EFFECT OF DISINFECTANTS, PARTICULARLY QUATERNARY AMMONIUM COMPOUNDS, ON BACTERIA ISOLATED FROM SOIL-ASPHALT INTERFACES

by

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INTRODUCTION

It appears that Hundeshagen (1935) was one of the first workers to observe the microbial degradation of asphalt. Though few reports have appeared in the literature, other workers have undoubtedly noted similar activity. The work of Burgess (1956), though incomplete, seems to be the most provocative, if only because of its practical aspect.

Burgess was able to show that crude bacterial cultures in soil solutions were capable of oxidizing at least a portion of the hydrocarbons contained in asphalt, thus causing visible changes in its appearance. For this reason Burgess postulated indirectly that soil microorganisms might be a contributing factor in the breakdown of asphalt surfaced roadways.

All are familiar with "chuckholes" and broken shoulders on both rural and urban roads and have usually attributed these faults to the effect of weathering. But it is not impossible to visualize microorganisms which can radically change the properties of this asphalt as being an unknown quantity in the deterioration of asphalt mats.

Scott (1921) and Novelli (1943) both expressed a belief that microorganisms were of utmost importance in the genesis of petroleum. It would follow that if these organisms could synthesize such a complex material, certainly they should be able to break down almost any component part.

On the supposition that it could be shown that organisms having the ability to break down long chain hydrocarbons in asphalt were present at the soil-asphalt interface of a road surface, it was thought desirable to study the effect of certain disinfectants on their activity.

Accordingly, experiments were outlined and performed to establish the presence of these organisms in and around road surfaces together with their

relative numbers, to determine the effectiveness of various disinfectants on representative cultures of isolated organisms, and study the action of the disinfectants in the presence of materials found in conjunction with road surfaces.

REVIEW OF LITERATURE

Slightly more than a half century has passed since Söhngen (1906) and Kaserer (1906) were able to show, independently, that certain bacteria were capable of utilizing methane as their sole source of carbon. Söhngen isolated Bacillus methanicus, now known as Methanomonas methanica (Söhngen) Orla Jensen (Breed, et al. 1948) in pure culture; Kaserer, however, was only able to show methane oxidation with mixed cultures.

Three years later, two Italian workers, Giglioli and Masoni (1909) confirmed the results of Söhngen and Kaserer that methane was utilized by certain organisms in the presence of atmospheric oxygen and noted that 30° C. appeared to be the most favorable temperature for this biological oxidation. These same workers (1917) discovered that many different species and varieties of methane oxidizers existed, each with its own optimum temperature. Münz (1915) employed the methods of Söhngen and Kaserer for the culture of the organism B. methanicum and found that it was capable of multiplication in all proportions of methane and oxygen, with two per cent oxygen being the optimum. Münz also noted that the organism could grow either autotrophically or heterotrophically.

Störmer (1908) first demonstrated the microbial assimilation of cyclic hydrocarbons, isolating Bacillus hexacarbovorum which utilized both toluene and xylene. The use of more complex hydrocarbons such as petroleum, paraffin oil and paraffin by microbes was reported by Söhngen (1913). Isolating his

cultures from garden soil, manure and pond water, he found that the selected organisms could break down hydrocarbons completely to carbon dioxide and water.

Several early workers were able to show a microbial breakdown of paraffins under varied conditions. Grieg-Smith (1914), Gainey (1917), and Fleming (1927) are only a few who observed the utilization of these hydrocarbons, particularly by soil microorganisms.

After Störmer (1908) first reported that bacteria could use aromatic compounds, other men made similar studies. Wagner (1914) obtained seven species of organisms from the soil which utilized benzene as their sole carbon source. In addition, the organisms used toluene and xylene, grew well on ordinary culture media, and could proliferate easily when certain fatty acids and aliphatic hydrocarbons were substituted in the media. This worker was also one of the first to record the use of crude oil by an organism -- Bact. benzoli used one gram of crude oil in eight days in 100 cc of medium. Coobin (1923) and Tausson (1929) both isolated benzene utilizing bacteria, the former, a non-sporeforming rod, Bact. hidium, which used coal oil equally as well as benzene, and the latter, four strains of B. toluolicum that utilized derivatives of benzene, like toluene and ethyl benzene, with apparent ease.

Tausz and Donath (1930) set down some general rules governing hydrocarbon utilization by bacteria. Having studied a number of organisms on various compounds they stated that, in general: the ease with which a hydrocarbon is attacked increases with the length of the chain; when a given member of a hydrocarbon series is attacked by a given organism, then it may be assumed that all higher members will be attacked by that same organism. Thus for the methane bacterium studied, the usable hydrocarbons began with

methane, for Bacterium aliphaticum liquefaciens available hydrocarbons start with pentane, and for the paraffin bacterium hexadecane initiates the series.

Twenty years later ZoBell (1950) added some additional rules of thumb to Tausz and Donath's contribution. He stated that, in general, aliphatic or paraffinic hydrocarbons are attacked more readily and by more microbial species than aromatic compounds, long chain hydrocarbons are attacked more readily and by more species than those of low molecular weight, hydrocarbons with double bonds seem more susceptible to microbial oxidation than their saturated counterparts, and branched chain hydrocarbons are more susceptible to microbial oxidation than straight chain members.

The literature over the past years contains many noteworthy references concerning microbial oxidation of hydrocarbons. To mention a few, there are Haas, et al. (1941), Bushnell and Haas (1941), Johnson, et al. (1942), Strawinski and Stone (1942), Grant and ZoBell (1942) and Cribbins (1949). These workers studied many phases of hydrocarbon utilization but seemed to concentrate on enumerating the various species of organisms capable of this feat. In a recent work, ZoBell (1950) stated that since 1895 more than 100 species, representing 40 genera of bacteria, yeasts, and molds have been shown to be able to utilize one or more kinds of hydrocarbons.

Several workers have undertaken studies involving petroleum compounds or their derivatives and the effect that bacterial populations have upon them. Tausson (1928) and Hessel (1924) both felt that bacteria played a major role in the alteration and modification of crude oils or their by-products. Waskenhut (1936) isolated a hydrocarbon utilizing organism directly from crude oil while Tausz (1919) noted that a layer of petroleum oil over a quantity of ditch water with salts added decomposed in a matter of weeks leaving a markedly turbid solution. From this water he was able to isolate

three new bacterial species.

Related substances such as cutting and industrial oils have received some attention in this respect, also. Duffett, et al. (1945) discussed the normal bacterial flora of cutting oil emulsions. Stone, et al. (1940) mentioned that oils containing more paraffinic hydrocarbons are attacked with greater facility than those containing more aromatic hydrocarbons. Growth of bacteria in soluble oil emulsions was discussed by Fabian and Pivnick (1953). Lee and Chandler (1941) found that cutting compounds in machine shops have high counts of bacteria due to Ps. oleovorans in almost pure culture. Total counts varied from 15-50 million per milliliter.

Very little can be found in the literature about the effect of bacteria on asphaltic preparations. A reference was made by Stone, et al. (1942) in which it was noted that bacteria could attack asphalt in moist garden soil. However, Hundeshagen (1935) may have been the first to notice microbial degradation of asphalt when he stated that cement slabs covered with an asphalt coating and stored in a moist place had been attacked by fungus mycelium which formed a slimy fibrous coating over the asphalt surface. More recently Burgess (1956) has done considerable work in an effort to show that microbial oxidation of asphalt could play a part, if only a small one, in breaking down the structure of road surfaces.

Since, in the experimental work particular emphasis was placed on the disinfectant activity of quaternary ammonium compounds on asphalt utilizing bacteria, a brief survey of the literature concerning the bactericidal characteristics of these compounds seems in order.

It is difficult to say who was first to synthesize a quaternary ammonium compound (QAC). It is unlikely that they occur naturally. Komatsu (1915) gives one of the earliest recorded accounts of the preparation of QACs.

discussing both their formation and decomposition.

The initial observation of the germicidal properties of quaternaries was made by Jacobs (1916), Jacobs, Heidelberger, and Amoss (1916), and Jacobs, Heidelberger and Bull (1916). These men studied the effect of the hexamethylene tetramine group on bacteria; however, these classical studies did not strike a responsive chord for practically no work was done on the use of QACs as bactericidal agents until Domagk (1935) reported on a new class of disinfectants. Domagk's work with Zephirol, an aqueous solution of high molecular weight alkyldimethylbenzylammonium chlorides seemed to open the door for further study of this versatile group of compounds.

