discussed later in Section I,B,2–3.

2. *Mutagenicity*

There is a considerable body of evidence demonstrating mutagenic activity of FA against both eukaryotic (Auerbach, 1951; Auerbach *et al.*, 1977; Ashby and Lefevre, 1982) and prokaryotic species (Englesberg, 1952; Temcharoen and Thilly, 1983). The reactivity of FA described above is undoubtedly the basis of subsequent genetic damage. The earliest reports (reviewed by Auerbach *et al.*, 1977) on studies with *Drosophila* are most interesting and have a bearing on potential applications as well as mutagenicity. Initially reported by Rapoport in 1946 (Auerbach *et al.*, 1977), *Drosophila* fed FA-treated food underwent mutations. This initial study involved eggs or young larvae and has been confirmed several times since. Only young male larvae showed mutational effects. Toxicity was negatively correlated with mutagenicity. That is, poor survival and delayed development preempted mutagenicity.

Food composition has a bearing on mutagenicity; adenosine or adenylic acid were necessary, while adenine was not. Treatment of adults with FA vapor was ineffective, and injection with formalin only produced

mutations at low frequency. These food findings in *Drosophila* may have significance in interpretation of data on mode of action, especially as it may relate to the moiety delivering FA to the sensitive site. Perhaps among the most active reactants with FA are free primary amino groups, forming amino methylol adducts; these subsequently react with other biologically active groups (e.g., nucleotides) as the basis for mutagenicity (Feldman, 1973).

3. *Toxicological and Environmental Considerations*

The estimated production of FA in the form of 37% solution in the United States reached 5.7 billion pounds in 1983 (Greek, 1984). Formaldehyde is a major building-block compound used in industrial processes and consumer products. Its major end uses include adhesives (60%) and plastics (15%), with the main derivatives being urea-FA resins, phenol-FA resins, polyacetal, and butanediol. Formaldehyde-derived resins are used primarily in manufacturing particle board, insulation, appliances, and automobiles, and residual FA vapors are known to off-gas from some of these products. The potential for both occupational and environmental exposures to this chemical is thus considerable (Starr and Gibson, 1985).

There are many reports on toxicity of FA. The Chemical Industry Institute of Toxicology (CIIT) reported in 1980 and 1983 that FA is carcinogenic in rats and mice (Swenberg *et al.*, 1980; Kerns *et al.*, 1983).

The National Institute of Occupational Safety and Health (NIOSH) has revised its standards and recommendations regarding levels of FA concentrations in the working environment. The standard for the workplace air is that it shall be controlled so that no employee is exposed to FA at a concentration greater than 5 ppm with acceptable ceiling, 10 ppm maximum ceiling for any 30-minute sampling period (U. S. Dept. of Health and Human Service, 1986). Furthermore, NIOSH refers to FA as a potential human carcinogen and recommends a limit described as 0.1 ppm ceiling (15-minutes); that represents the lowest reliably quantifiable concentration for recommended exposure limit (REL).

In setting the threshold limit values (TLVs) for chemical substances in the work environment, the American Conference on Governmental Industrial Hygienists (ACGIH) also put FA as a suspected human carcinogen. The time-weighted average (TWA) threshold limit values for a normal 8-hour workday and 40-hour workweek are given as 1 ppm. The short-term exposure limit (STEL) concentration adopted is 2 ppm (TLVs for chemical substances in the work environment adopted by ACGIH in 1986).

In an article published in 1979, Loomis described the recognizable levels of FA concentrations by individuals in the workplace and their reaction.

The threshold for recognition of the odor of FA in the air varies between individuals from 0.12 to 1.2 mg/m^3. As the concentration of FA is increased above the detectable concentration, exposures of 0.5 to 1 hour produce primary irritation to the respiratory tract. Numerous studies suggest that as the concentration rises above 2–5 ppm, complaints of irritant effects and signs of irritation of the pharynx, lungs, and eyes and some erythema of exposed areas of skin such as the face and neck will be seen. Acute exposures much greater than the maximum threshold of detection would be expected to produce severe acute pulmonary edema after only a few minutes of exposure.

Allergic dermatitis from skin contact as well as from exposure to gaseous FA in the air has been demonstrated (Loomis, 1979).

The National Institute of Occupational Safety and Health has listed 52 occupations that involve potential exposure to FA. Continuous exposure in the working environment to biologically active quantities of FA is evident in the laminating, textile garment, and urea-FA resin molding industries. There are also studies reported on employees in wood processing and filter manufacturing plants. Professionals such as pathologists, embalmers, and nurses working with FA-treated equipment are also referred to as examples of FA-exposed subjects in many reports (Loomis, 1979).

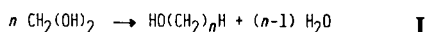
Risk estimation studies indicate the importance of additional factors to be considered in FA toxicity. Most important is the relationship between administered dose and delivered dose. The administered dose is an external measure of exposure directly controlled in laboratory studies of toxicity. In contrast, the delivered dose is an internal measure of exposure referring to the quantity or concentration of the biologically active form of a test chemical present in specific target tissues (Starr and Gibson, 1985; Starr and Buck, 1984; Hoel *et al.*, 1983; Starr, 1983).

II. Structural Relationships of Formaldehyde Condensates

A. Paraformaldehyde and Methoxy Derivatives

1. *Paraformaldehyde*

Paraformaldehyde was first prepared by Butlerov in 1959 (Walker, 1964) by vacuum distillation of FA (I). Its structure was erroneously reported



$$n\ CH_2(OH)_2 \longrightarrow HO(CH_2)_nH + (n-1)\ H_2O \qquad \text{I}$$

as trioxymethylene (CH_2O_3) but in truth is a mixture of various linear polymers of FA from $n = 8$ to $n = 100$. It is commercially available containing the equivalent of 91–93% FA. The polymer is completely and quickly vaporized to FA vapor at 224°C and gradually vaporized at room

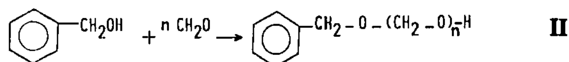
temperature. Both dilute alkali and acid induce polymer hydrolysis but by slightly different mechanisms; at the former pH values, hydroxyl end groups are attached followed by stepwise release of FA, while with the latter pH values, internal ether linkages are also attacked. Hydrolysis of paraformaldehyde is more rapid at lower pH. Dissolution and depolymerization are more readily accomplished in water at higher temperatures, and solutions are equivalent to those prepared by FA gas. Paraformaldehyde solutions have the recognizable pungent odor of FA.

Paraformaldehyde is a major source of high concentration FA for generating vapor phases (Figs. 6 and 7) (Tulis, 1973) and for synthesis of other derivatives.

2. *Methoxy Derivatives*

Alcohols react with FA to form hemiformal or methyoxy derivatives. In a number of cases, polyoxy compounds may be formed from nCH_2O.

The reaction of benzylalcohol with FA (II) yielded an adduct with



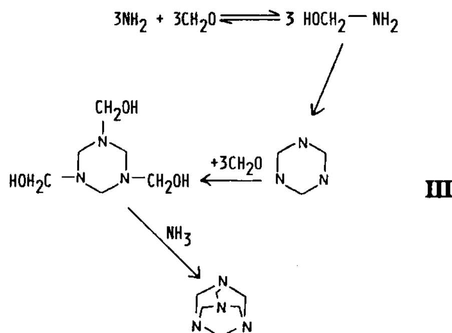
greater activity than either the FA or the benzylalcohol (Paulus, 1976).

In the synthesis of these hemiformals, care must be taken to keep the pH between 6 and 9. Above pH 9, the aromatic ring is methylolated, resulting in a compound with less than expected activity.

B. Cyclic Aminals

1. *Hexamethylenetetramine and Derivatives*

Hexamethylenetetramine (HMT) was first prepared by Butlerov in 1859 (Walker, 1964) from paraformaldehyde and ammonia (III). Although our



major interest is in its antimicrobial activity, this is only a minor portion of its use. It is a convenient form of solid anhydrous and stable FA. The

compound can be prepared by both FA solutions or gaseous FA with NH_3 added. It is readily soluble in H_2O, and the reaction is exothermic with slight decreases in solubility as temperature is increased (47% solubility at 0°C and 45% at 50°C). It is extremely soluble in aqueous solution at room temparature, hydrolysis being affected by pH below, but not above, 6.

In terms of its reactivity, it behaves either as FA after hydrolysis or as a tertiary amine and is quaternized (Graymore, 1938). It is this latter reaction that was subsequently exploited by Jacobs and Heidelberger (1915; Jacobs *et al.*, 1916a,b) in a series of adducts based on halogenated organic compounds. In addition, HMT readily forms salts with organic acids; this has proven important in the construct of derivatives used in treating urinary tract infections where the organic acid component is purported to lower urine pH and thus facilitate hydrolysis of HMT (Musher and Griffith, 1974).

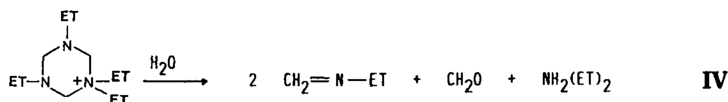
To this end, a clinical study involving HMT hippurate (HP) (Miller and Phillips, 1970) was studied in a simulated clinical trial. Subjects were given the HP over a 24-hour period, and pooled filtered urine was collected at 4 and 12 hours postmedication. Pooled urine was challenged with four test organisms associated with urinary tract infections. Of most interest is the relationship between urine pH and bacteriostatic activity. Regardless of HMT levels, there was no activity at 6.5 pH or above.

Two other points of interest in this article relate to FA mode of action. A greater amount of FA (25 μg/ml of urine) is needed for inhibition when added directly to the urine than when derived from medicating (18 μg/ml). It would appear that at appropriate pH values favoring hydrolysis of HMT, the FA generated would be more economically used in reactions involving biologically sensitive molecules. The externally added FA is probably more prone to nonspecific reactions unrelated to biological activity. The authors also noted that FA activity was greatly diminished when tryptic soy broth was used as a diluent for the urine samples. The reactivity of FA with nucleophiles associated with protein hydrolysates is now well established.

A most interesting group of derivatives based on quaternized HMT (Scott and Wolf, 1962) has been described. The reaction of a series of aliphatic and aromatic halohydrocarbons with HMT resulted in these quaternary adducts which were compared with both parent compound and FA against six organisms (two grams positive and four gram negative). The best of these were equal in effectiveness to FA and 10 times more effective than HMT. Three of the adducts were evaluated with and without a lecithin-Tween mixture to neutralize putative activity from the quaternary ammonium cation. In an earlier series of reports (Jacobs *et al.*, 1916a,b), activity of a related group of compounds was imputed to the cation as well as the side chain of any hydrolysis products. The lecithin-

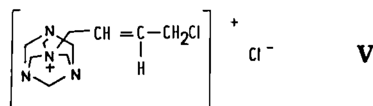
Tween mixture successfully neutralized an established quaternary germacide but had no effect on the activity of the HMT adducts.

Polarigraphic measurements of FA levels at several pH levels (Table I) showed that the quaternized adducts did release FA above pH 6 while HMT itself was entirely refractory at those pH levels. Hydrolysis was slow, not stoichiometric, and less complete at higher pH. The levels of FA obtained were comparable to those reported by Graymore (1938) with quaternized hexahydroethyltriazine (**IV**, where ET denotes).



IV

Notice that the highly reactive methyleneimine derivatives should react with H_2O to yield N-methylolethanolamine, a secondary source of FA. It would appear that quaternizing results in potential products with slow FA release capabilities which could increase the utility of HMT as a FA reservoir. One such product currently commercialized is used in a variety of applications, including paints, cosmetics, and metalworking fluids. This compound, 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride



V

(Q) (V), was one of 15 compared to FA in a cross-resistance study with a genotypically resistant and sensitive strain of *Pseudomonas aeruginosa* (Sondossi *et al.*, 1986a).

Especially in contrast with the hexahydrotriazines (Fig. 1), the stoichoimetric relationship between FA and Q does not exist based solely on the total number of FA molecules involved (i.e., 6/1 Q/FA). Rather, a dose reflecting both slow release and partial release (Scott and Wolf, 1962) was needed. In fact, cells induced with 3 mM FA were completely insensitive to 4.64 Mm Q (potentially about 28 Mm FA) or nine times the induction FA level. Bear in mind that this compound in 24 hours only released about one-third of the potential FA (Scott and Wolf, 1962). This dose is in the range recommended by the manufacturer and approved in its EPA registration.

However, it should be emphasized that although slow release may be a blessing in certain preservative applications with minimal initial contamination, use in grossly contaminated situations could lead to early resistance development to this compound as well as others based on FA. When assayed with Nash reagent (Table IV), almost all of the FA was

TABLE I

EFFECT OF pH ON FORMALDEHYDE RELEASE FROM HEXAMETHYLENETETRAMINE (HMT)/HALOHYDROCARBONS[a,b]

Compound[c]	Recovery based on 6 mol formaldehyde per compound (%)							
	pH 4		pH 6		pH 8		pH 10	
	24 hours	1 week	24 hours	1 week	24 hours	1 week	24 hours	1 week
Formaldehyde	104	104	98	97	97	90	97	80
HMT	17	44	4	21	0	0	0	0
HMT-3-chloropropene	74	105	52	86	46	66	35	35
HMT-1,3-dichloropropene	32	70	31	46	28	36	23	35
HMT-chlorotoluene	31	64	31	43	26	39	24	38

[a]Modified from Scott and Wolf (1962); see Rossmoore (1979, 1983).
[b]Measured polarigraphically for free formaldehyde.
[c]500 ppm.

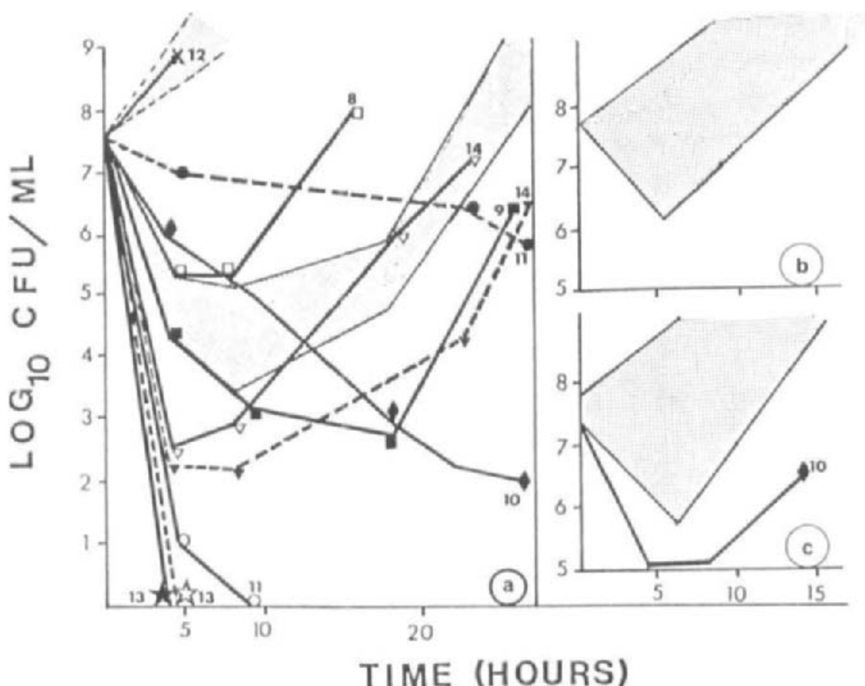


FIG. 1. (a) Exponentially growing sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to 3 mM of formaldehyde (FA) and equimolar concentration of the following biocides (3 mM available FA): (1) FA, (2) Hexahydro-1,3,5-trisethyl-s-triazine, (3) Hexadryo-1,3,5-tris(2-hydroxyethyl)-s-triazine, (4) 4,4-Dimethyloxazolidine and 3,4,4-trimethyloxazolidine, a commercial mixture, (5) 2-(Hydroxymethyl)aminoethanol, (6) 2-(Hydroxymethyl)amino-2-methylpropanol, (7) A commercial mixture of (3) above and sodium 2-pyridinethiol-1-oxide, (8) 1,3-(Dihydroxymethyl)-5,5-dimethylhydantoin, (9) A commercial mixture of 5-hydroxymethoxymethyl-1-aza-3,7-dioxabicyclo[3.3.0]octane, 5-hydroxymethyl-1-aza-3,7-dioxabicyclo[3.3.0]octane, and 5-hydroxypoly[methyleneoxy(C_2,C_3,C_4-,C_5)]methyl-1-aza-3,7-dioxabicyclo[3.3.0]octane. Shaded areas show the range of results with the preceding biocides. Area bounded by solid line, S strains; dotted line, R strains. Exceptions: (8) S (□——□) and (9) S ■——■).] (10) 1-(3-Chloroallyl)-3,5,7-triaza-1-azoniaadamantane, 4.64 mM (1000 ppm active); S (♦——♦). (11) *N*-Methylolchloroacetamide, 8.16 mM (1000 ppm active); S (o——o), R (●---●). (12) Tris(hydroxymethyl)nitromethane, 13.24 mM (2000 ppm active); S (×——×). (13) 2-Bromo-2-nitro-1,3-propanediol, 1.5 mM (3000 ppm active); S (★——★), R (☆---☆). (14) 4-(2-nitrobutyl)-morpholine and 4,4'-(2-ethyl-2-nitrotrimethylene)dimorpholine (585 ppm active), a commercial mixture; S (△——△), R (▼---▼). (b) The S strain was induced with 3 mM of FA. Then cells were exposed to the above biocides at the given concentrations [except that (12), (13), and (14) are not included; see text]. (c) The S strain was induced with the above biocides at 3 mM available FA, and then exposed to 3 mM of FA. [Exceptions: 3.64 mM (750 ppm) of biocide (10), 6.12 mM (750 ppm) of biocide (11), 16.55 mM (2500 ppm active) of biocide (12), and 585 ppm of biocide (14) was used, and cells then exposed to 3 mM of FA. Biocide (13) was not included; see text.]

detectable, a result emphasizing the distinction between free FA and repository FA.

One other variation of the adamantanes available commercially, 1,1-(2-butylene)bis(3,5,7-triaza-1-azoniaadamantane) chloride (**VI**), has been used primarily for preservation of latex paints.

VI

2. *Hexahydrotriazines*

The reaction of primary amines with FA yields as a primary adduct a saturated symmetrical triazine. Several will be discussed in the following section. The first of these, hexahydro-1,3,5-triethyl-S-triazine (HET; **VII**) was synthesized in 1904. Although HET is demonstrable as the major

VII

product with NMR data (Fig. 2), there is evidence that qualitatively the resultant may be an equilibrium mixture of several compounds (H. Eggensperger, personal communication). In the presence of even low levels of equilibrium products, the potential exists for interactions with competing reactions (**VIII**).

VIII

The hexahydrotriazine molecule is purported to be extremely stable at neutral to alkaline pH levels, presuming no hydrolytic release of FA under those conditions. This presumption makes it difficult to explain biocidal activity at all pH ranges. In an attempt to establish the source of FA activity vis-à-vis pH, a typical FA reagent was used qualitatively to detect FA release with pH variation, (DeMare *et al.*, 1972; Rossmoore, 1979). None of several FA condensates were FA positive at alkaline pH (Table II).

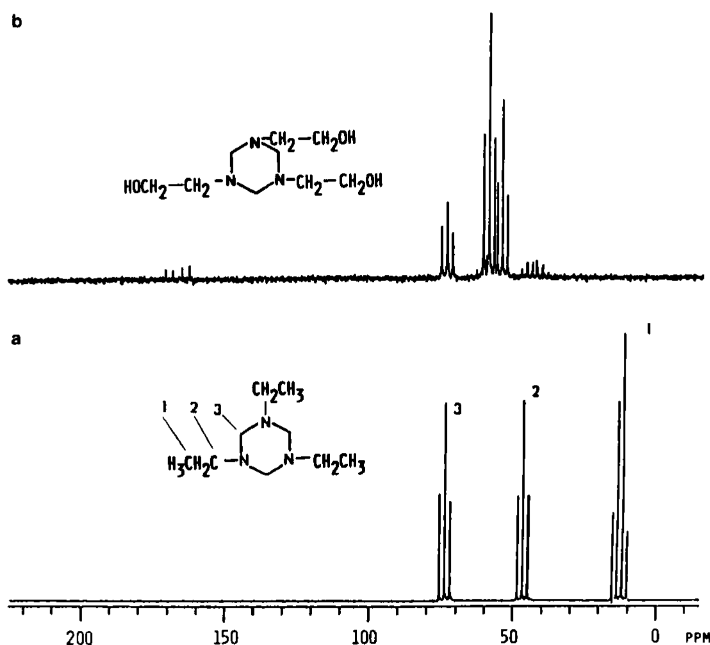


FIG. 2. 75 MHz, ^{13}C NMR spectrum of (a) hexahydro-1,3,5-triethyl-s-triazine and (b) hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine. The numbered signals of (a) correspond to the numbered carbons shown in the figure. The signals of (b) indicate presence of several species of carbon.

TABLE II

FORMALDEHYDE DETECTION FROM HEXAHYDRO-1,3,5-TRIALKYL-S-TRIAZINE BY DIMETHONE COMPLEXING[a]

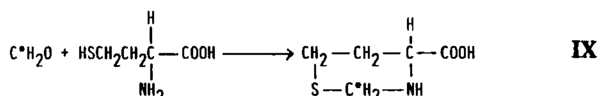
Triazine side chain[b]	pH 5	pH 9
EtOh	+	–
PropOH	+	–
Et	–	–
XOH[c]	+	–
YOH[c]	+	–
None (HMT)	+	–

[a]For method, see DeMare *et al.* (1972). +, Formaldehyde detected; –, formaldehyde not detected.

[b]EtOh, 2-hydroxyethyl; PropOH, 2-hydroxypropyl; Et, ethyl; HMT, hexamethylenetetramine.

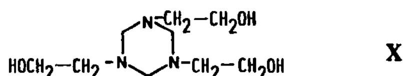
[c]Based on NMR data.

The controversy concerning the primary or exclusive mode of action of these compounds (i.e., FA or not FA) was based on the reluctance of manufacturers of commercially viable products to concede (for toxicological considerations) that FA was significantly involved. The failure to demonstrate FA release qualitatively in the absence of a microbial population did not help in the dilemma regarding mode of action. Neely (1963a,b) reported on the metabolic effect of FA in *Enterobacter aerogenes*. He showed that low levels of CH_2O interfered with protein synthesis by blocking methionine biosynthesis. Using homocysteine (a methionine precursor) and $^{14}CH_2O$, a stable six-membered ring was formed (**IX**). The assumption was that the FA did not react with the cell envelope component.



IX

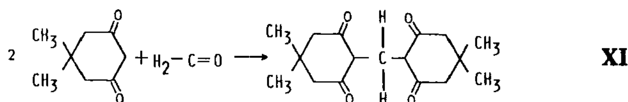
In a subsequent study (Holtzman and Rossmoore, 1977), homocysteine was added to FA or hexahydro-1,3,5-tris-(2-hydroxyethyl)triazine (**X**)



X

(HEAT) at pH 4–9. The homocysteine inhibited the activity of both the FA and HEAT (Fig. 3). This appeared to be convincing evidence that FA was both released and involved in lethality. Although these results appear to contradict the earlier study with 1,3-cyclohexanedione, it should be emphasized that this latter study was done in the absence of organic substrate or bacteria. The specificity and sensitivity of analytical reagents for FA have been well documented (Turoski, 1985; Walker, 1964; Sawicki and Sawicki, 1975).

The dimethone reaction (**XI**) may not be sufficiently avid for FA to cause



XI

an equilibrium shift with resulting hexahydrotriazine hydrolysis. That is, the propensity for cyclization, especially at alkaline pH, precludes release of detectable FA. The analytical paradox is to separate free and combined FA and to be convinced that the technique for measuring "free" is not dependent on the relative avidity of the free and the adduct for the reagent involved in detection (Feldman, 1960, 1973). Thus, there is

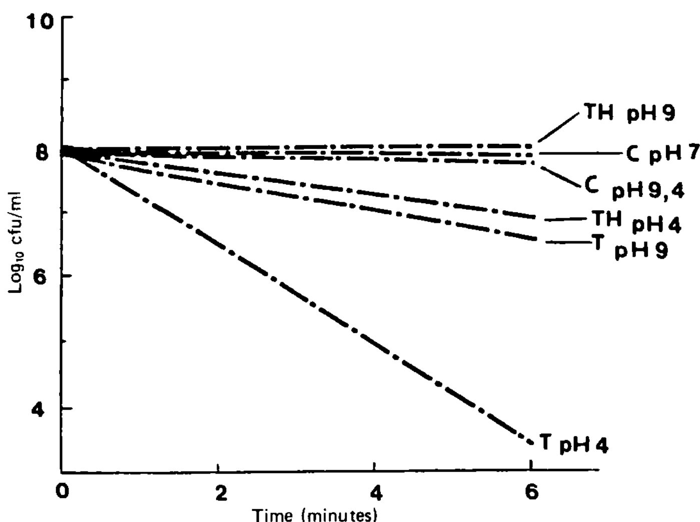


FIG. 3. Neutralization of hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine by homocysteine. Inoculum was 8.78 × 10^{10} *P. aeruginosa*/ml. T, Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine; H, homocysteine; TH, T and H; C, 0.9% NaCl. From Holtzman and Rossmoore (1977).

sound evidence that FA is involved in the HEAT results reported by Hotzman and Rossmoore (1977). The question remains only in what form and how much of the condensed FA is available.

From a number of unrelated studies (Auerbach *et al.*, 1977; Ratner and Clarke, 1937; Neely, 1963b) involving reactions with cysteine and homocysteine, it would appear that N-methylol groups would be good candidates for the intermediary donor form of FA. They are putative equilibrium reactants (H. Eggensperger, personal communication) in HEAT synthesis and transient adducts in fruit fly food treatment. There is potential that the active moiety is a Schiff base, also a probable member of the equilibrium mixture. However, the bulk of the evidence points to the hydrated form $[CH_2(OH)_2]$ as the major repository of FA. Despite the consensus suggesting that the saturated triazine is the representative structure, there is strong evidence that under conditions of use or in the presence of reactants that have more affinity for "formol," ring opening is encouraged.

In a series of studies by Eagon and associates (Hall and Eagon, 1985; Candal and Eagon, 1984), plasmid-mediated resistance to HET in *Pseudomonas putida* was definitely demonstrated. No mention was made of the relationship of FA to this resistance process. Concomitantly, Sondossi *et al.* (1985) found that resistance to HET coexisted with resistance to FA. Cultures of *P. aeruginosa* exposed to equimolar FA concentrations

of HET and FA (5.25 m*M* FA and 1.75 m*M* HET) and recovered after regrowth (Sondossi *et al.*, 1984) showed cross-resistance to the alternate biocide (Fig. 4). This relationship was reinforced by demonstrating enhanced FA dehydrogenase activity induced by HET.

Barnes and Eagon (1986) confirmed that FA activity paralleled HET activity in resistant and sensitive strains of *P. putida*. This sensitivity was induced by plasmid-curing with novobiocin. They correlated HET activity with analytical evidence for FA content and concluded on the basis of this evidence and additional metabolic results that HET acts primarily through release of its degradation product, FA. In a companion study, Eagon and Barnes (1986) confirmed the role of FA dehydrogenase in resistance to HET which was also demonstrated by the work of Sondossi *et al.* (1985). Resistance was transferred by conjugation to both FA and HET and was coupled to high levels of FA dehydrogenase.

Loss of resistance occurred simultaneously to both agents. Eagon and Barnes (1986) concluded that a single gene was involved in both instances and in all probability was solely FA based. Resistant isolates subsequent to their original *P. putida* strain were not curable, suggesting either recalcitrant plasmids or a chromosomal location. A related study with FA resistance in *Serratia marcescens* was also reported to be plasmid

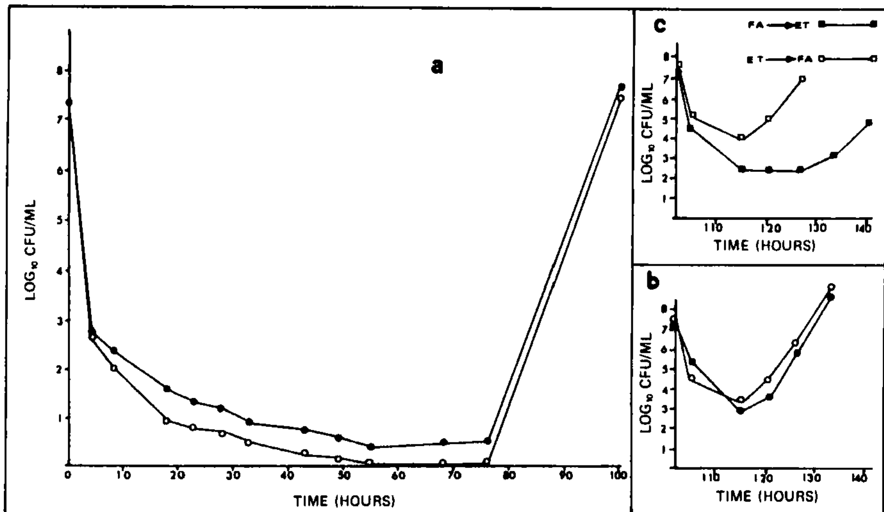


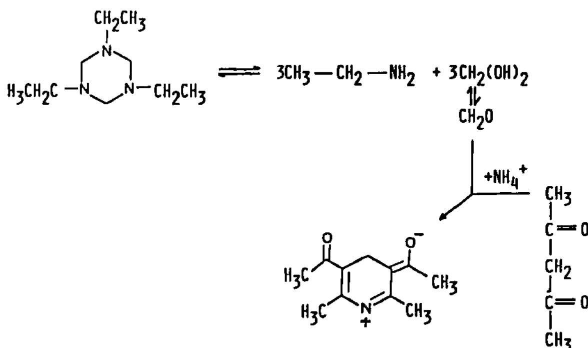
FIG. 4. (a) Initial treatment of *P. aeruginosa* with (●) formaldehyde (FA), 5.25 m*M*, or (○) hexahydro-1,3,5-triethyl-s-triazine (HET), 1.75 m*M*. (b) Subsequent treatment with same concentrations of FA and HET. (c) Treatment with alternative biocides: (■) FA → HET; (□) HET → FA. From Sondossi *et al.* (1985).

mediated (Kaulfers and Laufs, 1985). Two plasmids, 58 and 90 MDa, were found to be both curable and transferable with FA resistance. Resistant strains bind more FA (^{14}C) than do susceptible strains.

In contrast to other studies (Hall and Eagon, 1985; Candal and Eagon, 1984; Barnes and Eagon, 1986; Eagon and Barnes, 1986) in which resistant isolates recovered from the field were used, Sondossi *et al.*, (1985, 1986a,b) began with a prototroph of *P. aeruginosa* sensitive to HET and FA. These authors demonstrated two types of FA/HET resistance, both induced by HET (Fig. 5) (Sondossi *et al.*, 1986b). This resistance was coupled to activity of three distinctive FA dehydrogenases (Table III). The permanent resistant strain had a high constitutive NAD+-linked, glutathione-dependent dehydrogenase activity also reported by Eagon and Barnes (1986). This constitutive enzyme activity did not increase with further induction; however, a dye-linked dehydrogenase also involved did increase. This latter activity was also inducible in cells pregrown in methanol. The transient resistant strain had no detectable dye-linked enzyme but showed a significant increase in the glutathione-dependent enzyme, although at all levels much lower than the permanently resistant strain. Both strains responded after induction with low levels of glutathione-independent NAD^+-linked enzyme (Table III).

There remains no doubt that for HET there is no qualitatively demonstrable difference between its activity and FA. Stoichoimetric equivalence of FA and HET based on FA synthesis content in cross-resistance studies was demonstrated in a study of 15 biocides based mostly on FA (Sondossi *et al.*, 1986a). This result is of much interest since Barnes and Eagon (1986) and we (Table IV) found 75–100% of expected FA using the 2,4-pentanedione Nash reagent (Nash, 1953), one of the most sensitive of FA reagents.

The equivalency data of Sondossi *et al.* (1986a) imply that triazine hydrolysis is completed as reactive "FA" is consumed by more avid reactants, while in the assay with Nash reagent (**XII**) this is confirmed



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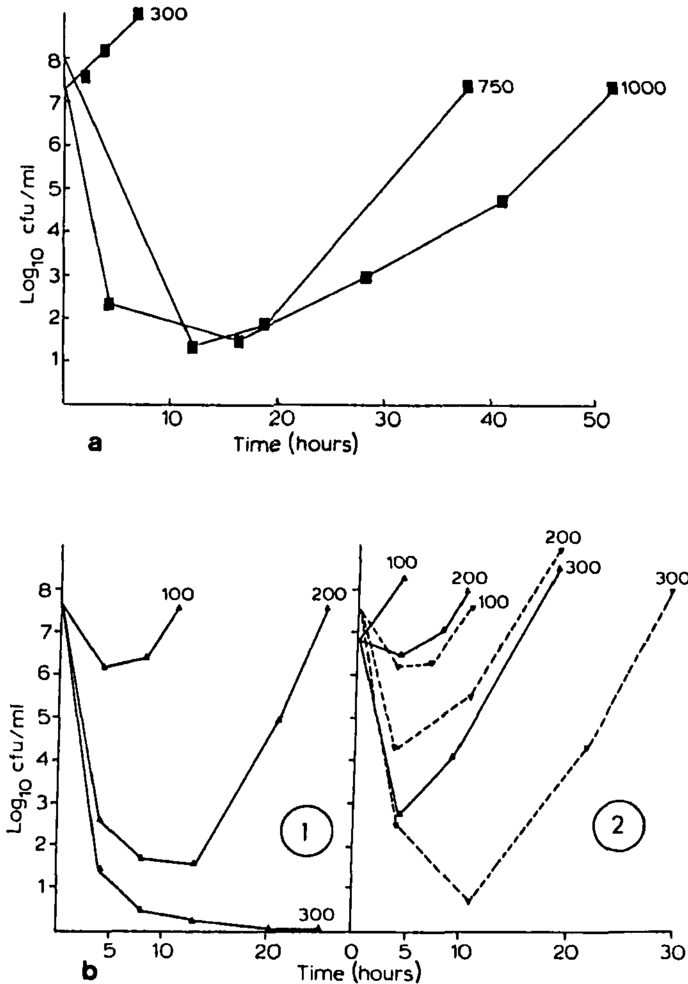


FIG. 5. (a) The resistant (R) strain of *P. aeruginosa* was treated with various concentrations of hexahydro-1,3,5-triethyl-s-triazine in tryptic soy broth (TSB): 300 ppm, 1.67 m*M* HET or 5 m*M* formaldehyde (FA) equivalents; 750 ppm, 4.17 m*M* HET or 12.5 m*M* FA equivalents; 1000 ppm, 5.56 m*M* HET or 16.69 m*M* FA equivalents. (b) 1. The sensitive (S) strain was treated with three levels of HET (100, 200, and 300 ppm). After the survivors of the 100 ppm treatment had recovered to approximately 1 × 10^8 cfu/ml, they were harvested and resuspended in fresh TSB. 2. The survivors from the 100 ppm HET treatment (above) were divided into two parts. One part was immediately exposed to 100, 200, or 300 ppm HET (▲——▲), and the other part was subcultured in TSB for 50 hours with several transfers to fresh media prior to exposure to HET (▼---▼). From Sondossi *et al.* (1986b).

