INTERRELATIONSHIPS BETWEEN BIOCHEMICAL PROPERTIES AND SUSCEPTIBILITY TO ANTIBIOTICS AND SULFATHIAZOLE OF STRAINS OF VIRIDANS STREPTOCOCCI AND ENTEROCOCCI

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Interrelationships between Biochemical Properties and Susceptibility to Antibiotics and Sulfathiazole of Strains of Viridans Streptococci and Enterococci.

Thesis directed by Dr. William E. Clapper.

The purpose of this work was to determine the susceptibility of strains of viridans streptococci and enterococci to sulfathiazole, aureomycin, bacitracin, penicillin, and streptomycin; and to determine whether their susceptibility to these agents was related to other biochemical properties. Since previous work had indicated that trained sulfathiazole-resistant viridans streptococci acquired properties similar to those characteristic of enterococci, it followed that strains trained to resistance to penicillin, streptomycin, aureomycin, and bacitracin should also be compared in biochemical properties to naturally-resistant enterococci. Investigation of cross-resistance among these strains seemed of further importance because of its possible clinical applications.

A study of the interrelationship between susceptibility and other biochemical properties commonly used for the identification of the organisms might produce several useful results. New and better tests for identification might be discovered, possible agents for trial in treatment of the changed organisms might be indicated, and possible changes in organisms subjected to the chemotherapeutic agents might be anticipated. Finally, it was of interest to compare all changes in both the parent and the trained resistant strains for a possible

explanation of the increased resistance. Gale had found larger amounts of free glutamic acid in strains of staphylococci naturally resistant to sulfathiazole than in susceptible strains, if the young cells were examined. Less glutamic acid was found in penicillin-resistant cells. He did not examine cells resistant to other antibiotics. Therefore, the glutamic acid content of cells trained to resistance to sulfathiazole, penicillin, streptomycin, and aureomycin was determined in this study.

The work was carried out in two parts. First, the biochemical properties and susceptibility to sulfathiazole, aureomycin, bacitracin, penicillin, and streptomycin was determined for 40 strains of viridans streptococci and enterococci. Thirty-seven strains of the organisms were isolated from pathological material and 3 strains were used as controls, one Streptococcus fecalis (A.T.C.C. #9790) and two viridans streptococci, Streptococcus "MG" and Streptococcus "SBE". Secondly, the biochemical properties and susceptibility to the chemotherapeutic agents was determined for trained resistant strains to sulfathiazole, aureomycin, penicillin, and streptomycin. No bacitracin-resistant strains were studied, as even after prolonged exposure to bacitracin, no resistant variants developed. The glutamic acid content was determined manometrically for viridans streptococci, enterococci, and resistant variants to sulfathiazole and the antibiotics.

The biochemical characteristics best suited for differentiating between viridans streptococci and enterococci, as indicated in this study, are: growth in broth incubated at 45°C for 24 hours, the reduction of 0.1% methylene blue milk, growth in 6.5% NaCl broth, and growth

on 40% bile agar. The enterococci were more resistant to all of the chemotherapeutic agents used than were the viridans streptococci. The much greater resistance of the enterococci to sulfathiazole and penicillin indicated the possibility of using definite concentrations of these in broth as added criteria for identification. It was found that all of the enterococci and none of the viridans streptococci would grow in concentrations of 10 mg/cc of sulfathiazole in broth and 2 units/cc of penicillin in broth. Results of the in vitro tests for susceptibility to the five agents indicate that infections due to viridans might respond to all of the chemotherapeutic agents tested, but those due to enterococci might best be treated with bacitracin or aureomycin.

The most interesting findings in the development of resistant strains were the cross-resistance and the difference between the streptomycin-resistant variants as compared to the others. Some increase in resistance to all the agents was shown by the sulfathiazole-resistant variants. Increase in resistance to other agents was also shown by those strains trained to resistance to penicillin and aureomycin, but this was not generally true of the streptomycin-resistant strains. The variant strains selected by sulfathiazole, aureomycin, and penicillin all showed the same biochemical properties as a typical enterococcus. Those selected by streptomycin varied in their biochemical properties and often did not have the properties of enterococci.

The results of the glutamic acid determinations showed that appreciable amounts are found free inside those cells which have acquired sulfathiazole resistance. Whether this resistance has been

induced by sulfathiazole or accompanies increased resistance to other agents makes no difference. Whether the resistance is due to the increase in internal glutamic acid or whether this is merely a result of other changes which determine the organism's resistance, is not answered by this study. The viridans streptococci tested, and the streptomycin-resistant variants showed no free glutamic acid at any stage of growth.

This abstract of about 750 words is approved as to form and content.
I recommend its publication.

Signed William E. Clapper

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INTERRELATIONSHIPS BETWEEN BIOCHEMICAL PROPERTIES AND SUSCEPTIBILITY TO ANTIBIOTICS AND SULFATHIAZOLE OF STRAINS OF VIRIDANS STREPTOCOCCI AND ENTEROCOCCI

I. INTRODUCTION

It has previously been shown (Clapper and Heatherman, 1948 and 1950) that trained sulfathiazole-resistant strains of Streptococcus mitis possessed biochemical properties similar to those characteristic for enterococci. It seemed of interest, therefore, to determine the susceptibility of strains of viridans streptococci and naturally-resistant enterococci not only to sulfathiazole but to the better known antibiotics as well, and to determine whether their susceptibility to these agents was related to other biochemical properties as sulfathiazole resistance had been found to be in the trained strains. As a corollary to this study, it followed that strains trained to resistance to penicillin, streptomycin, aureomycin, and bacitracin should be compared in biochemical properties to sulfathiazole-resistant strains and to the naturally-occurring enterococci. Investigation of cross-resistance among these strains seemed of further importance because of its possible clinical applications.

Changes in biochemical properties that accompany changes in resistance to chemotherapeutic agents may make for even greater confusion in identification or classification than that which already exists in the viridans group. A study of the interrelationship between susceptibility and other biochemical properties commonly used for the identification of the organisms may produce several useful

results. New and better tests for identification may be discovered, possible agents for trial in treatment of the changed organisms may be indicated, and possible changes in organisms subjected to the chemotherapeutic agents may be anticipated.

Finally, it was of interest to compare all changes in both the parent and the trained-resistant strains for a possible explanation of the increased resistance. It was for this reason that the determination of glutamic acid in cells of all classes was made. Gale had found larger amounts of glutamic acid in strains of staphylococci naturally resistant to sulfathiazole than in susceptible strains, if the young cells were examined. Less glutamic acid was found in penicillin-resistant cells. Cells resistant to other antibiotics were not examined.

The glutamic acid content of cells trained to resistance to sulfathiazole, penicillin, streptomycin, and aureomycin was therefore determined in this study.

II. REVIEW OF THE LITERATURE

Viridans Streptococci and Enterococci

There have been many tests used for the differentiation of the viridans streptococci from the enterococci and much disagreement among the workers in this field as to the value of certain tests. Since Houston and McCloy (1916) first noted the great resistance of the enterococci to temperature changes, investigators have tried, with little success, to correlate the resistance of enterococci with fermentative ability, serological reactions, cultural characteristics, biochemical characteristics, and susceptibility to certain bacteriostatic agents. Studies have been made on the sensitivity of both the enterococci and viridans streptococci to the various antibiotics and the sulfonamides with each investigator often using different criteria for the classification of each group.

Enterococci were first separated from the better known pyogenic group of streptococci by MacCallum and Hastings (1899). They isolated a streptococcus from a case of acute endocarditis, described its appearance on different media, and separated it from the pyogenic streptococci by the reaction it produced in litmus milk. They attributed the reaction in litmus milk to milk-curdling and proteolytic enzymes, which were separable from the cell and capable when isolated of producing their effects in milk. It is interesting that litmus milk still is used today by many investigators as a differential test between viridans streptococci and enterococci, although in the experimental data given later, this test was found to be quite variable in each group.

Probably the first attempt to place the viridans streptococci into a group apart from the enterococci and other streptococci was made by Andrewes and Horder (1906). They studied 1,200 strains of streptococci from various sources and made seven divisions. One of these divisions is called S. mitis, a non-committal term picked to express its non-virulent character; and another division which included intestinal streptococci, called S. fecalis. The term enterococci was first used by Thiercelin (1899), a Frenchman, to describe the same type of organism that Andrewes and Horder called S. fecalis. These two investigators used what they referred to as "Gordon's Metabolic Tests" which consisted of changes in litmus milk, neutral red broth, and the production of acid from seven different carbohydrate media. In discussing these "Metabolic Tests", Gordon (1905) states that here is a series of definite tests for differentiating streptococci by their biochemical properties. Thus, as early as 1905, the biochemistry of the bacterial cell was considered important in classification. Holman (1916) combined Gordon's fermentation tests and Shottmueller's descriptions of growth on blood agar (1903) to separate the streptococci into two large groups, the hemolytic and non-hemolytic, with each group having eight subgroups. S. mitis and S. fecalis were included under the non-hemolytic group and were differentiated by the fermentations of mannitol and salicin. Today it is known that hemolytic strains can be found among the S. fecalis, Evans and Chinn (1947), and that fermentation of salicin and mannitol are variable for each group, Swift (1948). However, this is one of the first attempts of an investigator to correlate bio-

chemical with cultural characteristics of streptococci.

