THE ACTION OF ANTIMICROBIAL SUBSTANCES ON SOME ENTERIC AND RELATED ORGANISMS

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THESIS

This is to certify that the

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Introduction

Antimicrobial agents are of chemical or biologic origin. The latter includes the products of a variety of higher plants, higher animals and microorganisms; to the substances obtained from the microorganisms is specifically ascribed the term "antibiotic". From the plants are obtained quinine, emetine, and chaulmoogra oil; from the animals, lactenin, lysozyme, histones and protamines.

The study of antibiotic substances is a comparatively new one, the initial impetus having been given by Fleming (14) in 1929 with his discovery of penicillin. The phenomenon of antagonisms exhibited among microorganisms has led to the discovery of these antibiotic substances, for it is known that certain organisms cannot grow in the presence of another species of organism. Such substances have been obtained from both spore-forming and non-spore-forming bacteria, actinomycetes and fungi. The antibiotic substances differ from the ordinary bactericidal agents because of their selective action upon different bacteria. Some bacteria may be affected by a very low concentration of a given substance whereas others are not acted upon at all or only by very high concentration. Each substance therefore is characterized by a specific bacteriostatic or antibiotic spectrum. The other antimicrobial agents also seem to show definite spectra of action.

Knowing that the antimicrobial agents, whether chemical

or biologic in origin, do show selectivity in their action, it was thought that a study of the action of several of these agents on some enteric organisms might lead to a method of early differentiation of the various organisms. Special interest was laid on the salmonellae because that group is exceedingly complex.

The Salmonella

Cultural and Biochemical Characteristics

Since the isolation of Salmonella choleraesuis by Salmon and Smith in 1885, paratyphoid bacilli have been found by many workers in a variety of diseases of animals and in enteric fever and gastroenteritis of man. The organisms have been recovered from many sources; isolations from such diverse sources have led inevitably to confusion. The International Association of Microbiologists in 1940 (30) defined the genus thusly: "A large genus of serologically related, gram-negative and non-sporing bacilli; 0.4-0.6 microns by 1-3 microns in usual dimensions but occasionally forming short filaments; showing, with certain exceptions, a motile peritrichous phase in which they normally occur; in fact, adhering to the pattern of B. typhosus in staining properties and morphology. Failing to ferment sucrose or to clot milk and rarely fermenting lactose, liquefying gelatin or producing indol, they regularly attack glucose with, but occasionally without, gas production. All the known species are pathogenic for man, animals, or both."

The bacteriologist normally finds little difficulty in identifying an organism as belonging to the genus Salmonella, for he can utilize various cultural, biochemical and agglutination reactions. Final species or type identification of the culture involves the use of known specific sera and what agglutinin absorbed sera necessary to distinguish one species

from another. In an average laboratory the preliminary identification of Salmonella-like colonies is based on the reaction of the organism on Kligler's iron agar with 0.1% sucrose, a gram stain, tests for motility, Voges-Proskauer reaction, indol and hydrogen sulfide production, fermentation of glucose, lactose, sucrose, salicin and other carbohydrates. A positive indol, Voges-Proskauer test or fermentation of lactose or sucrose would exclude the organism from the Salmonella group. Mere identification of the organism as belonging to the genus Salmonella may be sufficient information but in the larger laboratories, such as the laboratories of the state health departments, it is of especial epidemiological value to know of what species the organism is.

The following is a review of the methods described by various authors for the differentiation of the Salmonella organisms. In 1924 Brown, Duncan and Henry (4) advocated the substitution of organic salts for "sugars" in cases where sugar reactions are untrustworthy or fail altogether to differentiate certain serologically well defined types of bacteria. Salts of a few organic acids are utilized as food by some members of the paratyphoid group. Utilization of the salts was measured in a liquid medium by the precipitation of the residual acid with lead acetate; thus, observing the reactions on tartaric, fumaric, citric and mucic acids, they were able to find seven different groupings of Salmonella types as compared to the four types differentiated by the sugar re-

actions.

In 1926 Simmons (36) put forth a medium; his was a citrate agar medium with brom-thymol blue added as an indicator, resulting in an olive-green medium. He found that Sal. paratyphi and Sal. pullorum failed to grow in the medium and did not change the color of the medium, whereas Sal. schottmuelleri, Sal. enteritidis and Sal. typhimurium grew luxuriantly and formed translucent greenish blue-green colonies on the citrate agar and produced sufficient alkali to color the medium a deep Prussian blue.