TABLE III

SPECIFIC ACTIVITY OF FORMALDEHYDE DEHYDROGENASE IN CELL-FREE EXTRACTS OF *Pseudomonas aeruginos*[a]

Strain	Inducer	NAD⁺-linked FADH (μmol NADH/minute/mg protein)		DCPIP-linked FADH (nmol DCPIP/minute/mg protein	
		Glutathione independent	Glutathione dependent	PMS independent	PMS dependent
ATCC 27853	None	N.D.	11.20	N.D.	1.047
ATCC 27853	HET	7.90	82.44	N.D.	0.71
Sensitive	None	N.D.	2.65	N.D.	N.D.
Sensitive	HET	4.08	47.19	N.D.	N.D.
Resistant	None	N.D.	219.59	2.26	9.86
Resistant	HET	11.79	200.23	5.94	26.49
Resistant	Methanol	2.375	260.13	3.5	13.48

[a]Modified from Sondossi *et al.* (1986b). FADH, Formaldehyde dehydrogenase; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; HET, hexahydro-1,3,5-triethyl-s-triazine; N.D., not detectable.

TABLE IV

CHEMICAL DETECTION OF FORMALDEHYDE (FA) RELEASE FROM FA ADDUCTS[a]

Adduct	FA molecules in chemical structure based on synthesis	Number of FA molecules detected (Nash reagent) (range of different experiments)
Hexahydro-1,3,5-triethyl-*s*-triazine	3	2.65–3.09
Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine	3	2.75–2.97
A commercial mixture containing hexahydro-1,3,5-tris(2-hydroxyethyl)-*s*-triazine	3	<2.916>
1,3-(Dihydroxymethyl)-5,5-dimethylhydantoin	2	1.10–1.6
5-Hydroxymethyl-1-aza-3,7-dioxabicyclo-[3.3.0.]octane (one of three cyclooctanes in commercial mixture)	2	1.26–1.29
4,4-Dimethyloxazolidine (one of two oxazolidines in commercial mixture)	1	0.939–1.04
2-(hydroxymethyl)aminoethanol	1	0.824–0.979
2-(Hydroxymethyl)amino-2-methylpropanol	1	0.832–0.857
Tris(Hydroxymethyl)nitromethane	?	0.063–0.281
Hexamethylenetetramine	6	0.91–0.970
2-Bromo-2-nitro-1,3-propanediol	?	0.2–0.5
N-Methylolchloroacetamide	1	0.16–0.18
1-(3-Chloroallyl)-3,5,7-triaza-1-azoniaadamantane	6	4–4.5
Sodium 2-pyridinethiol-1-oxide	0	<0.008>
4-(2-Nitrobutyl)morpholine (70%) + 4,4'-(2-ethyl-2-nitrotrimethylene)-dimorpholine (20%)	1	0.05–0.08
3,5-Dimethyltetrahydro-1,3,5-(2*H*)thiadiazine-2-thione	2	1.68–1.8

[a]The assays were conducted at pH 7 in water by the method of Nash (1953).

chemically, implying that triazine hydrolysis is induced by the reagent's avidity for FA. The Nash procedure is relatively mild, highly selective, and apparently conducive to producing FA from some adducts. Treatment of saturated triazines with acid may produce more rapid hydrolysis with recovery of 3 mol FA/mol triazine, but there appears to be biological equivalency.

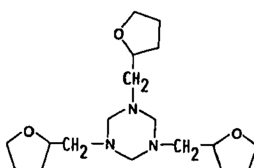
There has been very little commercial interest in any other hexahydrotriazine derivatives other than the ethylamine and ethanolamine adducts discussed above.

A comparison of the ethyl, ethanol, and propanol N-alkyls (DeMare *et al.*, 1972) showed them to be approximately equal in activity against *P. aeruginosa*. In a study conducted in six metalworking fluids (Bennett, 1973), again no great differences were noticed in effectiveness between the ethyl and ethanol derivatives. Using the same protocol, Borchert (1974) introduced an ethoxyethanolamine adduct with more activity than the other ethyl-based compounds.

A comment should be made about comparative data, especially involving compounds with presumptively similar modes of action (i.e., FA). Frequently (Bennett, 1973; DeMare *et al.*, 1972; Borchert, 1974; Paulus, 1976), recommended dosage levels are based on commercial product without regard to active component concentration. This is compounded by ignoring the potential amount of FA available and formula weight of the biocide molecule. A comparison with these factors considered (Sondossi *et al.*, 1986a) showed their significance in determining relative efficacy.

A major microbiological problem with all the FA adducts, including the triazines, relates to their relative lower activity against fungi (DeMare *et al.*, 1972; Rossmoore and Holtzman, 1974; Bennett, 1973; Rossmoore *et al.*, 1971; Paulus, 1976). This fact was previously noted for FA (Sykes, 1965). Variation of structure to increase antifungal activity was the basis for the synthesis of a series of tetrahydrofurfuralamine adducts of FA (Grier and Witzel, 1978, 1979).

This compound, more liphophilic than the ethanolamine derivative, was presumed to have greater potential for uptake by fungi with subsequent increased sequestration and release of FA. Whether this is a defensible explanation or not, the furanyl compound, hexahydro-1,3,5-tris (tetrahydro-2-furanylmethyl)-S-triazine (XIII), proved more effective in metalworking fluids in controlling fungi (Grier *et al.*, 1980).

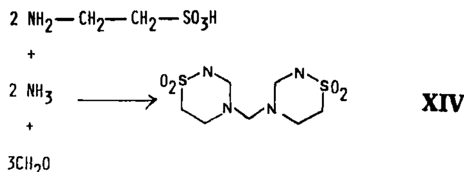


XIII

A practical concern for hydrophobic/hydrophilic partitioning and activity was presented by Jones *et al.* (1972). Preservation success with a series of alkylamine adducts (methyl to hexyl) in jet fuel reflected the greater solubility of the methyl and ethyl derivatives.

3. *Hexahydrothiadiazines*

After HMT, taurolin [bis-(1,1-dioxo-perhydro-1,2,4-thiadiazinyl-4) methane] is only the fourth FA adduct used as a chemotherapeutic agent (Browne *et al.*, 1976). It is based on taurine, a naturally occurring aminosulfonate originally isolated from bovine bile. It is described as a nontoxic, water-soluble formaldehyde carrier with a lower affinity for the formaldehyde than for bacteria, fungi, or toxins. Tauroline is formed from reaction (**XIV**).



The successful application of taurolin in controlling *P. aeruginosa* respiratory infections and *Salmonella typhimurium* infections in mice established a new dimension for FA condensates. The relationship between structure and activity of taurolin (Myers *et al.*, 1980) revealed that it exists in equilibrium with tauraltam, its carbinolamine derivative, and methylene glycol based on NMR and HPLC data. The intact tauroline is only a minor component in the mixture; presumptively, as FA is consumed by reaction with bacterial nucleophiles, the equilibrium is reestablished.

Equal levels of taurolin (35 m*M*), however, are much more active than either tauroltam or FA. Myers *et al.* (1980) conclude that taurolin or the tauraltam carbinolamine is more active than equimolar levels of FA. If indeed activity is based on FA content and release, this is an erroneous interpretation. Equimolar levels of taurolin have three FA equivalents, not one, as suggested by the authors. Their interpretation differs from Browne *et al.* (1976), who assume 3 mol FA/mol taurolin.

The same authors (Gidley *et al.*, 1981) have concluded that activity is primarily based on the release of an active methyleneimminium ion from the carbinolamine adduct with potential ring opening offering additional methyleneimminium ions. This conceptually accounts for the greater activity of taurolin. However, hydrated methyleneimminium becomes methylolamine (i.e., FA). The minimal activity they report for the

unsubstituted single thiadiazine ring may be due to the very short exposure time (8 minutes) in their study.

The versatility of taurolin was shown (Pfirrmann and Leslie, 1979) by neutralization of lipopolysaccharide (LPS) endotoxin from a number of gram-negative bacteria, as reflected in the inhibition of the pyrogenic response and in clinical trials in mitigation of established bacterial peritonitis (Browne *et al.*, 1978). In all probability, the clinical utility of taurolin is formed by its nonirritating quality; the final pH of saturated aqueous solutions (35 m*M*) is 6.5.

In a survey of 12 FA condensates, Bennett (1973) declared tetrahydro-3,5-dimethyl-1,3,5-(2*H*)-thiadiazine-2-thione (XV) the most active of those

CH_3–N(ring with S, S=)N–CH_3 **XV**

evaluated. Since only two FA equivalents per one of thiadiazine are involved, there may be an additional active moiety. Hydrolysis may yield a thiocarbamate derivative to account for the extra activity (Ross and Hollis, 1976; Paulus, 1976).

4. *Oxazolidines*

Oxazolidine compounds are primarily condensation products of FA and selected alkylamines (Sidi and Johnson, 1975), especially aminohydroxy compounds. Reaction (**XVI**) occurs rapidly at room temperature.

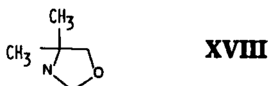
$$H_2C(NH_2)-CH_2OH + CH_2O \xrightarrow[-H_2O]{} \text{oxazolidine} \qquad \textbf{XVI}$$

When the compound contains more than one hydroxy group reaction (**XVII**) takes place.

$$HOCH_2-C(CH_2OH)(NH_2)-CH_2OH + H_2C{=}O \longrightarrow \text{bicyclic oxazolidine (}CH_2OH\text{)} \qquad \textbf{XVII}$$

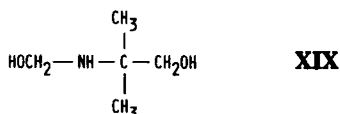
A review of oxazolidine chemistry, including stability as well as potential reaction products, detailed the permutations possible for FA and aminoalcohols (Bergmann, 1953). Although single chemical species are frequently reported for both types of oxazolidines, even with careful attempts at stoichoimetric reactions, impurities as well as unreacted starting materials yield mixtures. There are a number of oxazolidines

currently used as biocides based on the reactions described above. One of these is 4,4-dimethyloxazolidine (**XVIII**), present at about 98% in the



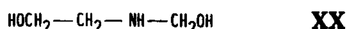
commercial product Bioban CS-1135 with 2% contributed by the condensation product of N-methylaminomethylpropanol. This compound has one equivalent FA on hydrolysis (Sondossi *et al.*, 1986a), making it relatively inactive based on yield per mole.

Another compound commercially available as Troysan 192 and chemically reported as 2-(hydroxymethyl)amino-2-methylpropanol (**XIX**)

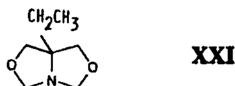


is really the first step prior to cyclization to the oxazolidine. Although there is probably some of the Schiff base in tautomeric equilibrium with the oxazolidine, the latter is prbably the stable adduct in the reaction (Bergmann, 1953).

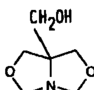
An analogous duplication exists with the HEAT (see Section II,B,2). This symmetrical triazine is in equilibrium (H. Eggensperger, personal communication) with a number of intermediates including 2-(hydroxymethyl)aminoethanol derived from ethanolamine and FA (**XX**).



The bicyclic oxazolidines are commercially available in two forms. One is the adduct of aminoethyldihydroxypropane and FA, 5-hydroxyethyl-1-aza-3,7-dioxabicyclo[3.3.0.]octane (**XXI**), which is sold as Bioban CS-1246



(about 98% pure). The second bicyclic is a mixture of 5-hydroxymethyl derivatives based on the reactions between trisamino(hydroxymethyl) methane and FA and is a representative adduct. In the presence of excess FA, a mixture of polymethoxy adducts is formed based on the C_5 position. Bioban N-95, the commercial product (85% active), is a 50% aqueous mixture of 5-hydroxymethyl (**XXII**) (35%) and 5-hydroxymethoxymethyl (49%) derivatives (Table VIII). The methoxy and polyalkoxy components may contribute to the activity of the mixture (Table VIII).



XXII

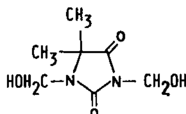
In the comparative study (Sondossi *et al.*, 1986a), relative FA levels were based on putative N-methylol content (after hydrolysis). Using this criterion, it appears (Fig. 1) that Bioban N-95 has greater activity than predicted.

C. N-Methylol Derivatives

Two commercially available N-methylol compounds (Troysan 174 and Troysan 192) are both probably in the cyclic form (see Section II,B,2) (Table VIII). The first two compounds in this series are N-methylolamide derivatives. Paulus (1980) reported that the amides release less FA than equivalent amines. This is substantiated by results presented in Fig. 1 and Table IV.

1. *N-Methylolhydantoin*

The methylolation of the diketoimidazole, dimethylhydantoin, yields 1,3-(dihydroxymethyl)-5,5-dimethylhydantoin (DMDMH) (**XXIII**).

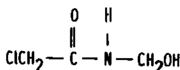


XXIII

Theoretically, this compound should yield two FA molecules from the N-methylol substituents. The cross-resistance studies (Sondossi *et al.*, 1986b) (Fig. 1) show that less appears to be available. The sensitive (to FA) strain grows back faster in a concentration of FA providing the putative two FA. Chemical analysis with the Nash method (Table IV) confirms only 1–1.5 mol FA/mol hydantoin. This is suggestive of the relative avidity of Nash reagent and the cell-sensitive molecule for pulling FA from the N-methylol adduct (at least the second FA). The compound DMDMH has had limited applications in cosmetics.

2. *N-Methylolchloroacetamide*

Methylolation of haloalkyl amines (Paulus *et al.*, 1967) yields an active compound, N-methylolchloroacetamide (**XXIV**), apparently hydrolyzable to yield one FA molecule, probably only at alkaline pH. The commercial



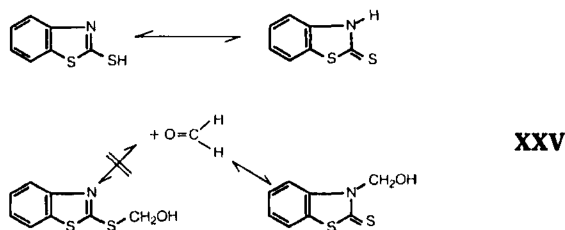
XXIV

product sold as Grotan HD has an extremely short half-life at alkaline pH. This compound at neutral pH has very little FA activity although a FA-resistant strain is more refractory to FA than the FA-sensitive strains (Sondossi *et al.*, 1986a) (Fig. 1); and to achieve even that distinction, about three times the FA equivalent was used.

In chemical analysis with Nash reagent (Table IV), a negligible amount of FA is detected. The parent compound, chloroacetamide, is a sulfhydryl reagent and possibly is responsible for some of the activity noticed at neutral pH.

3. *N-Methylolmercaptobenzothiazole*

Reaction of FA with mercaptobenzothiazole yields the N-methylol derivative (**XXV**) which significantly increases the antibacterial activity

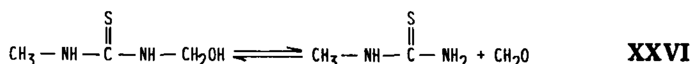


XXV

over the parent compound (Paulus, 1980). This improvement in activity may be real (that is, more active than the FA alone) or illusory, giving no practical improvement. The methylolation of O-phenylphenol and p-chlorometacresol drastically reduced activity of the parent compound. The hydroxymethyl additions were attached to the aryl ring carbon, but nevertheless the results suggest that structure modification with FA should proceed with caution.

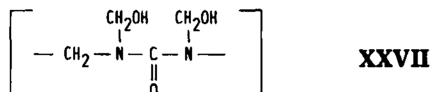
4. *Noxythiolin and Polynoxylin*

Noxythiolin and polynoxylin should be considered together. The former is N-methyl-N-methylolthiourea and presumably releases 1 mol of FA (**XXVI**). The latter is chemically polyoxymethylurea, a condensate



XXVI

of urea and FA (**XXVII**). The antimicrobial activity of polynoxylin was



XXVII

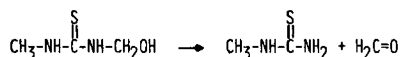
first reported in 1961 (Haler and Aebi, 1961). The compound is fairly insoluble (about 0.3%) in water. Its *in vitro* activity, specifically against gram-positive cocci, resulted in clinical trials with intractable topical infections.

A later report (Haler, 1963) reiterated the effectiveness against staphylococcal infections but reported minimal success against monilial vaginitis. Although the author stated that activity does not appear to be limited to release of free FA, no alternative was offered.

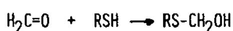
Noxythiolin, the thiourea derivative, has the advantage of H_2O solubility and has had greater subsequent use, especially clinically. Both compounds were evaluated chemically for FA release (Kingston, 1965) by analytically determining the uptake of FA into water in a closed system. It was concluded that antimicrobial activity was attributable to FA, although release rates as measured by uptake were rather slow. The stability and low toxicity of noxythiolin resulted in a number of *in vivo* trials in laboratory animals and human subjects for treating fecal peritonitis (Browne and Stoller, 1970).

A dose of 2.5 g/100 ml in gastric lavage was successful in a majority of otherwise intractable cases. A most interesting activity of noxythiolin apparently unrelated to FA was reported recently (Gorman *et al.*, 1985a,b, 1986). The compound itself, but not FA or methylthiourea, inhibited adhesion of *Candida albicans* pseudohyphae or blastospores to buccal epithelial cells. No alternative moiety was suggested except the intact noxythiolin. This is perhaps another example of the minimal understanding of the mode of action of FA itself. The quantitative comparisons were made between noxythiolin and FA, the amount of the latter determined as free FA due to spontaneous hydrolysis. This does not consider the equilibrium shift as "free" FA is continually consumed biologically. When the level of FA was increased to four times the level found from hydrolysis, there was a significant decrease in adhesion to buccal cells. This demonstrates that FA is qualitatively involved in this process.

It would appear that all N-methylol compounds release "FA" with an equilibrium shift to the right as the "free FA" is consumed by more avid biological molecules (**XXVIII**).



XXVIII

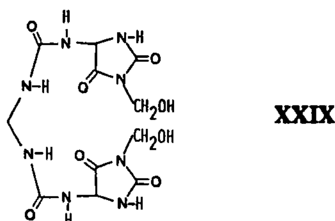


The same rationale (Gorman *et al.*, 1986) was used to conclude that noxythiolin was superior to "equivalent" FA levels as a candidicide. The authors erroneously use the level produced by spontaneous hydrolysis

during a contrived 30-minute detection time at 32° C as equal to the clinical contact time and temperature.

5. *Imidazolidinylurea*

Methanebis[*N*,*N*'-(5-ureido-2,4-diketotetrahydroimidazole)-*N*,*N*-dimethylol] (**XXIX**) was first evaluated in 1970 in cosmetics (Berke and



XXIX

Rosen, 1970) and was found to be effective in a number of cosmetic formulations, especially against *Pseudomonas* as well as other bacteria and some yeasts and fungi. However (Berke and Rosen, 1978), the 0.3% dose required for preservation is somewhat high. Based on the formula, two *N*-methylol groups should yield two FA molecules and the methylol bridge should yield one FA, totaling three. Under some conditions, all important species involved in cosmetic spoilage appeared controllable in some products. However, yeast and mold control was marginal. The advantage of this compound appears related (Elder, 1980b) to its formulation compatibility, general lack of acute toxicity, and potential synergism with other acceptable preservatives for cosmetics.

A study on the dynamics of disinfection for preservation systems (Hurwitz and McCarthy, 1985) emphasized the importance of rate of bactericidal action. Similar *H* values (*D* x concentration) and *n* values (dilution coefficients) for imidazolidinylurea and Bronopol were obtained. Both dilution coefficients are close to reported values for FA (about 1). The authors suggested that this relationship between kinetics of kill of imidazolidinylurea and Bronopol may be associated with FA as a mode of action.

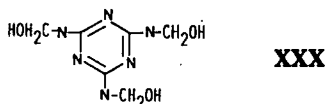
The relative ineffectiveness of imidazolidinylurea based on FA content was much less than FA itself when used against imidazolidinylurea-resistant bacteria isolated from cosmetics (Bowman and Lindstrom, 1985). This may be related to an alternate mode of action or poor FA release from amide-N-methylol groups.

More recently (Berke and Rosen, 1982), an additional compound based on imidazolidinyl-urea has become available. Its chemical name is *N*-(hydroxymethyl)-N-(1,3-dihydroxymethyl-2,5-dioxo-4-imidazolidinyl)-*N*'-(hydroxymethyl) urea and its trivial name is diazolidinyl urea. Initial studies suggest that it is about twice as effective as the compound described earlier (Berke and Rosen, 1970). Based on their structures, the

more active compound has also about twice the amount of potential formaldehyde on a molar basis.

6. N-Methylolmelamine

Tris(*N*-methylol)-2,4,6-triamino-1,3,5-triazine (**XXX**) is primarily used to impart wet strength to textiles, but its FA content seemed to be an



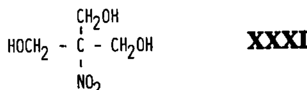
XXX

intriguing repository for antimicrobial activity of nascent FA. An experimental design evaluated FA release from paraformaldehyde and FA-melamine (Fig. 6) (Tulis, 1973). Even at 6 mg of product/100 cm^3 air, there was not sufficient FA release to come close to equaling paraformaldehyde at 1 mg/100 cm^3 air. Perhaps for long-term self-sterilizing of some products (e.g., textiles, potting soils), there may be some value since potting compound (RTV) impregnated with FA-melamine seemed to lose activity at a slower rate than paraformaldehyde (Fig. 7) (Tulis, 1973).

D. C-Nitro Derivatives

1. *Tris Nitro*

The addition of FA to nitromethane yields a trimethylated compound, tris(hydroxymethyl)nitromethane (**XXXI**). This was found to have



XXXI

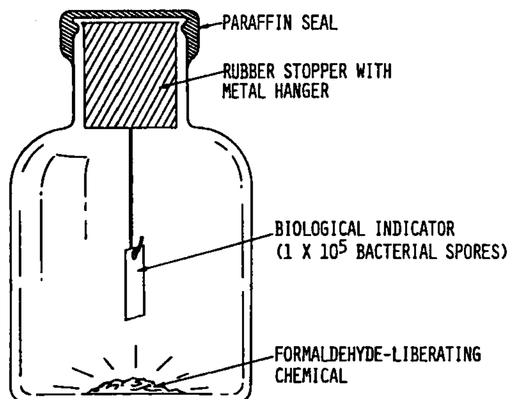


Fig. 6. Test procedure for evaluation of formaldehyde-liberating chemicals. From Tulis (1973).

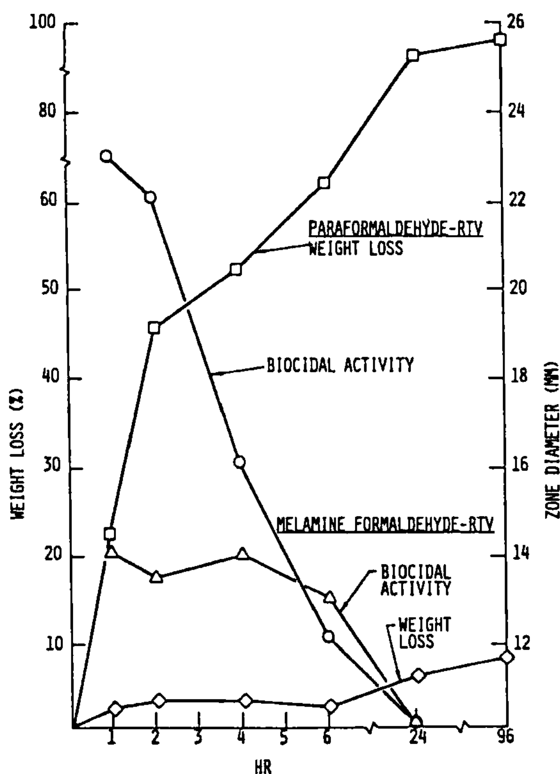
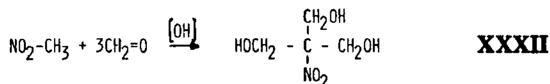


FIG. 7. Loss of formaldehyde (FA) and biocidal activity from paraformaldehyde-RTV and melamine-FA-RTV at 90°C. From Tulis (1973).

antimicrobial activity (Bennett and Hodge, 1961), especially in alkaline metalworking compounds (Wheeler and Bennett, 1956). This compound, essentially a nitroalcohol (Dewey and Bollmeier, 1981), is formed by replacement of 1–3 H atoms on the C atom attached to the nitro group. The reaction is carried out in the presence of base (XXXII).



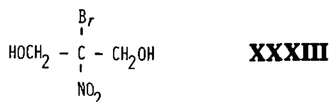
$$NO_2\text{-}CH_3 + 3CH_2{=}O \xrightarrow{[OH]} HOCH_2\text{-}C(CH_2OH)(NO_2)\text{-}CH_2OH \qquad \text{XXXII}$$

Nitromethane is the most reactive nitroalkane, FA is the most reactive aldehyde, and their reaction equilibrium strongly favors the adduct tris(hydroxymethyl)nitromethane. Its slow FA release is exemplified by the cross-resistance results of Sondossi *et al.* (1986b) in which four times the FA equivalent was ineffective against both the resistant and sensitive strains of *Pseudomonas* (Fig. 1; Table IV).

Two additional nitroalkane-formaldehyde adducts likewise behaved differently than most of the FA condensates tested.

2. *Bronopol*

The bromination of tris(hydroxymethyl)nitromethane yields an extremely reactive adduct, 2-bromo-2-nitro-1,3-propanediol (**XXXIII**)



XXXIII

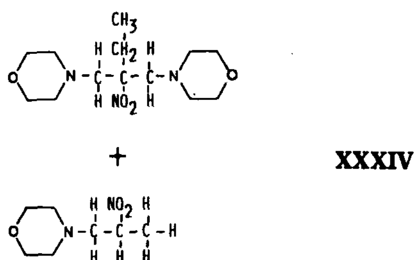
(Bronopol), with a suspect chronic toxicity profile. This compound is reasonably stable at acid pH but unstable at alkaline pH and appears to be twice as effective as FA (0.005% vs 0.01%) for preservation of protein solutions used in cosmetics. Reports (Elder, 1980a) of its breakdown at neutral or alkaline pH to FA and mixed bromine compounds (Rosen and Berke, 1973) are not compatible with results of Sondossi *et al.* (1986a). In this study, 0.03% at pH 7 produced an apparent 100% kill in 5 hours (Fig. 1) in both FA-sensitive and FA-resistant strains of *P. aeruginosa*. This was at 1.5 mM, the FA equivalent by structure of 3 mM FA. In addition, the analytical level of FA by Nash reagent (Table IV) was less than anticipated, about 0.2–0.5 molecules out of a possible 2.0.

Alternative mechanisms of action for this compound have been offered (Stretton and Manson, 1973; Bowman and Stretton, 1972). Proof of sulfhydryl oxidation to disulfides with subsequent reversal by thiols actually would remove from reactivity a potential nucleophile for FA interaction. There is also evidence for damage to membrane function with leakage of 260 nm absorbing material after exposure to the nitrobromopropanediol. Nevertheless, the highly electronegative nature of the NO_2-C-Br group may play a large part in the reactivity of this compound, exclusive of any FA involvement.

Unfortunately, the major use for this compound is in cosmetics. Formulations with alkylamines are readily nitrosated, being influenced by electronegativity of the bromine group, and thus result in potentially carcinogenic nitrosamine formation (Ong and Rutherford, 1980; Fan *et al.*, 1978).

3. *Nitroalkylmorpholine*

A third compound (**XXXIV**) based on nitroalkane chemistry involves the condensation of nitropropane, FA, and morpholine. It is partly water soluble and is useful where migration into the nonpolar phase is favorable. This mixture, 4,4'-(2-ethyl-2-nitrotrimethylene)dimorpholine and 4-(2-nitrobutyl)morpholine, is not reported to be a FA releaser even though FA is involved in the synthesis. However, in cross-resistance studies (Sondossi *et al.*, 1986a) (Fig. 1), both the FA-sensitive and



FA-resistant strains of *P. aeruginosa* behaved similarly; a commercial dose reduced viable levels by about 4 log units. Most interestingly, survivors of the morpholine biocide treatment were more resistant to FA itself (3 m*M* producing less than 1 log kill compared to about 4 log units prior to induction). Cells induced with FA did not respond differently to doses of the morpholine compound. These results are in agreement with chemical studies (Table IV) and indicate an additional mode of action for this compound.

E. Biocide Mixtures

Antimicrobial agents are used in combination for a number of valid reasons: (1) to broaden the antimicrobial spectrum; (2) to minimize physical and chemical incompatibilities; (3) to minimize toxicity; and (4) to produce biochemical synergism.

The use of formaldehyde adducts for inhibition and control of mixed populations (i.e., fungi and bacteria) revealed a differential response demonstrating that fungi were more resistant than bacteria (Rossmoore *et al.*, 1971; Rossmoore and Holtzman, 1974; Paulus, 1976), agreeing with an earlier observation (Sykes, 1965). A number of putative fungicides were mixed with HEAT (Rossmoore *et al.*, 1971; DeMare *et al.*, 1972) with some success. One of them, pyridinethione N-oxide, not only proved to be a complementary fungal control agent but also appeared to be a synergist against bacteria with HEAT (Rossmoore *et al.*, 1979) (Table V). This combination is now an EPA registered and marketed product.

Another combination, ostensibly to handle the problem of antifungal inadequacy of the frank FA releasers, is based on tris(hydroxymethyl)nitromethane and nitromorpholine mixture (Purcell and Selleck, 1978). The latter has greater oil solubility and appears to have better antifungal activity than the tris nitro, although as mentioned previously, a part of the morpholine activity may also be based on FA.

More recently, FA-releasing compounds—no doubt biocides in their own right—have been exploited for nucleophilic reactivity to protect a

TABLE V

LOG REDUCTION IN MICROBIAL COUNTS IN 5% METALWORKING FLUIDS AFTER 48 HOURS EXPOSURE[a,b]

Biocide	ppm	Soluble oil		Semisynthetic		Synthetic	
		B	F	B	F	B	F
P_1	20	2.1	1.2	2.0	0	2.1	3.3
$HEAT_1$	200	1.8	0.6	2.2	0	2.1	0
P_1 + $HEAT_1$		9.4	1.7	10.0	0.8	10.0	4.8
P_2	45	1.6	4.3	2.0	3.2	2.0	3.3
$HEAT_2$	450	2.6	1.6	2.5	3.0	5.4	1.6
P_2 + $HEAT_2$		9.4	4.9	10.0	4.5	10.0	4.8
P_3	91	1.6	3.9	1.9	3.2	2.0	3.3
$HEAT_3$	910	7.6	3.9	5.5	1.5	5.2	3.3
P_3 + $HEAT_3$		9.4	4.9	10.0	4.5	10.0	4.8

[a]Modified from Rossmoore *et al.* (1979).
[b]Sodium 2-pyridinethiol-1-oxide; HEAT, hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine; B, bacteria; F, fungi.

more sensitive primary biocide from degradation. Tris(hydroxymethyl)nitromethane (Table VI) appears to extend the activity of a chloromethylisothiazolone in an unfriendly primary amine environment (Hahn, 1984). The FA molecule in this mixture is also a commercially marketed biocide.

Other mixtures also capitalize on this nucleophile reactivity (e.g., using dimethylolurea and an ethyleneglycol-FA adduct as protectants for the susceptible isothiazolone molecule) (Hahn, 1979). However, in spite of what appears to be novel via patent process is far from unique. There are about seven combinations of what appear to be isothiazolone-FA adduct mixtures (Allsopp and Allsopp, 1983) (Table VIII). Unfortunately, total information is not forthcoming from manufacturers, especially outside of the United States.

Synergistic interactions, specifically where there is more than additive effect when two or more agents are used together, have been reported in several cases. Reports (Rossmoore, 1979; Izzat and Bennett, 1979) involving the use of HEAT showed that tetrasodium EDTA significantly increased the antibacterial activity of HEAT in a water-based metalworking fluid. Whether this synergism is due to outer membrane disorientation by EDTA with more FA-sensitive sites exposed or if cation sequestration contributes to increased FA activity was not resolved. Several phenols were additionally synergistic with the benzylhemiformal against both bacteria and fungi provided that the pH is kept between

TABLE VI

SYNERGISM BETWEEN TRIS(HYDROXYMETHYL)NITROMETHANE AND METHYLCHLOROISOTHIAZOLONE[a,b]

	Yeast						*Pseudomonas aeruginosa*						*Aspergillus niger*					
	24 hours			72 hours			24 hours			72 hours			24 hours			72 hours		
%	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
0.0075	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
0.01	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+
0.02	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−

[a]Modified from Hahn (1984).

