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Studies on Restaurant Sanitation and the Effect of Sodium Azide on the Physiology of *Streptococcus salivarius*

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TABLE OF CONTENTS

PART I

STUDIES ON RESTAURANT SANITATION

PART II

EFFECT OF SODIUM AZIDE ON THE PHYSIOLOGY OF STREPTOCOCCUS SALIVARIUS

PART I

Studies on Restaurant Sanitation

Whether restaurant eating utensils serve as vectors in the transmission of respiratory and related diseases is still a matter for question, in spite of the overwhelming evidence in support of this contention. This is due largely to the lack of epidemiological evidence, the tracing of an outbreak of a disease directly and unquestionably to contaminated eating utensils. The very nature of the vectors concerned obviates any possibility of obtaining this epidemiological evidence, since the contaminating agent exists as such only momentarily at the best. This research problem was undertaken with the hope that a technique designed to demonstrate the appearance of streptococci of unquestionable human origin on improperly washed glasses would further the contention that eating utensils are highly potent carriers of disease.

LITERATURE SURVEY

Techniques for sampling total bacteria on restaurant ware: Techniques for the determination of total bacteria counts on eating utensils are almost as numerous as the publications concerning restaurant sanitation. In a pamphlet published by the Cup and Container Institute, methods of 19 public health departments are listed, all varying in some phase of the procedure. In the main, all follow the procedure first introduced by Manheimer and Ybanez (1917) which consisted of swabbing the rim of a glass with a sterile cotton swab and plating out aliquots of the

saline washings from the swab in nutrient agar. Modifications of this technique have been numerous. Cumming and Yongue (1936) outlined a "multiple utensil test" in which one swab was employed in the testing of ten separate utensils, the swab being agitated between utensils in 10 ml. sterile saline. Both glasses and plates were tested in this manner. Silverware was tested by agitating ten utensils in a sterile mason jar containing 200 ml. saline. Suspensions thus obtained were plated in the usual manner. Marden, et al (1938) streaked one glass from each establishment around the lip, one inch down, and suspended the swab in 10 ml. sterile water. After agitation, aliquots were placed into nutrient agar and nutrient broth, the latter being used for inocula into various differential media after incubation overnight. MacDonald and Freeborn (1933) sampled a spoon, fork, cup, tumbler, knife, plate, table top, drainboard, and shelf, each with a sterile swab which was then placed in beef infusion broth and incubated one hour at 37°C prior to plating in agar. Mallmann (1940) standardized his procedure by streaking three times around the inside and outside of each glass or on a two square inch area of a plate. The swab was suspended in a test tube with 0.75 ml. of water and the suspension later plated into nutrient agar. It is apparent that such a diversity of methods could only lead to confusion in an interpretation of results.

Fellers, et al (1936) critically studied 18 various techniques then in use, comparing them as to their utility, accuracy, speed, and simplicity. The methods studied included the swab, agar disc (Olson and Hammer, 1933), rim impressions on soft and hard agar, direct plating of bits of glass, and microscopic examination of stained bits of glass rim. The sw ab method was found to be best, although percentage recovery of

artificially inoculated bact eria varied from 40 to 70 percent. A technique was suggested in which swabs were inserted through cotton plugs in 25 ml. culture tubes containing 3 ml. of physiological saline. After a glass was sampled, the swab was dropped into the tube and the tube agitated 25 times to obtain a suspension. The swab was then used to inoculate Loeffler's medium for the detection of pathogens, while the entire 3 ml. was plated in agar. Speck and Black (1937) critically studied the swab technique and compared it with the agar disc and rinse methods. The latter consisted of adding a known amount of water to a glass, rinsing the glass, returning the water to a sterile bottle, and subsequently plating aliquots of the rinse water (Dearstyne, 1920). They confirmed the results of Fellers, et al (1936) showing that the agar plate method was impractical, and the swab technique was the best. They reported a moist swab, followed by a dry one, gave 83% recovery of a rtificially inoculated bacteria. Walter and Hucker (1941) tested a wire swab technique suggested in the New York State sanitary code and found that a stiff wire swab (63 guage) recovered consistently more organisms than did the contact plate or cotton swab methods. The American Public Health Association committee on food and utensil sanitation (Hitchens, et al, 1937) suggested a standard multiple utensil technique apparently based on the method of Cumming and Tongue (1936)