The same year that Domagk reported his findings, Hornung (1935) completed a similar study which can be summed up by stating that Zephirol is a chloride of a basic phenol derivative showing high disinfectant activity. Other workers reported on additional properties of this disinfectant. Dunn (1937) noted that it was readily soluble in water, had low surface tension, and was most effective at an alkaline pH. Lousden and Döring (1938) observed that it was insoluble in fats but aided in emulsification, its stability was unaffected by low temperature, but broke down at high temperatures. Using a new name for the same compound, Corker (1939) stated that Zephiran was, on the whole, better than any other antiseptics which were used for sterilizing instruments and skin areas. Jerchel (1947), in a review of German research from May of 1939 to May of 1946 stressed the antibacterial properties of quaternary ammonium salts, also referred to as invert soaps. Thirteen compounds of the quaternary ammonium type were tested by Hucker, et al. (1948a) against both sporeformers and non-sporeformers. They reported that concentrations required to kill spores were five times greater than those which were lethal to vegetative cells; however, the rate of kill for both vegetative cells and spores

was similar. They maintained that the rate of kill rather than the total kill by these germicides was the important criterion of activity. Work with spores also occupied the attention of Klosmann and Wright (1950), who found that since concentrations of less than one per cent were not effective against spores of C. sporogenes, tetani, and welchii, they should not, therefore, be relied upon for sporicidal action. Mueller, et al. (1947) reported similar findings.

Additional information as to effectiveness of QACs against specific organisms was contributed by Lund (1950), Fisher (1949), and Zemits (1950). All were impressed with the relative ease with which these compounds killed yeast and bacteria alike. Katznelson and Sutton (1951), favorably compared Roccal and Hyamine 1622, both QACs, to chloromycetin and other antibiotics in the treatment of plant pathogenic bacteria.

Numerous reports in the literature have appeared in an attempt to explain the mechanism by which QACs kill microorganisms. Rawlins, et al. (1943) concluded that the general configuration of the QAC molecule is as important in the development of germicidal activity as is the exact chemical status of its constituents. Randles and Birkeland (1944) in studies on gram negative organisms in synthetic media advanced the theory that inhibition of growth was due to selective adsorption of the detergent cation at sites of the cell which normally adsorb the cation of the nitrogen compounds that bear a structural relationship to the detergent cation; thus the QAC prevents the adsorption of necessary nitrogen compounds. Knox, et al. (1949) observed that the inhibition of glucose oxidation and glycolysis in E. coli by QACs paralleled the per cent killed. A cell free lactic acid oxidase was found to be inhibited by the same amount of QAC required to kill the intact cell. Therefore, they continued, it follows that specific inhibition of essential

enzymes by a QAC can account for metabolic inhibition and death of the cell. It had been previously determined by Roberts and Rahn (1946) that the effect of a QAC on bacterial enzymes was not reversible.

A theory was propounded by McCulloch (1947) which differed greatly from other ideas on the subject of disinfection by QACs. He hypothesized that the very rapid initial decrease in bacterial numbers (plate count determinations) upon addition of QAC was due to agglomeration of the exposed organisms and their adherence to the sides of the tube as well as actual killing. Agglomerated cells may not be picked up with a loop; they may adhere to the loop and not remain in the subculture medium; and in the absence of particulate matter in the subculture medium, the bacteria may stay coated with the QAC and remain in a condition of bacteriostasis. This idea was strengthened somewhat by some studies of factors responsible for irregular results in testing QACs done by Gershenfeld and Brillhart (1950) in which they found that the washing and rinsing of tubes used, the order of addition of culture and test solution, and the size of the tubes all affected the germicidal test results.

Exhaustive experiments by Büchi, et al. (1951) gave evidence that substitution of one or more of the chains surrounding the nitrogen atom in the QAC reduced its disinfectant properties.

Recent years have seen a change from studies of the disinfectant action of QACs to studies on the development of resistance by organisms or studies on inhibition of disinfectant activity by other substances. Chaplin (1952) showed that a culture of S. marcescens, normally killed by 100 ppm, could be adapted to grow in a concentration of 100,000 ppm alkyldimethybenzylammonium chlorides. The resistance was shown to be due to an increased lipid production in the cells. Malman, et al. (1948) demonstrated that organisms grown in the presence of a fermentable sugar showed increased resistance to

QACs, but not to phenolic compounds. They suggested that this change in resistance was limited to the QACs and other compounds characterized by long chain hydrocarbons.

Inhibition studies by Schneider (1935) gave an indication that QACs might be inactivated bactericidally by many substances. He found that Zephirol's activity was not appreciably diminished by protein but that the addition of soap solutions had an adverse effect. Lewsden and Döring (1938) found that blood serum inhibited these compounds. That QACs could be adsorbed on particulate matter was seen by Rahn (1946). Mueller, et al. (1947) observed a significant decrease in the germicidal potency of a 200 ppm quaternary solution when 0.5 per cent non fat milk solids were present. It was determined by Hucker, et al. (1946b) that 40°F. retarded and 102°F. accelerated germicidal activity and that, in general, quaternaries are least effective at or near a neutral pH. Agreeing with those workers just cited on the effect of pH and temperature, Ridenour and Armbruster (1948) added that an increase in organic matter, calcium ions or magnesium ions decreased the killing power of QACs. Upholding ionic inhibition of QACs was work done by Shere (1948), in which he demonstrated that the QAC was tied up (adsorbed) by the precipitate formed by the water softening alkalis, by Butterfield, et al. (1950), that demonstrated that increased water hardness decreased the killing effect, and by Johns (1948), in which he noted that Roccal dilutions made in tap water were less effective than those made in distilled water.

Weber (1949) pointed out that a QAC is readily inactivated by anionic agents such as soaps and synthetic detergents. A number of workers have studied the effect of various organic materials on the bactericidal properties of the QACs. Adding skim milk to a Roccal solution decreased its activity according to Johns (1948); and Chaplin (1951) reported that substrate

protein removed the quaternary compound from a solution. Horse serum, skim milk, cottonseed oil, and soluble starch were tested on nine QACs by Van Eseltine and Hucker (1948), using the kill of E. coli as an endpoint. All inhibited disinfectant activity to a varying degree, milk proteins being most effective and cottonseed oil the least.

SECTION I
ISOLATION OF ASPHALT UTILIZING BACTERIA AND DETERMINATION OF THEIR RELATIVE NUMBERS IN ROAD SURFACE SAMPLES

Experiment 1

In order to establish the presence of asphalt utilizing organisms at the soil-asphalt interface of roadways, fifteen random samples of road material were inoculated into individual Erlenmeyer flasks containing an asphalt emulsion enrichment medium (Harris, et al., 1956). This medium consisted of a mixture of Ashby's salts medium and an asphalt-clay emulsion.

Ashby's salts medium was made by mixing 1.8 grams K_2HPO_4, 0.7 grams KH_2PO_4, 0.2 grams $MgSO_4$, 0.2 grams NaCl, 0.2 grams $CaCl_2$, 2-3 drops of a 10 per cent $FeCl_3$ solution and 1 cc of a 0.5 per cent solution of the following: $ZnSO_4$, $CoCl_2 \cdot 6H_2O$, H_2BO_3, MoO_3, $CuCl_2 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$. This solution, when diluted to 1000 cc volume, had a pH of 6.8. One gram of NH_4NO_3 was added per liter before use. To prepare the asphalt-clay emulsion 400 cc of the salts medium were placed in a Waring blendor and 30 grams of Wyoming bentonite clay were added slowly. When blending was completed, the blendor, with its contents was heated to 55° C; at the same time a container of SC-3 asphalt was heated to the same temperature. The heating completed, the blendor was restarted and 30 grams of the hot asphalt were pipetted dropwise into the clay suspension; blending was continued for 2-3 minutes. Finally 200 cc of heated salts base were added to bring the volume to 610-615 cc, yielding

roughly a 5 per cent asphalt and clay emulsion suspended in Ashby's medium.

The enrichment medium was made by mixing 900 cc of the salts base with 150 cc of the emulsion; to prepare a solid medium 1000 cc of the salts base were mixed with 100 cc of emulsion and 2 per cent agar added. Both media were stable to autoclaving at 121° C. and 15 pounds pressure for 20-25 minutes.

The 18 inoculated flasks of enrichment media were placed on a rotary shaker operating at approximately 60 rpm and incubated at 30° C. for three days. At that time an inoculum of 1.0 cc was subcultured from each flask into new flasks of medium and reincubated. After one week a specimen from each flask was streaked onto both yeast extract peptone glucose agar (YEPG) and asphalt agar.

Growth was profuse on the YEPG agar. This was expected since this medium contained an abundance of nutrients capable of supporting most heterotrophic organisms. Predominant colonies were picked to YEPG agar slants, incubated one day, and refrigerated for use as stock cultures. Limited morphological studies were made of each of the 17 colonies chosen; gram stains revealed that 13 were gram negative, the remainder gram positive. Variations in size and shape of the organisms ranged from small cocci to rather large rods. A majority of the cultures appeared to fall into either the family Pseudomonadaceae or Micrococcaceae.