[b]A, Tris(hydroxymethyl)nitromethane (20% in water); B, mixture of 2-methyl-3-oxo-5-chlorothiazoline (1.2) and 2-methyl-3-oxo-thiazoline (1.2) (1.6% in water); C, tris(hydroxymethyl)nitromethane (20%), 2-methyl-3-oxo-5-chlorothiazoline (1.2) and 2-methyl-3-oxo-thiazoline (1.2) (1.6%), and water (78.4%); +, growth; −, no growth.

6 and 9. Above pH 9, the aromatic ring is methylolated, reducing activity (Paulus, 1976).

The use of pyridinethione-triazine mixtures for controlling mixed bacterial and fungal populations (Rossmoore *et al.*, 1979) also demonstrated that the two agents appeared synergistic against bacteria (Table V).

Another example of combinations designed to control multiple microbial populations (Rossmoore, 1977) involved a complex mixture of HEAT, tributyltin oxide, and EDTA. Three distinctive microbial populations were monitored in metalworking fluid: aerobic bacteria, anaerobic sulfate-reducing bacteria, and fungi. There was expected reduction of all three populations but quantitatively unexpected reduction of sulfate reducers with the mixture not obtained with any one of the components alone. No explanation was offered for the synergism.

True synergism was identified (Rossmoore, 1987) between five FA adducts and copper citrate. Not only did the Cu^{2+} markedly reduce the amount of FA compound for bacterial control (Table VII), it was subsequently shown that FA, under controlled conditions, was synergistic with Cu^{2+} (Fig. 8). Evidence was also presented (Wireman *et al.*, 1986) that Cu^{2+} reacts *in vitro* with glutathione, the cofactor in glutathione-dependent formaldehyde dehydrogenase (Sondossi *et al.*, 1986b; Eagon and Barnes, 1986). These results are a prime example of the ideal approach to chemical selection with more than empirical knowledge of mode of action.

Some inadvertent combinations may not contribute to a favorable conclusion. The finding (Keefer and Roller, 1973) of catalysis of nitrosamine synthesis at alkaline pH by FA (0.14 *M*) was an important explanation for nitrosamine presence in many environmental situations. Nitrosamine in metalworking fluid (Fan *et al.*, 1977) and the potential contribution of formaldehyde-condensate biocides to the catalysis of the former led to their investigation (Keefer and Hansen, 1982). At a dose (0.05 *M*) five times normally used in field situations, hexahydro-1,3,5-trimethyl-s-triazine increased levels of nitrosamine by as much as 15% over the control. The level used was extremely high and may have contributed to the levels of FA needed for catalysis (equivalent to the Keefer and Roller, 1973, finding). The practical implication in occupational health has not been assessed. However, in field surveys of metalworking fluid for nitrosamines including some containing FA condensates (Rounbehler and Fajen, 1983), no inordinately high levels of nitrosamines were reported.

A more direct interaction (synergism) involves genotoxicity of FA in combination with N-methyl-N-nitrosourea (Grafstrom *et al.*, 1985). The latter compound methylates DNA at O^6-guanine and is rapidly repaired by O^6-alkylguanine-DNA alkyltransferase. Formaldehyde inhibits this

TABLE VII

SYNERGISTIC EFFECT OF Cu^{2+} WITH SELECTED FORMALDEHYDE ADDUCTS[a]

	Bacteria cfu/ml	Apparent synergistic effect (log difference)	Fungi cfu/ml	Apparent synergistic effect (log difference)
Control (nothing)	3×10^7		9.1×10^4	
Cu citrate[b]	3.5×10^7		8.1×10^4	
Tris(hydroxymethyl)nitromethane				
2 m*M*	8.5×10^7	3 +	1.6×10^5	3 +
2 m*M* + Cu	1×10^4		$<10^2$	
4 m*M*	2×10^7	4 +	2×10^5	3 +
4 m*M* + Cu	$<10^3$		$<10^2$	
4-(2-Nitrobutyl)morpholine (70%) + 4,4'-(2-ethyl-2-nitrotrimethylene)-dimorpholine (20%)				
1 m*M*	3.5×10^7	3 +	1.6×10^5	0
1 m*M* + Cu	1×10^4		1.2×10^5	
4 m*M*	2×10^6	3 +	2.4×10^5	3 +
4 m*M* + Cu	$<10^3$		$<10^2$	
N-Methylolchloroacetamide				
0.8 m*M*	2.5×10^7	0	1.5×10^5	0
0.8 m*M* + Cu	4×10^7		9.6×10^4	
3.2 m*M*	8×10^7	4 +	1.1×10^5	2 +
3.2 m*M* + Cu	$<10^3$		7×10^2	
Hexahydro-1,3,5-tris(2-hydroxyethyl)-*s*-triazine				
0.9 m*M*	5.5×10^7	4 +	6.1×10^4	0
0.9 m*M* + Cu	$<10^3$		6×10^4	
1.8 m*M*	$<10^3$	0	1.4×10^5	3 +
1.8 m*M* + Cu	$<10^3$		$<10^2$	

Hexahydro-1,3,5-triethyl-s-triazine				
0.56 mM	6×10^7	3+	8.4×10^4	2+
0.56 mM + Cu	1.6×10^4		$<10^2$	
1.4 mM	1.6×10^4	1+	2×10^2	0
1.4 mM + Cu	$<10^3$		$<10^2$	

[a]From unpublished data presented by L. A. Rossmoore at the Annual Meeting of the American Society of Lubrication Engineers (1985). The experiments were conducted in a 5% oil/water metalworking fluid emulsion at pH 8.5 with an inoculum derived from contaminated field samples of metalworking fluid. *Pseudomonas* species dominated (99% +) the bacterial population, while *Fusarium*, *Cephalosporium*, and *Candida* shared the fungal inoculum. The time zero count was 10^7 cfu bacteria/ml and 1×10^5 cfu fungi/ml. Incubation was carried out at 25°C with shaking for 72 hours.

[b]Monocopper oxycitrate (1 mM Cu^{2+}).

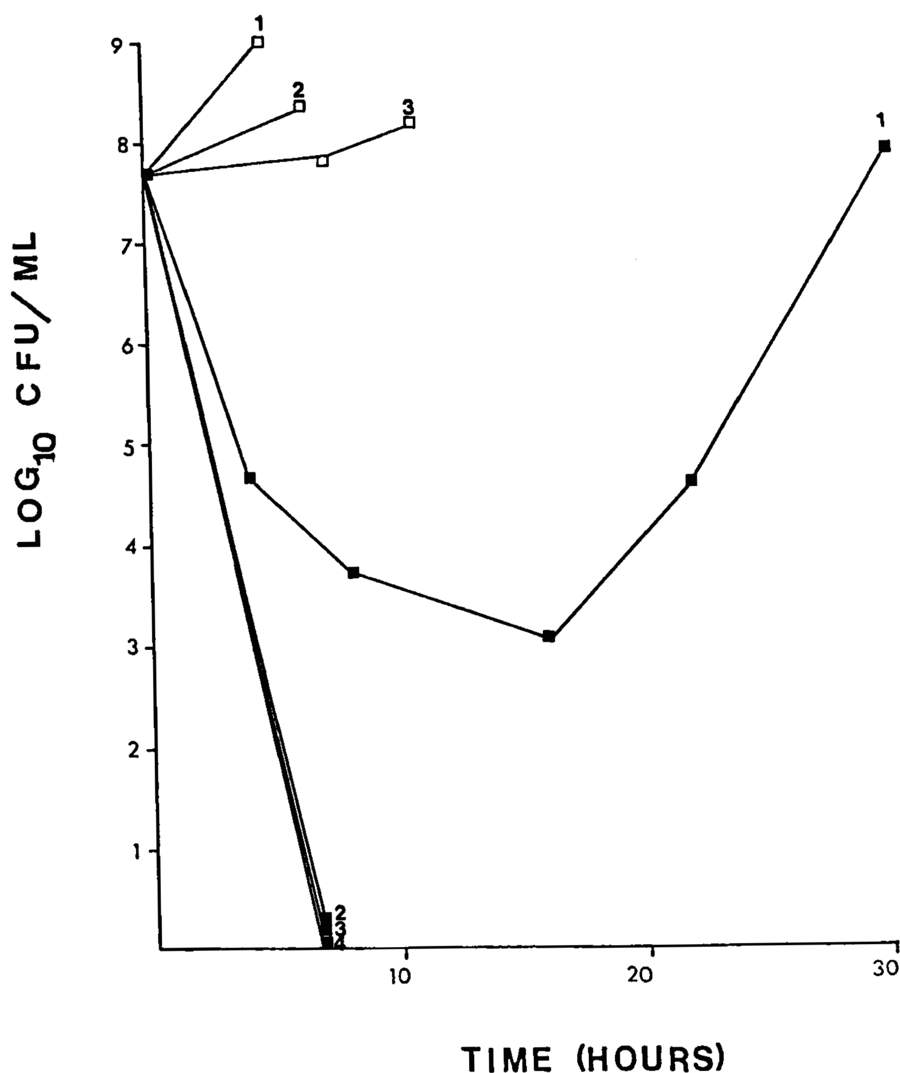


FIG. 8. Exponentially growing *P. aeruginosa* suspensions in tryptic soy broth were exposed to, for (□), (1) 2 mM; (2) 4 mM; (3) 6 mM of $CuSO_4$ and to, (■), (1) 3 mM of formaldehyde (FA) or (2) 2 mM; (3) mM; (4) 6 mM $CuSO_4$ plus 3 mM FA.

repair, as demonstrated by both an increase in mutations produced and cytoxicity. It is suggested that the enzymes responsible for aldehyde metabolism may also be involved in the susceptibility of organs in nitrosamine carcinogenicity.

Cosmetic lotions exemplify a group of products with all of the challenges most demanding of preservatives (i.e., biocide function, variation in pH, net charge, hydrophobic/hydrophilic phase shifts, readily available organic source, broad deteriogen spectrum, and frequency of microbial reinoculation). It is not surprising that biocide combinations offer the best chance for success, especially for the limited number of compounds that are toxicologically acceptable for cosmetics. Among these are a number of FA adducts. A study was conducted (Jacobs *et al.*, 1975) with a number of the above and in combination with non-FA compounds. A mixture of imidazolidinylurea in combination with methyl- and propylparahydroxybenzoates appeared synergistic in all permutations evaluated.

In the evaluation of the imidazolidinylurea, a concentration of 0.5% failed to control the two fungal organisms *Candida albicans* and *Aspergillus niger*. This is not unexpected for FA-releasing compounds. The bacteria *P. aeruginosa* and *Streptococcus faecalis* were controlled. A mixture of methyl- and propyl-P-hydroxybenzoate was not successful universally against bacteria or fungi. When added to the imidazolidinylurea, all four organisms were inhibited.

F. Toxicological and Environmental Considerations

The potential chronic toxicity of FA has already been discussed as has been the propensity for nitrosamine production by some FA adducts. The overt acute and subchronic problems associated with the FA adducts are limited to the expected alkaline corrosivity of some of the aminals and delayed-type hypersensitivity reported for several others (Keczkes and Brown, 1976; Robertson and Storrs, 1982). The responses are minimal.

The extent to which FA adducts are used leads to a wide variety of environmental impact conditions intrinsic as well as extrinsic to the application. They react with metals with mixed results (Rossmoore, 1979; Bennett *et al.*, 1982). Nucleophilic interaction certainly is a factor in environmental dissipation of FA generated. The wide variety of microorganisms in waste treatment facilities, industrial as well as municipal, ensure that released FA will be dissimilated.

In the United States, all of the products commercially available are subjected to a battery of environmental impact studies as well as a series of acute toxicology tests. These have been required for Environmental Protection Agency (EPA) registration (U. S. EPA, 1975, 1978). Current

TABLE VIII

USE OF COMMERCIALLY AVAILABLE FORMALDEHYDE-BASED BIOCIDES[a]

Active ingredient (chemical name when known)	CAS Reg. No.	Trade name and/ or generic name	Manufacturer	Use	Recommended level of use	Availability and regulatory status
Paraformaldehyde (90–99% active)		Polyoxymethylene; Formagene		Vapor-phase fumigant		Universal
Arylmethanolmono-(poly)hemiformal	[30525–98–4]	Preventol D2	Bayer	Paints, adhesives, textiles, metal-working fluids	0.05–0.25%	Western Europe
Hexamethylene-tetramine	[100–97–0]	Urotropin; methenamine		Urinary tract antiseptic	1–2 g/day	United States Pharmacopoeia (USP)
Hexamethylene-tetramine mandelate	[587–23–5]	Mandelamine		Urinary antiseptic	1–2 g/day	USP
Hexamethylene-tetramine hippurate	[5714–73–8]	Hiprex; Urex			1–2 g/day	
1-(3-Chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride (67%) active	[4080–31–3]	Dowicil 75	Dow	Preserves raw latex emulsions, joint cement, biode-gradable deter-gents, floor-wax emulsions and polishes, inks, laundry starches, and polyvinyl alcohol; adhesives preservative, metalworking fluids (microbiocide),	0.015–0.2% concn.; 0.01–0.1% in metal-working fluids	EPA #464–403 preser-vative; CFR 121.2520 adhesives; CFR 121.2522 polyure-thane resins in contact with bulk food areas; CFR 121.2526 latexes used as pigment binders in paper and paperboard for use in contact with dry and fatty food

				in-can water-based latex paints; pulp and paper-based coatings; textile spinning and finishing		
Cis isomer of above		Dowicil 200	Dow	Cosmetic preservative	0.05–0.1%	United States Food and Drug Administration (FDA)
1,1-(2-Butylene)-bis(3,5,7-triaza-1-azoniaada-mantane)chloride (90%)	[51350–84–6]	Cosan 265	Cosan Chemical Co.	Antibacterial agent in latex paints, resin emulsions, adhesives, dispersed colors	62–260 ppm	EPA #8489–22; U.S. Patents 3,928,607 and 3,959,276; bactericide highly effective against both gram-positive and gram-negative organisms
Hexahydro-1,3,5-tris(2-hydroxy-ethyl)-s-triazine (78%)	[4719–04–4]	Grotan BK	Schülke & Mayr	Metalworking fluid	0.15%	EPA #10.001–1
		Onyxide 200	Onyx-Stepan	Metalworking fluid	0.15%	EPA #1839–59
		Glokill 77	A.B.M. Chemic	Emulsion systems (e.g., metalworking fluids, latex, adhesives)	0.04–0.1%	
		Keytrol T (hydroxy-triazine)	Cochrane & Keane	Metalworking fluids, paint, aqueous-based systems	0.03–0.25%	United Kingdom; European Economic Community (E.E.C)

(continued)

TABLE VIII (continued)

Active ingredient (chemical name when known)	CAS Reg. No.	Trade name and/ or generic name	Manufacturer	Use	Recommended level of use	Availability and regulatory status
		Bakzid 80 (hexahydro-triazine)	Bacillol-fabrik Dr. Bode, Bode Laboratories	Metalworking fluids	0.1–0.15%	
		Margold KM 200 (hexahydro-triazine)	Hoechst UK; Riedel-de Haën	Metalworking fluids, in-can preservation	0.05–0.15%	
		Bacillat 35 (1,3,5-hexa-hydrotriazine)	Bacillol-fabrik Dr. Bode, Bode Laboratories	Metalworking fluids	0.1–0.15%	
Hexahydro-1,3,5-triethyl-s-triazine	[7779-27-3]	Vancide TH	R.T. Vanderbilt	Alkaline aqueous systems: paint, metalworking fluids	0.01–0.1%	EPA #1965-55; Canada; Japan; E.E.C.
Tetrahydro-3,5-di-methyl-2*H*-1,3,5-thiadiazine-2-dione (98%)	[533-74-4]	Metasol D3T	Merck Chemical Co.	Slimicide and preservative for coatings, clay, slurries, adhesives, glues, latex emulsions, casein and titanium slurries	0.01–2.5%	EPA #2079-28AA; antimicrobial (bacteria and fungi)

		Biocide N-521	Stauffer Chemical Co.	Fungicide and bactericide: leather, paint, starch, paper processing, drilling muds	0.01–0.25%	FDA approved biocide for use as antimicrobial agent in formulations of slimicides, animal glues and adhesives, for the treatment of paper, paperboard, foodboard, and coatings
		Sepacid CE 5232	BASF			Europe
		Dazomet	Tenneco			FDA
		Cosan S	Cosan Chemical Co.			EPA
		Biosperse 210	Drew Chemical			EPA
Bis (1,1-dioxo-perhydro-1,2,4-thiadiazinyl-4)-methane	[19388–87–5]	Taurolin	E. Geistlich Sons, Ltd. (Switzer-land, United Kingdom	Mucus membrane antiseptic; antiendotoxic	N/A	N/A
4,4-Dimethyloxa-zolidine (74.7%) + 2.5% 4,4-tri-methyloxazolidine	[51200–87–4]	Cosan 101	Cosan Chemical Co.	Paints, adhesives, cements, secondary oil	0.05–0.5%	United States (EPA); Europe (ECPIN)

(continued)

TABLE VIII *(continued)*

Active ingredient (chemical name when known)	CAS Reg. No.	Trade name and/ or generic name	Manufacturer	Use	Recommended level of use	Availability and regulatory status
		Bioban CS 1135	ANGUS Chemical Co.	Metalworking fluids		
		Bakzid 2 (oxazolidine)	Bacillol-fabrik Dr. Bode, Bode Laboratories	Metalworking fluids	0.15%	
		Bodenil	Bacillol-fabrik Dr. Bode, Bode Laboratories	Metalworking fluids	0.05%	
5-Hydroxymethoxy-methyl-1-aza-3,7-dioxabicyclo-[3.3.0]octane 24.5%; 5-hydroxy-methyl-1-aza-3,7-dioxabicyclo-[3.3.0]octane 17.7%;5-hydroxy-poly[methyl-eneoxy(74% C_2, 21% C_3, 4% C_4, 1% C_5)]methyl-1-aza-3,7-dioxa-bicyclo[3.3.0]-octane 7.8% (50% active)	[56709–13–8]	Nuosept® 95	Tenneco	Paints, adhesives, metalworking fluids, other aqueous systems	0.1–0.5%	EPA #1100-82-4830; Western Europe
		Nuosept 95	Durham Chemicals			
		Bioban N-95	ANGUS Chemical Co.			

1-Aza-3,7-dioxa-5-ethylbicyclo[3.3.0] octane (90% active)	[7747–35–5]	Bioban CS-1246	ANGUS Chemical Co.	Metalworking fluids, fuel	0.05–0.2%	United States; Wester Europe
Exact chemistry not available		Grotan (bisoxazolidine)	Sterling Industrial Schülke & Mayr	Oils, fuels, adhesives, cutting emulsions, spin finishes	0.05–0.2%	Western Europe
N-Methylolchloracetamide (39% active)	[2832–19–1]	Grotan HD	Sterling Industrial Schülke & Mayr; Lehn & Fink	Aqueous systems	0.075–0.2%	United Kingdom; Western Europe; United States; Canada
(Hydroxymethyl)-5,5-dimethyl-2,4-1,3-bisimidazolidine-dione (55% active	[6440–58–0]	Glydant; Dantoin DMDMH-55 (dimethyloldimethylhydantoin)	Lonza	Cosmetics	0.05–0.2%	FDA
Methanebis[N,N′-(5-ureido-2,4-diketotetrahydroimidazole)-N,N-dimethylol]	[39236–46–9]	Germall 115 (imidazolidinyl urea)	Sutton (U.S.A.)	Cosmetics	0.3%	FDA

(continued)

TABLE VIII *(continued)*

Active ingredient (chemical name when known	CAS Reg. No.	Trade name and/ or generic name	Manufacturer	Use	Recommended level of use	Availability and regulatory status
Poly[methylene-di(hydroxymethyl(urea]	[9011-05-6]	Polynoxylin; Anaflex; Ponoxylan; Larex	E. Geistlich & Sons, Ltd. (Switzerland, United Kingdom)	Topical antibacterial	0.2%	United Kingdom; Western Europe
N-Hydroxymethyl-N'-methylthiourea	[15599-39-0]	Noxyflex; Noxythiolin (N-methylol-thiourea)	E. Geistlich & Sons, Ltd. (Switzerland, United Kingdom	Peritoneal antiseptic	2–5% lavage	United Kingdom; Western Europe
2-(Hydroxymethyl)-amino-2-methyl-propanol	[522-99-20-4]	Troysan 192	Troy Chemical Co.	Latex paints, resin emulsions	0.05–0.15%	EPA
2-(Hydroxymethyl)-aminoethanol	[34375-28-5]	Troysan 174	Troy Chemical Co.	Paints, resin emulsions	0.05–0.15%	EPA
Tris(hydroxy-methyl)nitro-methane	[126-11-4]	Tris Nitro	ANGUS Chemical Co.	Metalworking fluids	0.1%	EPA #271-18
4-(2-Nitrobutyl)-morpholine (70%) + 4,4'-(2-ethyl-2-nitrotrimethyl-ene)dimorpholine (20%)	[1854-23-5]	Bioban P-1487	ANGUS Chemical Co.	Metalworking fluids, distillate fuels	0.01–0.3%	EPA #271-30

2-Bromo-2-nitro-1,3-propanediol	[52–51–7]	Bronopol; Onyxide 500	ANGUS Chemical Co.; Boots; Onyx-Stepan	Cosmetics	0.01–0.1%	United States; United Kingdom
N-Methylochloro-acetamide + glycols + isothia-zolinones		Parmetol K50	Sterling Industrial Schülke & Mayr	Aqueous emulsions, adhesives, paints	0.05–0.2%	
Hexahydro-1,3,5-tris(2-hydroxy-ethyl)-s-triazine (63.6%) + sodium 2-pyridinethiol-1-oxide (6.4%)		Triadine 10	Olin Corp.	Used in oil/water emulsion concen-trates with care; compatible with most other concen-trates	0.7% synthetic fluids; 0.1% in oil-containing fluids	EPA #1258–990; Western Europe
Hexahydrotriazine + S,N-hetero-cyclic compounds		Margold KM 203	Hoechst UK; Riedel-de Haën	Metalworking fluids	0.05–0.15%	Western Europe
Bisoxazolidine + isothiazolinone		Grotan OD	Sterling Industrial Schülke & Mayr	Water-based emulsions, oils, fuels	0.05–0.2%	Western Europe
Isothiazolinones + formals		Grotan TK2	Sterling Industrial Schülke & Mayr	Metalworking fluids, aqueous systems	0.05–0.2%	Western Europe

(continued)

TABLE VIII (continued)

Active ingredient (chemical name when known)	CAS Reg. No.	Trade name and/ or generic name	Manufacturer	Use	Recommended level of use	Availability and regulatory status
N-Methylochlor-acetamide + heterocyclic compounds		Mrthsl K 6 N	Hoechst UK; Riedel-de Haën	In-can preservation	0.1–0.3%	Switzerland
N-Methylochlor-acetamide + isothiazolinone		Parmetol A23	Sterling Industrial Schülke & Mayr	Aqueous systems, paints, dyes, adhesives	0.1–0.3%	United Kingdom; Western Europe
N-Methylochlor-acetamide + isothiazolinones		Parmetol DF12	Sterling Industrial Schülke & Mayr	Paints, in-can film, fungicidal wash	0.10–0.2%; 1.0–3.0%; 1.0–2.0%	
Semiacetal		Bodoxin	Bacillol-fabrik Dr. Bode, Bode Laboratories	Metalworking fluids, emulsion paints, synthetic polymer dispersions, slimicide in paper manufacture oil field	0.05–0.1%	West Germany (BGA) for polymer dispersions, and as slimicide (paper for food packaging)
Semiacetal (with glutaraldehyde/ formaldehyde reaction product)		Sepacid CE 5265	BASF	Oil field	0.001–0.005%	

[a]The major sources for information in this table were Allsopp and Allsopp (1983; Sharpell (1980); and Rossmoore (1979). This list is only complete within the limits of the search conducted; the absence of any product and/or manufacture implies neither a lack of importance nor lack of existence.

guidelines may impose more stringent requirements for registration or re-registration, including mutagenicity tests as well as other subchronic and chronic tests (U. S. EPA, 1987).

III. Mode of Action

A. Formaldehyde Role

At the outset, there should be agreement that the mode of action of all the FA adducts is partially or exclusively attributable to FA. What must be defined are the limitations of the concept of "mode of action." In dealing with antimicrobial agents used in chemotherapy, we are usually concerned with some specific metabolic site, e.g., organelle assembly (peptidoglycan synthesis with β-lactams), protein synthesis (tetracyclines), purine synthesis (sulfonamides). The high degree of reactivity of FA with all the nucleophiles in biological molecules precludes any facile prediction of degree of importance in causing loss of viability. Thus, ultimate "mode of action," a vital target in the metabolic machinery of the cell, may not be discernible.

What should be the most obvious mode of action, or perhaps the most pragmatic, relates to the release of FA from the putative biocide. Certainly this means how much and under what condition. Does synthesis input equal action output? Product reactivity must always be a function of environmental conditions. Many are pH dependent. Once "available," the FA has a number of indiscriminate targets. The amount available for the microbial cell is dependent on the amount of nucleophiles extrinsic to the cell. In this regard, FA is no different than other biocides (e.g., halogens, heavy metals).

The secondary mode of action involves the hit; the reaction with the cell envelope. We cannot assume that FA is the magic bullet in any environment containing primary amines which may be the most abundant reactive chemical species with FA. They form methyloamines which will subsequently react with the cell. There must be a great deal of reaction with envelope proteins and LPS prior to actual entry into the cell. Gidley and Sanders (1983) investigated the role of FA in *N*-methylolthiourea mode of action and concluded that so-called "masked" activity based on imminium ion function (Gidley *et al*, 1981) was not relevant. Formaldehyde was directly related quantitatively to N-methylolthiourea activity.

Formalin-killed *Pasteurella multocida* affected the extractability of LPS and although no antigenic differences were noted, toxicity for chick embryo was less than for LPS from cells not formalin treated (Rebers and Rimler, 1984). Since the LPS came from FA-treated intact cells, this is

indeed proof of first hit with envelope sites, probably amino groups. The authors report also of the use of $^{14}CH_2O$ yielding ^{14}C-LPS in *Salmonella*. Both *Salmonella* and *Pasteurella* LPS contain 2-aminoethanol. Methylene bridge formation would permanently immobilize the FA and apparently affects the biological properties of the cell envelope (e.g., toxicity).

Methylol derivatives of amino acids serve as methylene donors to bridge neighboring reactive compounds (e.g., other amino acids, including sulfhydryls as well as free amines). Denaturation of porin proteins by recommended levels of biocide would preclude any meaningful concerns with the nonlethal effects of lower levels. Barnes and Eagon (1986) reported on studies on the mechanism of action of HET against several strains of *P. putida* and *Escherichia coli*. Proline uptake and induced enzyme synthesis were inhibited by both formaldehyde and an equivalent amount of HET. Levels of inhibiters used were equivalent ot calculated MIC.

There is no indication that any effects or metabolic lesions produced by FA adducts reflect anything but the action of FA. The report cited above (Barnes and Eagon, 1986) suggests that a major mechanism of HET action is via FA. They stated that any contribution of HET to mode of action still lacked evidence. There are several approaches to demonstrate the relative contribution of FA and an intact FA adduct to antimicrobial activity. One obvious method is based on the putative FA content with appropriate comparisons made to similar levels of FA itself. This approach has a number of drawbacks. Under actual use, the adduct must be less stable (i.e., release FA to an appropriate molecule).

The biological equilibrium of FA and another adduct, HMT, has also been demonstrated (Plesner and Hansen, 1983). Transformation of baby hamster kidney cells (Styles' cell transformation) at the rate of about 35 per 5×10^5 cells was found with 4 μg FA/ml and 100 μg HMT/ml after 3 hours exposure. Considering the FA content of the HMT molecule at 6 FA/HMT on a molar as well as dose basis (using above doses), almost 30 times as much FA is potentially available for HMT. This low rate of FA release from HMT in biological pH is in agreement with measurements with Nash reagent (Table IV).

B. Role of Other Active Groups

All of the FA adducts, no matter how tested, appear to owe at least part of their activity to FA (Sondossi *et al.*, 1986b; Paulus, 1976). Conceded that relative differences in activity of different adducts can be due to environmental conditions that affect hydrolysis (pH) or availability (neucleophiles, metals), there are compounds that demonstrate additional qualitative effects. This is essentially so with the 2-nitro-2-bromo-1,3-propandiol

which is extremely active whereas tris(hydroxymethyl) nitromethane has minimal activity. Induction of resistance with FA to the nitrobromo adduct was not demonstrated at pH 7. Minimal levels of FA were detected with the Nash assay (Table IV) for both adducts. However, both are referred to as FA active compounds.

Hydrolysis of β-bromo- β-nitrostyrene yields bromonitromethane, the possible active moiety of the precursor styrene (Friend and Whitekettle, 1980). This suggests that there is an antimicrobial contribution from the non-FA portion of the 2-bromo-2-nitro-1,3-propanediol. Similarly, the cross-resistance studies with the nitromorpholine derivative (Sondossi *et al.*, 1986a; see also Section II,D) suggest that the "nitro" group may very well have independent activity.

It should be emphasized that complexing FA with another putative biocide does not guarantee an increase in either the qualitative or quantitative activity of the adduct (Paulus, 1976). THe thiadiazine FA-dithiocarbamate adduct is cited for its high relative activity (Bennett, 1973; Paulus, 1976) which Ross and Hollis (1976) stated may be related in part to the dithiocarbamate fraction. Recent analytical results (Table IV) with Nash reagent at pH 7.0 give close to 100% FA yield (2 FA/adduct). The more complete release or greater availability of FA should be considered the basis for increased activity until there is positive evidence for the contribution of the non-FA moiety. The approach taken by Sondossi *et al.* (1986b) using cross-resistance to all the structural components of the FA adduct appears the only valid mechanism to establish the contribution of each to the mode of action.

There is strong evidence that other residues of FA adducts (e.g., hydrogen bonds between carbonyl and methylol groups) will influence FA release and availability. A prime example of this influence results in the relative low detectability and poor release of FA from amide-N-methylol compounds (Paulus, 1976; Sondossi *et al.*, 1986a) (Table IV).

Bowman and Lindstrom (1985) recently reported that a imidazolidinylurea-resistant *P. putida* strain was inhibited by FA, concluding that resistance to imidazolidinylurea is unrelated to FA. Based on their data, this conclusion is untenable. They neither challenged FA-resistant cells with imidazolidinylurea nor did they choose stoichiometric equivalents of FA for their reported studies.

If hydrogen bonds are involved in stabilizing amide-N-methylol compounds, then pH may have an effect on FA release. This seems to be the case for imidazolidinylurea (Engelhardt and Klinkner, 1985).

The role of neighboring substituents on FA release was demonstrated in another type of study. THe efficacy of HEAT and not HET was reduced by Al^{3+} (Rossmoore, 1979). It was suggested that the AL^{3+} formed a coordination complex with the hydroxyl oxygen and ring nitrogen, stabilizing the triazine molecule and inhibiting hydrolysis.

C. Role of Analytical Methods in Determining Formaldehyde Activity

Because of legal constraints on (free) FA levels, concerns for long-term preservation and perhaps an unwillingness on the part of manufacturers to equate FA adducts with FA release, there has been an interest in measurement of so-called free FA in the presence of FA donors. This is no easy task since the avidity of the analytical reagent for FA must not exceed the avidity of the FA condensate for FA. The use of 2,4-pentandione, the Nash (Nash, 1953) reagent, at pH 6.5 competes favorably with aminals for FA (Table IV), giving yields of FA approaching the amount structurally available. At that operational pH, the N-methylols and C-methylols do not as readily yield FA.

Chemical reagents cannot be relied upon to separate nondestructively free from bound FA (Turoski, 1985). Polarigraphic methods are useful in this regard as is use of NMR with and without D_2O. The combined use of HPLC separation and column reaction detection followed by reaction with 2,4-pentandione made it possible not only to determine "free" FA in the presence of the adduct but also to monitor the decomposition kinetics at different pH levels (Engelhardt and Klinkner, 1985).

The analytical techniques have some relationship to biological activity since the sensitive biological site must have greater avidity for the FA than the FA donor. This also may be true for vertebrate toxicity since toxicity must reflect reactivity and relative avidity. Stability of FA generators was related to both tissue lipophilicity and carcinogenic potency (Ashby and Lefevre, 1982). Genotoxicity of labile FA generators, if any, will only be evident locally, related to their rate of hydrolysis. These would unlikely be systemically toxic based on the noncarcinogenic response in rats fed only with HMT and dinitrosopentamethylenetetramine (DNPT). Compounds stable to hydrolysis and lipophilic as well [e.g., hexamethylphosphoramide (HMPA)] may elicit a systemic effect due to their distribution prior to metabolism. All three compounds (HMT, DNPT, and HMPA) are sources of FA, but only the latter yields FA via enzyme-mediated conversion to a methylol. It occurs slowly *in situ* and is genotoxic, whereas the HMT and DNPT are too stable for any consequential FA release *in vivo*.

IV. Uses and Applications

The potential for the use of FA adducts in a variety of preservative and biocidal applications is a function of the nature of the substrate to be treated or preserved, the pH of the environment, the level of nucleophile present, the variety and extent of microbial contamination, and the relative toxicity of the FA progenitor.

Essentially, the reactivity of FA itself with amines and sulfhydryls has

extended its utility in microbiology not only for reducing viability but also for reducing toxicity of biologically active proteins by modifying their structure.

What Ramon in 1923 referred to as diphtheria anatoxin is now called toxoid (Wilson and Miles, 1946). Ramon reported the first effective use demonstrating the subtle reactivity of FA with diphtheria and tetanus (exo) toxins, reducing toxicity without modifying antigenicity. It is conceivable that methylene bridge formation between free amino groups induces polymerization and thus increases immunogenicity. At the same time, active sites of toxicity could be blocked (Bizzini and Raynaud, 1974). A specific case for toxicity reduction with enhanced immunogenicity after FA treatment was made for staphylococcal δ-hemolysin (Stearne and Birbeck, 1979). At pH 5 or 7.5, hemolytic activity was rapidly lost but antigenic reactivity was enhanced at pH 5. There was no evidence, however, of polymerization.