Jordan and Harmon (21) in 1928 described another differential medium for the paratyphoid group. Sodium potassium tartrate was the salt incorporated into the medium which contained phenol red as an indicator. An acid reaction was given by 87 strains of Sal. typhimurium and an alkaline reaction was given by 45 strains of Sal. schottmuelleri.

Following the idea set forth by Brown, Duncan and Henry, Mallman (26) in 1931 reported that he could differentiate Sal. pullorum from Sal. gallinarum with the addition of d-tartaric and mucic acids in the culture medium. Sal. pullorum constantly gave an alkaline reaction on sodium d-tartarate and sodium mucate whereas Sal. gallinarum yielded acid reactions on both.

In 1934 Vogelsang (43) experimented with Sal. schottmuelleri in western Norway and described its definite cultural and biological characteristics, such as "slime wall" formation at

room temperature, formation of papillae on raffinose agar, formation of hydrogen sulfide in lead acetate agar, negative Bitter reaction, negative Pesch-Maschke reaction, and the decolorization of neutral red agar.

More recently in 1941 Bornstein and Strauss (2), while utilizing the agar strip method in their in vitro experiments, found that sulfanilyl-guanidine inhibited Sal. choleraesuis quite markedly and affected Sal. paratyphi to some slight degree but was ineffective against the other organisms of the group. Hinshaw (18) then reported that 89 out of 91 strains of Sal. gallinarum produced a characteristic yellowish-white or grayish turbidity when incubated at 37° C for 72 hours in a gelatin medium containing 0.15% cysteine hydrochloride. None of 19 other types of Salmonella studied gave this typical reaction.

The sequence of phenomena in litmus milk (47) is also a valuable guide in the differentiation of the Salmonella organisms. No member of the group sets up sufficient acidity to produce clotting of the milk although all of the members cause the indicator to change within 24 hours at 37° C from the violet of neutrality to a more or less definite pink, registering the formation of acid in small amounts due to the presence of traces of readily fermentable and glucose-like sugar. Further events then divide the Salmonella into two groups. One group is composed of Sal. schottmuelleri, Sal. typhimurium, Sal. enteritidis, Sal. abortivoequina and Sal.

choleraesuis. In the case of these organisms, the acid indicator undergoes the so-called "chameleon" change; in the course of two to five days it assumes the blue color of alkalinity. Associated with the definite alkalinity there also occurs a clearing of the hitherto turbid medium with the formation of a deposit. The second group of strains is composed of Sal. paratyphi and Sal. abortusovis. In the case of these organisms the acid reaction developed during the first 24 hours is maintained.

Neutral red agar (Rothberger) has already been mentioned for its purpose in Salmonella differentiation. The reactions seemingly run parallel with the aerogenic or anaerogenic behavior of the organism. The gas-forming species such as Sal. schottmuelleri and Sal. typhimurium cause first fluorescence and then decolorization of the medium. Anaerogenic forms such as Eberthella typhosa, Sal. gallinarum and anaerogenic strains of types which normally form gas cause no indicator change.

Differentiation of the Salmonella is also possible by their production of or lack of hydrogen sulfide as detected in media using soluble salts of lead or iron as indicators. Sal. schottmuelleri, Sal. enteritidis and Sal. typhimurium are hydrogen sulfide positive while Sal. paratyphi and Sal. abortivoequina are hydrogen sulfide negative.

The carbohydrates for which differential value has been claimed are the pentoses -- arabinose, xylose, and rhamnose; the disaccharides -- maltose and trehalose; the trisaccharide

raffinose; the polysaccharide dextrin, and the alcohols -- mannitol, dulcitol, inositol, and glycerol. The outstanding reactions of differential value may be noted. Sal. paratyphi does not ferment xylose while most of the other members do. Sal. schottmuelleri does not ferment d-tartrate while Sal. typhimurium and most of the food-poisoning bacilli do. Dulcitol is almost always readily attacked by members other than Sal. pullorum and Sal. choleraesuis. Maltose is apparently attacked by all types save occasionally Sal. pullorum. Sal. choleraesuis is unable to ferment trehalose whereas Sal. enteritidis, Sal. paratyphi and Sal. schottmuelleri do so readily. Inositol is fermented by the great majority of strains of Sal. schottmuelleri and Sal. typhimurium but not by Sal. paratyphi, Sal. abortivoequina, Sal. choleraesuis or Sal. enteritidis. Sal. schottmuelleri and Sal. enteritidis have been termed "Stern-positive", for when they are cultured in fuchsin-sulphite-glycerol-meat-extract broth they cause the indicator to assume a deep lilac-red color while other forms such as Sal. paratyphi, Sal. choleraesuis and Sal. abortivoequina determine at most a pale-pink coloration.