Growth on the asphalt agar plates was not nearly so marked as that on YEPG agar. In addition the colony types were completely different from any observed on YEPG agar, for here, with rare exception, all colonies were small, convex, watery and transparent. The most obvious exception proved to be an actinomycete which grew rather typically, forming white colonies of filamentous strands radiating from a central point.

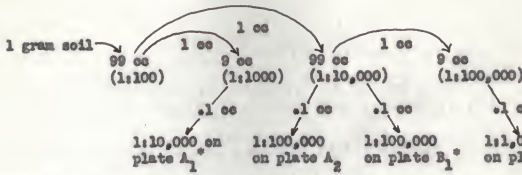
It was interesting to note that when each of the 17 isolates from YEPG agar was streaked on asphalt agar plates, all pigmentation and density were lost, save for the actinomycete mentioned, so that each became watery and transparent to the eye.

Five organisms from the group were selected for use in measurement of disinfectant activity. Selection was based on largest amounts of growth on asphalt agar, thus guaranteeing the ability of the organism to use asphalt as their only carbon source. The organisms chosen were designated Nos. 40, 45, 48, 53, and 54, being a bacillus, coccus, bacillus, coccus, and actinomycete, respectively. Cultures of each were carried on asphalt agar slants in the refrigerator throughout the experimentation.

Experiment 2

The purpose of this experiment was to determine as accurately as possible how many microorganisms were present at various soil-asphalt interfaces and also what per cent of this total were capable of utilizing asphalt as their sole carbon source.

Fifteen soil samples were collected along Highway 13, north of Manhattan. The samples were equally distributed in three categories, loose soil-asphalt interface, asphalt saturated soil interface and the interior or cracked asphalt aggregate. One sample from each category was used to make serial dilutions for preliminary plate counts to determine the range of microbial flora in each. When the respective ranges had been determined the following dilution scheme in sterile distilled water was established.



* A -- asphalt; B -- YEPG

The 0.1 cc inocula were pipetted onto the surface of the two kinds of agar used and were spread with a sterile bent glass rod. Duplicate plates of all dilutions were made and then incubated, inverted, at 30° C. for five days.

Results and Discussion

It was anticipated that if specimens of the soil asphalt interfaces were taken along areas of the road surface where obvious degradation was apparent that the use of differential media would distinguish between total numbers[1] of bacteria present and the per cent thereof that were asphaltic hydrocarbon utilizers.

From Table 1 the average counts for each set of specimens were calculated. The average total count on specimens 1 through 5 was 2.5 million, on 6 through 10, 2.37 million and on 11 through 15, somewhat less than 1.6 million. The average count of organisms which could apparently utilize asphalt as their carbon source was: on specimens 1 through 5, 0.6 million, on 6 through 10, 0.36 million, and on 11 through 15, 0.92 million.

A comparative ratio can be established for each set of specimens or category from this information. In the loose soil-asphalt interface group

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[1] No attempt was made to determine numbers of psychrophiles, thermophiles, or organisms requiring anaerobic conditions or special atmospheres.

Table 1. Colony counts* on asphalt agar and YEPG agar.

Specimen**	YEPG agar			Asphalt agar		
	No. of colonies in		ave/gm in millions	No. of colonies in		ave/gm in millions
	1-100 T	1-1 M		1-10 T	1-100 T	
1	20	3	2.5	67	9	.785
2	29	1	1.95	48	4	.44
3	32	3	3.1	51	7	.605
4	21	4	3.05	64	7	.67
5	15	2	1.9	39	6	.495
6	12	2	1.6	33	5	.415
7	26	4	3.3	24	3	.27
8	---	2	2.0	29	2	.245
9	17	3	2.35	36	4	.39
10	32	2	2.6	37	6	.485
11	3	0	3.0	96	12	1.08
12	9	2	1.45	84	7	.77
13	1	0	1.0	93	7	.815
14	1	-	1.0	104	8	.92
15	---	0	<1.0	90	11	1.00

* Average of two determinations.

** Specimens 1-5, incl., are from loose soil asphalt interface; 6-10, incl., from oil saturated soil; 11-15 from broken aggregate.

calculations showed that one of every four organisms (25 per cent) was an asphalt utilizer. For category two or the asphalt oil saturated soil-asphalt interface only one of six (16 per cent) could grow on asphalt, while in the final category which included samples from within cracked aggregates on the roadside more than one half (60 per cent) showed evidence of being adapted to asphalt for their energy.

It was interesting to note that the colony morphology of organisms isolated on the asphalt plates was almost identical to that mentioned in the previous work. Virtually all colonies, with the exception of the actinomycetes which were white filamentous colonies, were watery and transparent as previously described. No explanation other than a possible genetic trait, characteristic of the actinomycetes, was offered to satisfy this prominent difference in colony types.

Because of this difference, however, it was observed further that relative numbers of actinomycetes varied in each category. The asphalt agar plates which had the loose soil-asphalt interface dilutions spread over their surfaces showed very few actinomycetes; the oil saturated interface material dilutions gave colonies predominantly of this type and the dilutions made of material from broken asphalt aggregate exhibited approximately one-half actinomycetes. All other colonies, with few exceptions, were of the clear, watery type already described. Little can be concluded from this but perhaps it may be inferred that there exists a correlation between numbers of actinomycetes and origin of sample material.

Oddly enough, on all the plates of YEPG agar used for total count determinations, only two actinomycetes could be identified -- and these only by the water soluble, melanin-like pigment produced. It is somewhat difficult to visualize but from this one might state that these organisms preferred hydrocarbons over glucose as a carbon source.

Finally, from this experimental work, some generalizations can be made. Along most, if not all, road surfaces, there exists a flora composed of widely variant microorganisms, some of which have the facility for using asphalt as a major energy source. The numbers of asphalt utilizers may, as compared to total numbers, depend on the location from which the sample is taken. Evidently a selective process is involved, the selection resulting from a diversity of conditions existing along the edge of a road surface. The degree to which the oils from the asphalt have saturated the immediate surroundings makes a marked difference in the type of bacterial life found.

In the face of inadequate information on the chemical composition of asphalt, it is difficult to say whether observed results are attributable to

particular hydrocarbons, other types of organic matter, or inorganic constituents in the asphalt.

SECTION II
DISINFECTION ACTION OF QUATERNARY AMMONIUM COMPOUNDS AND OTHER SELECTED COMPOUNDS IN THE PRESENCE AND ABSENCE OF CLAYS

Having determined that a one per cent bentonite clay suspension had the same effect on disinfectant activity as a five per cent asphalt-clay emulsion, the latter was eliminated in all subsequent work.

All disinfectants used were diluted according to a predetermined scheme, unless otherwise noted.

Table 2. Disinfectant dilution scheme.

Materials* in cc	Tube No.** 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Dist. water	0	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
10% Disinfectant	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	4 cc *** Discard
Clay suspension	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

* In some instances, all volumes were doubled. Control dilution series contained no clay suspension, therefore amounts of distilled water and disinfectant were increased by 1 cc to keep volumes uniform.

** Final dilutions --
Disinfectant; Tube 1, 1-12.5, Tube 2, 1-25, ... Tube 15, 1-204,800
Clay; Tube 1-15 incl., 1-1000 if 0.5% clay used, 1-500 with 1.0%, and 1-250 with 2.0% clay.

*** 4 cc were transferred from tube 2 to tube 3, 3 to 4, etc.

The dilution scheme set forth in Table 2 was not followed exactly in all cases. In beginning work with the various disinfectants, seldom was a dilution of greater than 1-800 used. Only after it had been determined that the quaternary ammonium compounds were effective even in higher concentrations was the above table fully utilized.

Preliminary work was required to ascertain just how concentrated the

various disinfectants had to be in order to kill the bacteria being used. The work was actually separated into two experiments, the first using all the quaternary compounds available plus Vancide 51Z, phenol and bichloride of mercury, the second using pentachlorophenol, creosote and several fractions thereof.

Experiment 1

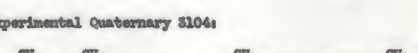
A quaternary, by definition is a substituted ammonium ion, the four hydrogens being replaced by organic side chains of varying length. They exist either in a salt or hydrated form, thus being capable of ionizing in an aqueous solution. The quaternaries, together with the other compounds used, and their formulae, are as follows:

1. Roccal:

A mixture of high molecular alkyl dimethyl benzyl ammonium chlorides in which the alkyl groups range from C_8H_{17} to $C_{18}H_{37}$.