Reactivity is not limited to protein toxins but also has been reported for lipopolysaccharide (LPS) endotoxins (Pfirmann and Leslie, 1979). Using an animal model (rabbits and mice), tauroline was effective both *in vitro* and *in vivo* in preventing the lethal and pyrogenic activities of a number of gram-negative endotoxins. The authors make no speculation about a possible mechanism of action.

Inhibition of enzyme activity by FA has also been exploited. The end result of microbial growth in cellulose ester latex paints is viscosity reduction from cellulolytic activity. A variety of FA-amine adducts are claimed to inhibit both bacterial and fungal cellulase (Nowack and Singer, 1977).

The recognized and accepted uses of FA adducts are listed in Table VIII.

V. Conclusion

The reactivity of FA has made possible the synthesis of a wide variety of adducts, including aminals, formals, and methylols. As a group, they exhibit compatibility with cosmetics, metalworking fluids, adhesives, paints, wood, etc. They exhibit stability from acid to alkaline pH depending on the nature of the adduct. Their selection is based on the environmental pH and the rate of FA release desired. For the most part, with a few exceptions as noted, the mode of action of the FA adduct is FA itself. The extent of activity is a function of the avidity of FA for the adduct and the biological molecule initially hit. Resistance development, especially to aminals, is stoichiometrically related to FA resistance. Microbial resistance to FA should be anticipated. Dissimilation of FA via intermediary metabolism is carried out by many nonmethylotrophs (e.g., *Pseudomonas* sp.). The presence of FA as a potential substrate, especially at sublethal levels, could be expected to increase the capacity for utilizing FA (e.g., FA dehydrogenase) levels.

The known mutagenicity of FA may also contribute to the increased resistance in the progeny of survivors.

There appears to be a divergence of opinion between medical-based (Allwood and Myers, 1981; Mandell and Sande, 1980) and industrial-based (Paulus, 1980; Druskeit and Eggensperger, 1971) reports concerning resistance development to FA among bacteria. The former state no resistance develops, while the latter state the opposite. The evidence published and reviewed here support this latter conclusion.

Knowledge of mode of action of FA makes it possible to utilize mixtures to extend the spectrum of these compounds.

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Occurrence and Mechanisms of Microbial Oxidation of Manganese

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I. Introduction

The oxidation of manganese by microbes has been a subject of great interest for many years, and much has recently been written concerning the biogeochemistry of manganese (Kuznetsov, 1975; Marshall, 1979; Ehrlich, 1981; Nealson, 1983a,b). There is little doubt that microbes are major catalysts of manganese cycling; their effects can be seen on scales that can range from microscopic to grossly macroscopic, and possibly even global. Many different organisms oxidize manganese (Ehrlich, 1981; Nealson, 1983b; Ghiorse, 1984b), including bacteria, algae, yeast, and fungi, and the mechanisms by which they do this may be as diverse as the organisms themselves.

Since several excellent reviews have been presented in the past few years on the biogeochemistry and microbiology of manganese, this article focuses on two aspects of the field in which recent progress has been made. First, we examine field studies of Mn(II) oxidation, including newly developed methods for measuring rates of Mn(II) oxidation and distinguishing biological from chemical oxidation. We then present a

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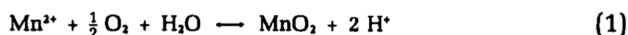
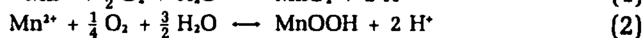
brief synopsis of some of the field data that unequivocally establishes the importance of microbes in Mn(II) oxidation in natural systems. Second, we review the recent physiological, structural, and biochemical studies of microbial manganese oxidation. To introduce these subjects, we first present an overview of the chemistry and biology of manganese which must be understood in order to properly appreciate the field and laboratory studies.

A. Manganese Chemistry

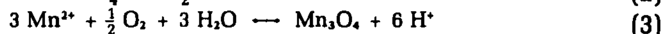
Manganese is the fifth most abundant metal (it is the second most abundant transition metal after iron) on the surface of the earth. Although it can exist in any one of seven different redox states, in nature Mn is most commonly found with an oxidation state of II, III, or IV (Table 1). Mn(II) forms many soluble salts with a variety of anions, and several insoluble carbonate and phosphate minerals, the most common being Mn(II) carbonate or rhodochrosite. In natural waters the soluble form occurs primarily as the biologically available free cation (Mn^{2+}), although in seawater the chloride species ($MnCl^{+}$) also occurs. Mn(III) and Mn(IV) form a variety of different insoluble oxides and oxyhydroxides (Balistrieri and Murray, 1982; Murray, 1973; Murray and Dillard, 1979), often referred to as particulate manganese. The most common form of these is usually given the formula MnO_x, where $1 < x < 2$. The oxidation of Mn(II) typically follows these three representative stoichiometric relationships, depending on the oxide product formed:



$$Mn^{2+} + \frac{1}{2} O_2 + H_2O \longrightarrow MnO_2 + 2\,H^+ \qquad (1)$$



$$Mn^{2+} + \frac{1}{4} O_2 + \frac{3}{2} H_2O \longrightarrow MnOOH + 2\,H^+ \qquad (2)$$



$$3\,Mn^{2+} + \frac{1}{2} O_2 + 3\,H_2O \longrightarrow Mn_3O_4 + 6\,H^+ \qquad (3)$$

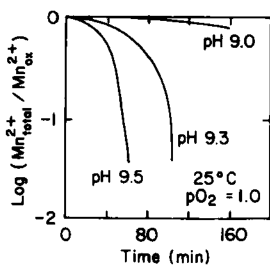
At the pH and E_h values characteristic of the Earth's surface, the equilibrium of these reactions is shifted to the right (Table I). However, since the activation energy of Mn(II) oxidation is large (Stumm and Morgan, 1981), these reactions proceed very slowly, and soluble manganese can be quite stable in natural waters. The oxidation of Mn(II) is, however, pH sensitive, and, as the pH rises, inorganic oxidation proceeds faster; at pH values above 9.0, the reaction is very rapid. Because the Mn(II)/Mn(IV) redox transformation is poised around the pH characteristic of the Earth's surface (and of most biological systems), manganese is probably one of the most interactive metals with which the biota interface. To further complicate matters, Mn(II) oxidation is autocatalytic; once oxidation begins, the freshly formed MnO_x adsorbs Mn(II) and greatly increases the rate of reaction. Thus, in order to predict the rate of Mn oxidation in any environment, pH, E_h, counterions, and

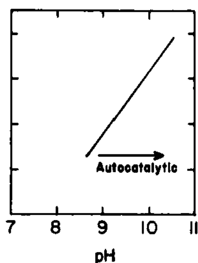
TABLE I

MANGANESE CEMISTRY AND BIOCHEMESTRY

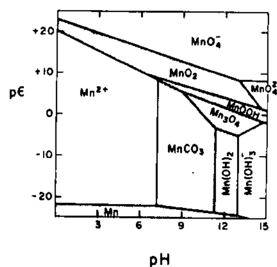
	high pH, E_h →		
General Notation:	Mn(II) ← – – – →	Mn(III) ← – – – →	Mn(IV)
	← low pH, E_h		
Cationic Forms	Mn^{2+}	Mn^{3+}	Mn^{4+}
Examples of Compounds Formed	$MnCl_2$, $MnSO_4$, etc.	Mn_2O_3, MnOOH, etc.	MnO_2
General Properties:	A. Forms soluble salts	A. Form insoluble oxides and oxyhydroxides, e. g., Manganates (MnO_x, $1<x<2$)	
	B. Forms insoluble minerals 1. $MnCO_3$ 2. $MnPO_4$	B. Manganese oxides adsorb trace metals and radionuclides, e.g., Cd, Co, Cu, Ni, Mn	
	C. Biologically available 1. Required trace element 2. Specific transport systems 3. Toxic at high concentrations	C. Unavailable as trace nutrients 1. Nontoxic 2. Potential electron acceptor	

KINETICS OF MANGANESE OXIDATION





THERMODYNAMIC STABILITY OF MANGANESE PHASES



manganese oxides present must be known. In theory, anything that leads to changes in E_h, pH, or binding of Mn(II) (which might alter its activation energy) could affect the rate of Mn(II) oxidation under the oxic conditions typical of the present-day Earth environment.

The ability of manganese oxides to adsorb cations is not restricted to Mn(II); they are capable of adsorbing many other cations, and have been

referred to as the "scavengers of the sea" (Goldberg, 1954) because of this capacity. When Mn(II) oxidation is occurring, there is always the possibility that other metals (e.g., Co, Cu, Ni, Cd) will be removed by adsorption to the freshly formed surfaces. For example, oxides of manganese have been used to remove radium quantitatively from natural waters (Moore and Cook, 1975).

It is generally believed that bacteria may use manganese oxides as electron acceptors for respiration of organic matter under anoxic conditions (Ehrlich, 1981). Both Mn(III) and Mn(IV) oxides have favorable electron potentials (E_h) for this process and it is hypothesized that biological reduction of manganese is of central importance in many sedimentary environments.

B. The Biology of Manganese

Manganese is a required trace element, and virtually all organisms require it in very small amounts. The most well known of the functions is that of catalyzing oxygen evolution in the photosynthetic reaction (see review by Dismukes, 1986). Thus, Mn(II) is required in relatively large amounts by phytoplankton and higher plants, and for many of these organisms, manganese limitation can be easily demonstrated (Shapiro and Glass, 1975; Sunda and Huntsman, 1985). Although Mn(II) is a required element for bacteria and nonphotosynthetic organisms, manganese limitation is often difficult to establish, since only trace amounts are usually required.

Some other functions of manganese include those shown in Table II. Many of the reactions in which manganese is involved require molecular oxygen, and the ways in which Mn(II) reacts with oxygen may well be the basis for much of its importance. For example, manganese is proposed to have a role in oxygen protection (Archibald and Fridovich, 1981; Kono and Fridovich, 1981). Other bacteria possess a Mn-containing superoxide intracellularly, where it acts as a scavenger of oxygen radicals (Archibald and Fridovich, 1985). Other bacteria possess a Mn-containing superoxide dismutase (Vance *et al.*, 1972) or Mn-containing pseudocatalase (Kono and Fridovich, 1983).

Since Mn(II) is often in very low concentrations, especially in oxic environments, it is perhaps not surprising that most organisms have very efficient systems for transporting Mn(II) into cells. Although very few bacteria have been examined, those that have been studied possess both low and high affinity transport systems that are induced in response to high and low manganese concentration, respectively, in the environment (Silver, 1978). Similarly, phytoplankton isolated from environments with

TABLE II

INVOLVEMENT OF MANGANESE WITH ENZYMES AND BIOCHEMICAL PROCESSES

Function or enzyme	Reference
Photosynthesis	Dismukes (1986)
Involved with oxygen evolution in all photosynthetic organisms possessing photosystem II	
Oxygen protection	
O_2^- scavenging by Mn(II)	Archibald and Fridovich (1981)
Manganese superoxide dismutase	Vance *et al.* (1972)
Manganese pseudocatalase	Kono and Fridovich (1983)
Nitrogen fixation	Yoch (1979)
Glutamine synthetase	Hunt and Ginsburg (1980)
Isopropylmalate synthetase	Weigel (1978)
Phosphoglycerate phosphomutase	Oh and Greese (1976)
Endonuclease activity	Scher and Dubnau (1976)

different levels of Mn(II) have been shown to possess transport systems that are adapted to the Mn(II) levels characteristic of these environments (Sunda and Huntsman, 1985, 1986).

At high concentrations, Mn(II) can be toxic, even to microbes. The basis for the toxicity is not understood, and is an area that needs more study. One area of recent interest is the observation that low concentrations of Mn(II) are mutagenic *in vitro* (Beckman *et al.*, 1985). The authors hypothesized that the mechanism of action involved binding of Mn(II) to single-stranded regions of DNA. This may provide a mechanism to explain the previous reports of Mn(II) mutagenicity (Demerec and Hanson, 1951) and carcinogenicity (Stoner *et al.*, 1976). There are many other examples of Mn(II) toxicity and inhibition that do not necessarily involve mutagenicity. Our own experiences have shown that when Mn(II) is added to natural lake water or seawater, even at concentrations that might be considered low (10–20 μM), the ability of the microbes in the water to take up and oxidize Mn(II) is markedly inhibited (Chapnick *et al.*, 1982; Tebo and Emerson, 1985), but like many other examples of Mn(II) toxicity, the mechanism of this inhibition is not understood.

For the organisms that oxidize Mn(II), the biochemistry of the processes are only beginning to be understood (see below). So far, no purified Mn(II) oxidases have been characterized. However, as will be shown, several examples are now known in which Mn(II)-binding proteins that catalyze Mn(II) oxidation are being purified and characterized, and progress is being made.

C. Manganese Cycling and Biogeochemistry

The global manganese cycle is not well understood, but some of the sources and sinks of manganese are shown in Fig. 1. The major reservoirs of manganese are in the soils and sediments, where they primarily exist as oxide-phase minerals and metal deposits. It is not unusual to find sediments where Mn oxides constitute 10% or more of the total weight percentage of the environment. Since MnO_x may be used by bacteria as an electron acceptor, Mn oxides can potentially play a major role in the redox chemistry and carbon cycling in sedimentary environments (see Reeburgh, 1983).

In oxic waters, manganese is concentrated in the biota, while in stratified marine basins, such as fjords, manganese also accumulates in the particulate matter just above the oxic/anoxic interface, where it then sinks down into the anoxic zones to be reduced again (probably by the sulfide just beneath the interface). Since Mn has no volatile phase, global cycling tends to be most strongly impacted by geological forces such as river transport, hydrothermal circulation, and tectonic activity.

For many years microbiologists have contended that Mn(II) oxidation by microbes was an important part of the biogeochemical cycle of manganese, and recent studies by geochemists and aquatic chemists have supported this view. The very existence of oxic/anoxic interfaces (such as those depicted in Fig. 1) that are established by biological activities tends to exemplify this. In anoxic zones, Mn(II) is thermodynamically

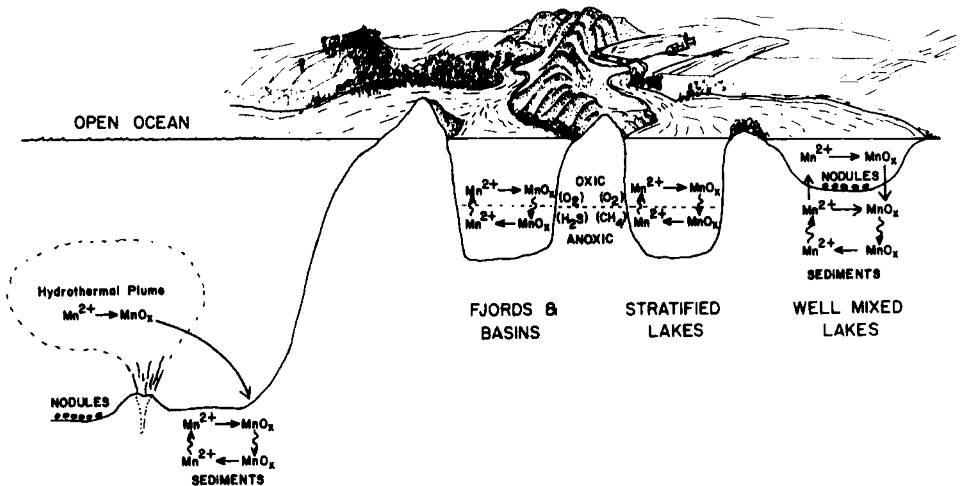


Fig. 1. Schematic of global sources and sinks of manganese.

favored; the potential at which Mn oxide reduction occurs is roughly the same as that of nitrate reduction, so soluble Mn(II) appears in sediment profiles soon after depletion of nitrate. Mn(II) then diffuses into the oxic zone where Mn(II) oxidation can occur. Because of the high activation energy of Mn(II) oxidation, this process is usually quite slow. When estimates of the rates of Mn(II) oxidation have been calculated from chemical models, however, the rates are often many orders of magnitude too fast to be explained by chemical mechanisms (Emerson *et al.*, 1979). It was the realization of this fact that led to much recent activity in attempting to understand the role of the biota in catalyzing Mn(II) oxidation in nature, and to attempt to separate biological from chemical oxidation.

D. CRITERIA FOR BIOLOGICAL INVOLVEMENT IN MANGANESE OXIDATION

Many studies of microbes isolated from naturally formed manganese oxides have concluded that the precipitates might be of biological origin. Microbiological analysis of manganese nodules (Ehrlich, 1978), manganese-coated rocks (Mustoe, 1981), desert varnish (Krumbein and Jens, 1981; Palmer *et al.*, 1986) manganese-clogged pipelines (Tyler and Marshall, 1967), and manganese-rich sediments (Nealson, 1978) commonly yield high numbers of Mn-precipitating bacteria. Furthermore, direct examination of natural precipitates (Tyler and Marshall, 1967; Ghiorse, 1980, 1984a; Ghiorse and Hirsch, 1979) often yields manganese-coated forms that lead one to strongly suspect that the bacteria are causally involved with the formation of the precipitates.

However, the criteria that must be met to prove the involvement of microbes in Mn(II) oxidation in nature include the actual determination of oxidation rates (Duinker *et al.*, 1979; Emerson *et al.*, 1979), preferably with controls that distinguish microbial from chemical oxidation (Wollast *et al.*, 1979; Emerson *et al.*, 1982; Rosson *et al.*, 1984). Much work has been done in the past few years to establish the importance of the biota in catalyzing Mn(II) oxidation in nature, as will be discussed later.

E. BIOLOGY OF MANGANESE OXIDATION

A wide variety of different bacteria, algae, and fungi have been reported to accumulate oxidized manganese (see reviews by Marshall, 1979; Ehrlich, 1981; Ghiorse, 1984b), but little is really known of the reason for this or of the mechanisms by which it occurs. Since the oxidation of Mn(II) to Mn(IV) is an exergonic reaction, yielding approximately −18.2 kcal/mol at 1 M concentrations of reactants (Table III), Mn(II) has always been suspected of being utilized by bacteria as an energy source for chemolithotrophic growth. The idea still predominates, and a reasonable

TABLE III

CALCULATED VALUES OF THE FREE ENERGY CHANGE OF MANGANESE OXIDATION UNDER VARIOUS CONDITIONS OF pH, Mn(II) CONCENTRATION, AND O_2 TENSIONS[a]

Reaction	pH	[Mn(II)]	[O_2]	ΔG [kcal/mol of Mn(II) oxidized][b]
$Mn^{2+} + \frac{1}{2} O_2 + H_2O \rightarrow MnO_2 + 2 H^+$	7	1 M	1 atm	−16.2[c]
	7	1 M	1 M	−18.2
	6	1 μM	225 μM	− 4.8
	7	1 μM	225 μM	− 7.5
	8	1 μM	225 μM	−10.2
	9	1 μM	225 μM	−12.9
	8	1 mM	225 μM	−14.3
	8	100 μM	225 μM	−12.9
	8	10 μM	225 μM	−11.6
	8	1 μM	225 μM	−10.2
	8	100 μM	225 μM	−12.9
	8	100 μM	100 μM	−12.7
	8	100 μM	50 μM	−12.5
	8	100 μM	10 μM	−12.0
$3 Mn^{2+} + \frac{1}{2} O_2 + 3 H_2O \rightarrow Mn_3O_4 + 6 H^+$	7	1 M	1 atm	−10.0[c]
	7	1 M	1 M	−10.6
	6	1 μM	225 μM	1.1
	7	1 μM	225 μM	− 1.6
	8	1 μM	225 μM	− 4.3
	9	1 μM	225 μM	− 7.1
	8	1 μM	225 μM	− 8.4
	8	100 μM	225 μM	− 7.1
	8	10 μM	225 μM	− 5.7
	8	1 μM	225 μM	− 4.3
	8	100 μM	225 μM	− 7.1
	8	100 μM	100 μM	− 7.0
	8	100 μM	50 μM	− 6.9
	8	100 μM	10 μM	− 6.8

[a]225 μM O_2 is about the value of oxygen-saturated seawater (most oceanic dissolved O_2 concentrations are lower). In aerobic environments, 1 μM Mn(II) is a generous value for the concentration of Mn(II).

[b]ΔG was calculated from the equation: $\Delta G = \Delta G^\circ + RT \ln K$. The ΔG° was determined from the free energy change of formation (ΔG_f°) given in Stumm and Morgan (1981), assuming an oxide phase of MnO_x where $1.7 < x < 2.0$.

[c]This is the standard free energy change for this reaction at 25°C per mole of Mn^{2+} oxidized.

amount of circumstantial evidence now supports it. However, no direct proof that Mn(II) is used as an energy source has been presented, and for many cases of microbial Mn(II) oxidation, there is little doubt that energy utilization is *not* accomplished during the oxidation (Ehrlich, 1984).

If energetics is not the reason for Mn(II) oxidation, then what is? Although many possible explanations exist (Grosovsky, 1982), few have been supported with data, and careful work is needed to understand why the process is so widespread and abundant among microorganisms. One of the more intriguing hypotheses is that Mn(II) removal is a way of dealing with toxic levels of Mn(II) that occur on an annual basis in environments that stratify. It may well be that when the mechanisms are more fully understood, the reason(s) for the oxidation will become obvious.

II. Manganese Oxidation in Natural Environments

Several years ago, the evidence for the importance of microbes in catalyzing the oxidation or reduction of manganese in nature was largely circumstantial. In many environments, both the high numbers of Mn(II)-oxidizing bacteria that were often cultured and microscopic analyses of natural precipitates by scanning and transmission electron microscopy (structural studies) suggested that microbes might be involved in manganese precipitation. Field studies were largely driven by the laboratory data, in the sense that most of the efforts concentrated on obtaining viable counts of Mn(II)-oxidizing bacteria and structural analyses of the isolated bacteria. In the past few years this situation has changed markedly, in part because of excellent geochemical data. For example, when the concentration gradients of oxidized and reduced forms of manganese across an O_2/H_2S interface in a reducing fjord were obtained and modeled to obtain Mn(II) oxidation rates (Emerson *et al.*, 1979), it was concluded that the mean residence time of manganese with respect to oxidation was on the order of days. These rates of oxidation were much too rapid to be accounted for by chemical oxidation (Stumm and Morgan, 1981) or by catalysis by particulates such as iron oxides (Sung and Morgan, 1981), and hence biological oxidation was hypothesized (Emerson *et al.*, 1979).

Largely on the basis of results like these, efforts were begun to develop methods to separate biological from chemical Mn(II) oxidation. Because there was a readily available radioactive isotope of manganese (^{54}Mn, a gamma emitter) that could be obtained at a high specific activity, it was possible to measure Mn(II) uptake or removal rates by adding ^{54}Mn(II) as a tracer and trapping the oxidized and bound Mn radioactivity on filters, much as one uses ^{3}H- or ^{14}C-labeled compounds to measure

heterotrophic uptake or carbon fixation. However, distinguishing biological from chemical oxidation proved to be very difficult. The chosen approach (Tebo, 1983; Rosson *et al.*, 1984) was to measure the uptake of $^{54}Mn(II)$ in the presence of poisons, fixatives, inhibitors, or antibiotics, to specifically inhibit biological activity, thereby gaining a measure of abiotic Mn(II) removal attributable to adsorption or inorganic oxidation. In order for this to be a viable approach, the poisons should not interfere in the solution chemistry of manganese. Poisons were tested with the goal of finding those that could meet the following criteria: (1) the poison should not catalyze Mn(II) oxidation; (2) the poison should not reduce MnO_x; (3) the poison should not displace Mn(II) adsorbed to MnO_x; (4) the poison should not interfere in adsorption of Mn^{2+} onto MnO_x.

After an exhaustive study, several poisons were selected that met these criteria, namely sodium azide, well-buffered formalin, and mercuric chloride. Virtually all others tested failed to meet one or more of the above criteria (Rosson *et al.*, 1984). It should be emphasized that using poisoned controls to inhibit Mn(II) removal measures Mn(II) binding or removal from solution, but not oxidation per se. To demonstrate oxidation requires additional tests (see below).

Using this approach, we initiated several studies of biological Mn(II) binding in different environments. In all of these, it was concluded that biological activity was important. The environments studied included Saanich Inlet, British Columbia, Canada (Emerson *et al.*, 1982; Rosson *et al.*, 1984; Tebo *et al.*, 1984; Tebo and Emerson, 1985), Framvaren Fjord, Norway (Tebo *et al.*, 1984), Oneida Lake, New York (Chapnick *et al.*, 1982), and Lake Washington, Washington (Maki *et al.*, 1987). Several other groups have reached the same conclusions for other study sites. Wollast *et al.* (1979), based on the results of poisoning experiments with chloroform, postulated that microbial activity was important in Mn(II) cycling in the Scheldt estuary. Vojak *et al.* (1984) presented evidence of the potential for microbial activity in the River Tamar Estuary in southwest England, and Cowen *et al.* (1986) have presented preliminary evidence for microbial activity in hydrothermal vent plumes. In no environment studied in which rates of Mn(II) removal were easily measured has the biological component been insignificant.

Perhaps the most intense Mn(II) binding was measured in Framvaren Fjord. This Fjord has a very shallow sill depth, and consequently the basin is permanently stratified, becoming anoxic around 18 m, a depth at which light still penetrates. In addition, because of the density gradient (fresh water overlying saline water), the gradients of redox species and metals are extremely steep. Consequently, the O_2/H_2S interface supports a large microbial biomass that can be seen as an increase in light scattering at this depth (Fig. 2). In water column experiments, manganese

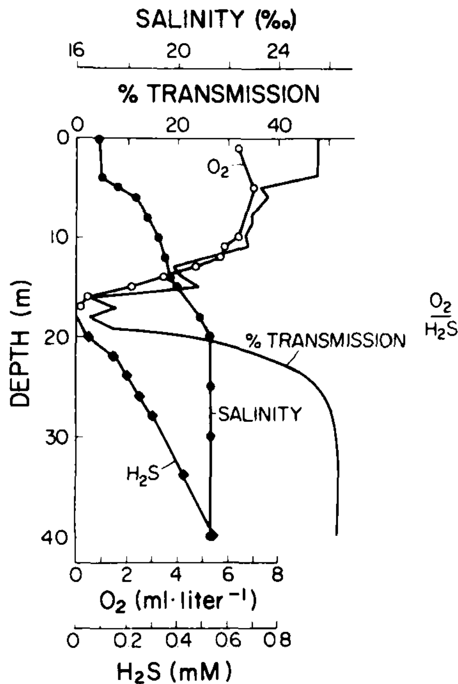


FIG. 2. Profiles of oxygen, sulfide, salinity, and percentage of light transmission in Framvaren Fjord, August 1981.

binding is maximal just above the oxic/anoxic interface, and addition of azide essentially stops all ^{54}Mn(II) binding, indicating the importance of biological catalysis in this process (Fig. 3).

Most of these environments are strongly stratified, and since the major removal of Mn(II) occurs just above the oxic/anoxic interface, oxygen tension varies widely in different samples. To circumvent this potential problem in Saanich Inlet, studies were done with an *in situ* sampling device (Taylor *et al.*, 1983; Tebo *et al.*, 1985). This device allowed the incubation of a water sample at depth with ^{54}Mn(II), with subsamples being removed and poisoned at various times via automated syringes. Mn(II) removal rates obtained with the *in situ* sampler were moderately slower than those obtained using the shipboard methods under air saturation (Tebo *et al.*, 1985).

As mentioned before, none of the methods described above proves that Mn(II) is being oxidized. However, when the amount of Mn(II) binding or removal is much too great to be accounted for simply by adsorption onto particulate matter and intracellular uptake by bacteria, it suggests

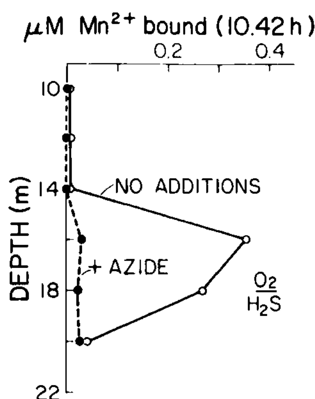
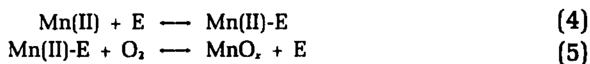


FIG. 3. Profile of ^{54}Mn(II) binding under air saturation conditions in the presence and absence of azide and as a function of depth in Framvaren Fjord.

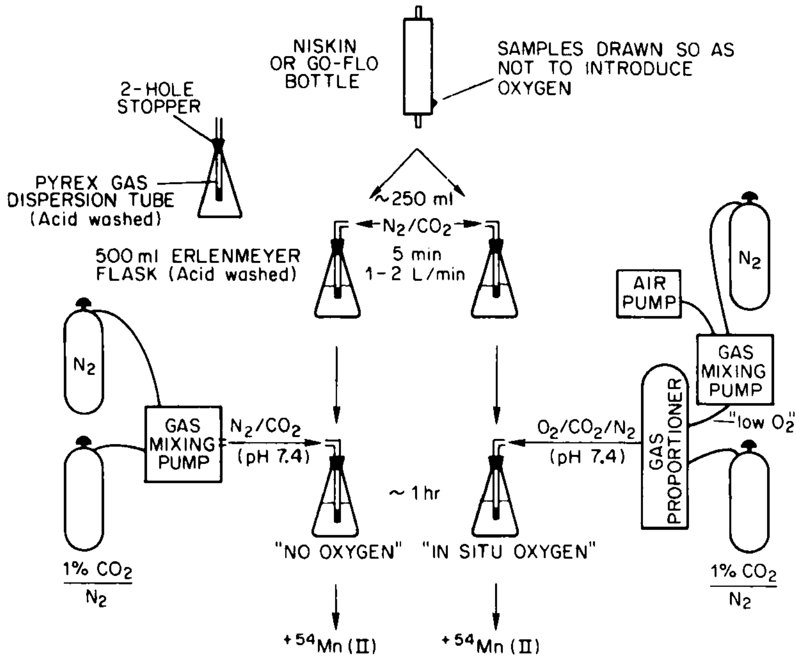
that oxidation is occurring. Measurements of the oxidation state or oxidizing equivalents performed by an iodiometric titration (Kalhorn and Emerson, 1984; Murray *et al.*, 1984) or by *o*-toluidine (Morgan and Stumm, 1965) of the particulate manganese in the water samples confirm the presence of oxidized manganese (Tebo *et al.*, 1984; Tipping *et al.*, 1984). At neutral pH, high oxidation states are not expected for Mn oxides formed chemically (Hem, 1981), so the presence of these highly oxidized Mn oxides further supports the notion that biological activity is important. However, despite the overwhelming evidence that biological catalysis is occurring, the derivation of rates of oxidation from this approach (i.e., measuring the oxidation state) is very difficult, primarily because of the insensitivity of the method, and the resulting requirement to filter large volumes of water.

Three different approaches have been used to try to determine Mn(II) oxidation directly in water samples. Two of these are modifications of the ^{54}Mn uptake experiments described above. First, Tebo and Emerson (1985, 1986) used the difference in particulate ^{54}Mn formation in the presence and absence of oxygen to estimate Mn(II) oxidation rates (Fig. 4). The data from the oxygen-containing and oxygen-less binding experiments were interpreted as a two-step mechanism in which dissolved Mn(II) is first adsorbed (bound) by an exchange site (E) and then oxidized:



$$\mathrm{Mn(II)} + \mathrm{E} \longrightarrow \mathrm{Mn(II)\text{-}E} \quad (4)$$
$$\mathrm{Mn(II)\text{-}E} + \mathrm{O_2} \longrightarrow \mathrm{MnO_x} + \mathrm{E} \quad (5)$$

Reaction (4) accounts for the adsorption and exchange of the radioactive label with the various dissolved pools of manganese [e.g., the Mn(II) that is adsorbed onto particulates or taken up into cells], while that



AS A FUNCTION OF TIME:

1 Filter subsamples in triplicate through 0.2 μm membrane filters (= particulate ^{54}Mn)

2 Remove subsamples to vials (= total ^{54}Mn)

COUNT RADIOACTIVITY

$$\% \text{ Bound} = \frac{\text{particulate } ^{54}\text{Mn}}{\text{total } ^{54}\text{Mn}} \times 100$$

$$\text{Mn(II) Bound} = \% \text{ Bound} \times \frac{[\text{Mn(II)}]}{100} \text{ measured}$$

FIG. 4. Experimental procedure for measuring *in situ* rates of ^{54}Mn(II) binding in Saanich Inlet.

measured in the presence of oxygen [reactions (4) and (5)] also includes the portion of the manganese that is oxidized. Typical results of a binding experiment are presented in Fig. 5a. By subtracting the no-oxygen experimental values from the oxygen values, the amount of Mn(II) oxidized at a given time can be calculated (Fig. 5b). Typical profiles of Mn(II) oxidation rates in the Saanich Inlet water column, determined by this method, show maximal oxidation rates just above the oxic/anoxic interface where maximal Mn(II) binding also occurs (Fig. 6).

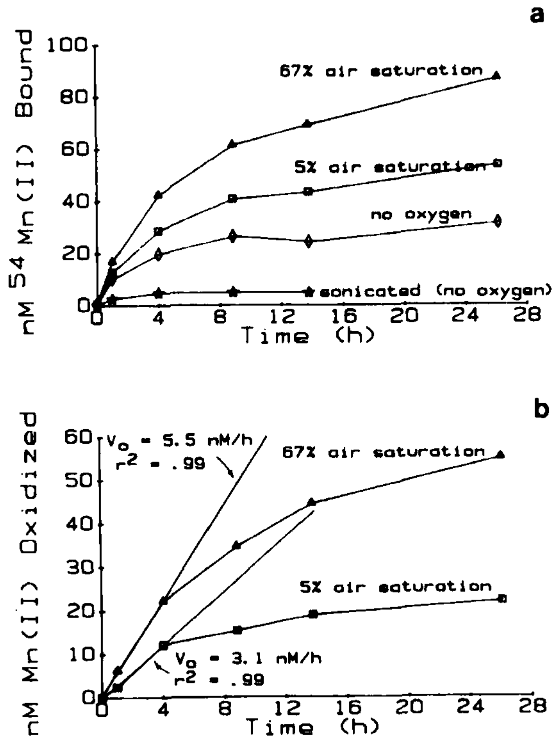


FIG. 5. (a) Time course of ^{54}Mn(II) binding under conditions of no oxygen and 5 or 67% air saturation, and in a sonicated (10 minutes) Saanich Inlet water sample in the absence of oxygen. Both temperature and pH were at their *in situ* levels (9°C and 7.4, respectively). This experiment is for a water sample collected at 95 m in August 1984. (b) The same data displayed in (a), except that the no-oxygen values have been subtracted from the 5 and 67% air saturation values; this is interpreted to be the amount of Mn(II) oxidized. The rate appears to be linear over at least the first 4 hours of incubation. The slope of the regression lines drawn through these initial time points yields the initial Mn(II) oxidation rate (V_o).