The substrate used for standard plating methods in restaurant sanitation studies has been nutrient agar, in spite of the knowledge that the fomites tested commonly carry an abundance of fastidious organisms, such as the streptococci, which are unable to grow to any great extent upon this medium. Consequently the results have been, in the main, indicative only of extraneous contamination and, therefore, of relatively little

sanitary importance. That an enriched agar was needed has become apparent only recently. (Hucker, et al. 1942), (Novak and Lacy, 1941), and Walter and Hucker, 1941)

Examination of the effectiveness of dishwashing techniques and factors influencing total bacterial counts from restaurant ware: For an elucidation of the significance of any routine technique, studies must be made of the effect of all possible factors which might influence the results. The following paragraphs contain a survey of the literature concerning, (a) factors influencing the total counts obtained from restaurant utensils, and (b) effect of dishwashing methods on the sanitary condition of the finished product.

MacDonald and Freeborn (1933) first called attention to the fact that towel-drying of utensils was more unsanitary than air drying. This conclusion was based on a study of the effect of wiping on bacterial counts of glassware. Their results have also been observed by others, (Horwood and Pesare, 1942) (Mallmann, 1940). However, data by Hucker, et al (1942) indicated that toweling greatly benefits the sanitary condition of utensils, provided of course that an adequate supply of clean towels were available.

Wash and rinse water temperatures markedly influence the kind and number of bacteria occurring on a washed utensil. Krog and Dougherty (1936) were able to correlate wash and rinse water temperatures with the number of organisms occurring on various utensils. In twenty establishments studied they found wash and rinse water temperatures ranging from 84° to 164°F and 60° to 166° respectively. The bacterial counts on forks, for instance, from the same establishments varied from 24,000 to 20 per utensil. The American Public Health Association committee for the

studied 28 restaurants in New York City, comparing bacterial counts of dishes in relation to washing techniques and temperatures of wash and rinse waters. In addition, a test organism, Bacillus pyocyaneus (Pseudomonas aeruginosa), was suspended in saline water, broth, or egg albumin solution and inoculated onto utensils. These were then run through the dishwashing procedures and counts resulting were compared with unwashed controls. Sanitary conditions of eating utensils was found to be extremely bad, but it was observed that even careful hand washing could not compare with machine washing for rendering an eating utensil bacteriologically clean.

The various phases of dishwashing have been critically examined since this report. Dearstyne (1920) called attention to the fact that machine washing, although usually better than hand washing, could not always be considered safe. He noted the effectiveness of any method varied with the conscientiousness of the operator. Cox (1938) studied 21 machine washing devices in daily use on a military reservation. His results indicated (a) bacteria from rinse or wash waters can be deposited on sterile dishes, the amount varying directly with the bacterial load of the water, (b) wash waters of 170°F prevent a rapid "build up" of bacteria in double tank machines during normal washing procedures, (c) in intermittent single tank machines, a wash water temperature of 120°F and a rinse water temperature of 140°F showed a great increase in counts, over a 90 minute washing time, as compared to an insignificant increase when temperatures were 170°F and 190°F respectively. Similar results were obtained by Ward and Dack (1939) on household washers.

The efficiency and germicidal power of a variety of detergents have been investigated in recent years. These studies were undertaken

once it became apparent that the bacterial load varied with the film left by soaps. Baker (1931) studied the relative efficiency of sodium silicate, sodium phosphate, sodium hydroxide, and sodium carbonate as to their wetting, emulsification, deflecculation, and dissolving powers and found sodium silicate consistently the best. Halvorsan, et al (1935) studied the germicidal efficiency of a variety of soaps and detergent mixtures as well as their etching effects on glasses. One of their best mixtures appeared to be #1000:

Sodium chlorothophenyl phenolate	1%
G. U. Brand sodium silicate	3%
Dry sodium resinate	15%
Sodium hexametaphosphate	13%
Sodium carbonate	<15.5%
Sodium bicarbonate	<57.5%

Germicidal tests with this mixture showed it rapidly killed a wide variety of pathogenic and non-pathogenic organisms likely to occur on utensils.

Schwartz and Gilmore (1934) presented evidence that a mixture of trisodium phosphate (45%), sodium metaphosphate (53%), and sodium hydroxide (2%) (hereafter referred to as the S & G mixture) was an exceptionally efficient detergent. Using the following equation:

Where E = Emulsifying power
S = Saponification power
P = Precipitation power (film-forming properties)
C = Corrosive power
D = Detergent efficienty

and $D = k \frac{ES}{PC}$

these workers studied the relative detergent efficiency of a variety of mixtures including the S & G mixture. Of 15 detergents tested, S & G mixture was most efficient in lowering the value PC and raising the value ES. Sodium hexametaphosphate forms soluble calcium and magnesium salts with these ions which are common to hard water, thereby preventing the formation of insoluble films.