2. Hyamine 1622:

3. Experimental Quaternary 3104:



4. Vancide 51Z:

$$\left[(CH_3)_2 N.\ C\ (S)\ S\right]_2\ Zn + (C_6H_4SNC.\ S)_2\ Zn$$

5. Hyamine 2389:

A mixture of alkyl tolyl methyl trimethyl ammonium chlorides with

alkyl groups ranging from C_9H_{19} to $C_{15}H_{31}$. The average equivalent weight is 331. It does not contain phenol, iodine, heavy metals, or available chlorine.

6. Arquad 18:

An alkyl trimethyl ammonium compound in which the alkyl group consists of 6 per cent hexadecyl chains, 93 per cent octadecyl chains and 1 per cent octadecenyl chains.

7. Bichloride of mercury: $HgCl_2$

8. Phenol:

To investigate the potential of each of the above described compounds with regard to killing effect on the representative organisms selected in Section I, ten tubes of each disinfectant were prepared, five of which were 1-500 dilutions of the disinfectant, and five that contained a 1-500 dilution of the compound in a 1-125 dilution of bentonite clay. A 0.1 cc inoculum of 24 hour broth cultures of microorganisms numbered 40, 45, 48, 53 and 54 was added to one pair of tubes of each disinfectant, one with clay, one without. After 10 and 20 minutes one loop of material was subcultured from each tube into nutrient broth tubes which were then incubated at 30° C. From the resultant growth in the subcultures it was seen that disinfection was complete in all tubes that contained no clay and also in the Vancide 51Z with clay, and bichloride of mercury and phenol with clay. All QACs with clay showed no kill.

It was noted in this work that in each instance where a QAC had been mixed with the bentonite clay, flocculation of the clay suspension occurred accompanied by rapid aggregation and settling out. This phenomenon was seen repeatedly in later work. A discussion of the possible cause will be included in the analysis of results.

Having determined that the various compounds tested were effective in their role as a disinfectant against these organisms it was decided that a titration of a kind must be established to determine the end point of killing power both in the presence and absence of clay.

Preliminary titrations showed that a dilution of 1-640 did not nearly approach a dilution of the disinfectant great enough to allow bacterial growth. Included in this preliminary work was a control check on the clay suspension being used; growth in the subcultures demonstrated that clay, at least in a one per cent suspension, was not bactericidal.

It was suggested that before any further trials were made in an attempt to locate the end point of bactericidal action, an experiment should be outlined and performed to assay the importance of the flocculation of the clay suspension when mixed with a QAC that was noted earlier.

Accordingly, a seven tube serial dilution of each QAC was made and 1 cc of a 1 per cent bentonite suspension added. Final concentrations of clay and disinfectant can be found in Table 2. After mixing thoroughly the tubes were allowed to stand for one hour at room temperature to allow for complete flocculation and subsequent precipitation. All tubes were then centrifuged for 15 minutes at 1500 rpm. Two cc of the supernatant (S 1) were removed aseptically from each tube and pipetted into another set of sterile tubes. The material left in the original tubes was brought back to volume with the addition of sterile distilled water, resuspended, and centrifugation repeated. Again, 2 cc of supernatant (S 2) were taken from each tube and treated in like manner to S 1. Finally, all tubes, the S 1 and S 2 series and the centrifuged clay suspensions, were inoculated with 0.1 cc of culture no. 48, agitated, and subcultured into broth after 15 minutes.

A bactericidal effect was exhibited in all tubes against this organism,

indicating that the flocculation was not completely effective in removal of the QAC or, put in other words, this concentration of clay did not exhibit the protective effect shown by the 1-125 dilution used in the initial work.

From this it appeared desirable to ascertain the effect of using varying concentrations of clay against serial dilutions of a QAC, which in this case was Roccal. Thus, four 7-tube serial dilutions of the disinfectant were made; to the first series was added 0.25 per cent clay, to the second, 0.50 per cent, the third 1.0 per cent and to the last series 2.0 per cent. The final concentrations of Roccal varied from 1-12.5 to 1-800, while the clay concentrations were 1-2000, 1-1000, 1-500, and 1-250, respectively. The tubes were thoroughly mixed, inoculated with 0.1 cc of culture no. 48 and subcultured after 15 minutes into sterile nutrient broth tubes which were incubated at 30° C.

No growth occurred in any subculture demonstrating again the need for much higher dilutions of the disinfectants in order to find the cessation of bactericidal action.

A trial experiment gave evidence that a 15 tube, two-fold dilution scheme as shown in Table 2 would carry a QAC well beyond an effective killing concentration. Therefore, a quadruplicate set of 15-tube serial dilutions was prepared with each of the seven disinfectants to be tested. One set was retained as a control as had been done in all previous work. To the remaining sets were added uniform quantities of clay suspension -- to one set, 0.5 per cent bentonite, to another 1.0 per cent, and to the last, 2.0 per cent.

It was thought worthwhile to study, at this same time, the effects of different clays on the killing dilution of a QAC. Employing suspensions of a kaolin and an illite clay in place of the bentonite, additional dilution sets were made of each QAC. All tubes were inoculated as in previous work,

and subcultured after 15 minutes into nutrient broth which was incubated at 30° C.

Results can be seen in Plates I-IV. Bentonite, kaolin, and illite are represented by B, K, and I, respectively.

The foregoing work constituted the major portion of Section II, having given results which indicated that there was little difference in the QACs regarding their efficiency as disinfectants; however, a marked difference was seen in the effect various clays had on disinfectant action.

It was considered advisable, however, to study further other possible contributing factors concerning the effect of the clays on this bactericidal action.

To determine if, perhaps, the order in which the materials came in contact with one another might appreciably change the results, an experiment was performed using Hyamine 1622 in which, after the 15 tube serial dilution was made, the tubes were inoculated with 0.1 cc inocula of culture no. 48 15 minutes prior to the addition of the clay. Upon adding the 1 per cent clay the tubes were agitated for five minutes and subcultured as in previous work.

Comparing the results with the counterpart in earlier work where the clay was added before inoculating, it was seen that the reversal of order of addition of bacteria and clay did not markedly influence the apparent bactericidal action of the QAC.

Another facet of study undertaken was to show the effect of reversing the volumes of QAC dilutions and clay suspension used in earlier experiments. In this case a ten-tube serial dilution of Roccal was made in a manner in which the final volume in each tube was only one cc. To each tube was added four cc of a 1 per cent bentonite clay suspension thus yielding a 1-125 clay

EXPLANATION OF PLATE I

Fig. 1. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of Roccal, as compared to a control containing no clay.

Tube numbers correspond to Roccal dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of Roccal under conditions existent, capable of killing inoculum.

Fig. 2. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of Exp. Quat. 3104, as compared to a control containing no clay.

Tube numbers correspond to Exp. Quat. 3104 dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of Exp. Quat. 3104 under conditions existent, capable of killing inoculum.

PLATE I

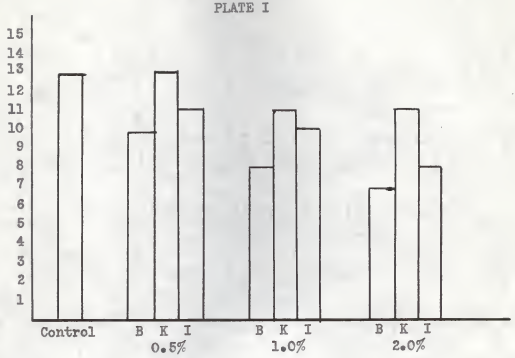


Fig. 1

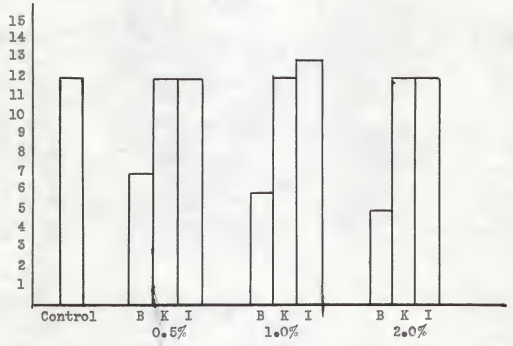


Fig. 2

EXPLANATION OF PLATE II

Fig. 1. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of Hyamine 2389, as compared to a control containing no clay.

Tube numbers correspond to Hyamine 2389 dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of Hyamine 2389 under conditions existent, capable of killing inoculum.

Fig. 2. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of Hyamine 1622, as compared to a control containing no clay.

Tube numbers correspond to Hyamine 1622 dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of Hyamine 1622 under conditions existent, capable of killing inoculum.