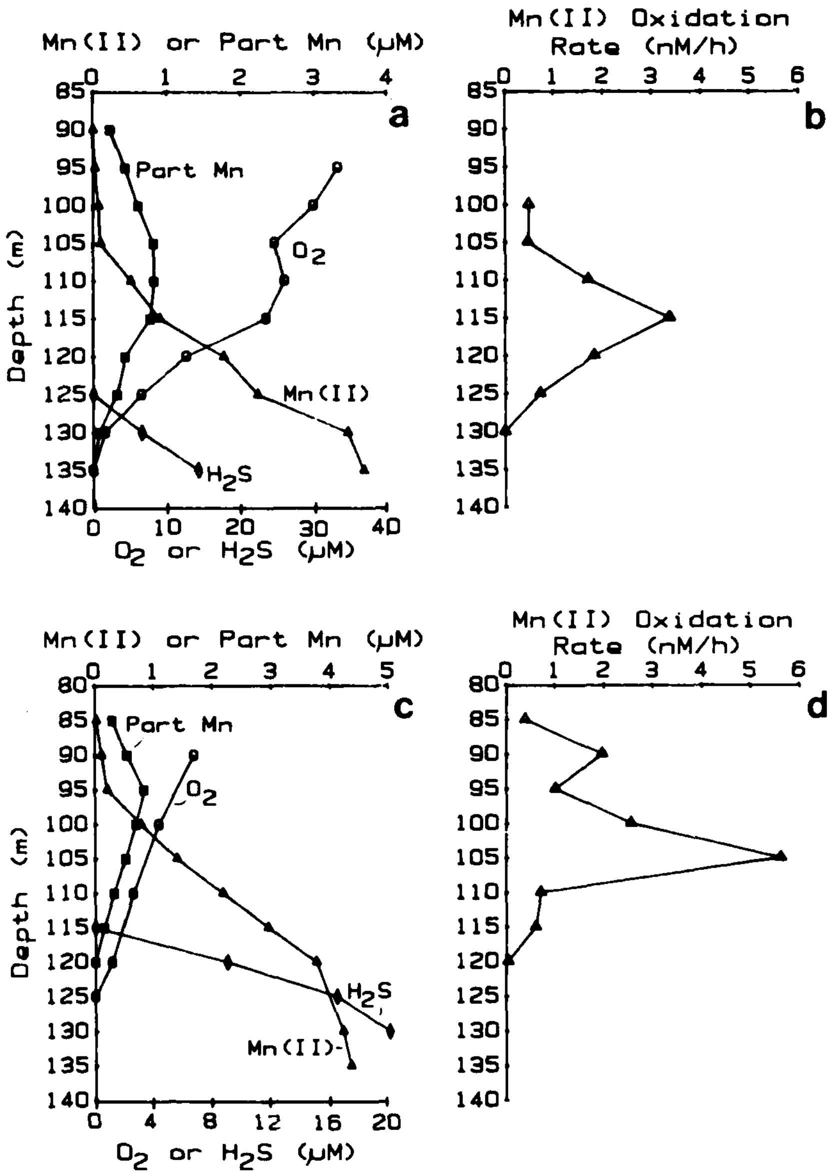


FIG. 6. Depth distributions of dissolved and particulate Mn, oxygen, hydrogen sulfide (a,c), and initial manganese oxidation rates (b,d) for July (a,b) and August (c,d) 1984. The initial manganese oxidation rates were determined as described in Fig. 5.

A second method to measure rates of oxidation was employed by Sunda and Huntsman (1987) in a North Carolina estuary. They estimated the proportion of particulate ^{54}Mn present as oxides from the difference in the amount of ^{54}Mn dissolved by ascorbic acid [a mild reducing agent that was not toxic and did not cause release of intracellular Mn(II) from phytoplankton] and that exchanged with excess nonradioactive Mn(II). The rates calculated using this method were in good agreement with those obtained using incubations in the presence and absence of oxygen (Sunda and Huntsman, 1987). This approach seems promising when combined with controlled incubations (i.e., in very short time courses where environmental conditions of pH, oxygen tension, and temperature are preserved as much as possible). In estuaries or open ocean environments, where gradients of oxygen and other chemical species may not be as steep as in stratified environments, this approach has several advantages: it is simpler, does not require gas mixtures, and yet can be quantitative.

In a third approach, Tipping (1984) was able to measure a change in oxidation state directly in incubations of lake water over a time period of 2–3 days. However, this required making additions of relatively high concentrations of exogenous $MnCl_2$ (4 to >100 times ambient), and rates were calculated from end-point determinations. Therefore, while the results clearly demonstrated biological oxidation, the rates can only be taken as approximate.

It is possible to estimate *in situ* rates of Mn(II) oxidation in a variety of natural environments and habitats by conducting experiments under controlled conditions of *in situ* oxygen, pH, and temperature (see for example Figs. 4, 5, 6). One should note, however, that these experiments are not easily adapted to pressure studies. Rates of Mn(II) oxidation determined with this method in Saanich Inlet, British Columbia, were in excellent agreement with the rates calculated from a simple chemical advection–diffusion model (Tebo and Emerson, 1986), lending credence to the idea that *in situ* rates can be measured directly. This approach also affords the opportunity to examine other environmental parameters that may affect the rates of oxidation, including depth from which the sample is collected, Mn(II) concentration, oxygen tension, and temperature. At the depth of the particulate manganese maximum in Saanich Inlet (about 100–110 m), Mn(II) oxidation is oxygen limited and inhibited by high Mn(II) concentrations (>10 μM), and the optimum temperature for oxidation is around 20°C (Tebo and Emerson, 1985).

Mn(II) oxidation rates in several marine and freshwater environments are listed in Table IV for those situations when rates have been determined by direct rate measurements, as described above. In general, rates of oxidation are much faster in freshwater than in marine systems, and in eutrophic environments compared to oligotrophic ones.

TABLE IV

ENVIRONMENTAL RATES OF Mn(II) OXIDATION DETERMINED BY DIRECT MEASUREMENT METHODS

Environment studied	Rates	References
Marine		
Saanich Inlet, B.C.	0–5.6 nM/hour	Tebo and Emerson (1985)
Newport River Estuary	0.62–3.5 nM/hour	Sunda and Huntsman (1987)
Sargasso Sea	0.04–37.5 pM/hour	Sunda and Huntsman (unpublished)
Juan de Fuca hydrothermal vent plume	0–1.7 pM/hour	Tebo and Rosson (unpublished)
Freshwater		
Rostherne Mere	0–0.35 μM/hour	Tipping (1984)
Estwaite Water	0–0.12 μM/hour	Tipping (1984)
Lake Washington	0–1.5 nM/hour	Maki *et al.* (1987)

Once accurate measurements in the field are made, it is possible to use these rate measurements to learn more about the biology of the system. For example, Mn(II) oxidation in at least two environments [Saanich Inlet (Tebo and Emerson, 1986) and Newport River estuary (Sunda and Huntsman, 1987)] has been analyzed using Michaelis–Menten enzyme kinetics. In Saanich Inlet, since there was a natural gradient of dissolved manganese with depth, it was possible to model both the Mn(II) binding rates in the absence of oxygen and the *in situ* oxidation rates as a function of Mn(II) concentration (Tebo and Emerson, 1986). It was concluded that (1) Mn(II) oxidation rather than binding was the rate-limiting step, and (2) the rate of Mn(II) oxidation was limited by the number of Mn(II) binding sites [or the number of Mn(II)-binding and -oxidizing bacteria]. Thus in Saanich Inlet, it appears either that some trace nutrient is limiting the growth of Mn(II)-binding and -oxidizing bacteria, or that Mn(II) oxidation is not providing the energy necessary for the growth of the bacteria that catalyze the reaction.

In the Newport Estuary, the V_{max} and half-saturation constants for manganese oxidation were found to be 11.6 nM/hour and 0.19 μM, respectively (Sunda and Huntsman, 1987). The half-saturation constant for manganese oxidation was comparable to the half saturation constant for uptake determined for two species of phytoplankton from different environments (Sunda and Huntsman, 1985), suggesting a possible involvement of phytoplankton in Mn(II) oxidation. However, as the authors point out, the kinetic parameters are also similar to those observed in Saanich Inlet (Tebo and Emerson, 1986) where bacteria are the known

catalysts. It is intriguing that the uptake and oxidation systems have similar affinities for Mn(II), but before generalizations can be made, more data of this kind are needed.

For the most part, studies of Mn(II) oxidation in water column environments have not concerned themselves with the activities of photosynthetic organisms, even though the environments studied are often in the photic zone. In their study of Framvaren Fjord, Tebo *et al.* (1984) studied Mn(II) binding rates in the presence and absence of inhibitors of photosynthesis, and concluded that little, if any, Mn(II) binding was due to the activity of photosynthetic organisms in this environment. In contrast, Dean and Ghosh (1978) and Dean and Greeson (1979), on the basis of measurements of the distribution of particulate manganese, postulated that phytoplankon were important in Mn(II) oxidation in Oneida Lake, New York; however, no studies of rates were presented. More recent studies of Oneida Lake have shown that phytoplankton can play a major role in the oxidation of manganese, and suggest that the mechanism involves changes in pH and O_2 levels in the microenvironment surrounding the phytoplankton (Richardson *et al.*, 1987). Three kinds of field evidence for the role of the phytoplankton have been presented: (1) visual and chemical identification of manganese-encrusted phytoplankton aggregates; (2) measurement of microgradients of pH and oxygen concentration during photosynthesis; and (3) correlation of Mn(II) removal in the lake with phytoplankton blooms (Richardson *et al.*, 1988). In addition, axenic cultures of algae from Oneida Lake exhibit light-dependent Mn(II) oxidation under controlled laboratory conditions (see laboratory studies; Richardson *et al.*, 1987).

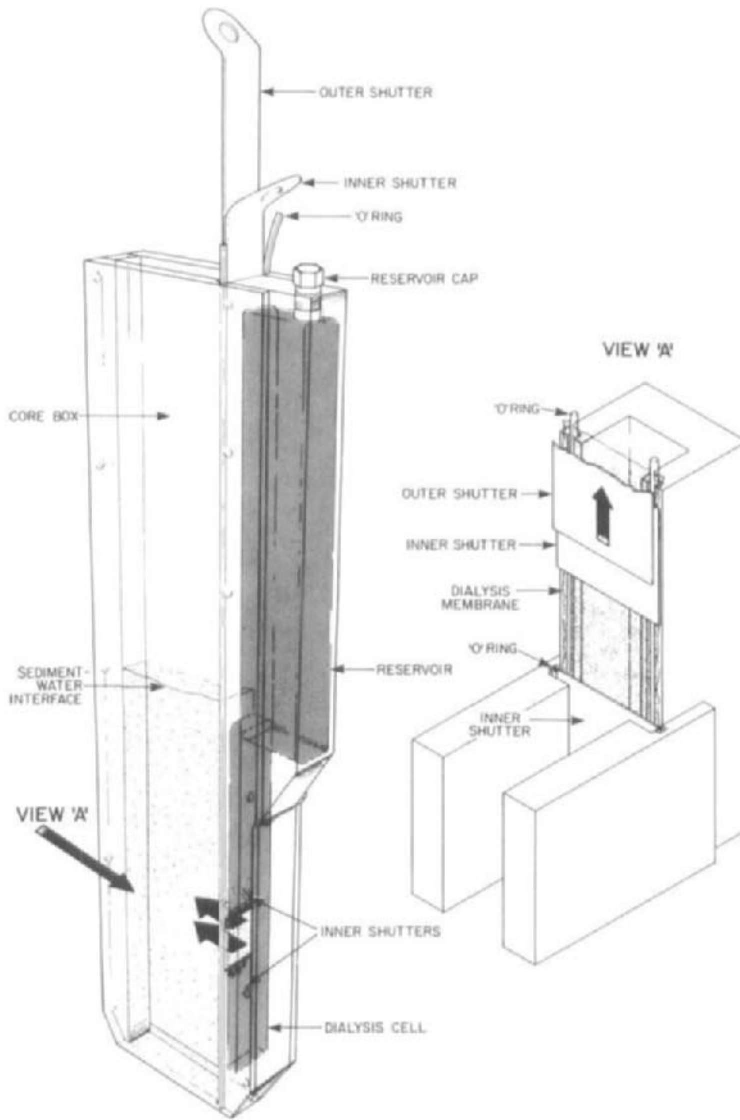
In all of the field work mentioned so far, the environments studied were aquatic, and the problems of working in sediments were thus avoided. While most sediments have well-defined oxic/anoxic interfaces, and are thus likely spots to encounter abundant populations of manganese-oxidizing microbes, they are difficult to work with experimentally. Manganese in particular is very difficult to work with in sediments, especially those sediments with high surface areas. Mn(II) is easily bound to almost any negatively charged surface, and once bound, may be either oxidized or made biologically unavailable. Furthermore, Mn(IV) oxides are also very surface active, and tend to adsorb a wide variety of cations, including Mn(II).

Edenborn *et al.* (1985) examined the microbial contribution to manganese oxidation in sediments of the Laurentian Trough of the St. Lawrence estuary. While there was evidence for the presence of Mn(II)-oxidizing bacteria in the surface sediments (top 30 cm), the authors concluded that bacteria were not the primary catalysts of Mn(II) oxidation. Over a range of mercuric chloride concentrations (5 μM to 10 mM),

glucose mineralization (CO_2 production) was completely inhibited, while Mn(II) removal from solution was not affected. With azide, Mn(II) removal was completely inhibited by >0.5 m*M* NaN_3, while inhibition of glucose mineralization did not occur until >5 m*M* azide was used. Because NaN_3 inhibited Mn(II) binding at concentrations that did not significantly inhibit glucose mineralization, the authors suggested NaN_3 interfered with Mn chemistry and they speculated that ferric oxides, which act as adsorption sites for Mn(II), may bind the azide group, thus making it unavailable to poison bacterial respiration.

Another interpretation of the data can be made however. Facultative anaerobes often ferment glucose, and hence CO_2 production may continue even if respiration is inhibited by NaN_3. Therefore, to determine the effectiveness of NaN_3 as an inhibitor of bacterial activity in sediments, a nonfermentable substrate would have been a better choice, Furthermore, NaN_3 can inhibit Mn(II) binding and oxidation by dormant, nonrespiring spores of a marine bacillus, *Bacillus* SG-1 (see below; Rosson and Nealson, 1984); inhibition of respiration is, therefore, not necessarily a prerequisite for inhibition of bacterial Mn(II) binding. Finally, Burdige and Kepkay (1983) and Kepkay (1985b) have demonstrated the usefulness of NaN_3 in some sediments (see discussion below). It is possible, therefore, that NaN_3 was, while $HgCl_2$ was not, an effective inhibitor of bacterial Mn(II) binding and oxidation, even though $HgCl_2$ effectively blocked glucose mineralization and NaN_3 did not. The data clearly show that additional controls, as well as caution in interpretation of the data, are required when using poisons to measure Mn(II) binding and oxidation in water or sediment samples.

In order to circumvent some of the difficulties of working with sediment slurries an *in situ* dialysis technique was developed that introduces a uniform, one-dimensional (rather than radial) horizontal diffusional concentration gradient of manganese into sediments through a dialysis membrane (Burdige *et al.*, 1983). This modified dialysis probe, or "peeper," is enclosed by an associated box core so that sediments and pore waters can be sampled perpendicular to the window of the dialysis cell after a fixed period of exposure to a constant flux of Mn(II) (Fig. 7). An inner shutter adjusts the position of the dialysis window, allowing selection of a specific horizon of sediment enclosed within the box core to be exposed to the constant flux of manganese. The sediment horizon to be probed with the peeper is selected by first measuring manganese binding potential of sediment slurries. Tracer ^{54}Mn is added to live and poisoned slurries of sediment from sectioned cores, and manganese binding is determined as a function of time. Greater than 90% of the adsorbed manganese could be solubilized from particles with $CuSO_4$ in poisoned samples, whereas in live samples the adsorbed manganese could only


OUTER SHUTTER
INNER SHUTTER
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RESERVOIR CAP
CORE BOX
SEDIMENT-WATER INTERFACE
RESERVOIR
VIEW 'A'
INNER SHUTTERS
DIALYSIS CELL
VIEW 'A'
'O' RING
OUTER SHUTTER
INNER SHUTTER
DIALYSIS MEMBRANE
'O' RING
INNER SHUTTER

be solubilized with hydroxylamine. This suggested that the linear uptake of Mn(II) in live samples was the result of microbial manganese oxidation and hence a potential for manganese oxidation could be calculated (Fig. 8b; Kepkay, 1985a,b). The horizon with the greatest manganese oxidation potential is chosen for probing with the peeper, in order to estimate actual *in situ* rates of manganese binding and oxidation.

Peeper experiments yield pairs of Mn(II) diffusion profiles. Equations (see Fig. 7, legend) describing the experimental profiles of Mn(II) diffusion for "diffusion under conditions of adsorption" (poisoned probes) and for "diffusion under conditions of adsorption plus metal binding" (live probes) can be solved. By using live (unpoisoned) and control (poisoned) probes, it is possible to separate biologically mediated Mn(II) removal from nonbiological components such as adsorption, ion exchange, and autocatalytic (e.g., surface-catalyzed) oxidation (Burdige and Kepkay, 1983). Curve fitting is first applied to poison peeper data to produce adsorption coefficients for abiotic metal binding, and then these coefficients are included as fixed parameters, to fit curves to live peeper data. The curve fits can be interpreted as the equilibrium coefficient (K) and an apparent coefficient (K_a) which includes both abiotic adsorption and the enhanced or apparent adsorption brought about by microbial activity. The technique can be further extended by expressing microbial binding or oxidation as the kinetic parameters K_m (half-saturation Michaelis–Menton constant) and V_{max} (maximum binding or oxidation rate).

A field test of the peeper in Lake Charlotte, Nova Scotia, confirmed that the peeper approach constitutes a valuable method to assess kinetics of metal binding, as well as to assess the relative importance of biological vs abiological processes in natural sediments (Kepkay, 1985a,b). Kinetically distinct microbial and abiotic processes were observed in the

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FIG. 7. Dialysis probe or peeper used to examine manganese oxidation and metal binding in sediments. The core box and dialysis cell are clear acrylic held together by stainless-steel screws. The enlargement of the front view of the dialysis cell shows how the dialysis membrane is held in place by an O ring and the inner shutters. The core box is 70 × 10 × 2 cm. Diffusion gradient equation for poisoned peepers (diffusion under conditions of adsorption):

$$\frac{\delta c}{\delta t} = \frac{D_s}{(1 + K)}\left(\frac{\delta^2 c}{\delta x^2}\right)$$

for live peepers (diffusion under conditions of adsorption plus metal bindings):

$$\frac{\delta c}{\delta t} = \frac{D_s}{(1 + K)}\left(\frac{\delta^2 c}{\delta x^2}\right) - \frac{1}{(1 + K)}\left(\frac{V_{max} c}{K_m + c}\right)$$

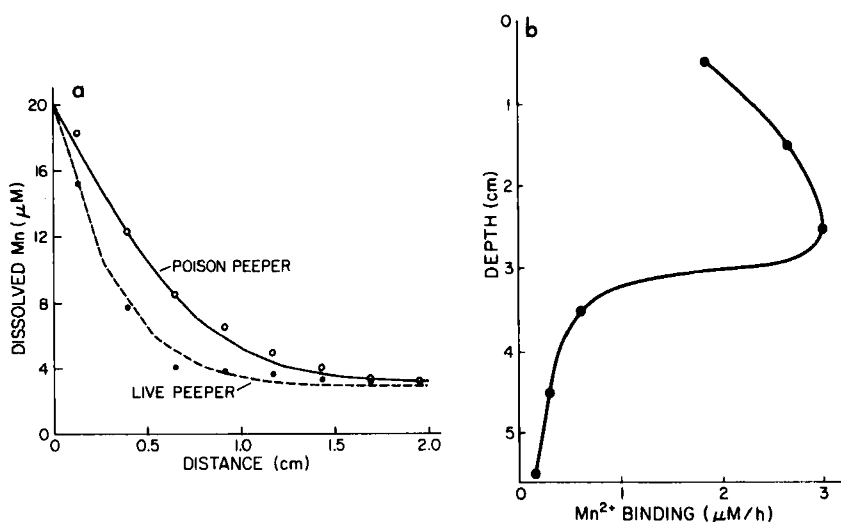


FIG. 8. (a) Best-fit curves for porewater Mn(II) in Lake Charlotte, Nova Scotia, sediments resulting from the diffusion of Mn(II) in live peepers from 20–25 October 1982 (●) and in poison peepers from 25–30 October 1982 (○). The horizontal axis defines distance from the dialysis cell to the center of a sediment slice 1 cm thick. (b) Profile of the potential for manganese oxidation at site M, Lake Charlotte, Nova Scotia.

poison and live peepers (Fig. 8a). More studies of this type are needed, especially in oxic sediments where layers of Mn(IV) oxides are formed. As mentioned earlier, the careful selection of poisons to be used with such peepers is critical. Burdige and Kepkay (1983) and Kepkay (1985a,b) used azide; as already discussed above, azide generally does not interfere with manganese chemistry (Emerson *et al.*, 1983; Rosson *et al.*, 1984), and it diffuses approximately three times faster than manganese in laboratory clays and natural sediments (Burdige and Kepkay, 1983).

In summary, work from several laboratories using different methods strongly supports the contention that microbes catalyze the oxidation of Mn(II). The questions that remain are now becoming more mechanistic, and the time is rapidly approaching when the field work will dictate the nature of the laboratory work. Prime questions that need to be answered include (1) what mechanisms really are used by microorganisms to oxidize manganese, and (2) under what conditions these mechanisms might be expected to predominate. When questions such as these are answered by laboratory studies (such as those discussed below), a new level of understanding of environmental manganese

oxidation will be possible. For now it can be confidently stated that the geochemical and biogeochemical data of the last 10 years force us to accept microbes as important catalysts of Mn(II) oxidation in many environments.

III. Laboratory Studies of Manganese Oxidation

A significant amount of new information has been published in the past few years which is leading to a better understanding of some of the diverse mechanisms by which microbes oxidize manganese. In this section, we focus on recent reports of both direct manganese oxidations, where oxidation is mediated by a cell component, and indirect manganese oxidations that result from changes of extracellular E_h and pH. Results for the following organisms are summarized: (1) spores of a marine *Bacillus* (SG-1); (2) sheathed bacteria of the *Leptothrix* group; (3) *Pseudomonas* S-36; and (4) photosynthetic microorganisms such as the cyanobacterium *Microcystis* and the green alga *Chlorella*. For each of these organisms, a considerable amount of information has accumulated on cell ultrastructure, physiology, and biochemistry of manganese oxidation, and in some cases, mineralogy and oxidation state of the MnO_x formed.

A. Direct Oxidation

The known genera of Mn(II)-oxidizing bacteria, and the general properties of this group, have been recently summarized in a review by Ghiorse (1984b). For example (but not meant to be inclusive), there have been many reports of enzymatic oxidation of Mn(II) by cell suspensions or by cell-free extracts for a variety of the Mn(II)-oxidizing bacteria, including two *Arthrobacter* spp. (Bromfield, 1956; Erhlich, 1984), *Leptothrix* sp. (Johnson and Stokes, 1966), and *Citrobacter* sp. and *Pseudomonas* sp. (Douka, 1977, 1978). There has also been at least one report of an intracellular protein of *Pseudomonas manganoxidans* that oxidizes manganese noncatalytically (Jung and Schweisfurth, 1979). However, in none of these studies was an enzyme purified or a Mn(II) oxidase identified.

When an enzyme or manganese-oxidizing component of a cell is implicated by physiological studies, further studies are necessary in order to answer the following questions: (1) what cell components are involved in Mn oxidation (e.g., enzymes, proteins, or polysaccharides); (2) how these compounds catalyze Mn oxidation (i.e., what is the mechanism); and (3) what the mineralogy of the biologically formed oxides is and how they compare to chemically (abiotically) formed oxides or

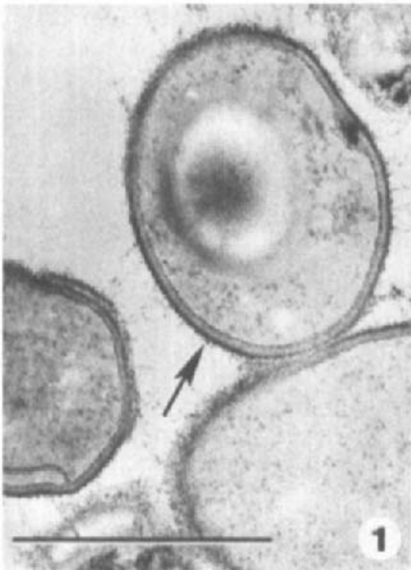
to those found in the natural environment. For several different bacteria, one or more of these questions have been answered, and we discuss some of these recent findings in the following sections.

1. *Bacillus* SG-1

A considerable amount of information is now available for the Mn-oxidizing system of the spores of a marine *Bacillus* (SG-1) isolated from a nearshore sediment (Nealson and Ford, 1980). Rosson and Nealson (1982) showed that Mn(II) was oxidized subsequent to binding, only by mature dormant spores, and not by the vegetative cells. The spores of SG-1 [as well as spores of other bacilli (Nealson and Tebo, 1980; Ghiorse, 1984b)] accumulated MnO_x externally (Fig. 9).

Mn(II) oxidation rates were maximal between 4 and 45°C; oxidation rates decreased at temperatures between 45 and 80°C, and were completely abolished at 100°C or greater (Rosson and Nealson, 1982). Potential for germination was not a prerequisite for Mn(II) oxidation, as spores treated with glutaraldehyde, formaldehyde, or ethylene oxide continued to oxidize manganese. Mn(II) oxidation was inhibited, however, by azide, cyanide, and mercuric chloride. It is important when characterizing microbial Mn(II) oxidation to interpret poison experiments with care, since inhibitors may interfere with the redox and surface chemistry of manganese (Rosson *et al.*, 1984). However, these poisons were shown in control experiments not to interfere with manganese chemistry. Furthermore, since the spores were dormant, interference of Mn(II) oxidation by these inhibitors was not due to inhibition of metabolism. Since these poisons are known to act by interfering with sulfhydryl or metal components of cells, it seems like that they may be interfering with sulfur or metal sites of coat proteins. The inhibitor studies coupled with ultrastructural studies of the location of precipitated manganese (Tebo,

FIG. 9. The life cycle of SG-1, a manganese-oxidizing marine *Bacillus* sp., as seen in thin section by transmission electron microscopy. (1) Vegetative cells in the process of sporulating (bar, 1 μm). (2) Vegetative cells stained with ruthenium red. The cells are covered by a polyanionic-rich outer layer (arrow) apposed to the gram-positive cell wall (bar, 0.5 μm). (3) A culture of SG-1 grown on agar medium and viewed without heavy-metal staining. Manganese, which is just beginning to be oxidized, is being precipitated on the spores and not on the vegetative cells. The outline of the presumptive remains of the vegetative cell wall can be faintly seen in the background of the thin section (arrows) (bar, 1 μm). (4) A micrograph of a developing SG-1 spore with manganese precipitated on its surface. The outer structure of the spore is characterized by ridges which appear to be formed by the outer spore coat or exosporium. Note that some of the channels in the ridges have not been completely closed, indicating the spore may not be mature (bar, 0.5 μm).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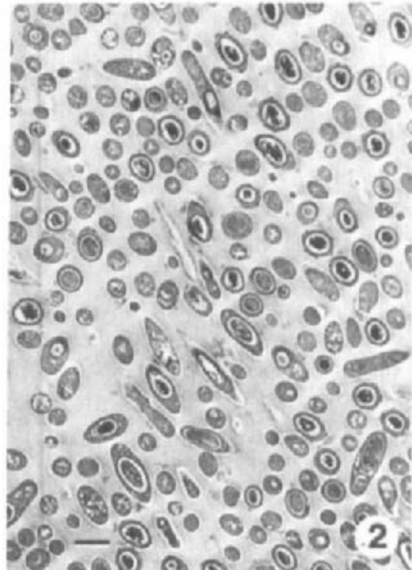
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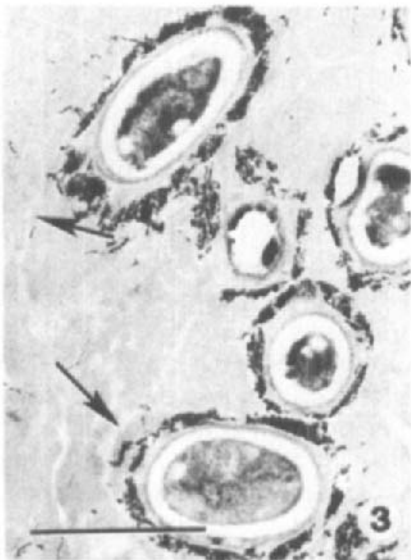
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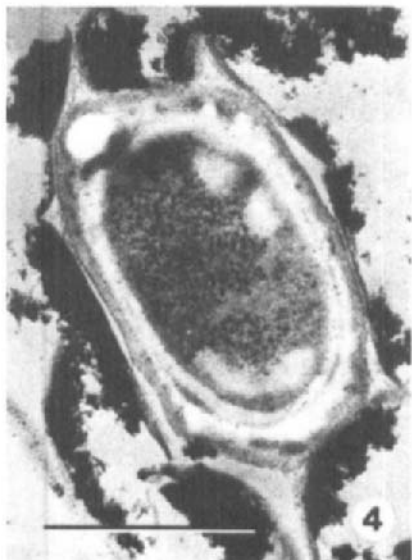
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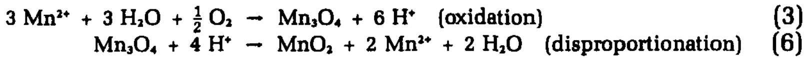
1983) have suggested that a spore coat protein or exosporium protein component is involved in Mn(II) oxidation (Fig. 9).

It has recently been confirmed that the SG-1 manganese-oxidizing activity is located in the spore coat; isolated and purified spore coats bind and oxidize Mn(II) at rates indistinguishable from those of whole spores (de Vrind *et al.*, 1986). For whole spores, the Mn(II) oxidation rate decreases with increasing amounts of MnO_x on the spore surfaces (de Vrind *et al.*, 1986; Hastings and Emerson, 1986). When this MnO_x is reduced with hydroxylamine and the hydroxylamine removed by washing, the kinetics of the Mn(II) oxidation by resulting spore suspensions are essentially identical to the original, untreated spores. This suggests that the Mn(II) oxidation is catalytic; it is unlikely that the spore coat component(s) is consumed stoichiometrically during oxidation of Mn(II). Under anoxic conditions, Mn(II) binding is minimal, unless preformed MnO_x is present, and in either case, there is no measurable oxidation of bound manganese. It would appear, therefore, that both the spore coat Mn-oxidizing component and oxygen are required for catalysis of Mn(II) oxidation.

The specific manganese-oxidizing component(s) of the spore coat has still not been identified and characterized. Tebo and Rosson (unpublished results) have preliminary evidence that a protein extracted from spore coats and electrophoretically separated from other proteins can catalyze Mn(II) oxidation. However, at this time there is not sufficient evidence to establish that this protein is the one responsible for Mn(II) oxidation by intact spore coats.