Later work by the same authors (Hall and Schwartz, 1937, 1938) presented further evidence of the value of sodium metaphosphate as a detergent. Mallmann (1937) studied the bactericidal efficiency of trisodium phosphate, sodium metasilicate, and S & G mixture, three commonly used detergents, against Staphylococcus aureus at various temperatures. His data indicated that both trisodium phosphate and sodium metasilicate have greater bactericidal properties than the S & G mixture. Experiments to determine the effectiveness of these detergents in actual practice resulted in the confirmation of data obtained by previous workers (Schwartz & Gilmore, 1934) (Hall and Schwartz, 1937) (Hall, 1932). It was concluded that the effectiveness of S & G mixture in lowering bacterial count of dishes was due to the detergent rather than the germicidal power of the mixture.

Gilcreas and O'Brien (1941) investigated various methods of dishwashing by varying the procedures as to temperature, detergent, and disinfecting agent. A standard laboratory technique for evaluation of detergents was offered involving the reduction of bacterial numbers on artificially inoculated glass slides and the effect of the detergent on the transmission of light, i.e. its corrosive power.

Many experiments have been made on the use of chlorinating agents in the sterilization of eating utensils. Mallmann and Devereux (1935) and Devereux and Mallmann (1936) tested organic and inorganic chlorine compounds against Escherichia coli and Staphylococcus aureus under simulated dishwashing conditions and found (a) Escherichia coli but not Staphylococcus aureus was killed by an organic chlorine residual of 200 ppm., (b) inorganic chlorine, 200 ppm., sterilized rinse water in sixty seconds. It was

suggested that the chlorine content of a rinse water should never fall below 100 ppm. and that inorganic chlorine compounds should be used. MacNabb, et al (1938) reported, however, that 100 ppm. in rinse water was insufficient. They suggested 400 ppm. in washwater or 100 ppm. in both wash and rinse waters. It was further suggested that when hypochlorites were employed, the temperature should never be allowed to rise above 120° F, in order to prevent the destruction of the hypochlorite.

Cumming and Yongue (1936) determined the chlorine consumption per dish in a three-tub vs. a four-tub procedure and found it to be 20 ppm. per hour and 10.6 ppm. per hour respectively. On the strength of these results they strongly advised inclusion of a fourth tub, interposed between the soapy wash and chlorine disinfection baths, as a soapy water rinse for economic reasons. Stone and Pomeroy (1936-37) found restaurants using 50-100 ppm. chlorine rinse showed markedly low counts from eating utensils. Norwood and Pesare (1942) suggested 50-100 ppm. with a temperature of around 50-80° F for the chlorine disinfection bath.

The use of chlorine in utensil sanitation is accompanied by several disadvantages, viz. (a) Inorganic chlorine compounds leave a taste and odor, which must be removed for esthetic reasons; are relatively unstable to heat; must be used in the cold; and are corrosive to ordinary metal washing tubs found in the average restaurant; (b) Organic chlorine compounds also leave taste and odor but are more stable to heat and less corrosive. Their rate of germicidal activity, however, is much lower than that of inorganic chlorine compounds. (Mallman, 1937).

A new type of germidice was reported by Dunn (1937), alkyl-dimethyl-benzyl-ammonium chloride, or commonly, "Zephiran". Krog and Marshall (1940) investigated the possibility of using this substance as a germicidal agent in restaurant sanitation, and the results of their

investigation seemed to indicate this substance may be the answer to the entire question, viz, (a) Detergents or soap have little or no effect on the potency of "Zephiran" when used in either two or three-tub methods; (b) It is stable, non-toxic to humans, odorless, tastless, and non-corrosive to metals; (c) The concentration can be checked colorimetrically; (d) Temperatures above 70° F do not effect its stability or bactericidal efficiency. The best temperature was found to be 74° F; and (e) One minute rinse in 1:50,000 solution is sufficient to reduce the count on glass rims to below 100. The value of a germicidal substance whose properties seem to be ideal must be taken with reserve. This substance is now being used in some restaurants and the results are being checked, although the literature is relatively silent on its effectiveness at this time. (Walters, 1942). Its present high cost is also a factor in limiting its wide use.