Because of the increasing occurrence of non-hemolytic streptococci in intestinal infections, Krumweide and Balentine (1916) studied the agglutination and cultural relationships of members of the so-called "Streptococcus viridans group". They compared organisms from endocarditis and other pathological conditions with strains from tonsils of healthy individuals, and concluded that this was a heterogenous group, and different strains might show some relationship one to the other, but there were no common properties shown by all the pathological types as compared to the control group. Dible (1921) described the enterococci more concisely than had been done up to that time. To differentiate them from the viridans group, he made use of their resistance to 60°C for 30 minutes. He also mentioned that he had found some S. mitis strains which were thermal-resistant by this test, and that they might be variants of the enterococci. This seems to be the first mention of a possible evolutionary relationship between the viridans streptococci and the enterococci. Later (1935) Kleckner in a study of fecal streptococci found that there were many strains which would not tolerate exposure to 60°C for 30 minutes. Lancefield (1925) studied immunological relationships and chemical constitution of the viridans group, and concluded that there was considerable difference in the protein from different strains. Although much work has been done on this phase since 1925, no successful serological classification of the viridans group has been made (Lancefield, 1933; Solowey, 1942 and 1945; Wheeler and Foley, 1945).

A very comprehensive study of 150 strains of enterococci was carried out by Bagger (1926). Fifty-eight strains were isolated from normal intestines and 92 strains from cases of appendicitis. In this investigation the resistance of the enterococci to antiseptics was pointed out. Phenol and formalin were the agents used. The resistance of the enterococci to bacteriostatic agents was again noted by Chapman (1936). He found they were more resistant than E. coli, staphylococci, and other streptococci to a 1:500,000 dilution of merthiolate and a 1:2,500 dilution of phenol. This is another early instance of the recognition of resistance of enterococci to antiseptics and chemotherapeutic agents. Two new tests, modifications of which are widely used today, were introduced in 1929. They have proven most valuable in differentiating the viridans streptococci from the enterococci. The first of these was the use of a 1:5,000 dilution of methylene blue in milk (Avery, 1929), and the second, the use of 40% bile blood agar. The latter test differentiated enterococci from viridans streptococci as well as certain other groups of streptococci (Belenky and Popowa, 1929). Today the use of 0.1% methylene blue milk and 40% bile agar plates differentiates very well between viridans streptococci and enterococci, and correlates with the resistance of the two groups, as will be shown later.

Sherman, Mauer, and Stark (1937) studied 434 cultures of S. fecalis and made use of more extensive tests for characterization than had ever been used before. They concluded that fermentation tests were of minor value, but that of primary differential value were thermal resistance, changes in litmus milk, growth in broth pH 9.6,

and with 6.5% NaCl, and in 0.1% methylene blue milk. In any definition of the S. fecalis today these tests are all still included.

The viridans streptococci were described as having at least two definite species by Safford, Sherman, and Hodge (1937): S. salivarius and S. mitis. Niven, Smiley, and Sherman (1941) showed that the property of S. salivarius to produce very distinctive large mucoid colonies on 5% sucrose agar seemed to be correlated with the ability to ferment inulin. Concerning the non-hemolytic streptococci of the viridans type, Sherman et al. (1943) wrote, "As compared with the other major groups of the streptococcus genus, the taxonomy of the viridans streptococci is in a highly unsatisfactory condition; but the common impression that the viridans streptococci compose a heterogenous conglomeration is largely due to the general failure to determine the basic nature of the organisms dealt with." Since this was written, much more has been learned about the basic nature of these organisms due to the use of the antibiotics which has stimulated intensive study of many phases of the metabolism of these organisms. Probably in the near future some very fundamental differences in metabolism between the viridans streptococci and enterococci will be disclosed.

Mirick et al. (1944) described a new type of viridans streptococci which they called Streptococcus "MG". This organism was consistently isolated from a group of patients who had primary atypical pneumonia. This viridans type of streptococci differed in some of its biological characteristics from S. mitis, S. salivarius, and from the enterococci. Two identifying characteristics of this group of organisms were the formation of small fluorescent colonies on 5% sucrose agar and capsular

swelling of all strains in the presence of specific immune rabbit serum.

Studying the serology of the enterococci, Foley and Wheeler (1945) found no correlation with biochemical tests but did find that the only characteristics common to the whole group were the reduction of 0.1% methylene blue milk and growth in 6.5% NaCl broth. They also found that only 10% of the group were thermal-resistant and, therefore, concluded that this property was unreliable to use as a differential criterion.

White and Niven (1946) described the biochemical characteristics of a new type of viridans first isolated by Loewe et al. (1946) from cases of subacute bacterial endocarditis. As with Streptococcus "MG" this group of organisms seemed to have characteristics intermediate between those of the viridans streptococci and the enterococci. Streptococcus "SBE" as this new type was named by Loewe, had the unique property of synthesizing a polysaccharide in 5% sucrose broth which caused a jelling of the medium. It also hydrolyzed arginine and fermented inulin but not raffinose, and grew on 40% bile agar but not in 6.5% NaCl broth. Swift (1948) presents all of the most widely used biochemical tests for the viridans streptococci and the enterococci in tabulated form, and this served as the basis for the differential tests used in this study.

Fleming (1929) noted that S. fecalis was very resistant to the antibacterial action of his penicillin cultures while viridans streptococcus was very susceptible. However, the first example of an investigator trying to show a relationship between biochemical reactions and sensitivity to antibiotics is found in the work of Bornstein

(1940). He studied the action of penicillin on enterococci and viridans streptococci and found that in penicillin broth all of his enterococci grew and the viridans streptococci did not. The exact concentration of the penicillin broth cannot be determined from his data, and no suggestions were given as to any practical application of this fact. The penicillin resistance of enterococci was made use of by White and Sherman (1944), who devised a selective medium containing 325 units/liter of penicillin that prevented the growth of most of the organisms in milk except the enterococci.

Dawson, Hobby, and Lipman (1944) determined the penicillin sensitivity of 50 strains of non-hemolytic streptococci isolated from cases of subacute bacterial endocarditis. Among the 50 strains, 19 were described as viridans streptococci, and 3 resembled enterococci. Penicillin in concentrations of .01 to .03 units/cc prevented growth. They concluded that the degree of sensitivity of any particular strain did not correspond with the cultural or serological properties of the strain. Watson (1944) determined the penicillin levels of 8 strains of Lancefield Group D Enterococci, and found 2.0 to 5.0 units/cc to be inhibitory. Massell, Meyeserian, and Jones (1946) carried out experiments with penicillin to see what effect varying the size of the inoculum would have on the sensitivity levels of viridans streptococci. They found that within wide limits, variations in the number of exposed organisms did not alter the concentration required to prevent growth. This concentration was found to be from .4 to .035 units/cc. They also found that by using both penicillin and different sulfonamides together, the concentration of penicillin necessary to inhibit growth was decreased by 25-75%.

Penicillin-resistant non-hemolytic streptococci were shown by Meleney and Johnson (1947) to be sensitive to bacitracin. Chandler and Bliss (1948) found that 6 strains of S. fecalis were more susceptible to aureomycin than penicillin. This was also shown by Bliss and Todd (1949), who obtained inhibiting concentrations of aureomycin of .62 to 1.25 mg/cc as compared to 2.5 units/cc for penicillin. Jawitz and Gunnison (1950) obtained penicillin sensitivity levels for viridans streptococci of 1.0 units/cc and 2.5 to 5.0 units/cc for enterococci.