Measurement of rates of Mn(II) oxidation and mineralogy of the oxides formed by SG-1 were reported by Hastings and Emerson (1986). SG-1 spores were used as a biological catalyst for Mn(II) oxidation under conditions of pH and Mn(II) concentrations typical of open ocean environments. Rates of Mn oxidation catalyzed by spores in seawater at pH 7.8 were four orders of magnitude greater than would be expected if only colloidal MnO_x surfaces were present. In agreement with the later results of de Vrind *et al.* (1986), these authors demonstrated that the rate of Mn(II) oxidation progressively decreased as the spores became coated with manganese oxide, ultimately approaching that of autocatalytic Mn(II) oxidation. Through studies of the Mn oxides formed, it was determined that the initial oxidation product of the spores was octahedral hausmannite (Mn_3O_4 or a MnO_x where $x = 1.33$), which aged to MnO_x ($x = 1.9$) over a period of weeks. This is in agreement with the two-step mechanism for inorganic Mn(II) oxidation hypothesized by Hem and Lind (1983), in which hausmannite is first formed by oxidation of Mn(II) by spores; hausmannite then more slowly disproportionates to MnO_2 and Mn(II):



$$3\,Mn^{2+} + 3\,H_2O + \tfrac{1}{2}\,O_2 \rightarrow Mn_3O_4 + 6\,H^+ \quad \text{(oxidation)} \qquad (3)$$

$$Mn_3O_4 + 4\,H^+ \rightarrow MnO_2 + 2\,Mn^{2+} + 2\,H_2O \quad \text{(disproportionation)} \qquad (6)$$

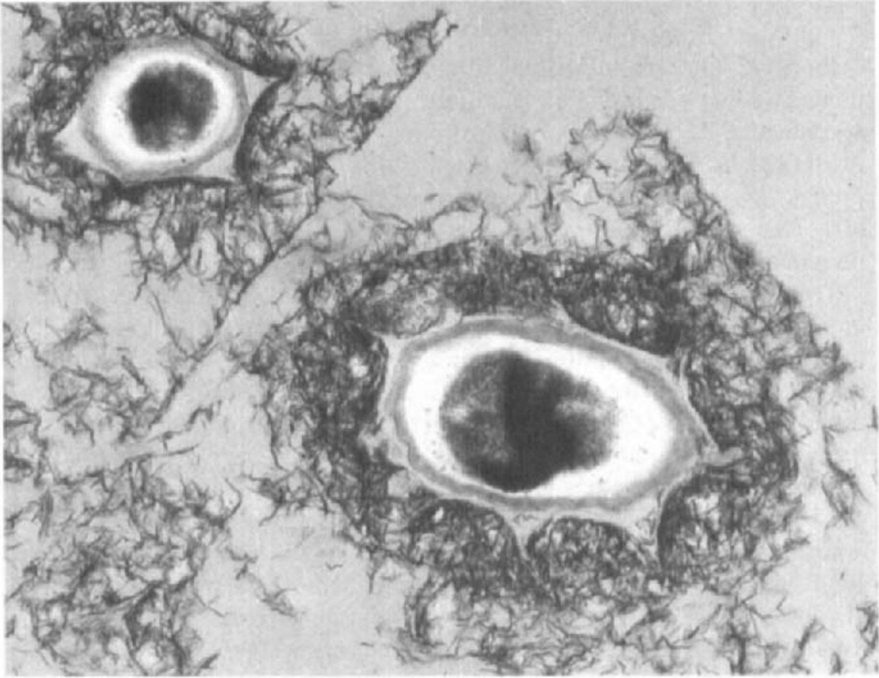
Rates of O_2 consumption, MnO_x formation, and H^+ release were measured by de Vrind *et al.* (1986) during oxidation of Mn(II) by purified spore coats. The pattern of proton release is consistent with the model presented by Hastings and Emerson (1986); an initial rapid release of protons [reaction (3)] is followed by a slower release [reactions (3) plus (6)]. The stoichiometry of the reaction, as well as the mineralogy of the product, suggests that oxidation follows Eq. (1).

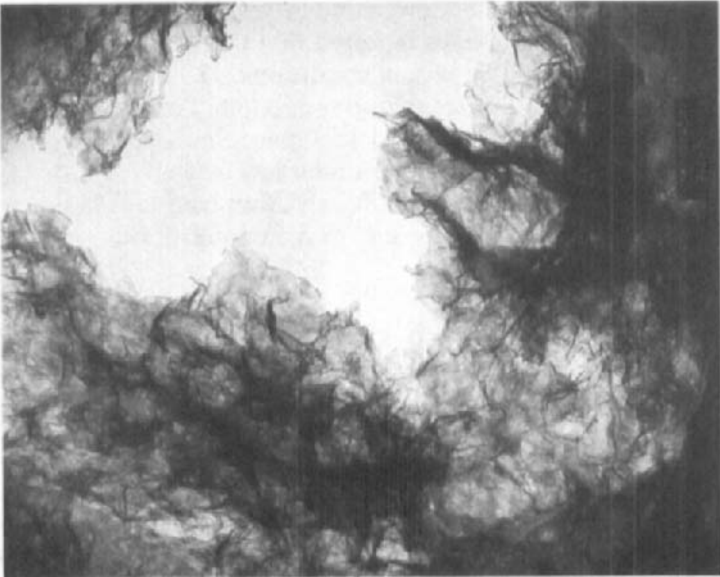
Transmission electron microscopy showed that the X-ray amorphous manganese oxides were crumpled, sheety microcrystalline solids which were not easily identifiable by electron microscopy. The morphology of the manganese oxide, both in thin section [Fig. 10 (top)] and as viewed directly on carbon-coated holey grids in the transmission electron microscope [Fig. (10 bottom)], has features similar to vernadite or δ-MnO_2 (Chukhrov *et al.*, 1979; Tipping *et al.*, 1984). Manganese oxides morphologically similar to those formed by SG-1 spores have also been reported to be formed by other *Bacillus* spp. spores (Ghiorse, 1984b; Tebo, 1983), and to be precipitated by sheathed *Leptothrix* spp. (Chukhrov *et al.*, 1979) as well as by a variety of manganese-oxidizing heterotrophs (Nealson and Tebo, 1980; Tebo, 1983). The morphology of the precipitates formed by pure cultures is similar to many natural manganese precipitates surrounding microbes in environmental samples (Emerson *et al.*, 1982; Tebo, 1983; Ghiorse, 1984b; Cowen and Silver, 1984; Cowen *et al.*, 1986).

De Vrind *et al.* (1986) have reported that the vegetative cells of SG-1 reduce δ-MnO_2 under low oxygen conditions. The data suggest electron transport is involved in manganese reduction. This is consistent with the suggestion of Tebo (1983) that MnO_x accumulated by spores might be utilized as an electron acceptor under low oxygen conditions during germination, and may hence confer an advantage to this organism by facilitating germination under low oxygen conditions.

2. *Leptothrix discophora*

The *Sphaerotilus–Leptothrix* group is a well-established and long-recognized group of manganese-depositing microbes (see Ghiorse, 1984b). Light and TEM studies of *L. discophora* (pure cultures) and of naturally occurring populations of sheathed bacteria (both *Sphaerotilus* and *Leptothrix*) have shown that either manganese or iron or both are deposited in copious amounts and that these are associated with an extracellular polymeric, structured sheath. Although early studies suggested that *L. discophora* utilized Mn(II) as a source of energy during mixotrophic growth (Ali and Stokes, 1971), it is now clear that protein(s) in the sheath are responsible for manganese oxidation (Johnson and





Stokes, 1966), and that oxidation of manganese by this protein is not used for energy generation (van Veen, 1972).

Recently, Boogerd and de Vrind (1987) and Adams and Ghiorse (1987) independently reported isolation, purification, and characterization of an extracellular manganese-oxidizing protein from *L. discophora* SS-1. SS-1 has lost its organized sheath upon subculture in the laboratory and produces structurally unorganized polysaccharide and protein exopolymers instead. It is apparent that both laboratories have characterized the same extracellular protein, which may normally be (in the wild-type parent) a component of the sheath. This protein has an apparent molecular weight of about 110,000, and has pH and temperature optima of 7.3 and 28°C, respectively. Manganese oxidation by this protein is sensitive to a variety of inhibitors including cyanide, azide, *o*-phenanthroline, and mercuric chloride. Manganese-oxidizing activity follows Michaelis–Menten kinetics (apparent $K_m = 7.0 \pm 3.2\ \mu M$; $V_{max} = 1.4$ nmol of Mn(II) oxidized/min - μg protein) between 1 and 200 μM Mn(II); oxidation is inhibited by greater than 400 μM Mn(II).

Boogerd and de Vrind (1987) also estimated the stoichiometry of manganese oxidation by this protein, by measuring rates of Mn(II) and O_2 consumption and the rate of release of protons. The stoichiometry is consistent with Eq. (1). This is the same overall stoichiometry that was estimated for manganese oxidation by *Bacillus* SG-1. As reported by Adams and Ghiorse (1987), the oxidation state of the initial MnO_x formed was greater than 1.65; whether this then aged to a higher oxidation state was not discussed.

The question remains whether this protein is normally a sheath component in the wild-type *L. discophora*. Adams and Ghiorse (1987) demonstrated that in the extracellular culture fluids, this protein is associated with polysaccharides, and that when purified by electrophoresis on SDS–polyacrylamide gels, high molecular weight polysaccharides [estimated by periodic acid–Schiff staining (PAS) technique] are coincident with the manganese-oxidizing protein. Additionally, it appears that the protein in culture supernatant fluids is normally associated with extracellular polysaccharides, and apparently with outer membrane polysaccharide aggregates (Adams and Ghiorse, 1987). These results are consistent with, but not definitive for, this protein also being involved in manganese oxidation in wild-type *L. discophora* sheaths.

FIG. 10. Comparison of manganese precipitates formed by spores of *Bacillus* SG-1 as seen in thin section (top) or as viewed directly on carbon-coated holey grids in the transmission electron microscope (bottom).

Should this protein be biologically important in the wild-type *L. discophora*, the questions still remain of the function of this protein and whether the organism benefits from manganese oxidation. While there is still no clear-cut evidence, Ghiorse (1984b) suggested that manganese oxidation is part of a detoxification mechanism, perhaps as a defense against peroxides formed during growth.

3. *Pseudomonas S-36*

Pseudomonas S-36 is a manganese-oxidizing heterotroph, originally isolated from a long-term (1 year) manganese enrichment culture of an offshore marine sediment that was plated onto K medium [an organic-rich medium supplemented with 1 mM Mn(II); Nealson, 1978]. The organism was identified as a *Pseudomonas* by standard numerical taxonomic analysis (Nealson, 1978), and analysis by 16 S rRNA methodology placed the organism in the purple alpha group (Woese *et al.*, 1984), closely related to the autotroph *Pseudomonas denitrificans*. When this organism was grown in a carbon-limited chemostat on succinate plus Mn(II), increasing the Mn(II) concentration in the reservoir growth medium increased the steady-state growth yield at all dilution rates less than the maximum growth rate (Fig. 11; Kepkay and Nealson, 1987). While this is suggestive that manganese serves as an additional energy source during carbon-limited growth in a chemostat (mixotrophy), the possibility still exists that manganese may stimulate growth in some other way (e.g., by increasing the efficiency of carbon utilization).

A similar pattern of steady-state growth yield was observed when S-36 was grown in a manganese-limited chemostat with CO_2 as the carbon source (Fig. 11; Kepkay and Nealson, 1987). Residual Mn(II) in the outflow of the culture vessel was generally less than 4% of the initial manganese concentration. Although the Mn(II) removed from solution was subsequently oxidized, the rate of ^{54}Mn(II) binding (absorption, exchange, uptake, and oxidation) was always much higher than the growth rate of *Pseudomonas* S-36. The actual rate of manganese oxidation was not directly quantified. Kepkay and Nealson calculated that CO_2 fixation rates by manganese-limited cells strongly correlated with cell carbon production at all dilution rates tested (Table V). However, the factors used to estimate the amount of carbon fixed per mole of Mn(II) oxidized are a liberal estimate of this value. The conversion factor (see footnote *b*, Table III) is based on 1 *M* concentration of reactants rather than actual values of Mn(II) concentration, oxygen tension, and pH. There is also the assumption that Mn(II) is oxidized to MnO_2. If, as has been demonstrated for *Bacillus* SG-1 spores or for *L. discophora* SS-1, Mn_3O_4 (hausmannite) rather than MnO_2 is the direct result of *Pseudomonas* S-36 catalysis, then the conversion factor would also decrease (see Table III). Taken together,

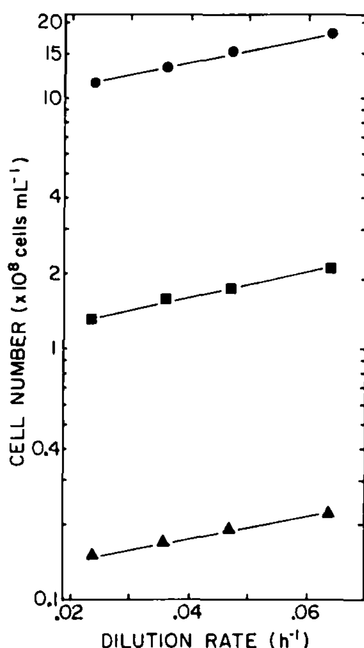


FIG. 11. Steady-state cell number of *Pseudomonas* S-36 in a basal-salts medium supplemented with (●) 10 m*M* Mn(II), (■) 1 m*M* Mn(II), or (▼) 100 μ*M* Mn(II). The maximal growth rate (μ_{max}) for all of the cultures was 0.066/hour.

the actual conversion factor may be lower by a factor of two or more; this would require a CO_2 fixation efficiency greater than 100%! Therefore, as the authors point out, while the data presented in Fig. 11 and Table V (Kepkay and Nealson, 1987) are consistent with autotrophic growth of *Pseudomonas* S-36 with manganese as the energy source, there is still considerable uncertainty about the actual function that manganese has on the growth of *Pseudomonas* S-36.

The studies with S-36 exemplify the problems encountered when trying to establish whether Mn(II) oxidation serves as a source of energy. Thermodynamically, the free energy change for the oxidation of Mn(II) to either MnO_2 [a Mn(IV) oxide; Eq. (1)] or Mn_3O_4 [a Mn(III) oxide; Eq. (3)] is negative for a variety of environmentally representative conditions (Table III). The amount of energy available changes as a function of O_2 tension, pH, Mn(II) concentration, and the oxide formed, with the latter three parameters having the most dramatic effects on energy yield (Table III). Clearly, for a wide range of conditions, Mn(II) oxidation could potentially provide energy for autotrophic or mixotrophic growth. In order to

TABLE V

CELL YIELDS OF *Pseudomonas S-36*[a]

Culture conditions	Dilution rate (hour^{-1})	Steady-state cell yield ($\times 10^7$ cells/ml)	Steady-state cell yield (μg C/ml)[b]	Residual Mn^{2+} (μM)	Mn^{2+} removed from solution (μM)	Cell yield from Mn^{2+} oxidation[c] (μg C/ml)	Rate of cell carbon production[d] (μg C/ml)	Rate of CO_2 fixation[e] (μg C/ml-hour)
Basal medium	0.024	1.5	0.25	1.8	98.2	0.35	0.006	0.006
+100 μM Mn^{2+}	0.036	1.7	0.25	3.8	96.2	0.38	0.010	0.009
(μ_{max} = 0.066/hour)	0.047	1.9	0.31	11.4	88.6	0.38	0.015	0.014
Basal medium	0.024	13.2	2.20	2.3	997.7	3.94	0.052	0.049
+ 1 mM Mn^{2+}	0.036	15.0	2.50	5.9	994.1	3.98	0.089	0.087
(μ_{max} = 0.066/hour)	0.047	17.1	2.80	7.8	992.2	3.97	0.132	0.133
	0.064	18.6	3.10	11.6	988.4	3.96	0.197	0.192

[a]Cell yields of *Pseudomonas* S-36 in relation to CO_2 fixed, Mn^{2+} removed from solution, and the cell yield predicted by assuming that the manganese taken up from solution was oxidized.

[b]Calculated from an average cell carbon conversion factor of 1.65×10^{-8} μg C/cell.

[c]Calculated on the basis that all of the Mn^{2+} removed from solution is oxidized (Kepkay *et al.*, 1984) and with a conversion factor of 3.9 g C/mol of Mn^{2+} oxidized. The conversion factor is based on 18.72 kcal/mol Mn^{2+} oxidized, 7.3 kcal/mol ATP, and 7.9 mol ATP/mol cell carbon (Schlegel, 1975).

[d]Calculated from steady-state cell yield $\times$ dilution rate.

[e]Calculated on the basis that the unlabeled CO_2 pool was 24 g C/ml (due to the presence of 2 mM $NaHCO_3$ in the basal medium).

prove whether this happens for any case, however, several parameters should be established: (1) the true oxidation rate [rather than the Mn(II) binding rate] should be determined and shown to be consistent with the rate of CO_2 fixation and growth; (2) the oxidation state and mineralogy of the Mn oxides formed should be determined; and (3) the actual concentration of substrates (Mn^{2+} and O_2) and the pH must be known. Further support would be aided by demonstration of activity in cell-free extracts of the enzymes involved in fixation of CO_2 and the oxidation of Mn(II). In no case so far reported have all of these criteria been met.

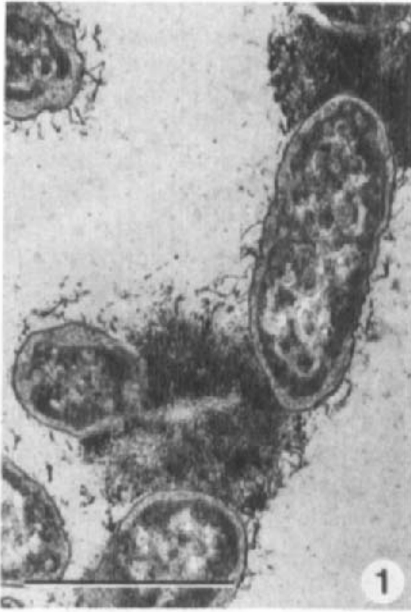
Ultrastructural evidence suggests that the mechanism of Mn(II) binding by S-36 involves an extracellular glycocalyx (Tebo, 1983). Electron microscopic examination of S-36 grown in a Mn-limited chemostat culture revealed that the cells were highly pleomorphic and synthesized a large amount of extracellular, ruthenium red-staining material on which manganese precipitates were located [Fig. 12(1–4)]. Only in aged batch cultures did Mn accumulate in any obvious crystalline form [Fig. 12(4)]. If Mn(II) is bound by polysaccharide as a prerequisite to oxidation, then the effective concentration of Mn(II) at the site of the reaction could be greatly enhanced; for this case, it will be difficult to measure accurately the Mn^{2+} concentration at the site of oxidation. With careful studies it should be possible to establish for S-36, as well as for other organisms, whether autotrophic or mixotrophic growth at the expense of Mn(II) oxidation occurs, but it will not be easy. Even if S-36 does *not* use Mn(II) as an energy source, the explanation for the data so far presented and discussed above will be most interesting.

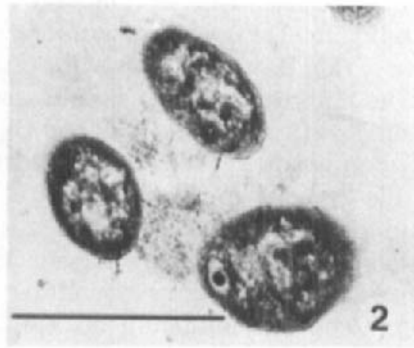
B. Indirect Oxidation

Indirect manganese oxidation, where the pH and/or the E_h of an environment is modified as a result of metabolism and growth of microorganisms, is well documented. Bacteria and fungi have long been recognized to catalyze this type of nonspecific manganese oxidation (see reviews by Ehrlich, 1976; Marshall, 1979). Recently, it has also been demonstrated that cyanobacteria and algae, both of which carry out oxygenic photosynthesis, can mediate Mn(II) oxidation via alteration of E_h (oxygen production) and pH (CO_2 consumption; Richardson *et al.*, 1988.

Chlorella sp. and Microcystis sp.

Pure cultures of a *Chlorella* species, a green alga, were found to catalyze a light-dependent oxidation of Mn(II) in the laboratory. This activity was inhibited when the medium was buffered to below pH 8.0 or by the addition of DCMU [3-(3:4-dichlorophenyl)-1:1-dimethylurea], an inhibitor of


1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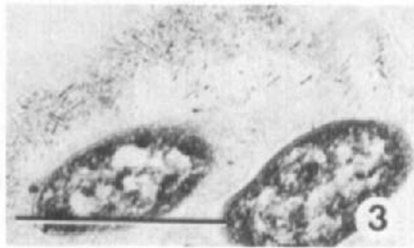
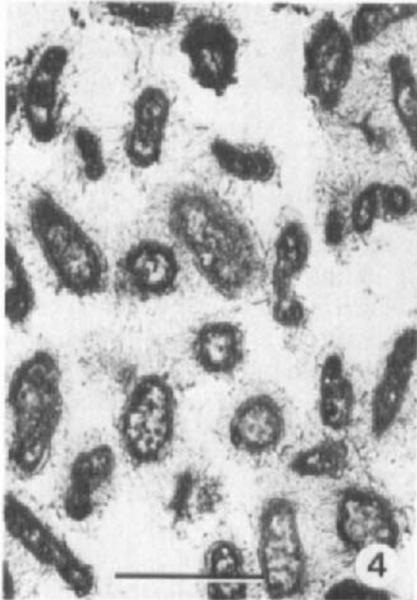
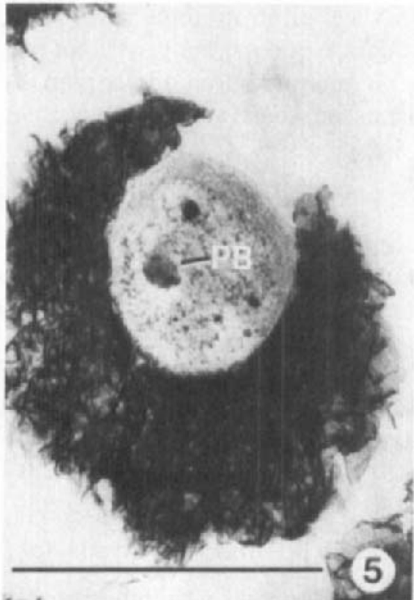

3


4


PB
5

photosytem II, and hence an inhibitor of oxygen production. Since oxygen evolution continues in buffered samples, an increase in pH rather than an increase in O_2 concentration seems to be the primary factor catalyzing manganese oxidation by *Chlorella*. Microelectrodes studies showed that in the microenvironment near a floc of *Chlorella* cells at the bottom of the experimental tube, the pH was as high as 9.9, and the oxygen concentration was above saturation levels (Richardson *et al.*, 1988). Manganese oxidation was very rapid under these conditions, even when the floc was dispersed.

Field experiments using microelectrodes demonstrated that the interior of naturally occurring, actively photosynthesizing aggregates of the cyanobacterium *Microcystis* can develop pH values of 10 or higher (Fig. 13; Richardson *et al.*, 1988). When the bulk pH of the lake water in which the aggregates were suspended was between 9.0 and 9.25, the pH within the aggregate increased sharply from the aggregate surface to its center. Under these conditions, manganese oxidation was extremely fast, and manganese oxides accumulated as extracellular precipitates at the surface of the aggregates.

One might imagine, therefore, that most aggregates of actively photosynthesizing cyanobacteria and algae catalyze manganese oxidation in surface waters of lakes. This oxidation would be expected to occur not only during the periods of blooms, but also within aggregates during nonbloom conditions, regardless of the bulk pH of the water. However, this does not appear to be the case. Many pure cultures of algae and cyanobacteria do not oxidize manganese, even under conditions of active photosynthesis (Richardson *et al.*, 1988), and removal of manganese from lake water by algae appears to be associated with only some species that bloom (C. Aguilar, personal communication).

As with all of the systems discussed so far, it is very important to measure the oxidation state and mineralogy of the manganese oxides

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FIG. 12. *Pseudomonas* S-36 grown in autotrophic media in the chemostat (1–4) or batch culture (5) with Mn(II) as the only known energy source, as viewed in thin section by electron microscopy (all bars, 1 μm). (1) S-36 cells precipitate Mn extracellularly on a holdfast material which is initially synthesized on one or two of the cell poles. (2,3) The cells have been treated with dilute acid after fixation to remove the precipitated manganese. Manganese deposition occurs on an extracellular matrix, which sometimes is barely visible (2) or at other times appears as a matrix composed of fine fibrils (3). (4) Cells have been fixed in the presence of ruthenium red and the extracellular matrix has been stained, indicating that the polymers are rich in polyanions, presumably polysaccharide or glycoprotein. (5) A cell from an aged autotrophic batch culture. The cells of such cultures often become surrounded with the dense cellophane-like crystalline Mn oxide as seen in SG-1 (Fig. 10) and other organisms. Occasionally, polyhedral bodies (PB), possibly carboxysomes, are visible in the cytoplasm.

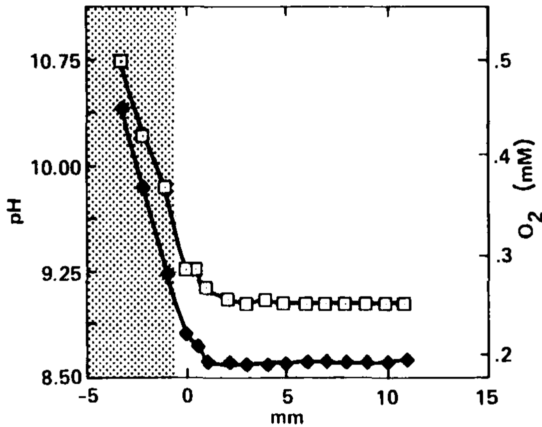

FIG. 13. Microprofiles of pH and O_2 associated with planktonic aggregates of *Microcystis* sp. These measurements were obtained with commercially available microelectrodes at Oneida Lake using freshly collected natural aggregates of individual cells of *Microcystis* (stippled area) under conditions simulating the pelagic lake environment. (□) ph; (♦) O_2 (m*M*).

precipitated by cyanobacteria and algae, especially considering that these organisms have high manganese requirements for photosynthesis. As more information is generated, it will be possible to compare the nature of these indirectly formed manganese oxides to the oxides precipitated directly by bacteria and to those formed abiotically; ultimately all of these can be compared to naturally existing manganates such as desert varnish, manganese nodules, and pipeline deposits.

IV. Summary

In recent years, much progress has been made in the area of microbial Mn(II) oxidation. We have tried to cover some of these areas that have not been previously reviewed. Work with pure cultures of Mn(II) oxidizers in the laboratory has led to a new appreciation of ultrastructural properties of these organisms and of their metal precipitates. The complex physiology and biochemistry of Mn(II) oxidation catalyzed by microorganisms are just now beginning to be unraveled. New techniques for field studies in aquatic environments have been developed to measure rates of Mn(II) oxidation and to allow the inhibition of biological Mn(II) oxidation without interfering with the chemistry of manganese. Other approaches have been developed for the analysis of Mn(II) oxidation rates in sedimentary environments. The results of these field studies have revealed that, in many environments with well-developed oxic/anoxic interfaces (fjords, lakes, and sediments), microbial Mn(II) oxidation is

an important catalytic factor which markedly affects the kinetics of metal removal.

When taken together, the laboratory and field data make a very strong case for the importance of microbes in the regulation of manganese chemistry in many environments. In a review such as this, one gets the rare opportunity to point to the areas in which research clearly is lacking, and in which, if knowledge were available, the entire system would benefit. During the past decade, the very preliminary laboratory data that were available had an immense impact on the field work that was done — field work that has unequivocably shown the importance of bacterial catalysis of Mn(II) oxidation. An important thrust which must now be made is to move back into the laboratory with pure cultures to learn more about the abilities of the organisms and thus be able to plan the next generation of field experiments properly.

It is important to extend our knowledge of the mechanisms of microbial Mn(II) oxidation, and to settle on representative organisms (that are reasonably well studied) as model systems for further study. The kinds of information that will be valuable include (1) careful kinetic studies of Mn(II) oxidation, and the factors that alter the kinetics; (2) studies of the relationship between Mn(II) oxidation and cellular metabolism (e.g., does the organism get usable energy from Mn(II) oxidation?); (3) studies of the mechanisms of Mn(II) binding and oxidation; and (4) studies of the chemistry and mineralogy of the manganese precipitates. Knowledge in each of these areas will be valuable in planning future field studies and for adopting strategies for isolation of new manganese-oxidizing microbes. For instance, the laboratory studies suggest that if manganese chemolithotrophs exist, they should be grown and studied under conditions of continuous culture, and the field studies suggest the environments from which it should be possible to isolate such organisms [e.g., environments where a continuous flux of manganese is available, steady-state Mn(II) concentrations are low, and rates of manganese oxidation are rapid and associated with bacterial activities].

Using these approaches, and insisting on meeting the criteria discussed above, we may begin to see the emergence of more answers to questions such as how microbes oxidize Mn(II); when, and at what rates microbes oxidize Mn(II) in natural systems; and why microbes oxidize Mn(II). Not until answers to such questions are found will we begin to understand the ecology of manganese oxidation and the organisms that catalyze it.

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Recovery of Bioproducts in China: A General Review

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I. Introduction
II. Cell Separation
III. Extraction
IV. Liquid-Liquid Two-Phase Extraction
V. Adsorption
VI. Precipitation
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VIII. Ion Exchange
IX. Affinity Chromatography
X. Protein Modification
XI. Dye-Ligand Chromatography
XII. Hydrophobic Chromatography

I. Introduction

Recent developments in biotechnology, especially genetic manipulation and cell engineering, have gained great attention from politicians, investors, and scientists worldwide. Ten years ago people believed that biotechnology would bring them tremendous economic and social benefits in the very near future, and hundreds of companies related to the business of biotechnology were set up within a short time. But so far, only a few products, such as human insulin, growth hormone, interferons, several kinds of monoclonal antibodies, and vaccines, have been industrialized even though we have heard of the development of many engineered strains with high expression in the laboratories.

Why? Several main factors have influenced the marketing of genetically engineered or other relevant products, but one of the most important factors is the neglect and, to a certain extent, underestimation of the importance and difficulties of downstream process development.

The technology of isolation, separation, and purification of bioproducts, especially very small amounts of biologically active substance from a large volume of fermentation broth, is the key problem of downstream process development. Bioproducts have special properties and requirements: (1) most are macromolecules; (2) they are often very unstable; (3) the products must be free from undesirable substances that may have similar physical and chemical properties; and (4) the

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products must be free from endotoxins, proteolytic enzymes, and pyrogens. These requirements make it more difficult to separate and purify the desired biologically active macromolecules, especially for food and medical uses. To overcome these obstacles, we need more technical innovations involving materials, media, and equipment.

Figure 1 is a diagram of the downstream process. The recovery of bioproducts can be divided into three stages. The first is a stage of preliminary treatment, including cell separation, cell disruption, and clarification. Of course, if the desired substance is extracellular, we can isolate it directly from the supernatant before or after the cell separation with no need for cell disruption.

The second stage is preliminary purification. Its purpose is to separate and concentrate the desired substance in a small volume from the large volume obtained after cell separation or cell homogenization and clarification, with high activity recovery and purification. Ultrafiltration is an efficient technology for separating substances with large molecular weight from those with smaller weight. Ion exchange and adsorption with natural or artificial materials; precipitation with salts, metal ions, organic solvents, and protein precipitating agents; extraction with organic solvents or liquid–liquid aqueous extractions—all of these kinds of technology

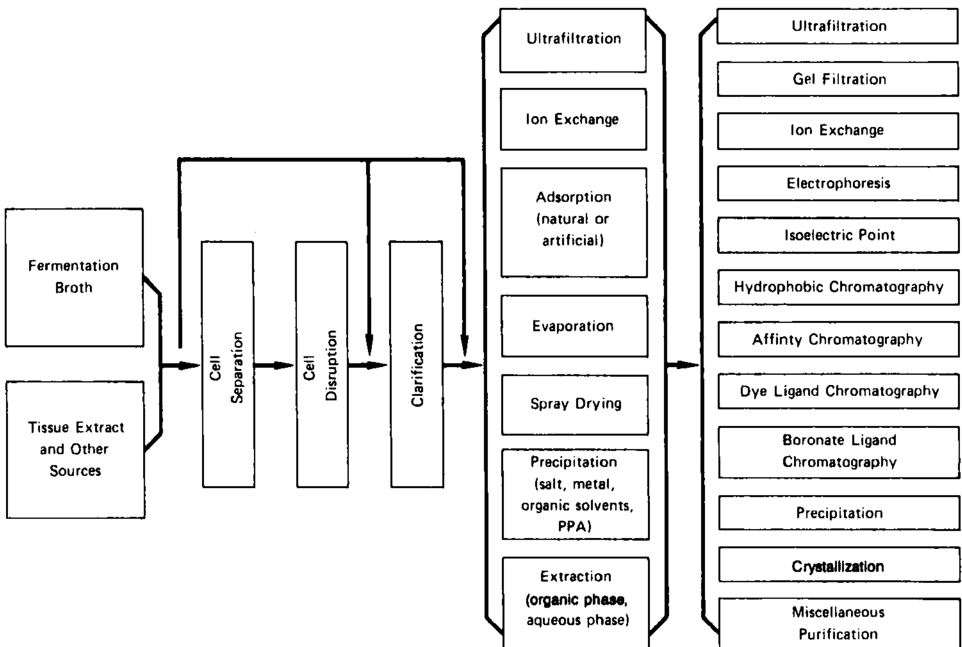


FIG. 1. Downstream process.

are now widely used for recovery of bioproducts in China. Spray drying and different types of evaporation such as vacuum, membrane evaporation, and rotary evaporation are still good methods to drive out most of the water, after which it is easier to apply the ensuing steps.

The third stage is final purification. According to different principles, there are different corresponding techniques. Ultrafiltration and gel filtration are based on differences in molecular weight of the desired substances. The Shanghai Institute of Pharmaceutical Industry (SIPI) was the first in China to develop an inexpensive ultrafiltration membrane, which is now used widely for desalting, ultrafiltration, and concentration. Ion exchange, electrophoresis, and isoelectric precipitation or focusing are based on the electric charge of the molecules. SIPI had the first workshop for producing ion-exchange resins in the early 1950s. Now ion-exchange techniques are the most widely used in China for the manufacture of antibiotics, amino acids, enzymes and coenzymes, nucleotides, nucleosides, and related bases, vitamins, and other bioproducts.

Because of hydrophobic regions in the macromolecules, we can use hydrophobic chromatography to purify bioproducts, especially for substances that cannot be purified well by ion-exchange technology. It is often unnecessary to desalt the eluents after using hydrophobic chromatography. Affinity chromatography is a useful tool based on the affinity between the substances and relevant ligands such as antigen–antibody; enzymes and their substrates, inhibitors, prosthetic groups or corresponding analogs; and hormones and their acceptors. Some of the laboratories and workshops in China have used this technique for research and production. In a broad sense, dye–ligand chromatography is also a kind of affinity chromatography; several kinds of dye–ligand chromatographic medium from Chinese-made agarose and reactive dyes have been developed at SIPI. We found them to be completely comparable to foreign products. Boronate ligand chromatography is most suitable for 1,2-*cis*-diol-containing substances, including most of the carbohydrates, glycoproteins, and nucleotides. The conventional methods of precipitation and crystallization are also useful for purifying biologically active substances. Of course there are many different types of purification techniques, but they are all based on the physical, chemical, and biological properties of the desired substances.

A few examples for each stage described above are given in the following sections.