A discussion on disinfecting agents would not be complete without mentioning the ultra-violet ray cabinet. Attempts have been made to sterilize glasses by ultra-violet rays either in connection with filling the glass with water (Devereux and Mallman, 1936) or in other ways. Appling and Tanner (1941) studied the bacterial load of artifically inoculated glasses before and after irradiation. A standard irradiation time of 30 seconds in a commercially produced "Sterile-Ray Cabinet" was used. They found irradiation of dry glasses was ineffective, whereas irradiation of wet glasses resulted in marked reduction of bacterial load. When initially inoculated with approximately 7,500 cells of Escherichia coli, sterilization occurred in two to three minutes. Soda fountain glasses were also irradiated for various times and their bacterial content compared with unirradiated controls. On the average, the results indicated 90% reduction after an irradiation time of four minutes. Others have studied the ultraviolet

cabinets in relation to their use in restaurants (Hucker, et al. 1942) (Tanner and Appling, 1941) (Garret and Arnold, 1938) and (Fellers, 1938), with essentially the same results. The high cost of ultra-violet cabinets has obviated their use in all but the most pretentious restaurants.

Reference to hot air sterilizers have occurred in the literature. A Hartford, Connecticut firm designed such a sterilizer for use in certain restaurants in that city. Marden, et al (1935) observed that glasses were sterilized in 15 minutes at 250° F. Tiedeman (1941) reported on the effectiveness of such a sterilizer, when employed routinely, in certain cities requiring its use. The instructions were to place glasses in the preheated oven and leave them not less than five minutes. A tray of heavy glasses placed in this oven required a full hour to reach the oven temperature as shown by tests with a thermocouple. At the end of five minutes, the glasses had only reached body temperature. The effectiveness of such a device was questioned.

Occurrence of organisms on restaurant ware: The demonstration of specific pathogenic organisms on washed restaurant ware is attended by obvious difficulties. The carrier rate of the etiologic agent or agents of any respiratory disease is usually low in the absence of an epidemic; the chance of a carrier depositing bacteria on the glassware of a restaurant is also low; and the chance that a sanitary inspector might select the specific glass is also quite small. Further, isolating organisms by means of the swab technique, if such is used, reduced the chance of recovery from 25 to 60%. (Fellers, et al. 1936). Many attempts have been made, however, some meeting with success.

Taylor (1921) tested unwashed spoons from active tuberculosis patients by rinsing them in sterile saline and inoculating the washings

table lists the investigators and their reported recoveries.

TABLE I

Investigators	Percent isolations from drinking glasses Strep. hemolyticus	Strep. viridans
Saelhof & Heinekamp (1920)	1.6	
MacNabb, et al (1938)	0 to 25	0 to 20
Cumming (1919)	91*	100.
Cumming and Sprunt (1920)	84	65.6
Horwood and Pearce (1942)	1.1	
Dick and Rucker (1941)		20 to 63
Carlisle & Hartinger (1939)	0	
Warden, et al (1938)	0 to 3.9	0.3 to 33.2
Wallman and Devereux (1935)	Numerous**	Numerous**

* In hospital wards, unwashed glasses.
** Unwashed glasses from restaurants.

These investigations, and others, have led public health officers to conclude that restaurant glasses must definitely be considered as vectors in the transmission of respiratory diseases. (Calver, 1935) (Smith, 1939) (Hitchens, 1941) (Bushong & Fletcher, 1940).

Organisms other than pathogens are most frequently isolated from restaurant ware. The microbial flora of washed utensils has been investigated by a host of workers. As an indication of the general types encountered and their universal occurrence see Table II, which lists the investigators and their results. The predominating organisms recovered were consistently staphylococci, spore-forming gram-positive rods, and coliform organisms. We have demonstrated the occurrence of a Proteus in many instances from washed glasses, especially from taverns. (Unpublished data).