PLATE II

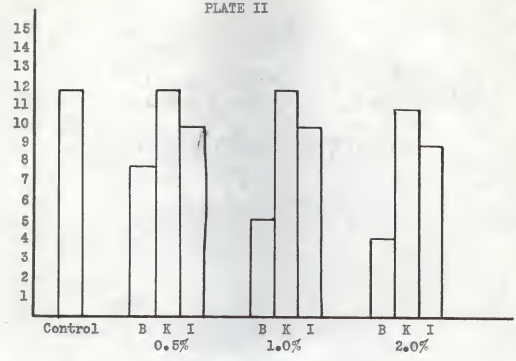


Fig. 1

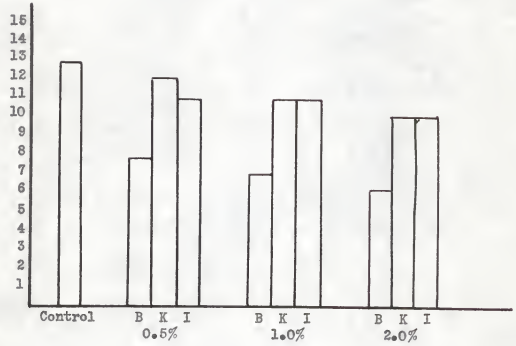


Fig. 2

EXPLANATION OF PLATE III

Fig. 1. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of Arquad 18, as compared to a control containing no clay.

Tube numbers correspond to Arquad 18 dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of Arquad 18 under conditions existent, capable of killing inoculum.

Fig. 2. Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of bichloride of mercury, as compared to a control containing no clay.

Tube numbers correspond to bichloride of mercury dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of bichloride of mercury under conditions existent, capable of killing inoculum.

PLATE III

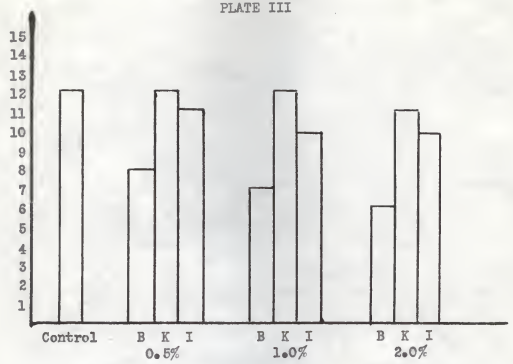


Fig. 1

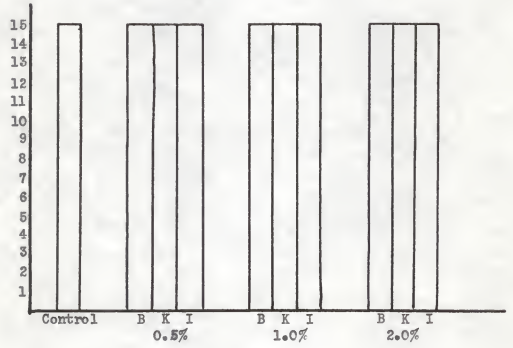


Fig. 2

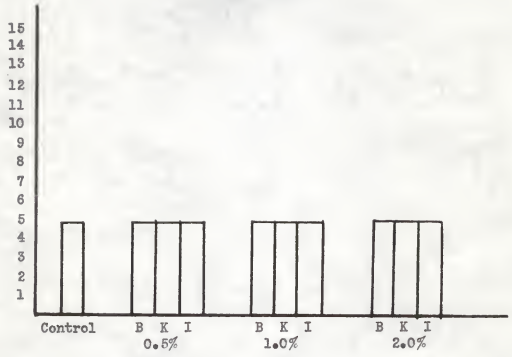
EXPLANATION OF PLATE IV

Illustration of the inhibiting effect which bentonite, kaolin, and illite had on the disinfectant activity of phenol, as compared to a control containing no clay.

Tube numbers correspond to phenol dilutions: Tube 1, 1-12.5, tube 2, 1-25, ...tube 15, 1-204,800. Clay dilutions were uniform in all tubes in a 15 tube set being 1-1000 when 0.5 per cent clay was used, 1-500 with 1.0 per cent and 1-250 with 2.0 per cent.

Tube number, therefore, indicates highest dilution of phenol under conditions existent, capable of killing inoculum.

PLATE IV



dilution in each tube and Roccal dilution ranging from 1-800 in tube 1 to 1-256,000 in tube 10. Each tube was inoculated with 0.1 cc of a 24-hour broth culture of organism no. 48 and after 15 minutes of mixing was subcultured in the usual manner.

Growth occurred in every subculture from tubes to which clay had been added; no growth was obtained from the control dilution set without clay.

Answers to questions concerning the effect that adsorbed ions might have on the clays' ability to inhibit disinfection by QACs were obtained by the following procedure. Six 20 cc aliquots of a 2 per cent bentonite suspension were treated with 1 per cent solutions of KCl, HCl, NaCl, $MgCl_2$, $CaCl_2$ and NH_4Cl by mixing equal volumes of the salt solutions with the clay. After centrifugation at 1500 rpm for 15 minutes, the supernatant fluid was decanted and the clay resuspended in another volume of the salt solution. This process was repeated four times, after which it was assumed that any ions previously adsorbed on the clay were replaced with the respective cations of the salts. The clays were then washed with neutral distilled water until the supernatants gave a negative test for the chloride ion when tested with 1 per cent silver nitrate.

The dilution scheme for this work can be found in Table 2. Seven sets of a 15 tube dilution of Roccal were made in order that one could be held as an untreated bentonite control. To each tube in one of the remaining sets was added 1 cc quantities of the 2 per cent H^+ clay. K^+, Na^+, NH_4^+, Ca^{++} and Mg^{++} clays were added to the other sets. Final concentration of clay in each tube was 1-250, and, as before, the QAC dilutions scaled up from 1-12.5 in tube 1 to 1-204,800 in tube 15. After mixing, each tube was inoculated with 0.1 cc of culture no. 48. Fifteen minutes later the tubes were subcultured into nutrient broth which was incubated at 30° C. All tubes

in the dilution series to which the ion saturated clays had been added and the control were saved for further studies.

As indicated in Table 3 growth in the subcultures revealed that the ion saturated clays did indeed react differently than the untreated bentonite clay.

Table 3. QAC inhibition with ion saturated clays.

Type of clay	Tube* 1...6	7	8	9	10	11	12...15
Bentonite	-	-	+	+	+	+	+
H^+ clay	-	-	-	-	+	+	+
K^+ clay	-	-	-	+	+	+	+
Na^+ clay	-	-	-	+	+	+	+
NH^+_4 clay	-	-	-	+	+	+	+
Mg^{++} clay	-	-	+	+	+	+	+
Ca^{++} clay	-	-	+	+	+	+	+

* See dilutions in Table 2.

The further studies carried out on the Roccal dilutions with the various ion treated clays involved the recording of a gross physical description of each tube, including amount and type of precipitate, and clarity of supernatant in addition to determining the pH of each tube. A Beckman pH meter was used throughout the observations which included pH readings on all starting materials together with the dilution sets. It was hoped that a correlation might be made between the differences observed in amount and type of flocculation or precipitation, and, indirectly, the bactericidal activity, using the various clays and the pH's observed with each. No such correlation could be made.

Experiment 2

To evaluate the bactericidal properties of the remainder of the compounds,

those not water soluble, it was necessary to find a material which would both act as a substrate for the organisms and as a solvent for the compounds to be tested.

Small tubes containing Ashby's medium were employed, the liquid being layered with various substrates -- kerosene, mineral oil, toluene, xylene, benzene, and motor oil -- for determining which best supported growth of the test organism. Marked turbidity at the juncture between the layers was used for selecting kerosene as the substrate material.

The kerosene also proved to be a satisfactory solvent for the disinfectants tested which were creosote, creosote fraction distilling up to 210° C., creosote fraction distilling off between 210 and 235° C., creosote fraction distilling off between 235 and 355° C., Sears-Roebuck Master-Mix (40 per cent pentachlorophenol in solvents), and pentachlorophenol. Ten per cent concentrations of the disinfectants were prepared in kerosene and used for making the serial dilutions which contained a final volume of 1 cc. Dilutions ranged from 1-10 in tube 1 to 1-20,480 in tube 12. After autoclaving, 3 cc of inoculated Ashby's medium were added to each tube of the different disinfectant dilutions. The inoculum, which consisted of a distilled water suspension of the test organism, was added directly to the Ashby's to eliminate the need for inoculating each tube individually thus decreasing the chances of contamination. The disinfectant dilutions rose immediately upon addition of the media and layered over its surface. All tubes were incubated at 30° C.

Growth at the layers' interface was observed in some tubes after five days. Two days later readings were taken; the effectiveness of creosote and its first distillation product was limited to a 1-80 dilution, that of the other two distillates ceased in a 1-160 dilution, while Master-Mix and

pentachlorophenol controlled bacterial growth only in the relatively concentrated dilution of 1-320.