Much work has been done by Kauffmann and some German workers on the Salmonella organisms. Their work is not readily translated by the writer of this paper and consequently no comments have been made about their findings.

The Serology of the Group

Cultural and biochemical reactions give the worker presumptive clues as to the species of the organism; final type identification of the suspected Salmonella must be based on a serological study which is by no means simple, for there are at least 155 types of Salmonella and also some 9 coliform organisms which have been described as containing Salmonella antigens (33). The method of subjecting an organism to a serological study will not be discussed here but is presented in Edwards' paper (13).

In 1926 White (46) recognized the importance of considering the bacterial variations in relation to the antigenic analysis of Salmonella. Later this pioneer work of White's was confirmed and extended by Kauffmann (22), efforts which resulted in the Kauffmann-White scheme of Salmonella classification.

In the serological identification of the Salmonella group one must keep in mind the variations that the organisms can undergo, variations which do cause discrepancies in the serology. There is first the H-O variation. In 1920 Weil and Felix (46) reported that the typical Salmonella presents two distinct classes of antigens, each stimulating in vivo production of its own particular antibodies with which it exclusively reacts. The H antigen is associated with the flagella and is therefore not found in the non-motile organisms. It is heat labile, being progressively inactivated by tempera-

tures above 60° C; after exposure to 100° C for 30 minutes or when treated with acids and alcohols it fails to function in serological tests or as an antigen when injected into an animal. Its development is inhibited by culture on certain media, notably the phenol-agar medium of Braun (3). When it reacts with its corresponding agglutinins it results in the agglutination of the bacilli in large, fluffy clumps which are readily dispersed. The O antigen is present in both motile and non-motile forms of the group. It resists prolonged heating at 100° C and treatment with alcohol and dilute acids. It agglutinates slower than the H antigen and it forms granular clumps which are dispersed with difficulty. In the reaction of an ordinary motile culture with its antiserum the agglutination determined by the flagellar reagents exceeds in titre and occurs more rapidly than that due to the somatic reagents. Also the somatic (O) and flagellar (H) agglutinins seem to be entirely independent because in correctly performed absorption tests either type may be withdrawn from the serum, leaving the other intact.

The S-R variation is not merely a morphological characteristic; the R colonies, besides possessing roughened surfaces and irregular borders, producing granular growth in broth, giving a saline suspension of organisms which is less stable than those of the S races, also have a tendency to cross-agglutinate. The typical rough variant is characterized by failure to respond to the O agglutinins of the corresponding

smooth serum and by failure to produce agglutinins acting on the somatic antigens of the smooth organism; however, it is marked by a special somatic antigen which reacts with the homologous rough antiserum to cause clumping of the bacilli in fine granules. Consequently only smooth cultures should be used to prepare O antiserums. In the change from a smooth to a rough form, the flagellar elements of the organism evidently suffer little or no qualitative modifications. The change is gradual.

There is also the V-W variation. Some cultures are inagglutinable in pure O antiserum but agglutinate with a Vi (virulence) antiserum. The virulence antigen is so called because the organisms possessing it are more virulent for mice; it is inactivated by heat and acids but is unaffected by absolute alcohol. The W colonies agglutinate with the O antiserum and not with the pure Vi antiserum.

So far as is known phase variation occurs only in motile Salmonella strains and not in other genera. Some organisms have specific and non-specific antigenic factors; the non-specific antigens cause different types to cross-agglutinate in high dilutions because these types are related; therefore a simple agglutination cannot be depended upon for differentiation. The following is an example of phase variation: Two Salmonella types with related H antigens are plated. Certain colonies will agglutinate only with their homologous serum whereas others will agglutinate with the serums of both. The

former is said to be in its specific phase and the latter in its non-specific phase or in phase 1 and phase 2 respectively. Not all Salmonella types are diphasic. Monophasic colonies have identical H antigens; Sal. enteritidis has that characteristic.

Certain minor O antigens of the genus overlap; that is, they occur in more than one of the major groups into which the salmonellae are divided on the basis of their O antigens. Antigens I and XII exhibit a phenomenon known as form variation. When certain types containing either of those two antigens are plated, some colonies contain large amounts of the antigen and some contain slight amounts of the antigen. S-R variation, V-W variation and form variation affect only the O antigens of the bacilli and their reactions in O serums. They have no effect on the H antigens except as roughness affects the stability of the bacterial suspensions and consequently their agglutination.