Increased Resistance and Biochemical Changes

Sulfonamide Resistance:

Several investigators have reported changes occurring in the properties of bacteria made resistant to the various sulfa drugs. One of the first studies made on the effect of increased resistance of bacteria to sulfonamide was carried out by McClean et al. (1939). They found that they could increase the tolerance of pneumococci to sulfonamide both in vivo and in vitro. The virulence of the parent and resistant strains was the same. MacLeod (1939) studied the differences in metabolism of "sulfapyridine-fast" strains and parent strains of pneumococcus Type I. He found no changes in morphology, type-specificity, virulence, or fermentations. However, the production of H_2O_2, a product of aerobic metabolism in the pneumococcus, was greatly reduced in the resistant strain. He found also that the resistant strain had lost dehydrogenase activity for glycerol, lactate, and pyruvate. Clapper and Heatherman (1948) found that viridans streptococci in becoming sulfathiazole-resistant no longer produced H_2O_2, and that the dehydrogenase activity of the resistant strains was

increased. Vivino and Spink (1942) observed sulfonamide-resistant strains *in vivo*. On treating two patients with bacteremias caused by *Staphylococcus aureus* with sulfonamide, cultures taken after a series of treatment were significantly more resistant than those taken before sulfonamide therapy. Hamburger (1942) also noted this *in vivo* development of resistance in a patient with pneumococcic endocarditis. The resistance of the pneumococcus went from 2.5 mg to 80 mg in 165 days. Seeley and Schmidt (1942) compared resistance developed to 3 different sulfonamides by pneumococci. They used sulfanilamide, sulfapyridine, and sulfathiazole and found that when their strains became resistant to one sulfonamide they were more resistant to the others, also. They found that the different strains developed resistance at different rates. However, Colebrook (1943) found that of 7 strains of hemolytic streptococci developing resistance to sulfanilamide, only one became more resistant to sulfapyridine, sulfadiazine, and sulfathiazole.

Interrelationships of drug-resistant strains were studied by McIntosh and Selbie (1943). They used a hemolytic streptococcus, Group A, and a *Staphylococcus pyogenes*, and developed resistant strains to proamidine, proflavine, quindoline, and penicillin. They found no cross-resistance between penicillin and sulfanilamide, but did find it between proamidine and proflavine. Tillett *et al.* (1943) found that penicillin was effective in mice against a sulfonamide-fast pneumococcus which had been isolated from a clinical case. The change in biochemical characteristics and resistance of penicillin-resistant cultures of staphylococci, pneumococci, and hemolytic streptococci was studied by McKee and Houck (1943). They found that over a period of 3 months by transferring their cultures in broth plus increasing

concentrations of penicillin, they obtained a six fold increase in resistance for the pneumococci, a 1,000 to 6,000 fold for the staphylococci, and a 30 fold increase for the streptococci. With increased penicillin resistance there was a marked decreased virulence for mice and a decreased rate of growth, while the bile solubility, carbohydrate fermentations, and sulfonamide-resistance remained the same. Felsenfeld (1943) found that with Shigella sonnei the colony forms with the greater fermentative activity were more resistant to the sulfonamides.

There have been many reports (reviewed by Sevag, 1946) of the reversal of the action of sulfonamides by PABA. This would suggest the possibility that sulfonamide resistance might be accompanied by an increased production of PABA. Spink et al. (1944) found that sulfonamide-resistant strains of staphylococci produced more PABA than sensitive strains. However, all resistant strains do not produce PABA. Yegian et al. (1946) studied changes which developed in Mycobacterium ranae with sulfonamide resistance. They found that the resistant variant grew slower and was more susceptible to $CuSO_4$, zephiran, and acriflavin. Clapper and Heatherman (1950) studied the biochemical changes of viridans streptococci made resistant, in vitro, to sulfathiazole. With increased resistance the viridans streptococci acquired ability to grow on 40% bile agar, in 6.5% NaCl, and to reduce 0.1% methylene blue milk, all of which are properties shown by enterococci. Sulfathiazole-resistant strains also increased in resistance to penicillin, streptomycin, aureomycin, and bacitracin. However, in streptomycin-resistant strains the biochemical changes were variable, and resistance to sulfathiazole and the other antibiotics did not take place.

This is confirmed in this study, as will be shown later. Harrison and Clapper (1950) found that the vitamin requirements for trained sulfa-resistant strains were different than for the parent-sensitive strains. The sulfathiazole-resistant variants and also a strain of S. fecalis required pteroylglutamic acid (PGA), while the parent strain did not.

Penicillin Resistance:

Changes accompanying the development of resistance to penicillin have also been reported. One of the earliest of these studies was reported by Abraham et al. (1941), who found that Staphylococcus aureus could become resistant to penicillin in vitro. By transferring the staphylococcus for 7 weeks in increasing concentrations of penicillin, the resistance was increased 1,000 fold. A decrease in growth rate and enzyme activities were noted. Rammelkamp and Maxon (1942) also increased the resistance of Staphylococcus aureus to penicillin in vitro from .04 units to 5.7 units/cc. Kirby (1944) extracted a penicillin inhibitor from 7 strains of resistant staphylococci isolated from clinical material and at the same time found that 7 strains of sensitive staphylococci did not produce any of this inhibitor. Bondi and Dietz (1946) in studying the properties of penicillinase found that only 14% of the more resistant staphylococci produced this enzyme and, therefore, came to the conclusion that the inability of an organism to produce penicillinase was not an indication of the organism's sensitivity to penicillin. Spink and Ferris (1947) studied penicillin-resistant staphylococci and found that resistant strains produced in vitro possessed no penicillinase while naturally-occurring resistant strains did.

Graessle and Frost (1948) studied the induced *in vitro* resistance of 6 strains of *Staphylococcus aureus* to penicillin and streptomycin. Two of the penicillin-resistant variants increased in resistance to streptomycin but there was no increase in penicillin resistance for the streptomycin-resistant variants. Some of the streptomycin-resistant variants showed a reduction in the rate of carbohydrate fermentations. Bellamy and Klimek (1947) found that a *Staphylococcus aureus* strain which had increased in resistance to penicillin 60,000 fold lost its ability to grow anaerobically and grew at a slower rate than the parent strain. They suggested that penicillin might interfere with an essential step in anaerobic metabolism.

Penicillin-resistant variants of Group A, B, and C beta-hemolytic streptococci which had been developed in vitro were examined by Gezon (1948). He found that with increased penicillin resistance there was a decrease in virulence for mice, a loss of specificity in the group antigen by some, and transient changes in the type of hemolysis. Bellamy and Klimek (1948) observed biochemical changes in penicillin-resistant staphylococci. They found that with an increase in resistance of from 250 to 60,000 fold, there was a progressive loss in physiological functions including the power to ferment sucrose, lactose, galactose, maltose, and mannitol. They also found that the strain which was 60,000 times more resistant than the parent strain no longer required nicotinamide as a growth factor. The resistant variant could be made susceptible again by growing in a medium deficient in amino acids. From results such as this it is very easy to see the rationale behind studies done on resistance in which the differences in biochemical characteristics and growth requirements are studied.

Hirsch et al. (1948) obtained from patients viridans streptococci which increased in resistance to penicillin, in vivo, from 2 to 2,048 fold. Changes in the general physical-chemical state of the organisms were noted but no specific changes were given. Gezon and Fason (1948) studied antigenic and enzyme changes in beta-hemolytic streptococci made resistant to penicillin, streptomycin, aureomycin, and bacitracin. After 40 transfers of the streptococci in suitable broth, the greatest increase in resistance was that for streptomycin. Penicillin and streptomycin were found to alter the behavior of the streptococci to a much greater extent than aureomycin or bacitracin. With increased penicillin and streptomycin resistance there was a decrease in general enzyme activity, while with aureomycin and bacitracin resistance there was little change from the parent strain. Changes in the production of streptolysin S, streptokinase, proteinase, and ribonuclease were studied. Nichols and Needham (1949) found that 54 penicillin-resistant staphylococci, isolated from bacteremias, were sensitive to aureomycin.

Streptomycin Resistance:

Miller and Bohnhoff (1946) developed streptomycin-resistant strains of gonococci and meningococococci in only 4 to 6 transfers with an increase over the parent strains of more than 5,000 fold. They found, however, that the streptomycin-resistant strains were susceptible to penicillin. In an investigation of streptomycin resistance in staphylococci Chandler and Schoenbach (1947) found that in the early logarithmic stage of growth, highly resistant mutants could be isolated by chance selection in a single transfer. Increased resis-

tance to streptomycin was maintained on subculture in streptomycin-free media and the organisms retained their original virulence. Berkman et al. (1948) studied the absorption of streptomycin by streptomycin-susceptible and resistant bacteria using strains of Staphylococcus aureus and Shigella dysenteriae. No difference between the resistant and susceptible strains was found. The metabolic properties of streptomycin-resistant and streptomycin-dependent strains of E. coli were studied by Smith et al. (1949). The resistant variants differed from the parent strains in that the resistant strains required lysine and no longer possessed an enzyme capable of causing the oxalacetate-pyruvate condensation in the citric acid cycle.