Discussion

Experiment 1. That quaternary ammonium compounds are good disinfectants is shown repeatedly in the data. Approximately a 1-40,000 dilution of the QACs tested was sufficient to kill the test organisms. This was far superior to the killing effect of phenol but fell considerably short of the bichloride of mercury which appeared to be effective in dilutions exceeding 1-160,000. The latter two compounds are common laboratory disinfectants and were included for comparison purposes.

As noted above, QACs are good disinfectants. This statement should be modified, however, in view of results obtained by previous workers and the writer, to read that QACs are good disinfectants only in the presence of distilled water or other "inert" material. For it has been, and was again, shown that the presence of certain materials greatly decreases the bactericidal powers of these compounds. One of the materials capable of performing this action is clay.

It was pointed out earlier that in the initial experiments in which bentonite was combined with the QACs, a flocculation occurred, followed by aggregation and rapid settling out of the particles. This observed flocculation was thought to stem from the reaction between the negatively charged clay particles and the positively charged quaternary ammonium ions -- a reaction which caused the formation of large aggregates which could no longer remain in suspension.

Further work indicated that with a known dilution of a QAC, a definite clay concentration was required to completely inhibit the disinfectant action

of the compound. From the results it appeared that one clay particle could adsorb a number of the quaternary ammonium ions; a 1-1000 dilution of clay particles completely negated the bactericidal action of a QAC which was only diluted to 1-1600 to 1-3200. On the basis of molecular weights there would be considerably more quaternary ammonium ions present than clay particles, even when the ionic concentration was half that of clay. Additional confirmation of this concept was apparent from results obtained by reversing the volumes of clay suspension and disinfectant dilutions which were normally used. Reversal of the materials accomplished a similar reversal of results, for the amount of clay contained in such a volume evidently was more than ample to complex with all available quaternary ammonium ions in the decreased volume of the diluted disinfectant, thus rendering them harmless to the bacterial cultures which were added. Applied to a practical case this would mean that under any conditions where clay concentration was excessive, bactericidal action with quaternary ammonium compounds would be almost non-existent. In order to overcome this inhibition, such high concentrations of the QAC would be required that cost would be prohibitive.

In the experiments in which various constant clay dilutions were added to sets of replicate dilutions a difference was seen in the flocculation pattern. The more concentrated the clay suspension, the more concentrated the QAC had to be to completely combine and precipitate the particles.

The inclusion of a kaolin clay and an illite clay in one experiment afforded an opportunity to observe the effect that a lesser degree of negativity might have on disinfectant inhibition. It is a reasonably well known fact that clays differ greatly in the amount of negative charge inherent in their structure. Bentonite clay has a high negative charge, kaolin almost no demonstrable charge, and illite is only slightly more

charged than kaolin. This structural charge, when sufficiently high, appears to have a definite suppressing effect on bactericidal potency. This is not remarkable because it follows that the more charged two materials are, the stronger the reaction between the two. Plates I-IV illustrate this situation well. The bentonite clay inhibited disinfectant action of all the QACs to a marked degree while illite and kaolin exhibited little or no inhibition. In addition it was noted that neither kaolin nor illite gave any visible flocculation when mixed with a QAC.

By way of comparison, the relationship between phenol and bichloride of mercury and the various clays should be pointed out. None of the three clays used had the least effect on the usual bactericidal effect of these two disinfectants. Both compounds ionize to some degree but neither seemed capable of reacting with the clays. Evidently the ionization is not sufficient to produce enough positively charged ions to satisfy the demands of the clay or the ions are not of sufficient chain length to cause aggregation.

Changing the order of addition of the bacterial inoculum and clay suspension from the usual indicated that whether the clay was added before or after inoculation the protective effect remained constant. The implication of this fact is that even though bacterial cells and clay particles possess an overall negative charge, the clay appears to either be more strongly charged or have a greater affinity for the quaternary ammonium ion than does the bacterial cell. Even after the ions had fifteen minutes in which to react with the cells, the clay that was added managed to remove the lethal ions allowing the cell to reproduce when subcultured. From this it can be concluded that the bactericidal effect of a QAC is reversible provided that the time factor is considered. One can go further along this line and state that if the disinfectant action of a QAC is reversible, then the ions are

probably not absorbed by the bacterial cells, but adsorbed, thus leaving them exposed to the attraction of materials possessing a larger overall negative charge.

Considerable evidence to substantiate such remarks was accumulated with data from other experiments. It was noticed in experiments where disinfectant dilutions were extended to a point at which bacteria were no longer killed as evidenced by growth in subcultures, that this growth (turbidity) did not become visible to the eye for at least four to five days. In contrast, nutrient broth inoculated with a loop of culture of the test organism was markedly turbid in 24 hours or less. This can be construed to mean that the quaternary ammonium ion is actually a competitive inhibitor of bacterial growth. The ions, when they were not removed by the clay, were adsorbed onto the bacterial cell at sites usually associated with adsorption of nutrients, perhaps the necessary nitrogen compounds such as amino acids. When the cell, in this adsorbed condition, was transferred to a subculture of nutrient broth which contained excess required nutrients, a time lapse was necessary for the excess nutrients to dislodge the inhibitor, thus allowing the cell to continue its metabolic functions. This postulation is in accord with that of Randles and Birkeland (1944).

Saturating the bentonite clay with various cations presented an opportunity to determine the effect this might have on the clays' ability to complex with the quaternary. By washing an aliquot of bentonite clay repeatedly with a cation it was possible to develop a situation where presumably all negative charges on the clay were satisfied. Using six different cations individual conditions could be produced that far exceeded the ionic saturation picture of naturally occurring clay soils.

It was thought that if the clay was saturated as fully as possible with

known cations, then, when the clay was mixed with a QAC, the complexing power would be diminished to some degree thus leaving more disinfectant available for adsorption onto the bacterial cells. This presumption was borne out by the results which showed that saturation with monovalent cations decreased the complexing ability of the clay; of the monovalent ions the hydrogen ion was most effective. The divalent cations were of no value in reduction of the clay's complexing activity. This may be explained on the basis of the molecular weights and hence the size of the ions. The hydrogen ion is small and therefore could get in between the layers of the clay micelle. The other monovalent ions, being larger, could not be absorbed into the structure as easily as the hydrogen and the divalent ions, being the largest, could not tie into the more inaccessible sites of the clay particle. On this basis it can be explained satisfactorily why the different ions affected the clay's complexing potential with a QAC. The divalent ions being adsorbed primarily on the surface sites of the clay were more easily replaced with quaternary ammonium ions. On the other hand the monovalent ions, being absorbed or more intimately adsorbed on the clay, were not as easily removed by the quaternary ammonium ions.

Since a bentonite clay with high adsorptive capacity was used for this work, it may be assumed that had illite or kaolin, with relatively low adsorptive capacity, been used in its stead, cation adsorption would have been negligible, and consequently no change would have been apparent in the already minute inhibition of the QACs disinfectant action.

Experiment 2. Evaluation of the efficiency of the remaining disinfectants tested is made rather difficult owing to the fact that conditions for testing varied markedly from the others. From the results it can be said that creosote,

its distillates, and the pentachlorophenol compounds were far inferior to the QACs. However, as just mentioned, the procedure for carrying out the two types of tests varied in every respect. Different organisms were used, different substrates were employed for ascertaining growth, and where distilled water was used in Experiment 1 as a vehicle in serial dilutions, here kerosene was used. Considering all these factors, no justifiable conclusion can be drawn. However, from empirical evidence it would seem that the disinfectants employed here were not as bactericidal as the QACs. It is possible that the six substances used here may have been only bacteriostatic; the method of assay followed did not enable distinguishing between organisms actually killed and organisms that would grow if transferred to more suitable media. The effect of clay in the presence of these compounds was not studied.

SUMMARY

A method for the isolation of asphalt utilizing bacteria from a soil-asphalt interface has been described. It was shown that the type of soil-asphalt interface influences the predominant bacterial population and also the ratio of asphalt utilizing bacteria to total bacterial numbers at a given site.

The efficiency of quaternary ammonium (QAC) and some other compounds as disinfectants was tested on bacteria isolated from asphalt surfaces.

The presence of certain clays was shown to reduce the bactericidal action of a QAC. The degree of ion saturation of the clays influenced the complexing ability of the clay with the positive ion of the QAC. No correlation could be observed between the flocculation of clay by QAC and the measurable pH. An explanation was offered both for the flocculation occurring when a clay and QAC are mixed and for the mode of action of a QAC disinfectant.

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LITERATURE CITED

Breed, R. S., E. G. D. Murray and A. P. Hitchens.
Bergey's manual of determinative bacteriology. Williams and Wilkins Co., Baltimore, 1948.

Büchi, J., R. Hirt, Th. Hofman, R. Luberherr and H. Burni.
Synthese und bakterizide Wirkung einiger quaternaren Ammonium-verbindungen. Helvetica Chim. Acta. 34 (7): 2162-2171, 1951. (from Biol. Abst. 26 (7): 18793. 1952.)