A survey of the cultural and biochemical characteristics and the serology of the Salmonella group has been briefly presented. It is easy to see that difficulty early arises in any attempt to differentiate among the species; the serological identification is a task demanding the best technique. If some of the organisms are sensitive to an antimicrobial substance, then it would be simple to eliminate quite early in the identification procedure the possibility of its being one which is resistant to the substance.

The Antimicrobial Agents

The following discussion on the antimicrobial agents deals largely with the antibiotic substances, since such information can be readily applied to the other antimicrobial agents.

The antibiotic substances may be classified according to their solubility, chemical nature, biological activity or toxicity to animals (44). They are selective in their action, even as regards specific types or strains of the group of bacteria being acted upon. The selectivity of the antimicrobial agent (11) may be due to the acidic and basic properties of the cell under consideration, the nature and property of its membrane, its permeability, the relative importance for metabolism and viability of the specific biochemical systems affected by the agent, and to the activity of the autolytic enzymes. Selectivity is exhibited not only with reference to the type of cell which is affected but also with reference to the metabolic systems or structural constituents with which the agents react.

Many mechanisms of action have been suggested. Some claim the antibiotic substance interferes with the enzymatic systems, with the vitamin utilization of the organism, with bacterial cell division, and with the metabolic processes of the microbial cell by substituting for one of the essential nutrients. It may bring about the oxidation of a metabolic substance which must be reduced in the process of bacterial nutrition or it

otherwise modifies the intermediary metabolism of the bacterial cell. The agent may combine with the substrate or with one of its constituents thereby rendering it inactive for bacterial utilization. The antibiotic substance may compete for an enzyme which is needed by the bacteria to carry out an essential metabolic process; it may favor certain lytic mechanisms in the bacterial cell or even affect the surface tension of the cell surface. It may also interfere with the sulfhydryl group in the makeup of the bacteria which is essential for cell multiplication.

Fleming (14) made his great discovery of penicillin in 1929 while studying the growth and properties of Staphylococcus. He grew the organism on a solid medium containing agar. In his examination of the culture plates he naturally exposed the surface to the air and the plates were thus contaminated with various microorganisms. Around a large colony of contaminating mold he observed that the staphylococcus colonies became transparent and were obviously undergoing lysis. This observation was the key to the discovery of penicillin, an antibiotic substance produced by various strains of Penicillium notatum and Penicillium chrysogenum and probably by a variety of other fungi.

In 1938 Florey and his group (17) pursued the initial work done by Fleming and developed a rapid assay method utilizing the glass cylinder cup. They uncovered properties of penicillin such as its exhibiting acidic properties, being

unstable in acid or alkaline media, being non-toxic for animals and leukocytes and tissue cultures, and its activity not being affected by pus, blood or breakdown products of dead tissue.

The salts of penicillin are inactivated by penicillinase; destroyed by acids and alkali, by oxidizing agents such as potassium permanganate, by some metals such as copper, lead, and mercury, and by methyl alcohol. Penicillin is soluble in ether, ethyl alcohol, acetone, esters and dioxane and in water at the rate of 5 mg. per ml. It forms water soluble salts with most of the heavy metals except iron; its barium salt is the most suitable form for use as it retains its antibacterial activity for an indefinite period. Penicillin's action on bacteria is chiefly bacteriostatic and not bactericidal. It exhibits a high degree of specificity, acting largely on the pyogenic cocci, anaerobic clostridia, certain pathogenic gram-negative cocci -- the meningococcus and gonococcus, and also the Salmonella; it has no effect on the organisms causing tuberculosis, malaria, typhoid, measles or mumps. Hobby (19) from the results of her experiments on the antibacterial action of penicillin against gram-negative organisms thinks that penicillin does exert that activity, it being more apparent in high potency penicillin preparations. Therefore possibly there may be a form of penicillin which shows greater activity against the gram-negative organisms than the present forms.

Fleming (14) found that penicillin was bacteriostatic with regard to streptococci, pneumococci, and diphtheroids and other organisms found in the respiratory tract while no inhibitory action was exhibited on the influenza bacillus. Thus he was able to isolate the hemophilic organisms, Hemophilus influenzae and Hemophilus pertussis, from specimens which also contained the aforementioned contaminating organisms. Potassium tellurite (16) has an extraordinarily selective bacteriostatic action on such gram-negative bacteria as the colityphoid group, Pasteurella, Brucella and the hemophilic bacteria whereas it exerts little action on the streptococci, staphylococci and diphtheroids. One can immediately note that the penicillin sensitive microbes are potassium tellurite insensitive and vice-versa. Fleming (15) also used gentian violet as a spread over a blood agar plate and was able to isolate a few streptococci from a multitude of staphylococci.