Aureomycin Resistance:

Paine et al. (1948) changed the resistance of Klebsiella pneumoniae in vitro from 6.2 mg to 25 mg/cc after 42 subcultures in broth containing aureomycin. One strain of S. mitis developed resistance to 12.5 mg/cc as compared to the 1.5 mg/cc in the parent strain after 21 subcultures. However, another strain showed no increase in resistance after 60 subcultures. They also found that aureomycin was as effective against penicillin-resistant as penicillin-sensitive staphylococci. The same was true for streptomycin-resistant staphylococci. Gezon and Fason (1950) studied aureomycin-resistant variants of beta-hemolytic streptococci developed in vitro. Resistance was increased among 23 strains of Group A, B, and C hemolytic streptococci 2 to 60 fold. Three strains lost their virulence for mice while the rest remained about the same. Only one of the 29 strains lost its ability to react with group specific serum. Increased aureomycin resistance

altered the enzymatic metabolism less than penicillin or streptomycin resistance. One strain had decreased streptokinase activity and 2 strains had decreased ribonuclease activity. No development of aureomycin resistance *in vivo* has been noted as yet.

Bacitracin Resistance:

Stone (1949) increased resistance to bacitracin *in vitro* in 4 strains of *Staphylococcus aureus* 50 to 60 fold. There was no increase in penicillin resistance in these strains. The bacitracin-resistant variants rapidly returned to their original sensitivity on subculture in bacitracin-free media. In this study after about 90 transfers, viridans streptococci strains did not show any increase in resistance to bacitracin.

Glutamic Acid and Resistance

Gale (1945) found that certain species of bacteria contain enzymes specific in decarboxylating six amino acids. *Clostridium welchii* SR 12 was found to contain only the enzymes capable of decarboxylating glutamic acid. By using this organism as the source of the decarboxylase it was possible to determine quantitatively the free glutamic acid inside any bacterial cell. Gram positive organisms, which require glutamic acid preformed, were found to accumulate the acid, while gram negative organisms which do not require it, did not show any evidence of its presence inside the cell (Gale, 1947 a). The possibility that certain growth inhibitory agents, particularly active against gram positive organisms, might be exerting their action by preventing the assimilation of glutamic, and other acids, through the cell wall, occurred to Gale. This was found to be the case with

penicillin (Gale and Taylor, 1947). With sulfathiazole, however, there was no interference with the passage of glutamic acid into the cell but the condensation of the acid into peptides and protein was prevented (Gale, 1947 b). Gale (1948) showed that in the early growth phase of staphylococci, two sulfathiazole-resistant strains had a higher content of glutamic acid than the two susceptible strains. This relationship did not hold in the later stages of growth. An attempt was made to correlate glutamic acid content with penicillin resistance, but no such relation could be shown. The penicillin-resistant strains which had been increased in resistance several thousand fold, however, became gram negative and lost their ability to concentrate free glutamic acid inside the cell (Gale and Rodwell, 1949).

III. EXPERIMENTAL DATA

Introduction

Evidence has been presented that strains of viridans streptococci at a certain level of sulfonamide resistance (developed *in vitro*) acquired the biochemical properties characteristic of enterococci. It seemed of interest, therefore, (A) to determine whether there was any relationship between biochemical properties and resistance to antibiotics and sulfathiazole of viridans streptococci and enterococci isolated from human sources; and (B) to study the same relationship among trained strains made resistance to the chemotherapeutic agents *in vitro*.

(A) Viridans Streptococci and Enterococci Isolated from Human Sources

Methods

Forty strains of non-hemolytic streptococci were used in this study. Thirty-seven strains were secured from the Bacteriology Laboratory of Colorado General Hospital, and 3 other strains were used as control organisms. The source of each culture is given in the following table:

Source	Viridans Type	Enterococcus	Total No.
Blood culture	34, 39, 40, 56	35, 42, 45, 54	8
Urine culture	27, 46, 70	52, 68, 69	6
Nose and throat cult.	1, 4, 6, 11, 16, 17, 18		7
Bronchial washings	48, 78	49	3
Uterine swab	25, 50, 58		3
Pus from thigh	23	47	2
Bone marrow	74		1
Colostomy opening		51	1
Pericardial swab	57		1
Pericardial sac		66	1
Rectal abscess	62		1
Spinal fluid		77	1
Vaginal swab	30		1
Heart vegetation	55		1
Amer. Type Culture Collection *S. fecalis*		9790	1
F. L. Horsfall	*Streptococcus* "MG"		1
H. W. Seeley	*Streptococcus* "SBE"		1

The tests for growth in broth and determinations of bacteriostatic concentrations were performed in Tryptose Phosphate Broth (TPB) (Difco) plus 0.1% Yeast Extract (Difco) at pH 7.2. In carrying out the various biochemical tests used for classification, one drop of an 18-24 hour culture was inoculated into the appropriate media and incubated for 24 hours or longer if needed, as indicated later. All tests were done in triplicate. The biochemical tests used for separating the viridans streptococci from the enterococci were the following:

(1) *Growth after exposure to 60°C for 30 minutes.* (Shattock and Mattick, 1944) Each organism was inoculated into 5 cc of TPB and put in a 60°C water bath for 30 minutes and then removed and incubated at 37°C for 18-24 hours. Positive tests were considered as any visible growth in the tube. If there was any doubt as to the turbidity indicating growth, the broth was subcultured on blood agar plates.

(2) Growth at 10°C. (Sherman, abs. 1937) Each organism was inoculated into 5 cc of TPB and placed in the refrigerator at 10°C for 24 hours. The test was considered positive by the same criteria as indicated for the 60°C test.

(3) Growth at 45°C. (Sherman, abs. 1937) Each organism was inoculated into 5 cc of TPB and incubated in a 45°C water bath for 24 hours. The test was considered positive by the same criteria as indicated for the 60°C test.

(4) Litmus milk. (MacCallum and Hastings, 1899) 5 cc were inoculated with 1 drop of the culture and the reaction was considered positive if the litmus milk became acid first and then curdled, followed by reduction of the litmus.

(5) Growth at pH 9.6. (Shattock and Hirsch, 1947; Sherman and Stark, 1934) 1N NaOH was added to Difco Brain Heart Infusion Broth and the pH adjusted. Tryptose Phosphate Broth was not used here, as the high pH precipitated out some of the phosphates and made it an undesirable media. Visible growth in 5 cc of pH 9.6 broth constituted a positive test.

(6) 0.1% methylene blue milk. (Sherman, Mauer, and Stark, 1937) Methylene blue was added to fresh skim milk to make an 0.1% concentration and the pH adjusted to 7.2. The test was considered positive if the milk curdled and the dye was reduced.

(7) Growth in 6.5% NaCl. (Sherman, Mauer, and Stark, 1937) NaCl was added to TPB to a concentration of 6.5%. Any visible growth was read as a positive test.

(8) Growth on 40% bile agar. (Evans and Chinn, 1947) Difco Bacto-Oxgall Solution was added to TPB agar to make a concentration of

40% bile. One loopful of a 24 hour broth culture was streaked on a plate and any visible growth on the agar plates after incubation was considered to be a positive test.

Four tests were used to differentiate the more common so-called "viridans" species: S. mitis (Sherman, 1937), S. salivarius (Sherman, Niven, and Smiley, 1943), Streptococcus "SBE" (White and Niven, 1946), and Streptococcus "MG" (Mirick, Thomas, et al., 1944, and Swift, 1948). These four tests were performed as follows:

(1) 5% sucrose agar. (Niven, Smiley, and Sherman, 1941) Five per cent sucrose was added to nutrient agar and plates made. Each viridans culture was streaked on the plate and incubated for 24 hours and the type of colony growth was noted. This test is diagnostic for S. salivarius as it is the only viridans type which forms very large typical mucoid-like colonies. It is also used for Streptococcus "MG" which grows in very small colonies that are fluorescent to ultraviolet light. However, the one culture of Streptococcus "MG" used did not do this.

(2) 5% sucrose broth. (Niven, Kizuita, and White, 1946) Five per cent sucrose was added to a nutrient broth and inoculated with 1 drop of an 18-24 hour culture of the organism and incubated for 7 days. "Jelling" of the broth, or increased viscosity, is considered to be a positive test. This test is diagnostic for Streptococcus "SBE".

(3) Fermentation of inulin. Inulin and brom cresol purple in the concentration of 1% each were added to nutrient broth. Production of acid was considered a positive test. This fermentation is

quite important when combined with 5% sucrose agar, 5% sucrose broth, and the production of ammonia in differentiating the types of viridans streptococci.

(4) Production of ammonia. (Mirick, Thomas, et al., 1944) A 4% peptone nutrient broth was inoculated and incubated at 37°C for 4 days. Ammonia was considered produced when the supernatant liquid mixed with Nessler's reagent gave a darker color than a control tube of uninoculated medium plus Nessler's reagent. This test was carried out on only 6 of the cultures, as it is of value in determining the difference between Streptococcus "SBE" and Streptococcus "MG", and only for these 6 cultures was this a possibility.