II. Cell Separation

Most of our factories now carry out cell separation using a vacuum filter drum and different types of filter press; sometimes an automatic washing filter press is used. Centrifugal separators are also widely used

TABLE I

CENTRIFUGAL SEPARATORS USED IN CHINA

Specification	Type		
	DRY-400	CA	OEP-10006
Sources	China	USSR	West Germany
Drum diameter (mm)	400	330	550
No. of disks	80–92	75	150
RPM	6650	4620	4060
Separation Factor (g)	9800	3900	5040
Operating Capacity (m^3/hour)	4	2.5	10
Motor power, (kW)	13	3.5	11

for cell separation. As shown in Table I, the Dry-400 separator was developed in our country; other types of centrifugal separator have been imported from Sweden, West Germany, the Soviet Union, and other countries.

The continuous foam plastic band press filter has been developed in pilot scale (Fig. 2). The fermentation broth adheres to the band and the solution goes outside by the effect of capillary osmosis. The liquid remaining in the band is pressed out by a set of rollers and the residue is cut off by a knife. Finally, a water sprayer washes and keeps the band

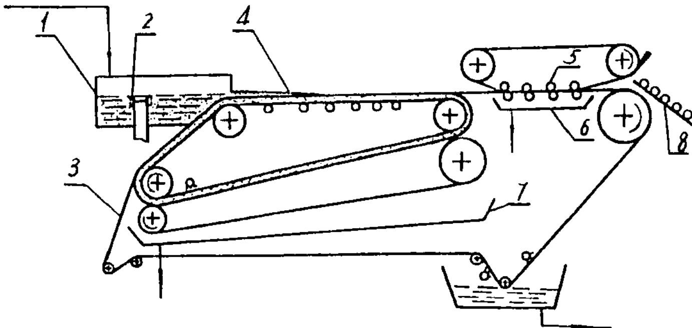


FIG. 2. Continuous foam plastic band press filter: (1) feed tank; (2) level control; (3) foam plastic band; (4) adsorb band; (5) press roller; (6) pressed liquid pan; (7) filtrate pan; (8) residue.

renewed. The filtration rates are 2.5 and 7 times as fast, respectively, as for the conventional filter press for citric acid fermentation broth and alcohol distilled wastes.

Flocculation is also used for separation of some extracellular enzymes. Cell separation in the alkaline proteinase fermentation, for example, is accomplished by polyacrylamide and aluminum sulfate (Table II). As the data show, the activity recovery is about 75–80%; the number of cells per milliliter decreases from 10^9 to 10^4.

III. Extraction

Figure 3 depicts a unit of a countercurrent distribution extractor for citric acid. It contains a stirring/mixing chamber for mixing the organic phase with the fermentation broth; the mixture then settles into two layers. We can connect several units to let the organic extracting phase (light phase) and aqueous phase (heavy phase) flow into the system from opposite directions. Thus the fermentation broth with a higher concentration of citric acid is mixed with the light phase with a lower concentration of extracted citric acid. The extracted broth with the lowest concentration of citric acid is mixed with the fresh light extracting phase. The extraction efficiency of each stage is then over 95% (Table III). The inverse extraction is done at 80°C. The product quality fulfills the specifications of the British Pharmacopoeia. Another kind of extractor is the vertical centrifugal extractor (Fig. 4). With type LC-500, developed in China, the heavy phase is mixed with the light phase at high speed and is finally separated by centrifugal force.

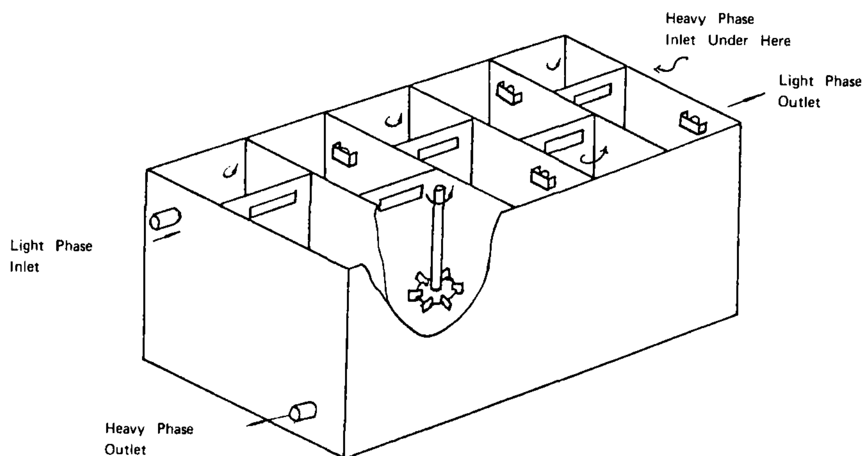


FIG. 3. Multiple mixer–settler construction.

TABLE II

CELL SEPARATION OF ALKALINE PROTEINASE (*B. licheniformis*) FLOCCULATING AGENT USING POLYACRYLAMIDE AND $Al_2(SO_4)_3$

	Fermentation				Filtrate					Residual			
No.	Volume (liters)	Total activity units	T	Cells/ml	Volume (liters)	Total activity units	Enzyme activity recovery (%)	Transparency (%)	Cells/ml	Weight (kg)	Total activity units	Enzyme activity recovery (%)	Moisture (%)
1	117	9.36×10^9	5	10^9	112	7.06×10^8	75	90	10^4	21	9.45×10^7	10	79
2	117	1.15×10^9	8	10^9	113	9.24×10^8	81	92	10^4	23	1.33×10^8	12	79
3	117	1.21×10^9	10	10^9	100	9.20×10^8	76	95	10^4	25	1.33×10^8	11	81
4	117	1.23×10^9	6	10^{10}	110	9.24×10^8	75	86	10^5	22	1.47×10^8	12	78
5	117	1.22×10^9	3	10^{10}	115	9.1×10^8	75	85	10^5	23	1.52×10^8	12	80

TABLE III

EXTRACTION OF CITRIC ACID FROM FERMENTATION BROTH WITH MIXER–SETTLER AND AP-4 AS AN EXTRACTING AGENT

Broth composition	
Citric acid	103.40 g/liter
Invertose	0.67%
Protein	0.38%
Co	14 ppm
Mg	31 ppm
Fe	16 ppm
Extraction conditions	
Number of stages	12
Temperature	Room temperature
Phase ratio (organic/aqueous)	2.5
Extracting agent	AP-4 (mainly TBP)

Results

Stage	Aqueous phase (g/liter)	Organic phase (g/liter)
I	5.75	1.60
II	5.90	1.67
III	5.92	2.10
IV	6.29	2.30
V	6.83	2.35
VI	6.90	2.50
VII	7.67	3.07
VIII	9.06	6.10
IX	12.0	7.70
X	18.3	19.6
XI	32.3	24.9
XII	56.6	41.3

Extraction efficiency of each stage ≥95%

Inverse extraction conditions	
Number of stages	8
Temperature	80°C
Phase ratio (organic/aqueous)	2.5
Extracting agent	Water

Results

Stage	Aqueous phase (g/liter)	Organic phase (g/liter)
I	102.6	27.5
II	77.5	19.9
III	53.6	14.5
IV	35.0	9.3

(continued)

TABLE III (continued)

Stage	Aqueous phase (g/liter)	Organic phase (g/liter)
V	23.0	7.0
VI	14.5	4.3
VII	8.4	2.4
VIII	3.6	2.1

Extraction efficiency of each stage ≥95%

	Product specifications	
	Per British Pharmacopoeia (1973)	Extraction product
Citric acid	99%	99.5%
As	<2 ppm	None
Fe	<50 ppm	3 ppm
Pb	<5 ppm	1 ppm
Ash	<0.1%	0.31%
Oxalate	Clear	Clear
Sulfate	<0.05 ppm	0.01
Carbonizable matter	OD 0.9	OD 0.37

Before discussing other instances of separation and purification technology, I would like to focus on the topic of antibiotics. The volume of antibiotics produced in China is the biggest in the world, 10,000 tons per year. Table IV lists the development of antibiotics in China since 1950. Between 60 and 80% of antibiotics are recovered from fermentation broth. Depending on the antibiotic, precipitation, solvent extraction, and ion exchange are used alternatively or in combination for recovery of antibiotics from fermentation broth (Fig. 5).

Penicillin G and penicillin V are recovered by butyl acetate extraction; ion-exchange resins have not been used in industry in China (Table V). Cephalosporin C is absorbed by the nonpolar macroporous resin developed in China. For tetracyclines we often first acidify the broth and then allow the tetracycline to form a complex with urea. Aminoglycoside antibiotics and rifamycins are recovered by weak acid ion-exchange resins and solvent extraction, respectively. Because the polyene and nonpolyene antibiotics are intracellular, we always use ethyl alcohol extraction to recover them. Polypeptide antibiotics are recovered by ion-exchange resins. Lincomycins, macrolide antiobiotics, antitumor antibiotics, and other kinds of antibiotics are recovered by ethyl acetate, butyl acetate, chloroform extraction, ion-exchange resins, or nonpolar macroporous

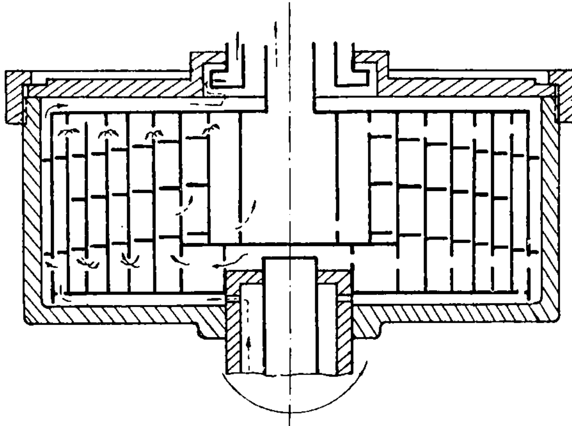


FIG. 4. LC-500 vertical centrifugal extractor.

resins. Three kinds of antibiotics first developed or commercialized in China are shown in Fig. 6.

IV. Liquid–Liquid Two-Phase Extraction

Liquid–liquid two-phase extraction is now utilized by many laboratories in our country. For example, we are successfully using this technique to purify α-amylase from contaminated cells in a pilot plant. Figures 7 and 8 outline the conditions studied for purification of glycerokinase and diaphorase with liquid–liquid two-phase extraction.

TABLE IV

DEVELOPMENT OF ANTIBIOTIC PRODUCTS

Products	Produced since
Penicillin	1951
Streptomycin	1957
Chlortetracycline	1956
Tetracycline	1958
Oxytetracycline	1959
Erythromycin	1961
Gentamicin	1967
Rifamycin	1970
Kanamycin	1982
Cephalosoporin C	

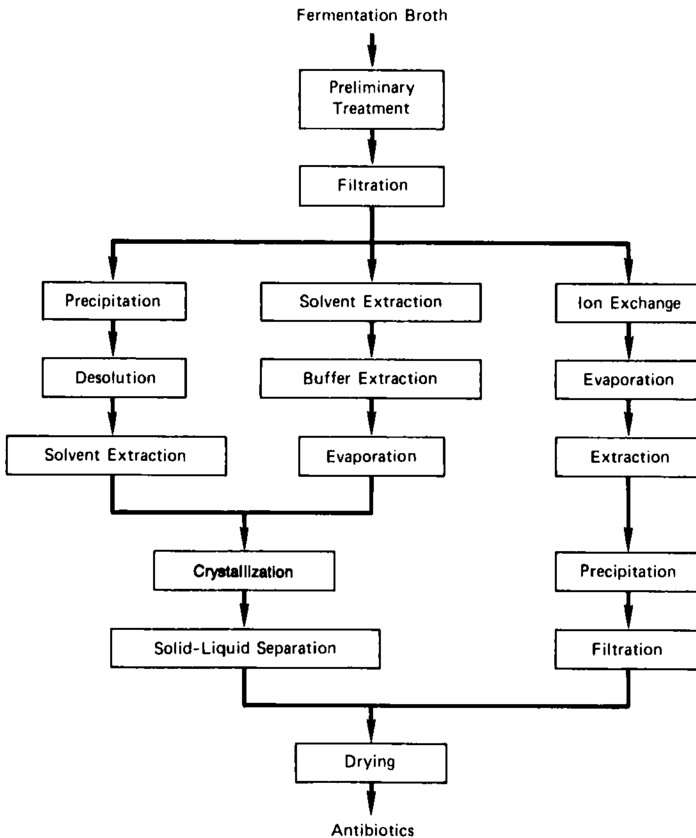


FIG. 5. Recovery of antibiotics: flow diagram.

Polyethylene glycol (PEG) of molecular weight 6000 can give a clear interface and good partial coefficient. Here we take the reciprocal of K, because we find that glycerokinase is not as common in the top phase as in the lower phase. Under a specific set of conditions (PEG 6000, 8–10% w/w; potassium phosphate, pH 7.0, 16% w/w), NaCl concentration, protein content, and pH are the most important factors governing the partial coefficient, purification, and activity recovery. I emphasize that in our laboratory we can deliberately adjust conditions such that the desired substance is in the top phase or in the lower phase. For convenience, we use the reciprocal of K instead of K.

The $1/K$, activity recovery, and purification are almost parallel under varying concentration of NaCl, but the optimum concentration of NaCl for glycerokinase and diaphorase are 0.2 and 0.1 M, respectively. Protein content is also important; for diaphorase, 16 mg protein/ml is the best

TABLE V

ANTIBIOTICS AND RECOVERY

Antibiotic	Recovery
Penicillins	
Penicillin G	Butyl acetate extraction
Penicillin V	Butyl acetate extraction
Semisynthetic	
Nafcillin sodium, cloxacillin sodium, dicloxacillin, flucloxacillin, ampicillin, pivampicillin, amoxicillin, carbenicillin, sulbenicillin, BL-P 1597 (furbenicillin), oxacillin, pipiracillin, ticarbillin, mecillinam, pivmecillinam	
Cephalosoporins	
Cephalosporins	Macroporous resin absorption
Semisynthetic	
Cephalothin sodium, cephaloglycin, cephaloridine, cephlexin, cefazolin, cephamandole, cephadroxil, cefotaxime, cefathiamidine	
Tetracyclines	
Tetracycline	Acidify–urea complex
Chlortetracycline · HCl	Acifidy–urea complex
Cexytetracycline	Acidify–urea complex
Semisynthetic	
Metacycline hydrochloride, doxycycline	
Chloramphenicols	
Organic; synthetics	
Chloramphenicol, chloramphenicol sodium succinate, thiamphenicol, synthomycin, synthomycin palmitate	
Aminoglycoside antibiotics	
Streptomycin sulfate	Weak acid ion-exchange resin
Dihydrostetraptomycin	Weak acid ion-exchange resin
Neomycin sulfate	Weak acid ion-exchange resin
Paromomycin sulfate	Weak acid ion-exchange resin
Lividomycin	Weak acid ion-exchange resin
Kanamycin B sulfate	Weak acid ion-exchange resin
Semisynthetic	
Gentamicin sulfate, saoamycin	
Rifamycins	
Rifamycin S	Solvent extraction
Rifamycin B	Solvent extraction
Rifamycin SV	Solvent extraction
Semisynthetic	
Rifampicin	

(continued)

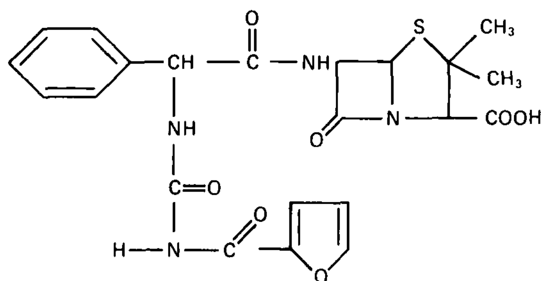
TABLE V (continued)

Lincomycins	
Lincomycin · HCl	Butyl acetate extraction or macroporous resins absorption
Semisynthetic Clindamycin · HCl	
Macrolide antibiotics	
Erythromycin	Butyl acetate extraction
Spiramycin	Butyl acetate extraction
Midecamycin	Solvent extractionn or macroporous resin absorption
Polypeptide antibiotics	
Polymyxin E sulfate	Ion-exchange resin
Polymyxin hydrochloride	Ion-exchange resin
Bacitracin	Ion-exchange resin
Vismycin pantothenate	Ion-exchange resin
Capreomycin sulfate	Ion-exchange resin
Vancomycin sulfate	Ion-exchange resin
Polyene and nonpolyene antibiotics	
Amphotericin B	Ethyl alcohol extraction
Lushanmycin	Ethyl alcohol extraction
Cannitracin sodium	Ethyl alcohol extraction
Trichomycin	Ethyl alcohol extraction
Aureofuscin	Ethyl alcohol extraction
Griseofulvin	Ethyl alcohol extraction
Cychloheximide	Ethyl alcohol extraction
Nystatin	Ethyl alcohol extraction
Antitumor antibiotics	
Bleomycin	Ion-exchange resin
Dactinomycin	Solvent extraction
Zhengdingmycin	Ion-exchange resin
Mitomycin C	Chloroform extraction
Streptomynigrin	Solvent extraction
Mithramycin	Solvent extraction
Adriamycin	Solvent extraction
Aclacinomycin	Solvent extraction
Miscellaneous	
Chuanghinmycin	Solvent extraction
Novobiocin sodium	Ethyl acetate
Gibberellic acid A_3	Solvent extraction
Vancomycin · HCl	Ion-exchange resin

concentration for getting highest activity recovery, highest purification, and lowest *K* value. The optimal pH for diaphorase is around 7.0; for glycerokinase it is preferably lower than 7.0 if the enzyme is stable in that pH range.

We have found that if we follow the operation conditions strictly, the desired enzymes can be moved from the upper phase (PEG phase) to the lower phase (phosphate phase). In such a case, it is unnecessary to

a



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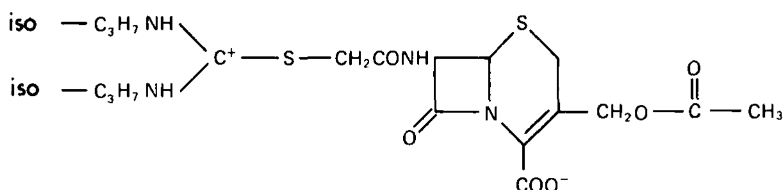

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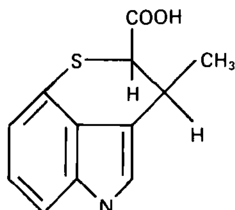


FIG. 6. Antibiotics: (a) furbenicillin (BL-P 1597); (b) cefathiamidine; (c) chuanghinmycin (*Actinomyces creaforus* sp.).

separate the PEG from the extracted substance, and the phosphate-extracting solution is easy to handle whether the cell homogenate comes from animal cells or microorganisms. Another advantage is that we can apply the extract directly to hydrophobic chromatography or different types of precipitation.

V. Adsorption

As an example of adsorption, consider streptokinase (Table VI). We have found that a weak acidic ion-exchange resin can selectively adsorb streptokinase in the fermentation broth without cell separation under

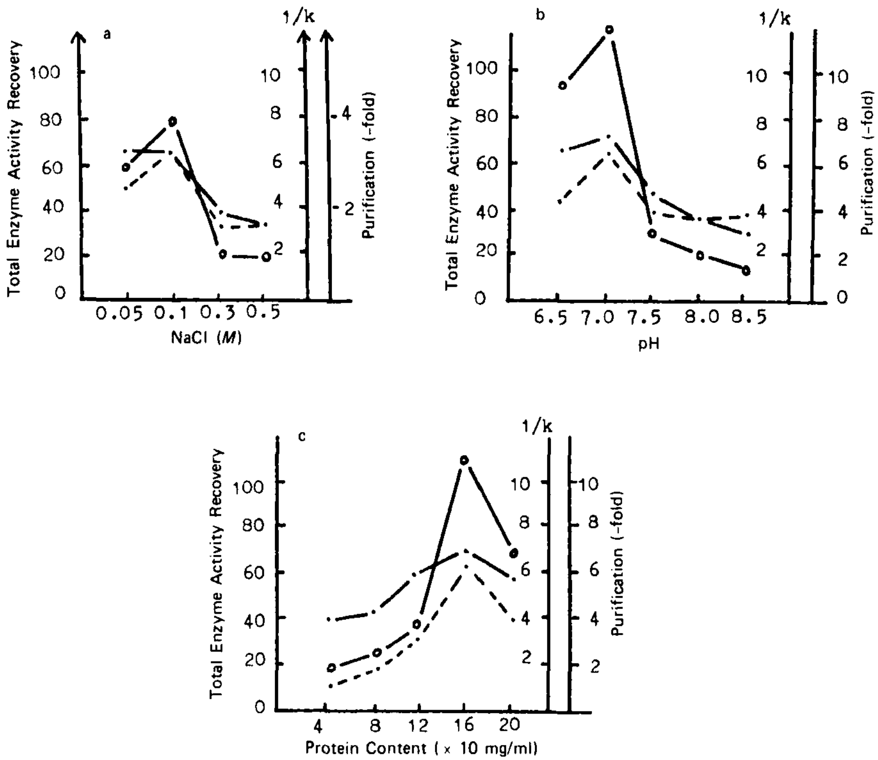


FIG. 7. Liquid–liquid two-phase extraction of diaphorase: (a) effect of NaCl concentration; (b) effect of pH; (c) effect of protein content on (— • —) activity recovery; (--- • ---) purification; (— o —) 1/K.

certain conditions with more than 90% activity recovery. After adsorption, we can separate the β-hemolytic streptococci from the fermentation medium by simple settling, decantation, and washing; complicated cell separation equipment is unnecessary.

Urokinase also can be adsorbed and exchanged from urine at the point of collection by a weak acidic ion-exchange resin with about 60–90% activity recovery (Table VII). We have designed a special device for urokinase adsorption, so the laborious transportation of urine is unnecessary.

Also, due to Chinese reality, pharmaceutical factories have asked some people using the adsorption technology in their family to collect human chorionic gonadotropin and human menapause gonadotropin as crude products. Many kinds of natural adsorbents have been used in the manufacture of enzymes and other active substances such as penicillin G acylase.

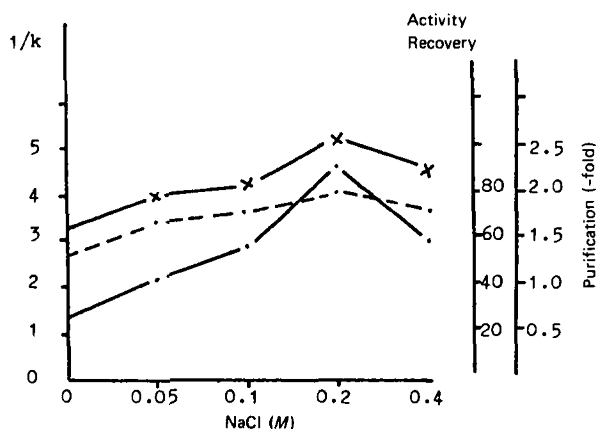


FIG. 8. Liquid-liquid two-phase extraction of glycerokinase: effect of NaCl concentration on (— • —) activity recovery; (-- • --) purification; (— x —) 1/K.

Nonpolar macroporous adsorbent resins developed at SIPI are shown in Table VIII. Other institutions and factories have also developed many kinds of adsorbent resins in our country.

VI. Precipitation

Many different kinds of prophylactic agents, human blood products, antisera, and diagnostics are produced by the Shanghai Institute of Biological Products (SIBP) (Table IX). All the biological products, especially the normal human plasma protein components and many kinds of vaccines, are recovered by precipitation methods. The Cohn fractionation procedure is still used for plasma protein fractionation (Table X).

Because meningitis ranks second among infectious diseases causing death in China, the production of group A meningococcal polysaccharide vaccine in our country is the biggest in the world. As shown in Fig. 9, by centrifugation we get the supernatant from 300 liters of fermentation broth; after use of a special cation detergent to precipitate nucleic acid and then alcohol precipitation and cold phenol treatment to remove the protein impurities, we get the final products, a capsular polysaccharide with molecular weight of 30,000. Thirty million doses are produced every year at SIBP.

Precipitation is widely used for recovery of bioproducts. Although it is a rather old method, we can make it efficient if we handle it carefully. The final pH after ammonium salt precipitation with a certain percentage of ammonium sulfate saturation usually lies between 5.0 and 6.0; this

TABLE VI

SEMIPURIFIED STREPTOKINASE (1000 LITER FERMENTATION)

Batch No.	Fermentation units (100 M.U.)	Cation resin absorption–desorption activity recovery (%)	Ammonium sulfate precipitation activity recovery (%)	Calcium phosphate activity recovery (%)	Methanol precipitation activity recovery (%)	Semipurified streptokinase	Total activity recovery (%)
1	7.8	91.0	64.0	95.5	91.5	4.24	54.3
2	6.27	89.5	66.0	94.5	90.0	3.12	50.0
3	6.75	81.5	60.0	77.4	71.0	1.80	26.8
4	6.6	87.2	75.5	75.0	92.4	2.88	43.5
5	8.1	103.2	86.0	123.6	52.5	5.12	63
6	9.8	73.8	83.0	91.0	72.0	4.5	45.8
7	9.16	90.0	66.0	73.0	71.5	2.9	32
8	7.2	90.4	51.5	109.8	89.2	3.3	45.8
9	7.2	99.8	89.0	74.8	83.0	3.58	49.8
10	8.08	105.6	91.0	56.0	88.3	3.98	47.7
Average		90.33	73.2	84.29	80.14		45.85

TABLE VII

CRUDE PREPARATION OF UROKINASE

Batch No.	No. of persons	Urine volume (liters)	Activity (μ/ml)	Resin amount (kg)	Yield (%)	Salt precipitation yield (%)	Total yield (%)
1	2529	506	5.9	6 + 4	82.4	81.2	66.9
2	1226	245.2	5.5	6 + 4	91.2	93.5	85.2
3	1613	322.6	5.0	5 + 5	84.9	107.3	91.1
4	1441	288.2	3.9	5 + 5	94.6	100.0	94.6
5	1056	211.2	5.0	8	78.9	89.2	70.5
6	1251	250.2	5.0	8	57.0	90.1	51.2
7	1714	342.8	5.0	8	60.0	99.0	58.7

TABLE VIII

NONPOLAR MACROPOROUS ADSORBENT RESINS

Catalog no.	SIP-1100	SIP-1200	SIP-1300	SIP-1400
Shape	Bead	Bead	Bead	Bead
Color	Milk white	Milk white	Semitransparent white	Milk white
Water regain (%)	58–64	64–68	48–52	58–62
Apparent density (g/ml)	~0.66	~0.66	~0.68	~0.67
Specific surface area (m^2/g)	450–550	500–600	550–580	600–680
Pore volume (ml/g)	1.2–1.3	1.5–2.0	0.85–0.92	1.0–1.1
Average pore diameter (Å)	~90	~120	~60	~70
Screen size (mesh)	16–50	20–60	20–60	20–60
Applications	CoA extraction; decolorizer; waste organic substance treatment	Decolorizer	Erythromycin; cephalosporin; lincomycin; waste organic substance treatment; aromatic substance adsorption; hemoperfusion	Stevioside; midecamycin; Chinese traditional; drug S-804; agricultural antibiotics

TABLE IX

BIOLOGICAL PRODUCTS PRODUCED BY SHANGHAI INSTITUTE OF BIOLOGICAL PRODUCTS

Types	No. of kinds
Prophylactics	21
Human blood products	24
Antiserum	7
Diagnostics	
Bacteriology	
Antiserum for agglutination test	80
Bacterial suspensions for agglutination test	8
Lyophilized staphylococcal protein A reagent	7
Miscellaneous	11
Immunology	
Reagents for human immunoglobulin detection	9
Antiserum to plasma proteins	16
Reagents for immunofluorescence	3
RPHA reagents	6
ELISA reagent kits	3
Reagent kits for radioimmunoassay	9
Reagents for experimental immunology	7
Reagents for clinical immuno-detection	4
THN monoclonal antibody reagents	6
Biotin–avidin system reagents	5
Clinical chemistry reagents	18

TABLE X

NORMAL HUMAN PLASMA PROTEIN COMPONENTS PRODUCED BY SIBP

Prealbumin	β-Lipoprotein
Albumin	Complement C_3
α-Lipoprotein	Complement C_4
α_1-Acid glycoprotein	Complement C_{1q}
α_1-Antitrypsin	Fibrinogen
α_1-Antichymotrypsin	Immunoglobulin G
α_2-Macroglobulin	Immunoglobulin A
Ceruloplasmin	Immunoglobulin M
Haptoglobin	C-Reactive protein
Hemopexin	Plasminogen
G_c-Globulin	Antithrombin III
Transferrin	Fibronectin

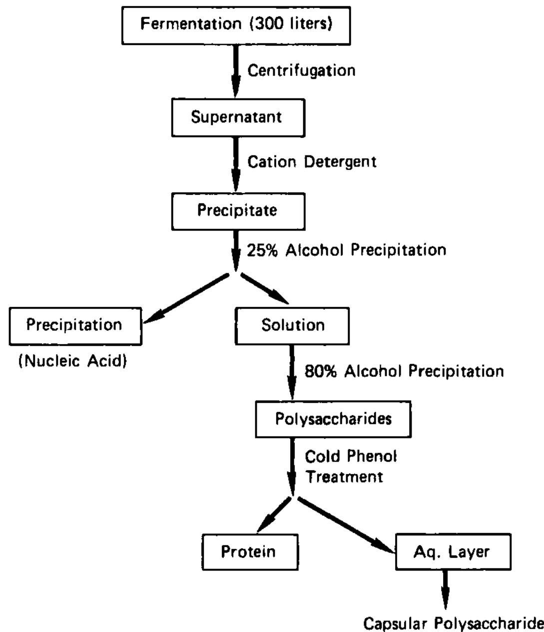


FIG. 9. Production of Group A meningococcal polysaccharide vaccine.

range of pH is the isoelectric point of most of the natural inert proteins. If we want to separate the inert proteins from the desired substance as completely as possible, we have to control pH carefully and we prefer to adjust pH during the salting out operation. For instance, if we can control the pH between 7.0 and 7.5 during ammonium sulfate precipitation, we can easily separate streptokinase from streptodornase in one step.

Isoelectric point precipitation is also an old but useful technique commonly used for precipitating proteins. During isoelectric point precipitation, we have to pay attention to the protein concentration. To prevent the overlapping of proteins with isoelectric points near that of the desired product, it is preferable to operate the precipitation with a protein concentration less than 1%, and it is better to do the precipitation at least twice, once from the acidic side of the isoelectric point of the desired component and once from the basic side. Sometimes we can add a solvent (ethanol, acetone), salts, or metals ions to accelerate the isoelectric point precipitation. Combinations of isoelectric point precipitation, salt precipitation, metal ion precipitation, and organic solvent precipitation are often used for purification of insulin, streptokinase, urokinase, penicillin acylase, and others.

VII. Ultrafiltration

SIPI was one of the first to develop minipore filter membranes and ultrafiltration membranes in China (Tables XI and XII). These membranes are now widely used for water treatment and sterilization. Different types of ultrafiltration equipment designed and developed by SIPI are now used in pilot plants for treatment of some fermentation filtrates and for concentration or separation of biological products. Membranes made at SIPI are inexpensive; for example, a 50-mm diameter ultrafiltration membrane costs only $0.20 or less.

VIII. Ion Exchange

Ion-exchange resins were developed in China during the early 1950s for streptomycin purification and for water treatment. Table XIII shows some of the ion-exchange resins developed at SIPI specifically suitable for certain bioproducts.

Figure 10 shows the purification of CoI, CoII, and CoA by ion-exchange resins. CoA is first separated from CoI and CoII, and then CoI and CoII are separated using other chromatographic conditions. Figure 11 depicts the purification of glycerokinase from a thermophilic bacillus using DEAE-Sephadex A-25. Glycerokinase is well separated from other contaminating proteins during elution with 0.15 *M* potassium phosphate buffer with 5.7-fold purification and a 67.6% recovery yield.

Another example of ion-exchange (Fig. 12) is the recovery of lysine using phenylvinylsulfonic resin. After using the resin (NH_4^+) to exchange

TABLE XI

MICROPOROUS FILTER MEMBRANES (MIXED ESTERS OF CELLULOSE)

Pore diameter (μm)	Water flow rate (ml/cm²-min)	Porosity (%)	Bubble-point pressure (kg/cm²)	Thickness (μm)	Shape
5.0	400	84	0.42	100–150	Flat
3.0	300	83	0.70	100–150	Flat
1.2	200	82	0.75	100–150	Flat
0.8	200	81	1.15	100–150	Flat
0.65	150	80	1.40	100–150	Flat
0.45	50	79	2.30	100–150	Flat
0.30	40	77	3.0	100–150	Flat
0.22	20	75	4.1	100–150	Flat
0.15	10	73	4.8	100–150	Flat

TABLE XII

ULTRAFILTRATION MEMBRANES

Structure	Type no.	Material	Molecular weight cut-off
Flat	CXA-10	Cellulose	10,000
	CXA-50	Acetate	50,000
Hollow fiber	LFA, LFB LFC, LFD, LFE	Polysulfone	50,000

the lysine from the fermentation broth, we can elute lysine by ammonia water with a high recovery yield (95%) and regenerate the resin simultaneously.

Not only ion-exchange resins but also ion-exchange cellulose has been used widely for the purification of bioproducts in China. For example, DEAE-cellulose has been used for purification of urokinase, streptokinase, and collagenase; conjugated carboxylmethylcellulose is used specifically for adrenocorticotropin (ACTH) purification.

IX. Affinity Chromatography

A few examples of the application of affinity chromatography in China are outlined in Table XIV.

CoA is specifically bound on the column by a CoA–binding protein obtained from a species of *Brevibacterium*. AMP cannot be absorbed; ADP and ATP can be adsorbed but to a much lesser degree than CoA. The purity of CoA can be increased from 5 to 92% in one step (Fig. 13). Neutral proteinase from *Bacillus subtilis* can be purified by a dipeptide ligand Sepharose medium with a 35-fold purification of trypsin and trypsin inhibitor. Affinity chromatography is used widely in China for these purifications. An extract containing trypsinogen and trypsin inhibitor is added successively to A and B columns containing immobilized trypsin and trypsin inhibitor, respectively. In the A column, trypsinogen is converted to trypsin by the immobilized trypsin and passed through the column; trypsin inhibitor is absorbed due to the affinity to trypsin. Trypsin from the A column is adsorbed on column B and all the contaminated proteins flow out the end of column B. Using these two steps of affinity chromatography (Fig. 15), trypsin and trypsin inhibitor can be easily recovered with high yield (400 and 600 units/mg protein, respectively). Sugarcane cellulose can be used as the carrier, greatly decreasing cost.