Following Fleming's statement that penicillin had a more pronounced action on the staphylococcus than on the diphtheroids and since the acne bacillus belongs to the diphtheroid group, Craddock (8) used glucose broth with penicillin double the concentration which inhibits the staphylococcus. In that way he was able to obtain pure cultures of acne bacilli from 47 specimens.

Osborn (28) in 1943 reported on the investigation for antibacterial substances of approximately 2300 species of plants belonging to 166 families. The test organisms were Staph.

aureus and E. coli. Of these plants 63 genera were found to contain substances which inhibited the growth of one or both of the test organisms. The inhibitory substances were sometimes distributed throughout the different parts of the plant and at other times, restricted to a single part.

Tsuchiya, et al (41), discussed an antibacterial substance isolated from a plant; it is called nordihydroguiaretic acid and was found to be active in vitro against Staph. aureus, Sal. paratyphi, Sal. schottmuelleri and Sal. enteritidis.

Both in legend and in the scientific literature Allium sativum, the common garlic, has been endowed with therapeutic virtues. The claims of its remarkable characteristic have been attributed to a number of substances in its makeup -- diallyl sulfide, the unstable sulfur in the alkyl polysulfides, a bacteriophage, acrolein or some other unstable aldehyde, and to some undefined group of substances designated as the phytoncides. Cavallito (5) has isolated a substance which he calls allicin and he reports that to be the garlic antibacterial substance. It is a colorless oil, approximately 2.5% soluble in water and relatively unstable. It is equally effective against gram-positive and gram-negative organisms. Garlic oil can achieve some interesting results, such as preventing meat decay in vitro, killing some types of worms, stimulating the intestinal mucosa and peristalsis, inhibiting peptic digestion, and oxidizing hemoglobin to methemoglobin in vitro.

Pederson and Fisher (29) discovered that certain undesirable gram-negative bacteria present on the surface of the cabbage leaves ordinarily disappear shortly after the cabbage is cut. The presence of a bactericidal substance in the cabbage tissues causes a marked reduction in the number of gram-negative bacteria within 6 to 24 hours. This substance is less active against gram-positive bacteria and is inactivated by heating. Osborn (27) stated in his article that the inhibitory substance of Brassica oleracea, the cabbage, was of greater concentration in the seed than other parts of the plant.

Thalhimer (38) in 1911 recognized the bactericidal action of quinones and thought they compared with other disinfectants. In the chemical analysis of the antibiotic substances some were found to have the quinone structure. Investigators therefore tested compounds with a known quinone structure to see what their effect on bacteria would be. Colwell and McCall (6) reported that synthetic 2-methyl-1-4-naphthoquinone was bacteriostatic and bactericidal for both the gram-positive and gram-negative organisms.

The introduction of the sulfonamide group of drugs as antibacterial chemotherapeutic agents and its clinical application dates back to the time of Domagk (10), when he reported on the curative effects of "prontosil" in streptococcal infections in mice in 1935; in the same year the Trefouels, Nitti and Bovet (40) announced the antibacterial activity

of the simple organic compound, sulfanilamide, which is related to prontosil.

Dubos (12) reports that the bacteriostatic action of the sulfonamides is due to the competition of the drug with p-aminobenzoic acid at some vital stage of metabolism in which the latter substance is concerned. P-aminobenzoic acid is an essential growth factor or metabolite for many species of organisms. Numerous in vitro studies (27) have been made of the effect of various sulfonamides against bacteria. It was found that the concentration of the drug necessary to produce bacteriostatic or bactericidal action in vitro may have absolutely no apparent relation to that concentration necessary for its in vivo activity. Sulfapyridine is about two to three times as active as sulfanilamide in vitro but the two drugs show an equal activity in vivo in the mouse against some strains of streptococci.

Sulfanilamide (23) is active for hemolytic streptococcal infections produced by Lancefield's Group A organisms, also for chancroid, Welch bacillus infections, meningococcal infections and for infections (other than subacute bacterial endocarditis) produced by the viridans streptococci. Sulfapyridine and sulfathiazole are superior to sulfanilamide in the treatment of gonococcal infections. Sulfathiazole is the drug of choice in most staphylococcal infections. It is the best of the group for the treatment of colon bacillary infection of the tissues, such as peritonitis and pyelonephritis.