The sensitivity of each organism to antibiotics and sulfathiazole was determined. The sensitivity tests were performed in 5 cc of TPB containing various concentrations of the antibiotics, dilutions being five fold. All determinations were done at least in triplicate. The antibiotics used were:

(1) Sodium sulfathiazole - Merck
(2) Aureomycin HCl - Lederle Laboratories *
(3) Bacitracin - The Upjohn Company *
(4) Penicillin G Potassium - Squibb
(5) Streptomycin sulfate - Squibb

An 18-24 hour culture of each organism was diluted 1:1,000 and 1 drop from a 1 cc pipette was used for inoculation. The inoculum

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* The author is indebted to the Lederle Laboratories for kindly supplying the Aureomycin and to The Upjohn Company for the Bacitracin.

size of the viridans streptococci and enterococci cultures was adjusted to the same turbidity by visual inspection before diluting 1:1,000. This equalization was necessary because of the much more rapid growth of the enterococci as compared to the viridans streptococci for the same amount of time. All tests were incubated for 18-24 hours at 37°C and the level of inhibition was considered as the lowest concentration completely preventing growth. In the case of very slow growing organisms the result was read whenever the control tube containing no antibiotic or sulfathiazole showed good growth.

Results

Biochemical Tests Used for Classification and Their Correlation

The results are shown in Table I. The 27 viridans streptococci are listed first, and then the 13 enterococci. The first 8 viridans streptococci listed are the ones used to make resistant variants, in vitro, which are discussed later in Part B.

From Table I it can be seen that of the 3 temperature tests for resistance, growth at 45°C is the only one which appears to be of much value under the conditions of our experiments. This does not agree with the findings of some workers, particularly for enterococci (Sherman, abs. 1937; Sherman, Mauer, and Stark, 1937; Sherman, 1938) but does agree with others (Shattock and Hirsch, 1947). All enterococci are usually listed as being resistant to 60°C for one-half hour, and growing at 10°C for 24 hours. Of the 13 enterococci listed here, only 7 or about 50% grew after holding at 60°C; and only 4 or about 30% grew at 10°C. Only 1 culture of the 27 viridans grew at 45°C

TABLE I

Biochemical Characteristics of Viridans Streptococci and Ent

	VIRIDANS STREPTOCOCCI																								
Culture # ..	1	4	6	17	23	27	34	57	11	16	18	25	30	39	40	46	48	50	55	56	58	62	70	74	78	MG
60°C - ½ hr	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
Litmus Milk	0	0	0	+	+	+	sl	0	0	0	0	sl	0	0	0	0	sl	+	sl	+	sl	+	+	+	+	+
pH 9.6 Broth	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	+	0	0	0
.1% Methylene Blue Milk	0	0	0	0	0	sl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6.5% NaCl Broth	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
40% Bile Agar	0	sl	sl	0	0	0	0	0	sl	0	0	0	0	0	sl	0	0	0	0	0	0	0	sl	0	0	0
5% Sucrose Agar *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0
5% Sucrose Broth **	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inulin ***	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	+	+	0	+	0
NH_4 ****											+											0	0		0	+

\+ indicates growth in 24 hours unless otherwise indicated.
0 " no " " " " " " " "

* indicates formation of mucoid colonies.
** " increase in viscosity of broth.
*** " fermentation.
**** " production of ammonia in 4% peptone broth.

BLE I

iridans Streptococci and Enterococci

CI										ENTEROCOCCI													
50	55	56	58	62	70	74	78	MG	SBE	35	42	45	47	49	51	52	54	66	68	69	77	9790	
0	0	0	0	0	0	0	0	0	0	+	0	0	+	+	0	0	0	+	+	+	0	+	
0	0	0	0	0	0	0	0	0	0	+	0	0	+	0	+	0	0	0	0	+	0	0	
0	0	+	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	
+	sl	+	sl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
0	0	0	0	0	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
0	0	0	0	0	0	0	0	0	sl	+	+	+	+	+	+	+	+	+	+	+	+	+	
0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	
0	0	0	0	0	sl	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	
0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	+	+	0	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	
				0	0		0	+	+														

which does not agree with the findings of Sherman (abs. 1937) or Mirick et al. (1944), who found most of the viridans group grew at 45°C. The explanation for this could be the difference under which the conditions of the test were carried out. Incubation at 45°C for 24 hours was used in this study, while the other workers used incubation times of 7 days.

The litmus milk test was 100% positive for the enterococci, but also almost 50% positive for the viridans group. Similar results were found in the broth at pH 9.6.

The next three tests listed--0.1% methylene blue milk, 6.5% NaCl, and 40% bile agar--seemed to give the greatest correlation for both groups in addition to 45°C as stated earlier. All of the group tentatively designated as enterococci were positive for all three tests, while the viridans gave only a few slight reactions for the 0.1% methylene blue milk and 40% bile agar, and one positive result for 6.5% NaCl broth.

It is interesting to note that the only organism found with the biochemical properties of an SBE streptococcus was from a nose and throat culture and not from any of the 8 blood cultures. Streptococcus "SBE" was first described and named as such because it was found in about 40% of the cases of subacute bacterial endocarditis caused by various streptococci (Loewe, et al., 1946). Since the only Streptococcus "MG" culture available to use as a control did not produce fluorescent colonies on 5% sucrose agar--a test described as diagnostic for this culture (Mirick, et al., 1944)--it did not seem worthwhile to test all the viridans group for this property.

In view of present knowledge it would seem best to use the 5% sucrose agar, 5% sucrose broth, and inulin for further subdivision of the viridans group. The main type at present which needs to be identified as soon as possible is Streptococcus "SBE". The concentration of penicillin necessary to inhibit growth of this organism in the test tube bears little relation to the effective dosage in the human body. Therefore, patients known to be suffering from an infection due to Streptococcus "SBE" are given a much larger dosage of penicillin at the beginning of treatment (Loewe, Alture, and Werber, 1946; Schlichter, McLean, and Milzer, 1949).

The enterococci were not classified further into species or types, as there is still a question as to there being more than one species, S. fecalis (Evans and Chinn, 1947; Topley and Wilson, 1946).

Sensitivity Tests to Sulfathiazole and Four Antibiotics

The results of the sensitivity tests carried out on the 40 organisms are shown in Table II. The results of the four best correlated biochemical tests are repeated in order to show the relationship between the biochemistry of the organisms under study and their sensitivity to certain chemotherapeutic agents.

The sensitivity levels in Table II agree quite well with those found by other workers for viridans streptococci and enterococci (Bliss and Chandler, 1948; Bliss and Todd, 1949; Jawitz, Gunnison and Coleman, 1950; Jawitz and Gunnison, 1950).

TABLE II

Relation of the Biochemical Properties of Viridans Streptococci to Growth-Inhibiting Concentrations of Sulfathiazole, Aureomycin, Bacitracin

VIRIDANS STREPTOCOCCI

CULTURE #	1	4	5	17	23	27	34	57	11	16	18	25	30	39	40	46	48	50	55	56	58	62	70	74	78	MG
Biochemical Reactions																										
0.1% Methylene Blue Milk	0	0	0	0	0	sl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6.5% NaCl	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
40% Bile Agar	0	sl	sl	0	0	0	0	0	sl	0	0	0	0	0	sl	0	0	0	0	0	0	0	sl	0	0	0
45°C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
Inhibiting Concentrations																										
Sulfathiazole *	.1	.1	.1	.1	.1	1	5	.1	1	.1	.1	5	1	.1	.1	.1	.1	.01	1	.1	.01	1	10	.1	.01	10
Aureomycin **	.1	.1	.1	.01	.1	.1	.1	.1	.1	.01	.1	.1	.1	.1	.1	.01	.1	.1	.1	.1	.1	.5	.5	.5	.5	
Bacitracin **	.1	.01	.01	.001	.01	.01	.1	.01	.1	.01	.01	.01	.1	.01	.1	.01	.01	.01	.1	.01	.01	.01	.1	.1	.1	.1
Penicillin ***	.5	.1	.5	.1	.1	5	.1	.1	.1	.01	.01	.1	1	.1	.1	.01	.1	.1	.01	.1	.1	.1	.1	.1	.1	.1
Streptomycin**	5	5	5	1	5	10	1	1	1	5	5	5	5	1	5	5	5	5	5	5	1	.5	1	50	5	1

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc
sl = slight reactions

TABLE II

Properties of Viridans Streptococci and Enterococci
ılfathiazole, Aureomycin, Bacitracin, Penicillin, and Streptomycin

PTOCOCCI													ENTEROCOCCI												
40	46	48	50	55	56	58	62	70	74	78	MG	SBE	35	42	45	47	49	51	52	54	66	68	69	79	9790
Biochemical Reactions																									
0	0	0	0	0	0	0	0	0	0	0	0	sl	+	+	+	+	+	+	+	+	+	+	+	+	+
0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+
sl	0	0	0	0	0	0	0	sl	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+
0	0	0	0	0	+	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+
nhibiting Concentrations																									
.1	.1	.1	.01	1	.1	.01	1	10	.1	.01	10	1	30+	30+	30+	30	30	30	20	20+	30	20	30	30	+30
.1	.01	.1	.1	.1	.1	.1	.1	.5	.5	.5	.5	.1	.5	.5	.5	.5	.5	.1	.5	.5	1	.5	.5	5	5
.1	.01	.01	.01	.1	.01	.01	.01	.1	.1	.1	.1	.001	.5	.1	.1	.1	.1	.5	.1	.5	1	.5	.5	1	.5
.1	.01	.1	.1	.01	.1	.1	.1	.1	.1	.1	.1	.1	5	5	10	5	5	20	10	5	.5	5	5	5	10
5	5	5	5	5	5	1	.5	1	50	5	1	5	100	50	50	50	50	100	50	100	100	100	100	100	50

Summarizing the results of Table II, in Table III are the median values of each antibiotic and sulfathiazole for the viridans streptococci and the enterococci.