TABLE XIII

ION-EXCHANGE RESINS

Catalog no.	SIP 1X3	S-22	SIP 1X12	SPG-5	SIP 1X25	SIP 201X8	101	110	C 107	SIP-4	D-211	330	Decolor 1
Type	Cation strong acid	Cation strong acid	Cation strong acid	Cation strong acid	Cation strong acid	Anion weak base	Cation weak acid	Cation weak acid	Cation weak acid	Cation weak acid	Anion strong base	Anion weak base	Amphoteric
Structure	Gel	Gel	Gel	Gel	Gel	Gel	Gel	Gel	MR	Gel	MR	Gel	Gel
Matrix	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Acrylic	Acrylic	Acrylic	Acrylic	Acrylic	Epoxy	Phenol-aldehyde
Shape	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Bead	Granular
Color	Brownish yellow	Brownish yellow	Brownish yellow	Brownish yellow	Brownish yellow	Light yellow	Milk white	Milk white	Milk white	Milk white	Milk white	Amber	Dark brown
Water regain (%)	75–85	65–70	45–55	40–50	37–42	40–50	50–60	50–60	50–60	70–75	70–75	50–60	55–65
Total capacity (meq/g)	4.5	5.0	4.5	4.5	4.3	3.0	9.0	12	8.0	12	3.8	9.0	3.0
Apparent density (g/ml)	0.75	0.72–0.8	0.75–0.85	0.80	0.89–0.93	0.65–0.75	0.75	0.75	0.72–0.78	0.65	0.65–0.68	0.60–0.70	–
Screen size (mesh)	16–50	40–60, 60–80, 80–100, 100–120	16–50	16–50	16–50	16–50	20–50	20–50	16–50, 40–80	30–60	16–50	16–50	16–50
Applications	Oxytetracycline, paromomycin, vancomycin	Neutral amino acid, valine	Kanamycin, gentamycin	Lysine	Streptomycin purification	Antibiotic decolorizer	Streptomycin, dihydrostreptomycin, UK	Streptomycin, bleomycin	Streptomycin, amikacin, daunomycin	Lysozyme, elastase	Kanamycin, decolorizer	Antibiotic purification	Decolorizer

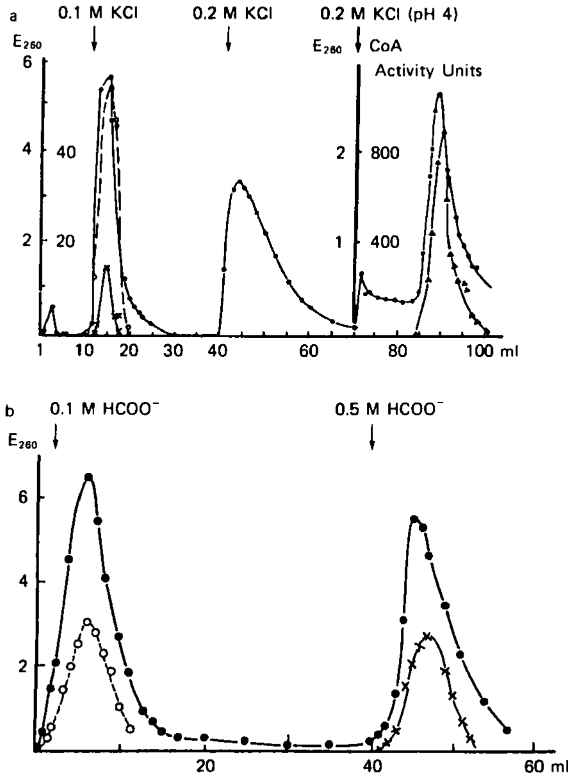


FIG. 10. Purification of (o) CoI, (×) CoII, and (▲) CoA: (a) separation of CoA by ion-exchange resin (Cl^-); (b) separation of CoI and CoII by acidic resin ($HCOO^-$). (●) E_{260}.

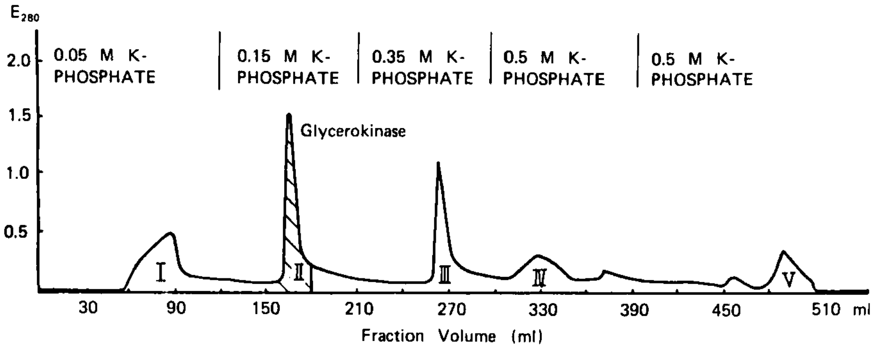


FIG. 11. Purification of glycerokinase (from *Bacillus stearothermophilus*) by DEAE-Sephadex A-25 ion-exchange chromatography (Pharmacia).

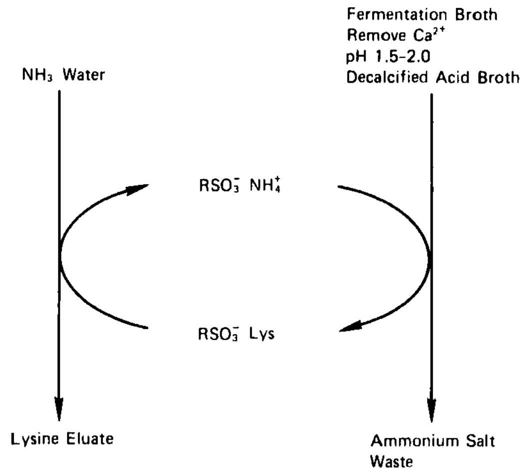


FIG. 12. Lysine ion-exchange resin extraction.

For the purification of trypsin inhibitor, different carriers and different trypsin immobilization methods have been used for preparing chromatographic media (Table XV). ABSE-agarose and Sepharose 4B are the best carriers, with about 90% activity recovery and more than 1000 T.I.U./mg protein on the purification of trypsin. ABSE is a red color reactive dye manufactured in China.

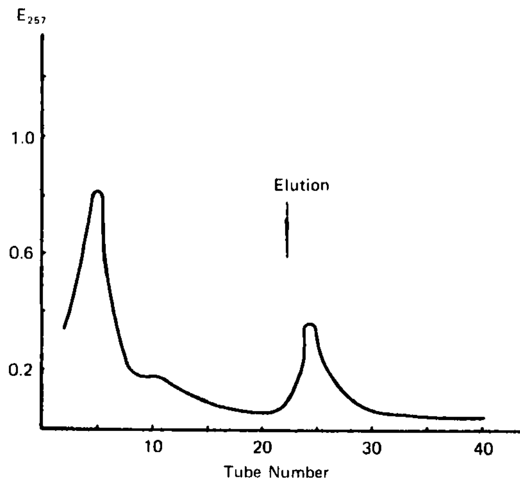


FIG. 13. Purification of CoA with affinity chromatography. Carrier: A BSE-Agarose CL; ligand: CoA binding protein from *B. reuibacterium ammoniagenes* 1844.

TABLE XIV

AFFINITY CHROMATOGRAPHY PRODUCTS

Substances purified	Ligands
α-Interferon	Anti-α-interferon
Hemoglobin	Anti-hemoglobin
Coagulant factor VIII	Anti-coagulant VIII
Complement C_3	Anti-complement C_3
Complement C_4	Anti-complement C_4
Alkaline phosphatase	Anti-alkaline phosphatase
Trypsin	Trypsin inhibitor
Trypsin inhibitor (pancreas)	Trypsin
Trypsin inhibitor (*Rheum palmatum*)	Trypsin
Haptoglobin	Lysine
Plasminogin	Lysine
Fibronectin	Gelatin
Anti-thrombin C	Heparin
Urokinase	p-Aminobenzamidine

Rheum palmatum is a Chinese traditional medicine, a plant root, which is used clinically for treatment of acute and chronic pancreatitis. We suspected that some trypsin inhibitor was present in the root and in fact found two kinds of trypsin and chymotrypsin inhibitor, designated inhibitor I and inhibitor II. The two have different molecular weights, the first about 200 and the second about 11,000. Each inhibits both trypsin and chymotrypsin activity by a competitive mechanism. The kinetic parameters of these two inhibitors are shown in Table XVI. We first separate them by gel filtration (Fig. 16) based on their molecular weight and then by affinity chromatography on an immobilized trypsin column. A very sharp peak of trypsin inhibitor I appears (Fig. 17). Both of the final preparations show only one band by disc electrophoresis.

X. Protein Modification

Because of instability, reaction conditions, kinetic properties, antigencity, and so on, most natural biologically active substances are unsuitable for direct use in industry or in the clinic. The most convenient and applicable method is combination of the enzyme molecule with a large biodegradable and nonantigenic molecule for clinical use, or with an insoluble carrier for industrial use. Because of the large size of the carriers, they can often more or less protect the active center and

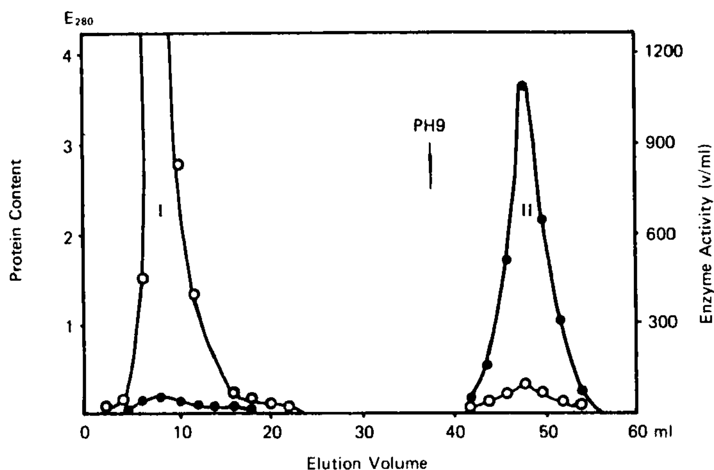


FIG. 14. Purification of neutral proteinase (*Bacillus subtilis* AS 1.398) by affinity chromatography: (○) protein concentration; (●) enzyme activity.

stereostructure of an enzyme from proteolytic enzymes, inactivators, inhibitors, or other harmful factors.

We have modified several enzymes and hormones, including elastase, insulin, and urokinase. I here take urokinase as an example. Human albumin is first succinylated with succinic anhydride, then converted to an active ester with p-nitrophenol in the presence of water-soluble carbodiimide, and then combined with urokinase (Fig. 18). The modified urokinase, which can maintain 95–100% activity, is isolated from natural urokinase and other impurities by gel filtration followed by affinity chromatography on a p-aminobenzamidine ligand carrier (Fig. 19). As Fig. 20 shows, the activity of albumin-modified urokinase is much more

TABLE XV

AFFINITY CHROMATOGRAPHY OF TRYPSIN INHIBITOR USING DIFFERENT TYPES OF IMMOBILIZED TRYPSIN

Carrier	Preparation method	Activity recovery (%)	Specific activity (T.I.U./mg protein)
ABSE-agarose	Diazotized	90	1200
Sepharose 4B	CNBr	86	1000
Polyacrylamide	Entrapped glutaraldehyde	50	1000
Silica gel	Adsorption glutaraldehyde	85	500

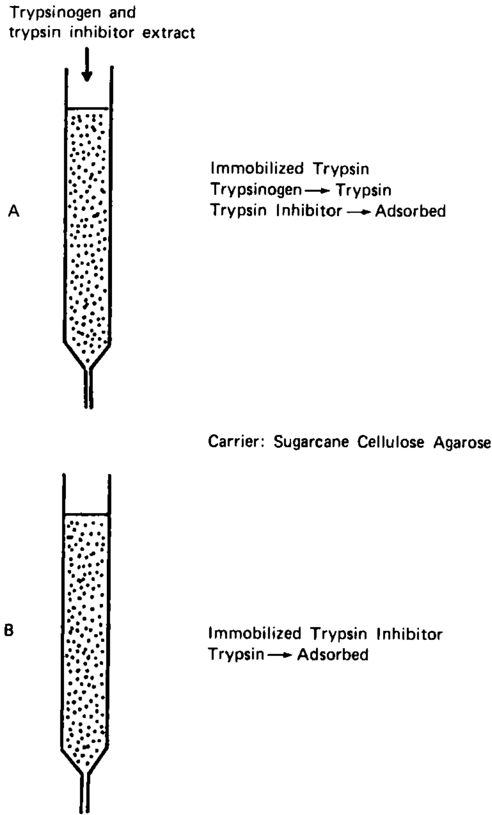


FIG. 15. Affinity chromatography of trypsin and trypsin inhibitor.

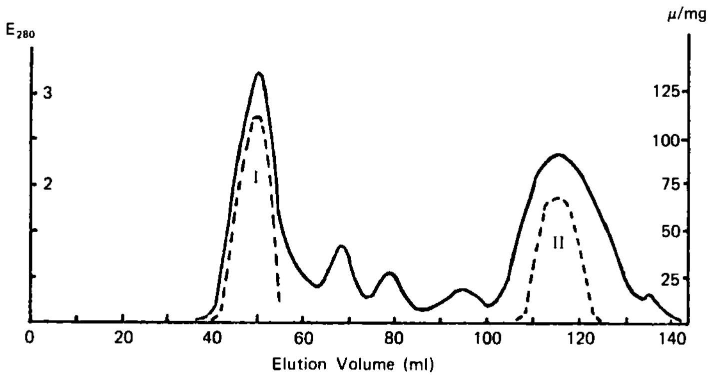


FIG. 16. Recovery of trypsin inhibitor I and II by Sephadex G-100: (——) E_{280}; (----) activity of inhibitor.

TABLE XVI

TRYPSIN INHIBITOR I AND II FROM *Rheum palmatum*

Substrate	Inhibitor I[a]				Inhibitor II[b]			
	Theoretical value		Complete inhibition	K_i	Theoretical value		Complete inhibition	K_i
	w/w	MW/MW	(w/w)	(*M*)	w/w	MW/MW	(w/w)	(*M*)
Bana	1:0.04	1:8.16	1:0.25	1.04×10^{-8}	1:1.35	1:0.63	1:0.33	9.22×10^{-8}
Casein	1:0.94	1:8.16		1.91×10^{-8}	1:1.32	1:0.61	1:0.25	1.99×10^{-7}

[a]Molecular weight of inhibitor I, 208,910.
[b]Molecular weight of inhibitor II, 11,220.

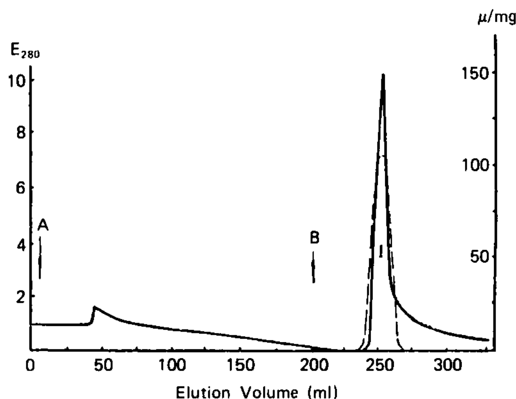


FIG. 17. Recovery of trypsin inhibitor I by affinity chromatography: (——) E_{280}; (--) activity of inhibitor. A, Equilibrium buffer; B, elution buffer.

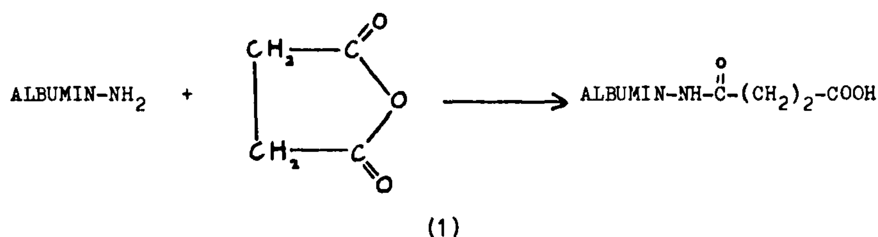
stable than that of dextran-modified urokinase. The heat resistance of the albumin-modified urokinase is 10 times that of natural urokinase at 60°C (Table XVII). After treatment with pepsin for 10 minutes, the activity of natural urokinase decreases to 50%, but the albumin-modified urokinase still maintains 70–80% of the original activity after pepsin treatment for 90 minutes. Resistance to placenta urokinase inhibitor is also increased 2- to 4-fold.

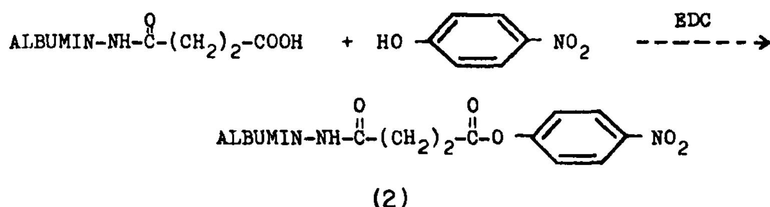
The stability of albumin-modified urokinase *in vivo* parallels the results *in vitro*. The half-life of the modified urokinase in mice, estimated by either the fibrin plate method or the specific substrate (S-2444) method, is 5-fold greater than that of the natural enzymes.

It may become possible to modify a new enzyme or to synthesize a new enzyme with much better characteristics by genetic manipulation after the structure–function relation becomes clearer. However, chemical or enzymatic modifications of enzymes and other biologically active substances are still useful for getting better enzymes. Some of the technology applied in modifying enzymes used in industry is the same as that applied for immobilization, but for the enzymes used in the clinic we must first consider factors such as toxicity, accumulation, side reactions, and biodegradability.

XI. Dye–Ligand Chromatography

SIPI has developed a few kinds of dye-ligand carriers by using Chinese-made reactive dyes. Because the structures of these kinds of reactive dyes are similar to the natural ligands or their chemical analogs, there is an





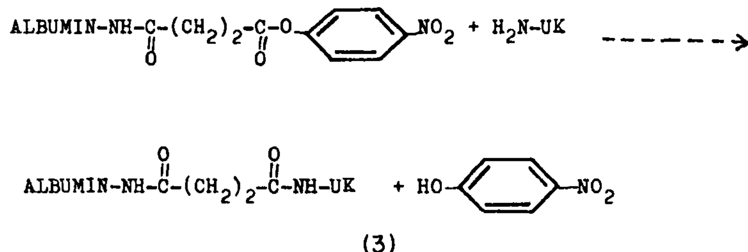


FIG. 18. Modification of urokinase. Reaction (1): succinylation of albumin, after which >90% of free amino groups have been acylated. Reaction (2): active ester formation; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide. Reaction (3): modification of urokinase.

affinity between the dyes and the enzymes or other biologically active substances. Although these affinities are not the same as those between enzymes and substrate, enzyme and inhibitor, antigen and antibody, or hormone and acceptor, because they are inexpensive and easy to prepare they are widely used for purifying dehydrogenases, kinases, phosphatases, nucleases, and so forth.

Diaphorase can be purified by Matrex Gel Blue A dye–ligand chromatography (Amicon) with a 2-fold purification and 68.5% yield (Fig. 21), and by K_2BP-Agarose 4B dye–ligand (Chinese made) chromatography with 2.6-fold purification and 87.8% yield (Fig. 22)

KPBR is a reactive dye similar to Cibacron blue. Glycerokinase (an important enzyme in serum triglyceride determination kits) can be easily purified

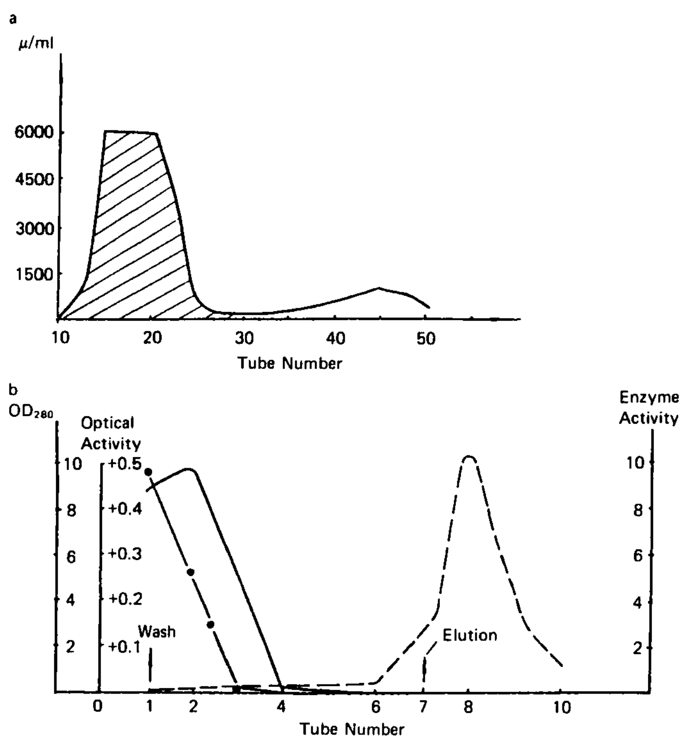


FIG. 19. (a) Isolation of modified urokinase (cross-hatched area) from natural urokinase. (b) Behavior of modified urokinase on a chromatographic column of Sephadex G-100: (--) enzyme activity; (●) optical activity; (——) absorbency.

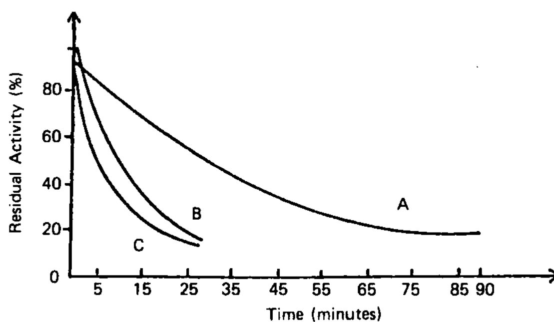


FIG. 20. Estimation of urokinase activity in time intervals using S-2444 (Pyr-Gly-Arg) from mice. (A) Albumin-modified urokinase; (B) dextran-modified urokinase; (C) natural urokinase.

TABLE XVII

PROPERTIES OF MODIFIED UROKINASE COMPARED TO NATURAL UROKINASE

Heat resistance	10-fold increase (60°C, 0.05 *M*, pH 8.0 PBS)
Resistance to pepsin	
Natural urokinase	50% activity remaining (at 10 minutes)
Modified urokinase	70–80% activity remaining (at 90 minutes)
Resistance to placenta urokinase inhibitor	2- to 4-fold increase
Activity against blood clot *in vivo* (rabbit)	3- to 5-fold increase ($p < 0.001$)
Kinetic behavior of activity disappearance	
Natural urokinase	First order
Modified urokinase	Zero order
Half-life in mice	
Fibrin plate method	5-fold increase (after 60 minutes)
Pyr-Gly-Arg (S-2444)	5-fold increase (after 30 minutes)
Half-life in rabbit with ligated liver and kidney	
Natural urokinase	Prolonged by 3- to 4-fold ($p < 0.001$)
Modified urokinase	No influence

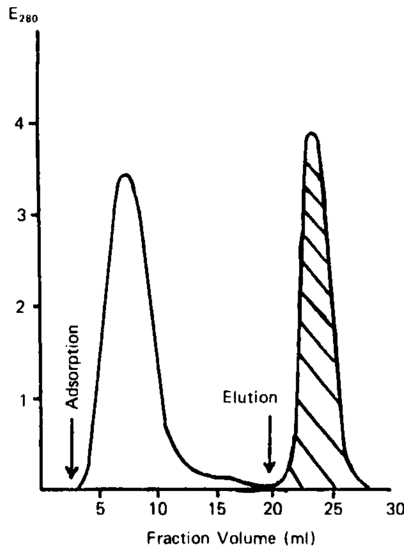


FIG. 21. Purification of diaphorase by Matrex Gel Blue A (Amicon) dye–ligand chromatography.

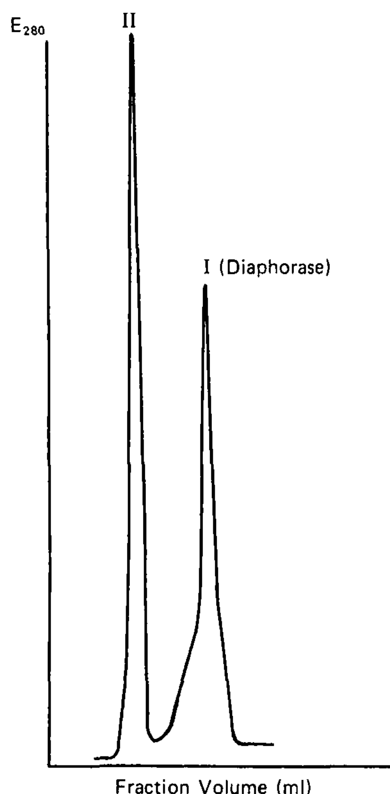


FIG. 22. Purification of diaphorase by K_2BP-Agarose 4B dye–ligand chromatography.

4-fold with 75.5% activity recovery. The results with KPBR-Agarose 4B are similar to those using Matrex Gel Blue (Amicon), AF Blue L-Toyopearl (Toyosoda), and AF Blue H-Toyopearl (Toyosoda), but Matrex Gel Red is not as good as the above four dye–ligand carriers. Perhaps anthraquinone dyes have stronger affinity with glycerokinase (Fig. 23 and Table XVIII).

In the purification of alkaline phosphates by Red Sepharose 4B (Fig. 24), the enzyme activity is well separated from other impurities, with 10.7-fold purification and 92.4% activity recovery.

D-Amino-acid oxidase is an important enzyme for splitting cephalosporin C, but it is difficult to separate the contaminating esterase, which will hydrolyze the side chain on the C_3 position of cephalosporin C. We found that D-amino-acid oxidase can be easily separated by Matrex Gel Blue A dye–ligand chromatography with 90% recovery yield (Fig. 25).

Hexokinase can be purified by the K_2G-Agarose 4B column. There is

TABLE XVIII

PURIFICATION OF GLYCEROKINASE (FROM *Bacillus stearothermophilus*) BY KPBR-AGAROSE 4B LIGAND–DYE CHROMATOGRAPHY[a]

Sample	Activity			Protein		Specific activity (U/mg)	Purification (-fold)
	U/ml	Total U	Yield (%)	Total (mg)	Yield (%)		
Crude glycerokinase	25.5	331	100	93	100	3.53	1
Dye chromatography wash					84.9		
Dye chromatography elution	8.87	250	75.5	17	18.3	14.7	4.16

[a]Matrex Gel Blue A, AF Blue L-Toyopearl, AF Blue A-Toyopearl, Matrex Gel Red (glycerokinase cannot be bound by this medium at the same condition).

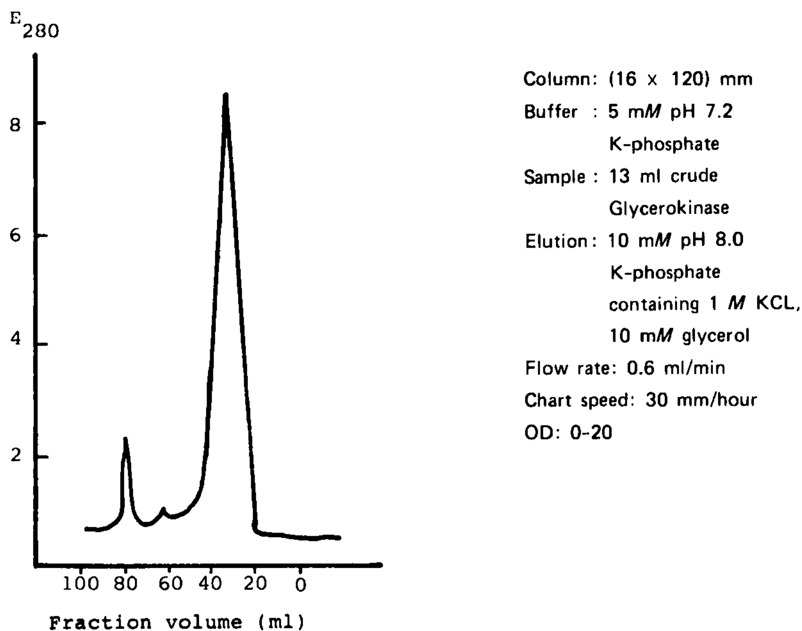


FIG. 23. Purification of glycerokinase (from *Bacillus stearothermophilus*) by KPBR-Agarose 4B ligand-dye chromatography.

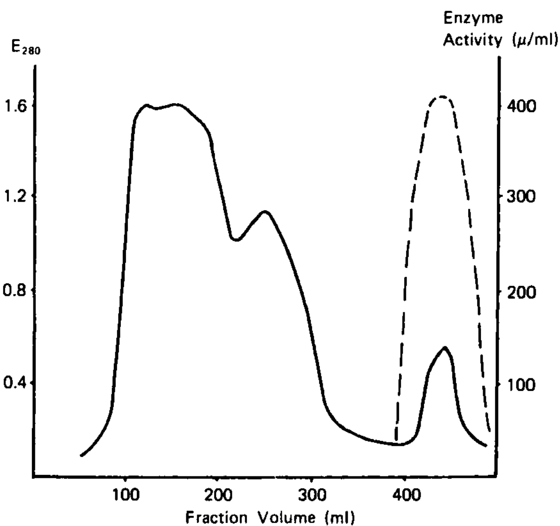


FIG. 24. Purification of alkaline phosphatase by Red Sepharose 4B dye-ligand chromatography: (——) protein concentration; (--) alkaline phosphatase activity.

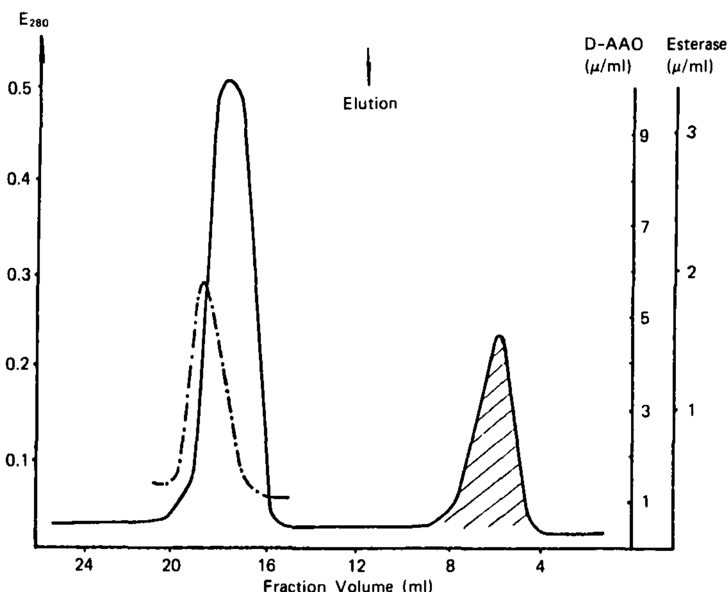


FIG. 25. Purification of D-amino acid oxidase (D-AAO) (from *Trisnopsis variabilis*) by Matrex Gel Blue A dye–ligand chromatography: (—) protein concentration; (– • –) D-AAO activity. Within the cross-hatched area, almost all of the esterase has been eliminated.

a very sharp peak of hexokinase activity with 3-fold purification and 78.5% recovery yield.

XII. Hydrophobic Chromatography

The charged groups between the desired substance and ion-exchange resins are often used to design a chromatographic procedure. But besides the hydrophobic portion, there are also some hydrophobic areas on the surface of biologically active macromolecules. This characteristic can be used to design a hydrophobic medium. For example, the substrate of lipase is hydrophobic; therefore, there should be some hydrophobic area on the surface of the lipase molecule and we should be able to purify a lipase by hydrophobic chromatography instead of the commonly used ion-exchange chromatography. A lipase purified by using a column containing octyl-Sepharose I-4B has a good separation, with 10-fold purification and 75% recovery yield.

Purification of cholesterol esterase by a macroporous acrylic acid cation-exchange resin, SIPI-D-20, also gives good results (Fig. 26).

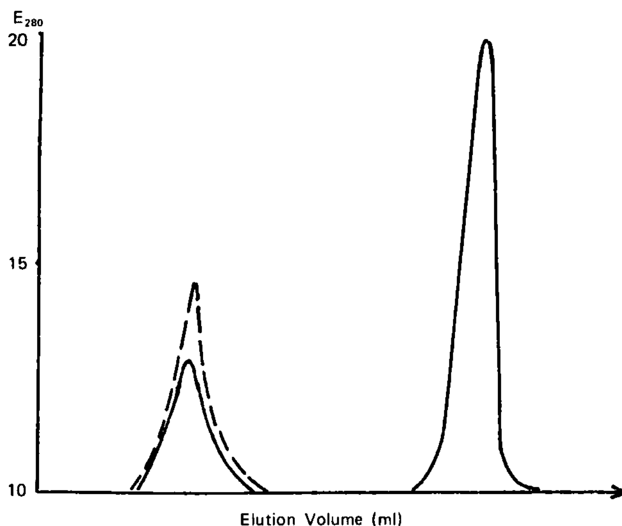


FIG. 26. Purification of cholesterol esterase by hydrophobic chromatography (SIPI-D-20 resin). Adsorb condition, 2 *M* (pH 4.5) HAC buffer; desorb condition, 1 *M* (pH 5.2) HAC buffer; (—) protein concentration; (---) enzyme activity.

Because the operation pH is rather low, no ion-exchange reaction can be used except the hydrophobic affinity between lipase and the medium. Cholesterol esterase was purified 6-fold with 75.7% activity recovery. Hydrophobic chromatography is especially suitable for products obtained by salt precipation; the desalting step can be omitted.

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