Burgess, S. J.
The action of microorganisms on petroleum asphalt fractions. Highway Research Board Bull. 118. Washington, D. C. 1956.

Bushnell, L. D. and H. F. Haas.
The utilization of certain hydrocarbons by microorganisms. J. Bact. 41 (5): 653-673. 1941.

Butterfield, C. T., Elsie Wattie and C. W. Chambers.
(Publ. Health Serv., Cincinnati, Ohio.) Bactericidal efficiency of quaternary ammonium compounds. Publ. Health Reports. 65 (33): 1039-1056. 1950.

Chaplin, C. E.
Observations on quaternary ammonium disinfectants. Canadian J. Bot. 29 (4): 373-382. 1951.

Chaplin, C. E.
Bacterial resistance to quaternary ammonium disinfectants. J. Bact. 63 (4): 453-458. 1952.

Corker, G. W.
An investigation into the efficacy of alkyldimethylbensylammonium chloride (Zephiran) compared with other antiseptics. Australasian J. Pharm. 20: 366-369. 1949 (from Chem. Abst. 33 (3): 5698[2].) 1939.

Cribbins, Lois R.
The bacterial oxidation of cyclic hydrocarbons. Bull. Pittsburgh Univ. 45 (8): 31-34. (from Biol. Abst. 24 (5): 12766. 1950.)

Domagk, G.
A new class of disinfectants. Deut. med. Wochschr. 61: 829-832. (from Chem. Abst. 29 (2): 7018[2].) 1935.

Duffett, N. D., S. H. Gold and C. L. Weirich.
Normal bacterial flora of cutting oil emulsions. J. Bact. 45: 37-38. 1943.

Dunn, Cecil G.
Antiseptic and germicidal properties of a mixture of high molecular alkyldimethylbensylammonium chlorides. Am. J. Hyg. 26: 46-62. 1937.

Fabian, F. W. and H. Pivnick.
Growth of bacteria in soluble oil emulsions. Applied Microbiol. 1 (4): 199-203. 1953.

Fisher, G.
Om desinfektion av rakknivar och kammar med bensalkonium-klorid. Nordisk Hyg. Tidskr. 30(10): 273-281. 1949 (from Biol. Abst. 24 (9): 27564. 1950.)

Fleming, W. E.
Effect of soil microorganisms on paraffin used as a coating to decrease the injurious action of lead arsenate on plant roots. J. Agr. Research. 34: 335-338. 1927.

Gainey, P. L.
Effect of paraffin on the accumulation of ammonia and nitrates in the soil. J. Agr. Research. 10: 355-364. 1917.

Gershenfeld, Louis and Russell E. Brillhart.
Some factors responsible for irregular results when testing quaternary ammonium compounds. Amer. J. Pharm. 122:464-473. 1950 (from Biol. Abst. 25 (9): 28514. 1951.)

Giglioli, Italo and Guilio Masoni.
New Observations on the biological absorption of methane and the distribution of methane organisms of Kaserer and Sohngen in soils and fertilizer. Staz. sper. agrar. ital. 42: 589-606. 1909 (from Chem. Abst. 5: 751. 1911.)

Giglioli, Italo and Guilio Masoni.
New observations on the biological absorption of methane and the distribution of the methane organisms of Kaserer and Söhngen in soils, mud, and farm manures. R. Univ. Pisa, Istit. Chim. Agraria, Studi Reserche. 22: 76-108. 1917 (from Chem. Abst. 14: 2930. 1920.)

Goobin, V. M.
Assimilation of hydrocarbons by bacteria of the Tembookansk and Petrovsk muds in reference to mud formation. Russian Health-Resort Service No. 5, 5-9. 1923 (from Chem. Abst. 19: 2685. 1925.)

Grant, Carrol W. and Claude E. ZoBell.
Oxidation of hydrocarbons by marine bacteria. Proc. Soc. Exp. Biol. and Med. 51 (2): 266-267. 1942.

Grieg-Smith, R.
The destruction of paraffin by Bacillus prodigiosus and soil organisms. Proc. Linnean Soc. N. S. Wales. 39: 538-541. (from Chem. Abst. 9: 1798. 1915.) 1914.

Haas, H. F., M. F. Yantzi and L. D. Bushnell.
Microbial utilization of hydrocarbons. Trans. Kansas Acad. Sci. 44: 39-45. 1941.

Harris, John O., R. M. Kline and Carl F. Crumpton.
A study of the presence of hydrocarbon utilizing bacteria at the soil-asphalt interface of Kansas highways. Trans. Kansas Acad. Sci. 59 (4): 495-499. 1956.

Hessel, F. A.
Action of bacteria on hydrocarbons and more particularly on petroleum. Mat. grasses. 16: 6936-6940. 1924 (from Chem. Abst. 19: 395. 1925.)

Hormung, H.
Zephirol, a new disinfectant. Z. Immunitäts. 84: 119-135. 1935.

Hucker, G. J., Dorothea Metcalf and Carolyn Cook.
The rate of germicidal action of the quaternary ammonium compounds. Tech. Bull. New York State (Geneva) Agric. Exp. Sta. 260. (1-20). 1948a (from Biol. Abst. 23 (3): 7902. 1949.)

Hucker, G. J., Shirley Watkins, Dorothea Metcalf and Jean Stone.
Effect of hydrogen ion and temperature on the activity of quaternary ammonium compounds. Tech. Bull. New York State (Geneva) Agric. Exp. Sta. 261 (1-21). 1948b (from Biol. Abst. 23 (3): 7903. 1949.)

Hundeshagen, R.
The behavior of asphalt bitumen and coal tar pitch toward microorganisms. Bautenschutz 6: 141-142; Chem. Zentr. I, 2995. 1936. (from Chem. Abst. 31 (2): 6860. 1937.)

Jacobs, W. A.
The bactericidal properties of quaternary salts of hexamethylenetetramine. I. The problem of the chemotherapy of experimental bacterial infections. J. Exptl. Med. 23: 563-568. 1916.

Jacobs, W. A., M. Heidelberger and H. L. Amoss.
The bactericidal properties of quaternary salts of hexamethylenetetramine. II. The relation between constitution and bactericidal action in the substituted benzylhexaminetetraminium salts. J. Exptl. Med. 23: 569-576. 1916.

Jacobs, W. A., M. Heidelberger and C. G. Bull.
The bactericidal properties of the quaternary salts of hexamethylenetetramine. III. The relation between constitution and bactericidal action in the quaternary salts obtained from halogenacetyl compounds. J. Exptl. Med. 23: 577-599. 1916.

Jerchel, Dietrich.
Inverseifen und Tetrasoliumsalze. In: FIAT Rev. German Sci., Biochemistry. I. pp. 59-65. 1947 (from Biol. Abst. 23 (9): 27019. 1949.)

Johns, C. K.
Influence of organic matter on the germicidal efficiency of quaternary ammonium and hypochlorite compounds. Canad. J. Res. Sect. F. Technol. 26 (2): 91-104. 1948.

Johnson, Frank H., Walter T. Goodale and John Turkevich.
The bacterial oxidation of hydrocarbons. J. Coll. and Comp. Physiol. 19 (2): 163-172. 1942.

Kaserer, H.
Uber die Oxydation des Wasserstoffes und des Methans durch Mikroorganismen. Zentr. Bakt. Parasitenk., Abt. II. 15: 573-576. 1906 (cited by Bushnell and Haas, J. Bact. 1941.)

Katznelson, H. and M. D. Sutton.
Inhibition of plant pathogenic bacteria in vitro by antibiotics and quaternary ammonium compounds. Canad. J. Bot. 29 (3): 270-278. 1951.

Klarmann, Emil G. and Eleanor S. Wright.
Are quaternary ammonium compounds sporicidal? Amer. J. Pharm. 122: 330-336. 1950 (from Biol. Abst. 25 (9): 28519. 1951.)

Knox, W. E., K. Zarudnaya Auerbach and M. Spirtes.
(Columbia Univ., N. Y. C.) The action of cationic detergents on bacterial enzymes. J. Bact. 58 (4): 443-452. 1949.

Komatsu, Shigiru.
Stereochemistry of Quinquivalent Nitrogen. I. Formation and decomposition of the quaternary ammonium bases and their salts. Mem. Coll. Sci. Eng. Kyoto Imp. Univ. 3: 371-426. 1913 (from Chem. Abst. 7: 1020. 1913.)

Lee, Melba and Asa C. Chandler.
A study of the nature, growth, and control of bacteria in cutting compounds. J. Bact. 41 (3): 373-386. 1941.

Leusden, Friedr. Pels and Reinhold Döring.
Studies on the new disinfectant RZ (crude "Zephirol"). Zentr. Bakt. Parasitenk. I Abt. Orig. 142: 197-208. 1938 (from Chem. Abst. 32: 8469[7]. 1938.)