TABLE III

Median Values of Growth-Inhibiting Concentrations of Sulfathiazole, Aureomycin, Bacitracin, Penicillin, and Streptomycin

	VIRIDANS STREPTOCOCCI	ENTEROCOCCI
Sulfathiazole*	.1	30.
Aureomycin**	.1	.5
Bacitracin**	.01	.5
Penicillin***	.1	5.
Streptomycin**	5.	100.

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc

A study of these values indicates that the enterococci are, in general, more resistant to all of the agents tested. The least increase is shown for aureomycin and streptomycin, the greatest for sulfathiazole. A comparison of these *in vitro* values to those clinically found treatable by other investigators (Neter, 1938; Rantz and Kirby, 1943; Fleming, 1929; Bornstein, 1940; Heilman and Herrell, 1942; Watson, 1944; Jawitz, Gunnison, and Coleman, 1950; Bliss and Todd, 1949) indicates that the enterococci would be resistant to treatment with sulfathiazole, penicillin, and streptomycin, but might be expected to respond to aureomycin and bacitracin. The viridans group show an *in vitro* level which indicates that any of the 5 agents might be effective for treatment.

The difference between the inhibiting concentrations of sulfathiazole and penicillin for the two groups was of great enough magnitude that it seemed some value might be chosen between that for viridans and that for enterococci which would make a useful test for differentiation. Different concentrations of sulfathiazole and penicillin were tried in preliminary experiments, and it was found that to differentiate between viridans streptococci and enterococci, TPB broth containing 10 mg/cc of sulfathiazole, and TPB broth containing 2 units/cc of penicillin seemed to give the most clear-cut results. The choice of these values may seem rather high in view of the definite antibiotic levels, but in these tests it was desirable to use 1 drop of an 18-24 hour culture of the organism without adjusting the size of the inoculum as was done in the sensitivity test.

Five cubic centimeters of TPB containing sulfathiazole or penicillin in the concentrations given above were inoculated and each tube was incubated for 37°C for 18-24 hours, and any turbidity read as a positive test. These two tests proved very successful in separating the viridans streptococci from the enterococci already classified by biochemical tests, and are highly correlated with the three better biochemical tests. This is shown in Table IV.

TABLE IV

Correlation of Three Biochemical Tests with Sulfathiazole and Penicillin Sensitivity

DIFFERENTIAL TEST	13 ENTEROCOCCI	27 VIRIDANS STREPTOCOCCI
0.1% Methylene Blue Milk	13 *	2 (sl)
6.5% NaCl	13	1
40% Bile Agar	13	5 (sl)
10 mg/cc Sulfathiazole	13	2
2 units/cc Penicillin Broth	13	1

* number with positive test
sl = slight reaction

Therefore, if these two tests were used in addition to the three biochemical tests--0.1% methylene blue, 6.5% NaCl, and 40% bile agar--one would have not only five correlated biochemical tests for differentiating enterococci from viridans, but also would have an indication at the very first of the resistance of the organism. This would prove valuable, both to the bacteriologist in the diagnostic laboratory and also to the clinician in starting chemotherapeutic measures.

(B) Resistant Strains Developed in vitro

Methods

Strains of viridans streptococci resistant to sulfathiazole, aureomycin, penicillin, and streptomycin were prepared by continuous

serial subculture in TPB in the presence of increasing concentrations of the chemotherapeutic agents named. The first 8 of the 27 viridans streptococci listed in Table I were used in developing these resistant variants. The final number of resistant variants developed were as follows: sulfathiazole 8, aureomycin 6, penicillin 5, and streptomycin 6. Strains resistant to bacitracin did not develop, even though subcultured many times. Each resistant variant was inoculated into 5 differential media in the same way as described previously for the streptococci isolated from human sources. However, the sulfathiazole broth of 10 mg/cc or the penicillin broth of 2 units/cc was not used, since preliminary sensitivity levels done on most of the resistant variants gave levels to both penicillin and sulfathiazole beyond the range of these tests. Sensitivity levels were determined to sulfathiazole and the 4 other antibiotics as before.

The free glutamic acid content of viridans streptococci, enterococci, and representative resistant strains was determined because Gale (1948) had shown with 4 strains of Staphylococcus aureus that there is a correlation between sulfathiazole resistance and the height of the level of free glutamic acid in the young cells. The method used for determining the glutamic acid content of the cells of the different resistant variants was that developed by Gale (1945). This method depends upon manometric measurement of CO_2 liberated from the amino acid by the specific decarboxylase for that acid at the optimum pH and temperature for that decarboxylase. The glutamic acid decarboxylase used here was obtained from a 12-16 hour culture of Clostridium welchii, SR 12, which had been shown by Gale (1945) to be specific for l-glutamic acid. The Clostridium welchii was grown in 800 cc of TPB

plus 0.1% yeast extract and 2% glucose. After 12-16 hours the organisms were harvested, washed twice in distilled water, and made up to 25 cc with M/5 acetate buffer of pH 4.5, which is the optimum pH for this decarboxylase. Each organism was grown in 300 cc of TPB plus 0.1% yeast extract, and harvested at different times, washed twice with distilled water, and made up in about 3.5 cc of distilled water. The cell suspension was divided into two parts--one was used as a control, the other was placed in boiling water for 10 minutes to release free glutamic acid inside the cell. One cubic centimeter of the control was put in a crucible, dried and weighed. Into the well of each Warburg flask was measured 1 cc of the cell suspension, 1.2 cc of M/5 acetate buffer, and into the side arm was measured 1 cc of the decarboxylase preparation. Each determination was done in duplicate.

Since Gale (1947) had shown that the free glutamic acid content of S. fecalis varied at different times in the growth cycle, by preliminary experiments covering the whole growth curve, it was found that maximum values of free glutamic acid would be found at the times chosen in our experiments.

Results

Biochemical Tests Used for Classification and Their Correlation

The aureomycin, penicillin, and sulfathiazole-resistant variants were all positive for 0.1% methylene blue milk, 6.5% NaCl, and 40% bile agar, thus resembling the enterococci in biochemical characteristics. However, the streptomycin-resistant strains showed considerable variation. These results are shown in Table V.

TABLE V

Biochemical Properties of Streptomycin-Resistant Variants of Viridans Streptococci

Culture #	1	4	6	17	23	57
Fold increase in resistance to Streptomycin	200	200	200	1,000	1,000	10,000
0.1% Methylene Blue Milk	0	0	0	0	0	+
6.5% NaCl	0	+	+	+	0	+
40% Bile Agar	+	+	0	0	+	+

Sensitivity Tests of Resistant Variants to Sulfathiazole and 4 Antibiotics

The results are shown as follows:

Sulfathiazole-resistant variants - Table VI
Aureomycin-resistant variants - " VII
Penicillin-resistant variants - " VIII
Streptomycin-resistant variants - " IX

TABLE VI

Sulfathiazole-Resistant Variants

Growth-Inhibiting Concentrations of Sulfathiazole, Aureomycin, Bacitracin, P

Culture Number	SULFATHIAZOLE*			AUREOMYCIN**			BACITRACIN**			
	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	P S
1	.1	+ 30	300	.1	1.	10.	.1	.1	0	
4	.1	+ 30	300	.1	.5	5.	.01	.05	5	
6	.1	+ 30	300	.1	.5	5.	.01	.05	5	
17	.1	+ 30	300	.01	1.	100.	.001	.05	50	
23	.1	+ 30	300	.1	.5	5.	.01	.05	5	
27	1.	+ 30	30	.1	1.	10.	.01	.05	5	
34	5.	+ 30	6	.1	.5	5.	.1	.1	0	
57	.1	+ 30	300	.1	1.	10.	.01	.5	50	