Cooper and Keller (7) obtained results from an *in vitro* test which indicate that sodium sulfathiazole is more bacteriostatic and bactericidal for the Flexner strains of *Shigella paradysenteriae* than for the Sonne strains and still less bactericidal for *E. typhosa* and *Sal. paratyphi*.

Streptomycin is a recent discovery by Schatz (31) in 1944. It is isolated from two strains of an actinomyces related to an organism described as *Actinomyces griseus*. Streptomycin resembles streptothricin, which is a substance produced by *Streptomyces lavendulae*, in its selective action against gram-negative bacteria; streptomycin differs in its greater activity against various gram-negative bacteria. Schatz (32) showed that streptomycin inhibits the growth of *Mycobacterium tuberculosis* and *Mycobacterium phlei*. Jones', et al (20), experiments on chick embryos indicate that streptomycin, if used in sufficient concentration, offers full protection to the chick against fowl typhoid caused by *Shig. gallinarum*; even the dead embryos showed complete freedom from the disease.

Schoenbach (34) was able to isolate gram-negative pathogenic organisms from the nasopharynx with the incorporation of tyrothricin in the medium, which selectively killed the staphylococcus.

Stokinger (37) also utilized tyrothricin in his work. He found that chocolate agar containing tyrothricin in a concentration of 1:15,000 aided in the primary isolation of *Neisseria gonorrhoeae*, since the tyrothricin acted against the

gram-positive organisms which are present in the genito-urinary tract.

There have been reports on the synergic action obtained when sulfonamides are used with certain other substances. Bigger (1) reported that the presence of sulfathiazole in the broth greatly increased the dilution at which the inhibitory action of penicillin on staphylococci could be demonstrated. Thatcher (39) noted a pronounced synergistic effect when sulfonamides which normally have little if any effect on gram-negative bacteria would in the presence of a 1:28,000 dilution of methylene blue or brilliant cresyl blue completely inactivate 10 million cells of a 24 hour culture of E. coli, the final concentration of sulfapyridine, sulfathiazole or sodium sulfathiazole being 1:14,000. Schwartzman (35) says that the addition of methionine in combination with threonine and methionine sulfoxide enhanced the activity of penicillin against gram-negative organisms in the presence of antipenicillin factors. He believes the enhancement is a result of synergistic rather than additive action of the amino acids.

Thus far investigators have utilized the antimicrobial agents for means of differentiating between groups of bacteria as an aid in the isolation of organisms. Little has been done to attempt to differentiate among species with the use of these agents.

Experimental Data

Organisms Used

The following salmonellae were used in the experiment: Sal. choleraesuis, var. Kunzendorf, Sal. paratyphi, Sal. schottmuelleri, Sal. enteritidis, Sal. abortivoequina, Sal. typhimurium and four strains of Sal. pullorum. Also tested were two strains of E. coli, three strains of Proteus and one strain of A. aerogenes. Sal. pullorum 3, Sal. pullorum 4, E. coli, Proteus and A. aerogenes were tested with three antimicrobial agents only, penicillin, streptomycin hydrochloride and 2-methyl-1-4-naphthoquinone. With the exception of three strains of Sal. pullorum, all the Salmonella cultures were obtained from the Michigan Department of Health; the other organisms were obtained from the Michigan State College bacteriology department.

Antimicrobial Agents Used

The antimicrobial agents tested were garlic oil, a cabbage seed extract, cabbage oil, hydroquinone, Hykinone -- a commercial product which contains a vitamin K derivative; 2-methyl-1-4-naphthoquinone, which is vitamin K; penicillin, sulfanilamide, sodium sulfapyridine, sodium sulfathiazole and streptomycin hydrochloride.

Procedure

A number of methods have been developed for determining

the activity of antibiotic substances. They vary greatly, each having its limitations and advantages. Results obtained with one method cannot be accurately compared with those obtained by another. Some of the methods are the agar plate-dilution method, serial dilution method, the classic agar diffusion or agar cup method, turbidimetric method and the filter paper disc method.

For its simplicity and practicability in the performance of the test in this paper, the filter paper disc method described by Vincent and Vincent (42) and with the modifications by Lucas, et al (24), was used. DeBeer and Sherwood (9) listed some worthwhile advantages of the filter paper disc method. Those applicable to this experiment are the following: Measurement of the volume of antimicrobial substance is accomplished simply by touching the edge of the paper disc to the material to be assayed because discs of the same size absorb surprisingly uniform amounts of solution; only small amounts of solution are necessary; and the labor of washing and caring for the small cylinders and pipettes is eliminated.