Lund, Aage.
Undersøgelser over en kvartær ammoniumforbindelse desinfektionsevne. Brygmesteren 7 (5): 173-180. 1950 (from Biol. Abst. 24 (11): 32293. 1950.)

Malman, W. L., Anita Leavitt and Dwight Joslyn.
Effect of medium on resistance of Staph. aureus to quaternary ammonium compounds. Proc. Soc. Exp. Biol. and Med. 68 (2): 401-403. 1948.

McCullouch, Ernest C.
The real and apparent bactericidal efficiencies of the quaternary ammonium compounds. J. Bact. 53 (3): 370. 1947.

Mueller, W. S., D. B. Seeley and E. P. Larkin.
Quaternary ammonium compounds. Amer. Milk Rev. 9 (10): 28, 30, 32, 57. 1947.

Münz, E.
Physiology of methane bacteria. Inaug. Diss. Halle 1915, 61 pp.; Zentr. Biochem. Biophys. 18: 400. 1915 (from Chem. Abst. 11: 349. 1917.)

Novelli, G. David.
Bacterial oxidation of hydrocarbons in marine sediment. Proc. Soc. Exp. Biol. and Med. 52 (2): 133-134. 1943.

Rahn, O.
Protection of dry bacteria by fat against cationic detergents. Proc. Soc. Exp. Biol. and Med. 62: 2-4. 1946.

Randles, C. I. and Jorgen M. Birkeland.
Mechanism of growth inhibition by cationic detergents. J. Bact. 47 (5): 454. 1944.

Rawlins, A. L., L. A. Sweet and D. A. Joslyn.
Relationship of chemical structure to germicidal activity of a series of quaternary ammonium salts. Sci. Ed. J. Amer. Pharm. Assoc. 32 (1): 11-16. 1943 (from Biol. Abst. 17: 16853. 1943.)

Ridenour, G. M. and E. H. Armbruster.
Some factors affecting the properties of quaternary ammonium compounds as sanitizers. Amer. J. Publ. Health. 38 (4): 504-511. 1948.

Roberts, Martha Holt and Otto Rahn.
The amounts of enzyme inactivation at bacteriostatic and bactericidal concentrations of disinfectants. J. Bact. 52 (6): 639-644. 1946.

Schneider, Gerhard.
Experiments with the disinfectant Zephirol. Z. Immunität's. 85: 194-199. 1935 (from Chem. Abst. 29 (3): 7379. 1935.)

Scott, J.
Petroleum under the microscope. Petroleum World. 18: 160-161. 1921 (from Chem. Abst. 15: 2542. 1921.)

Shere, L.
Some comparisons of the disinfecting properties of hypochlorites and quaternary ammonium compounds. Milk Plant Month. 37 (3): 66-69. 1948 (from Biol. Abst. 24 (8): 24562. 1950.)

Söhngen, N. L.
Ueber Bakterien, welchen Methan al Kohlenstoffnahrung Energiequelle gebrauchen. Zentr. Bakt. Parasitenk., Abt. II. 15: 513-517. 1906 (cited by Bushnell and Haas, J. Bact. 1941.)

Söhngen, N. L.
Benzin, Petroleum, Paraffinöl und Paraffin als Kohlenstoff und Energiequelle für Mikroben. Zentr. Bakt. Parasitenk., Abt. II, 37: 595-609. 1913.

Stone, R. W., M. R. Fenske and A. G. C. White.
Bacteria attacking petroleum and oil fractions. J. Bact. 44: 169-178. 1942.

Stone, R. W., A. G. C. White and M. R. Fenske.
Microorganisms attacking petroleum and petroleum fractions. J. Bact. 39: 91-92. 1940.

Störmer, K.
Ueber die Wirkung des Schwefelkohlenstoffs und ähnlicher Stoffe auf den Boden. Zentr. Bakt. Parasitenk., Abt. II, 20: 282-286. 1908 (cited by ZoBell, Bact. Rev. 10, 1946.)

Strawinski, R. J. and R. W. Stone.
The utilization of hydrocarbons by bacteria. J. Bact. 43 (1): 18-19. 1942.

Tausson, W. O.
Bacterial oxidation of crude oils. Neftyanoe Khozyaistvo. 14: 220-230. 1928 (from Chem. Abst. 23: 1451. 1929.)

Tausson, W. O.
The oxidation of benzene hydrocarbons by bacteria. Planta 7: 735-757. 1929 (from Chem. Abst. 23: 3945. 1929.)

Tausz, J.
Action of microorganisms on crude petroleum. Petroleum Z. 15: 553-555. 1919 (from Chem. Abst. 15: 3552. 1921.)

Tausz, J. and P. Donath.
The oxidation of hydrogen and of hydrocarbons by means of bacteria. Z. Physiol. Chem. 190: 141-168. 1930 (from Chem. Abst. 24: 5781. 1930.)

Van Escltine, William P. and G. J. Hucker.
The effect of organic matter on germicidal action of the quaternary ammonium compounds. Tech. Bull. New York State (Geneva) Agric. Exp. Sta. 282 (1-20). 1948 (from Biol. Abst. 23 (3): 7909. 1949.)

Wackenhut, A. M.
A hydrocarbon-oxidizing microorganism from crude oil. Arch. sci. biol. (U. S. S. R.) 43 (2-3): 55-56. 1936 (in German 62). (from Chem. Abst. 32 (2): 7509[2]. 1938.)

Wagner, Richard.
Benzene Bacteria. Z. Gärungsphysiol. 4: 289-319. 1914 (from Chem. Abst. 8: 3193. 1914.)

Weber, G. A.
Studies of quaternaries as bactericides. J. Milk and Food Technol. 12 (2): 107. 1949.

Zenits, Bernard L.
Quaternary ammonium salts as antibacterial agents. Science 112 (2911): 422. 1950.

ZoBell, Claude E.
Assimilation of hydrocarbons by microorganisms. Advances in enzymology. 10: 443-486. 1950.

EFFECT OF DISINFECTANTS, PARTICULARLY QUATERNARY AMMONIUM COMPOUNDS, ON BACTERIA ISOLATED FROM SOIL-ASPHALT INTERFACES

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From Söhngen's (1906) first work to one of the most recent works of Harris, et al. (1956), much study has been devoted to that field of bacteriology pertaining to the oxidation of hydrocarbons by bacteria.

Over a similar period of time, equal effort has been spent on determining the efficiency of quaternary ammonium compounds (QACs) as bactericidal agents both in the presence and absence of inhibiting materials.

In this work an attempt was made to isolate hydrocarbon utilising organisms from asphalt road surfaces, to determine their relative numbers, and to ascertain the effectiveness of QACs on these isolates in the presence of naturally occurring clays.

Using Ashby's basic salts medium, both liquid and solid media were prepared containing asphalt as the only known carbon source. Organisms were isolated in pure culture on these media from various road surfaces. In addition total counts of organisms present along roads were made and a correlation drawn between relative numbers of asphalt utilisers as compared to total numbers and type of soil-asphalt interface existing where samples were obtained. Type and degree of degradation of the road surfaces appeared to influence the kinds of microorganisms predominant.

Disinfectant studies were carried out with a number of compounds though particular stress was placed on QACs and their inhibition of clays.

Serial dilutions of the QACs were made in water and varying uniform suspensions of bentonite, illite and kaolin clays were added. After inoculation disinfecting ability was assayed by noting the highest dilution of QAC allowing no growth in subcultures. The presence of bentonite clay was shown to greatly decrease the bactericidal activity of all QACs tested by complexing with the positive ion of the QAC which resulted in flocculation and subsequent settling out of the particles. Illite and kaolin exhibited little or no effect

by comparison.

The clays used were then saturated with various cations to determine if degree of saturation prior to addition of QAC would appreciably change earlier observations. It was seen that complexing (inhibiting) ability of the clay was decreased in proportion to the size of the cation with which it was treated.

Studies made in an attempt to relate pH with amount of clay-QAC complex precipitation indicated that no such relationship between the two existed.

Overall consideration of results led to an explanation of the mode of bactericidal action of QACs. Those subcultures from the disinfectant experiments which showed visible growth did not do so for periods of four to five days while ordinary subcultures from aqueous suspensions showed growth in less than one day. It was concluded that QACs are adsorbed on the bacterial cells at sites which are ordinarily associated with required nutrients. Therefore, when the bacterial cells were removed to a medium in which excess nutrients were present, a time lag preceded growth during which the adsorbed QAC ions were dislodged and replaced with nutrients.

Work involving other test compounds' disinfectant activity was done for comparative purposes and, with the exception of bichloride of mercury, were found to be inferior to the QACs.