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc

TABLE VI

thiazole-Resistant Variants

thiazole, Aureomycin, Bacitracin, Penicillin, and Streptomycin

**	BACITRACIN**			PENICILLIN***			STREPTOMYCIN**		
Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase
10.	.1	.1	0	.5	10	20	5.	25	5
5.	.01	.05	5	.1	10	100	5.	25	5
5.	.01	.05	5	.5	10	20	5.	25	5
100.	.001	.05	50	.1	10	100	1.	25	25
5.	.01	.05	5	.1	10	100	5.	25	5
10.	.01	.05	5	5	10	2	10.	25	2.5
5.	.1	.1	0	.1	10	100	1.	25	25
10.	.01	.5	50	.1	10	100	1.	100	100

TABLE VII

Aureomycin-Resistant Variants

Growth-Inhibiting Concentrations of Aureomycin, Sulfathiazole, Bacitracin, Pe

Culture Number	AUREOMYCIN**			SULFATHIAZOLE*			BACITRACIN**			
	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Pa St
1	.1	10	100	.1	+ 30	300	.1	.1	0	
4	.1	10	100	.1	+ 30	300	.01	.5	50	
6	.1	30	300	.1	+ 30	300	.01	.5	50	
17	.01	20	2,000	.1	+ 30	300	.001	.5	500	
27	.1	10	100	1	+ 30	30	.01	.5	50	
57	.1	10	100	.1	+ 30	300	.01	.5	50	

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc

TABLE VII

ureomycin-Resistant Variants

omycin, Sulfathiazole, Bacitracin, Penicillin, and Streptomycin

E*	BACITRACIN**			PENICILLIN***			STREPTOMYCIN**		
Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase
300	.1	.1	0	.5	10	20	5	20	4
300	.01	.5	50	.1	5	50	5	30	6
300	.01	.5	50	.5	5	10	5	20	4
300	.001	.5	500	.1	5	50	1	3	30
30	.01	.5	50	.1	5	50	10	100	10
300	.01	.5	50	.1	5	50	1	100	100

TABLE VIII

Penicillin-Resistant Variants

Growth-Inhibiting Concentrations of Penicillin, Sulfathiazole, Aureomycin, Bac

Culture Number	PENICILLIN***			SULFATHIAZOLE*			AUREOMYCIN**			
	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Par Str
1	.5	200	400	.1	20	200	.1	1	10	
4	.1	100	1,000	.1	+ 30	300	.1	1	10	
6	.5	200	400	.1	+ 30	300	.1	.5	5	
17	.1	100	1,000	.1	+ 30	300	.01	1	100	
57	.1	100	1,000	.1	+ 30	300	.1	5	5	

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc

TABLE VIII

icillin-Resistant Variants

illin, Sulfathiazole, Aureomycin, Bacitracin, and Streptomycin

**	AUREOMYCIN**			BACITRACIN**			STREPTOMYCIN**		
Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase
200	.1	1	10	.1	.1	0	5	5	0
300	.1	1	10	.01	.05	5	5	5	0
300	.1	.5	5	.01	.05	5	5	5	0
300	.01	1	100	.001	.1	100	1	5	5
300	.1	5	5	.01	.5	50	1	100	100

TABLE IX

Streptomycin-Resistant Variants

Growth-Inhibiting Concentrations of Streptomycin, Sulfathiazole, Aureomycin, [illegible]

Culture Number	STREPTOMYCIN**			SULFATHIAZOLE*			AUREOMYCIN**			
	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Par Str
1	5	1,000	200	.1	.5	5	.1	.1	0	
4	5	1,000	200	.1	.5	5	.1	.1	0	
6	5	5,000	200	.1	.5	5	.1	.5	5	
17	1	5,000	5,000	.1	5	5	.01	.5	50	
23	5	5,000	1,000	.1	5	5	.1	.5	5	
57	1	+10,000	10,000	.1	+30	30	.1	1	10	

* inhibiting concentration in mg/cc
** " " " ug/cc
*** " " " units/cc

TABLE IX

tomycin-Resistant Variants

omycin, Sulfathiazole, Aureomycin, Bacitracin, and Penicillin

E*	AUREOMYCIN**			BACITRACIN**			PENICILLIN***		
Fold crease	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase	Parent Strain	Resistant Variant	Fold Increase
5	.1	.1	0	.1	.1	0	.5	.5	0
5	.1	.1	0	.01	.01	0	.1	.5	5
5	.1	.5	5	.01	.05	5	.5	.5	0
5	.01	.5	50	.001	.01	10	.1	.5	5
5	.1	.5	5	.01	.1	10	.1	.5	5
30	.1	1	10	.01	2	200	.1	5	5

Table VI shows that half of the sulfathiazole-resistant variants increased 10 fold or more in their resistance to aureomycin, and all but 1 of the 8 increased 20 fold or more to penicillin. The increased resistance shown to bacitracin and streptomycin is considerably less. Table VII shows a decided increase of resistance in most of the aureomycin-resistant variants to sulfathiazole, penicillin, and bacitracin, with a definitely smaller increase of resistance to streptomycin. In Table VIII we find the penicillin-resistant variants to show some increase in resistance to bacitracin and aureomycin, very similar in magnitude to that of the sulfathiazole-resistant variants. Again, there is in all cases but one little or no increase in resistance to streptomycin. The streptomycin-resistant variants (Table IX), while showing some small increases with individual strains, in general had little increase in resistance to sulfathiazole, aureomycin, bacitracin, or penicillin. It is interesting that it is streptomycin resistance which does not accompany other resistance increases with any of the variants, either the ones trained to resistance against streptomycin, or those trained to resistance to sulfathiazole or the other antibiotics. This difference in streptomycin-resistant trained strains was also observed in their biochemical properties which were variable, while those of all the other trained strains resembled the enterococci.

The results shown in these four tables may be of some value as a preliminary suggestion for the best antibiotic or combination of agents to treat a viridans or enterococcus resistant to any of the agents discussed. For example, bacitracin might be expected to be more effective than others against sulfathiazole and penicillin-resistant strains, and any of the four might still be active in inhibiting

growth of the streptomycin-resistant strains. They may also indicate what kind of resistance may be expected if such organisms emerge under treatment. The results do show that, although most reports have indicated no cross-resistance for sulfathiazole and the better known antibiotics, this group of organisms shows a great deal of cross-resistance, even though the magnitude of the increase in many cases was not very great. Finally, the survey shows that there is considerable variation in the response to other antibiotics and in biochemical properties, depending upon the agent which has brought about the increased resistance and the organism whose resistance has been increased.

Free Glutamic Acid Content

The results of the free glutamic acid content determined manometrically are given in Table X. The free glutamic acid content of the cells is greatest at the time when they have just reached maximum growth. This was also reported by Gale (1947 a). Gale had noted that a few sulfathiazole-resistant organisms appeared to have higher concentractions of glutamic acid. The results show all the susceptible viridans strains to have no free glutamic acid, while two strains of S. fecalis and all 8 sulfathiazole-resistant strains had very appreciable amounts. The aureomycin and penicillin-resistant trained strains also showed a high internal concentration of free glutamic acid, which might be expected if all sulfathiazole-resistant variants have a high content, since sulfathiazole-resistance did increase as aureomycin and penicillin-resistance developed. All but one of the streptomycin-resistant strains showed no glutamic acid. This single strain

was also the only one in which an increase in resistance to sulfathiazole was observed.

TABLE X

Glutamic Acid in Internal Environment
(ul/100 mg cells)

Hours of Growth at 37°C		6	8	10-12
CULTURE NUMBER				
Viridans	1		0	0
	4	0	0,0	0
	6		0	0,0
	17			0
	23			
	27			
	34			0
	57			
	18		0,0	0
	46		0	0
S. fecalis	9790	172, 163	432, 246	300,205
	47	68, 156	113, 260	173, 162
Sulfathiazole-Resistant Variant ...	1	168	129, 220	149, 145
	4	142	166, 128	321, 181
	6	228, 226	261, 311	234, 203
	17	137, 134		257, 168
	23		118	180
	27		170	201
	34	100		201
	57	284	399	353
Aureomycin-Resistant Variant	1		347	351
	4		208	234
	6		258	262
	17	158		187
	57	179	333	234
Penicillin-Resistant Variant	1		129	175
	4		372	378
	6		301	234
	17	290		204
	57		308	328
Streptomycin-Resistant Variant ...	1			
	4			
	6			
	17			
	23			
	57		388	309