The technique for the preparation of plates is as follows: Ten ml. of tryptose agar was pipetted into each specially selected flat-bottomed Petri dish. The plates were allowed to stand at room temperature for two hours so that some of the surface moisture may be removed. They were stored in the refrigerator at 4° C for at least 1 hour and for not more than three days before seeding. One ml. of an 18-24 hour tryptose

broth culture of the test organism was pipetted onto each plate. The excess of inoculum was drained off by placing the inverted dish on a metal rack at an angle of 45-50° for twenty minutes; the little drops of inoculum that collected on the lower rim of the inverted dish were wiped off with sterile gauze. Thus the plate was ready for the test.

Filter paper discs about 13.0 mm. in diameter were the conveyors of the antimicrobial substance. They were first sterilized in the autoclave and dried overnight in a 37° C incubator. With the use of sterile forceps each disc was placed on a seeded agar plate after having been immersed in the test fluid. The plates were incubated for 18-24 hours in a 37° C incubator and the zones of inhibition were then recorded.

Results

Tables 1 and 2 show the results of the action of various antimicrobial agents on all the Salmonella organisms, with the exception of Sal. pullorum 3 and Sal. pullorum 4, which were tested with only penicillin, streptomycin hydrochloride, and 2-methyl-1-4-naphthoquinone. The results of the organisms tested with only three substances appear on Table 3. Each result is an average of the zones of inhibition produced by eight separate filter paper discs. The results are the diameters of the inhibition zones recorded in millimeters; the results include the diameter of the filter paper disc

TABLE 1

	0.1% garlic oil A	0.1% garlic oil B	Cabbage extract	1% cabbage oil	0.5% garlic oil	1% Hykinone	5% Hykinone	1% 2-methyl-1-4-naphthoquinone
Sal. abortivoequina	22.8	39.9	31.5	-	39.1	-	21.9	19.6
Sal. choleraesuis	18.5	39.4	30.1	-	40.8	-	22.9	31.3
Sal. enteritidis	15.0	30.8	28.5	-	33.3	-	19.7	-
Sal. paratyphi	20.3	37.0	30.3	-	33.8	-	20.1	19.2
Sal. schottmuelleri	18.5	37.9	28.3	-	38.1	-	19.0	16.7
Sal. pullorum 1	25.9	approx 45	32.0	-	approx 50	-	22.9	29.4
Sal. pullorum 2		45.9	30.0	-	approx 48	-	22.7	25.0
Sal. typhimurium	16.0	34.2	30.6	-	33.9	-	18.7	-

- indicates no inhibition

TABLE 2

	2% Hykinone in 0.2% garlic oil	1% hydroquinone in 0.1% garlic oil	Penicillin 250 units	1% sulfanilamide	1% sodium sulfapyridine	5% sodium sulfapyridine	2% sodium sulfathiazole	0.2% (1:500) streptomycin hydrochloride
Sal. abortivoequina	-	28.6	24.1	-	-	-	-	20.8
Sal. choleraesuis	-	27.5	32.2	-	-	-	-	21.1
Sal. enteritidis	-	23.8	21.0	-	-	-	-	19.0
Sal. paratyphi	-	25.1	20.1	-	-	-	-	21.1
Sal. schottmuelleri	-	26.4	19.6	-	-	-	-	18.9
Sal. pullorum 1	-	29.0	32.9	-	-	-	-	23.0
Sal. pullorum 2	-	30.1	20.7	-	-	-	-	24.9
Sal. typhimurium	-	23.6	19.5	-	-	-	-	21.0

- indicates no inhibition

TABLE 3

	0.2% (1:500) streptomycin hydrochloride	1% 2-methyl-1-4-naphthoquinone	Penicillin 250 units
Sal. pullorum 3	24.1	24.6	19.0
Sal. pullorum 4	24.7	22.4	20.4
A. aerogenes	18.5	16.3	-
E. coli 1	19.4	16.5	-
E. coli 2	18.3	-	-
Proteus 1	17.6	18.9	19.7
Proteus 2	17.6	-	17.4
Proteus 3	18.6	19.6	19.4

- indicates no inhibition

which is approximately 13.0 mm.

The cabbage extract was an aqueous emulsion of the oils obtained from the cabbage seeds. There was a white zone of precipitate surrounding each filter paper disc; the zones of inhibition usually extended beyond that opaque zone. The salmonellae were not markedly differentiated since the largest zone of inhibition was no more than 3 mm. greater than the smallest zone.