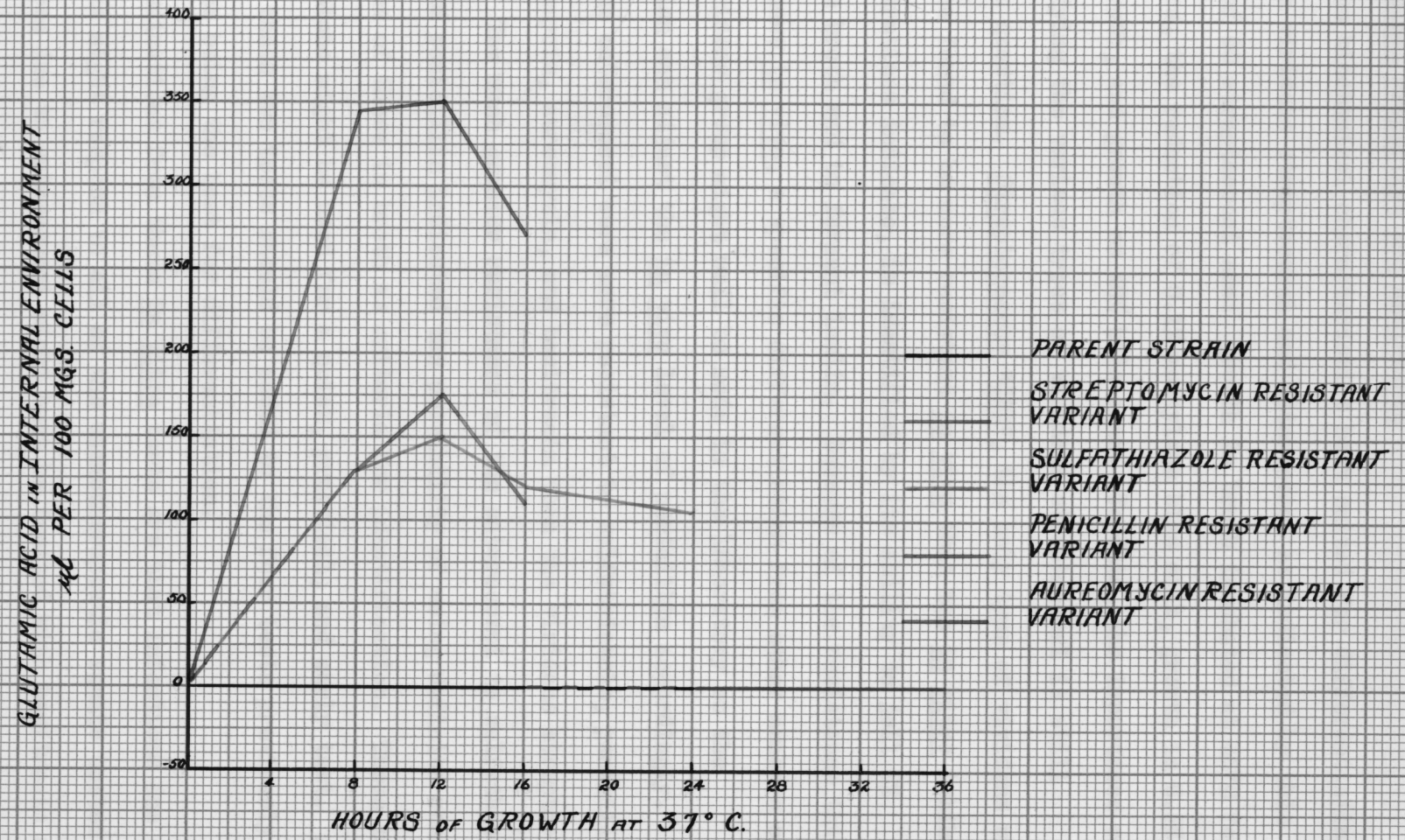
TABLE X

d in Internal Environment
/100 mg cells)

8	10-12	16-18	24	36
0	0	0,0	0,0	
0,0	0	0	0	
0	0,0	0,0	0	
	0	0	0,0	
			0	
			0	
	0	0	0,0	
			0	
			0	
0,0	0			
0	0	0	0,0	
432, 246	300,205	287	263	
113, 260	173, 162			
129, 220	149, 145	120	105	
166, 128	321, 181	124, 92		
261, 311	234, 203	118	68	
	257, 168	107,114	284	
118	180	113		
170	201	135		
	201	111		
399	353	162		
347	351	270		
208	234	80		
258	262	235		
	187	174		
333	234	139		
129	175	107		
372	378	360		
301	234	161		
	204	140		
308	328	183		
		0	0	0
		0	0	0
		0	0	0
		0	0	0
		0	0	0
388	309	147		

To show more clearly the relationship of glutamic acid content of the parent strains to the resistant variants, the values for organism #1 (which is typical of all other findings) are plotted graphically in Table XI.

FREE GLUTAMIC ACID CONTENT OF #1 VIRIDANS STREPTOCOCCI AND FOUR RESISTANT VARIANTS



IV. CONCLUSIONS

Biochemical Properties and Resistance

The biochemical characteristics best suited for differentiating between viridans streptococci and enterococci as indicated by this study of 40 streptococci are: growth in broth incubated at 45°C for 24 hours, the reduction of 0.1% methylene blue milk, growth in 6.5% NaCl broth, and growth on 40% bile agar. To date, the importance of identifying the various types of viridans streptococci is still open to question. Streptococcus "SBE", the only type of viridans which has been found to be more resistant in vivo than the in vitro inhibitory concentrations indicate, is identified by its ability to jell 5% sucrose broth and to ferment inulin. Because of the resistance this type shows in vivo, a specific differentiation from other members of the viridans group might well be made in the laboratory so the clinician can adjust the in vivo level of penicillin accordingly.

The enterococci were more resistant to sulfathiazole, penicillin, streptomycin, aureomycin, and bacitracin than the viridans streptococci. The much greater resistance of the enterococci to sulfathiazole and penicillin indicated the possibility of using definite concentrations of these in broth as criteria for identification. These two tests alone might be used for a quick, practical, and early test. At least, these two tests should be added to the four tests previously named as being the best for differentiation.

Indications from these in vitro tests are that infections due to viridans might respond to all of the chemotherapeutic agents tested but those due to enterococci might best be treated with bacitracin or aureomycin.

Resistant Strains Developed in vitro

The most interesting findings in the development of resistant strains was the cross-resistance, and the difference between the streptomycin-resistant variants as compared to the others. This may be related to the fact that streptomycin resistance develops rapidly, while resistance to sulfathiazole and the other antibiotics develops more slowly in step-wise fashion according to Demerac (1948). The variant strains selected by sulfathiazole, aureomycin, and penicillin all show the same biochemical properties as a typical enterococcus. Those selected by streptomycin vary in their biochemical properties and often do not have the properties of enterococci. This may be a further indication that the agent determines the kind of variant and does not select naturally-occurring mutants that would be found even in the absence of the agent.

Glutamic Acid Content

The results of the glutamic acid determinations show that appreciable amounts are found free inside those cells which have acquired sulfathiazole resistance. Whether that resistance has been induced by sulfathiazole or accompanies increased resistance to other agents makes no difference. It may be, as Gale has suggested, that the glutamic acid inside the cells reverses the sulfonamide action since it has been shown to be capable of doing this for some cells when added to the external environment. In the two resistant strains of Staphylococcus aureus studied by Gale and Rodwell (1948), a higher content of glutamic acid was observed only in the early growth phase. Later at the end of the active growth the content of two susceptible

strains was found to be higher than that of one of the resistant strains. The resistant strains used were naturally resistant, while those reported in this study had been trained to resistance. Whether the resistance is due to the increase in internal glutamic acid, or whether this is merely a result of other changes which determine the organism's resistance, is of course not answered by this study. This ability to concentrate glutamic acid was also shown by two strains of S. fecalis, and adds another property common to sulfathiazole-resistant organisms and S. fecalis. It is very interesting that the viridans group showed no free glutamic acid at any stage of growth, since Gale found all gram positive organisms to be capable of accumulating this acid inside the cell.

V. SUMMARY

1. Reduction of 0.1% methylene blue milk, growth on 40% bile agar, in 6.5% NaCl broth, in broth at 45°C, in sulfathiazole broth of 10 mg/cc, and in penicillin broth of 2 units/cc were the biochemical tests which correlated best for differentiating the viridans streptococci from the enterococci.

2. Sensitivity tests *in vitro* indicated that the enterococci were more resistant to sulfathiazole, penicillin, streptomycin, aureomycin, and bacitracin than the viridans group. The concentrations required to inhibit growth suggested that the viridans streptococci might respond to treatment with all of the chemotherapeutic agents, but the enterococci only to aureomycin and bacitracin.

3. Cross-resistance was observed in most resistant strains for all agents. However, streptomycin-resistant strains showed considerable variation, both as to cross-resistance and biochemical properties.

4. Organisms acquiring sulfathiazole resistance, either by growing in sulfathiazole or in the antibiotics, showed appreciable quantities of free glutamic acid, which was highest at the period when the organisms had just reached maximum growth. This was also observed with *S. fecalis*. All non-resistant strains of the viridans group showed no glutamic acid inside the cells at any growth phase.

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