The 1% cabbage oil solution used in this experiment showed negative results. That is contrary to what was anticipated, since the cabbage extract produced inhibition and the cabbage oil solution contains the essential oil from that extract. Dr. E. H. Lucas (25) explains the lack of action to an interruption during the extraction procedure, an interruption which may have cost the loss or destruction of the antimicrobial agent.

Different lots of 0.1% of garlic oil solutions -- A and B, were tested. B is the same as that used with the 1% hydroquinone solution in an attempt to illustrate synergistic action. A 0.5% solution of an oxalic acid preparation of garlic oil was also tested. The results for each organism, when tested with the separate solutions, show about the same degree of sensitivity. Sal. pullorum seems to be the most sensitive of the group.

A 1% Hykinone solution did not show any effect on any of the organisms, so a 5% solution was then prepared. There was

no marked sensitivity on the part of any one of the organisms to the substance although each one was inhibited.

The 1% acetone solution of 2-methyl-1-4-naphthoquinone (vitamin K) gave interesting results with several of the Salmonella organisms. Sal. enteritidis and Sal. typhimurium were not inhibited at all or had zones of inhibition which were too small to read. Sal. abortivoequina, Sal. paratyphi and Sal. schottmuelleri all had zones which were less than 20 mm. Sal. choleraesuis and the four strains of Sal. pullorum exhibited zones of inhibition of 20.0 mm. or more. The other organisms tested showed either a slight or no sensitivity to the antimicrobial agent. The sensitivity varied among strains of the same organism; thus one E. coli strain was inhibited slightly and the other was not. One strain of Proteus was also insensitive.

When 2% Hykinone in 0.2% garlic oil solution was tested with the organisms, there were no zones of inhibition. Normally a 0.2% garlic oil solution would give a noticeable zone of inhibition. It is surmised that the Hykinone behaves as a depressant rather than as a synergist.

The 1% hydroquinone in 0.1% garlic oil solution (B) inhibited the organisms but again, the quinone substance acted as a depressant, for the zones of inhibition obtained from the 0.1% garlic oil solution alone were approximately 10 mm. larger. Another phenomenon which may be of differential value was noticed. On all the plates, save those which were seeded

with Sal. pullorum, the pads and zones of diffusion of the substance into the agar were a definite brown color. Hydroquinone is a reducing agent and may have reacted with the products of those organisms, and Sal. pullorum may have been able to prevent the hydroquinone from reducing any such substance.

The penicillin results offer another worthwhile differentiation of the organisms. Sal. choleraesuis and one strain of Sal. pullorum are the most sensitive organisms of the group tested. The difference between the two strains of Sal. pullorum is quite remarkable and must be attributed to the marked selectivity of these antibiotic substances. A. aerogenes and E. coli were not inhibited by this concentration of penicillin.

All the sulfonamides gave no zones of inhibition although there were a few instances where an initial inhibition could be detected. If the plates had been read after fewer hours of incubation -- six hours, for instance, the zones of inhibition could possibly have been distinguished.

Reports from investigators indicate that streptomycin is strongly active against the gram-negative organisms. It is not known how much streptomycin there is in the streptomycin hydrochloride tested. Two lots of the streptomycin hydrochloride were obtained from Merck & Co. The 1:500 (0.2%) solution of streptomycin hydrochloride was arbitrarily used and gave no spectacular results. The two lots of streptomycin hydrochloride gave comparable results.

Summary

1. The sulfonamides and the cabbage oil solution were not active against the Salmonella. The solutions may have been too weak and the method of testing may not have been sensitive enough to detect any slight action of the substances.
2. The cabbage extract, Hykinone solution and streptomycin hydrochloride acted on the organisms but did not reveal any differential characteristics.
3. Sal. pullorum was the most sensitive to the garlic oil solution.
4. The 1% acetone solution of 2-methyl-1-4-naphthoquinone produced a variety of results. Sal. enteritidis, Sal. typhimurium, one strain of E. coli and one strain of Proteus were resistant to the substance. The other organisms were slightly, moderately or markedly inhibited.
5. Penicillin was most active against Sal. choleraesuis and Sal. pullorum. There was also a marked difference in the degree of sensitivity of the four strains of Sal. pullorum. A. aerogenes and E. coli were not inhibited by this concentration of penicillin.
6. Two per cent Hykinone and 1% hydroquinone in garlic oil solutions both acted as depressants to the activity of the garlic oil solutions.
7. With the exception of the two strains of Sal. pullorum,

the 1% hydroquinone in 0.1% garlic oil solution produced a brown color on the filter paper discs and the zones of diffusion on all the plates.

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