TABLE II

Types of Microorganisms Isolated
From Restaurant Ware By Various Investigators

Investigators	Staphylococci	Occurrence of Spore-formers	Coliforms	Streptococcus viridans
Saelhof & Heinekamp (1920)	P	P	P	-
MacDonald & Freeborn (1933)	-	-	P	-
MacNabb, et al., (1938)	P	P	P	P
Marden, et al., (1938)	P	-	P	-
Carlisle & Hartinger (1939)	O	P	-	O
Horwood & Pearce (1942)	P	P	P	-

P= Present on utensils
O= Absent
-= Not reported

Ecology of Streptococcus salivarius: The identity of the organism referred to in the literature as Streptococcus salivarius has been somewhat vague, due in part to the general confusion existing in the taxonomy of the non-hemolytic streptococci. The tests first used to identify this oral streptococcus have largely been altered or dropped, while new techniques for classification have appeared. Fermentation reactions and growth in litmus milk are the few tests which have survived. The inherent variability of the streptococci has only added to the confusion. As a consequence, several workers have identified strains as Streptococcus salivarius which, in some instances, have marked differences in character from those first described for this species by Andrews and Horder (1906). For lack of proper taxonomy, bacteria of the oral streptococcus group and others have often been classified simply as Streptococcus viridans. As a result, many of the characteristics of the organism, especially those concerning ecology and pathogenicity, has escaped notice. The review which follows briefly covers what is known regarding the taxonomy and ecology of Streptococcus salivarius.

The organism Streptococcus salivarius was first described by Andrews and Horder (1906). Its characteristics were given as follows: It occurs in short chains, ferments sucrose, lactose, and raffinose consistently, but inulin rarely; may or may not ferment salicin and coniferin, but never ferments mannitol; clots milk and reduces neutral red. In the practical classification of Holman (1916), which is based entirely upon hemolysis and fermentation reactions, Streptococcus salivarius is differentiated from the other strain commonly occurring in the human mouth, Streptococcus mitis, on the basis of its inability to ferment salicin. It is also classified as non-hemolytic, fermenting lactose, but not mannitol. Frost and Englebrecht (1940) list its characteristics as follows: Occurs usually in long chains, produces alpha-hemolysis on blood agar, not vigorous and dies out easily, ferments lactose, glucose, raffinose, and sucrose; does not ferment mannitol, salicin, inulin, sorbitol, trehalose, or arabinose; final pH in glucose broth is 4.9 to 5.13; litmus milk made slightly acid and rarely coagulated; sodium hippurate hydrolyzed by some strains; non-resistant to 50-60° C for 60 minutes. With the exception of its non-coagulation of milk and the formation of long chains, this organism seems to follow the original classification of Andrews and Horder fairly well.

Safford, et al (1937) reinvestigated the biochemical and physiological reactions of the non-hemolytic streptococcus found most abundantly in the human mouth. They found it to possess characteristics most nearly in accordance with those originally given by Andrews and Horder (1906) for Streptococcus salivarius. The following reactions were reported: Produces weak alpha hemolysis on blood agar*, occurs in short

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* Strains which we received from Dr. Sherman show gamma hemolysis.

chains, does not survive 60° C for 30 minutes, cannot grow at 10° nor 47° C, promptly acidulates and coagulates litmus milk followed by reduction of litmus; sodium hippurate is not hydrolyzed, final pH in glucose broth is 4.0 to 4.4, no ammonia is produced from peptone, starch is not hydrolyzed, and gelatin is not liquefied; ferments glucose, maltose, lactose, sucrose, raffinose (93%) and salicin (95%); frequently ferments inulin (80%) but does not ferment glycerol or mannitol; lacks viability with infrequent transfer. The organism described here seems to fit the original description of Andrews and Horder with the exception of inulin fermentation, but does not agree with the description of Frost and Englebrecht (1940) nor of Holman (1916). It is highly probable that the organism described by all these workers is essentially the same bacterium existing only as strain variants.

Niven, et al (1941) introduced the concept of a "typical" strain of Streptococcus salivarius. They noted all strains of Streptococcus salivarius which fermented inulin also produced a soluble levan when grown on substrates containing sucrose or raffinose. No other streptococcus tested possesses this ability, with the exception of a few strains of Streptococcus bovis, which produces an insoluble levan. They, therefore, called this gum-forming oral streptococcus "typical" Streptococcus salivarius since the majority of their strains (93%) produced levan. All other strains possessing the same general characteristics, but unable to produce mucoid colonies, were called "atypical" strains.

Gordon (1904) was the first to suggest streptococci as an index of oral polution. He demonstrated streptococci as the most abundant bacteria present in saliva. Streptococcus brevis was the most characteristic type. The species was limited to the short chain streptococci which

clotted milk and reduced neutral red, hence were probably the same as Streptococcus salivarius later described by Andrews and Horder (1906). These organisms were found to occur to the extent of 10 to 100 million per ml. of saliva and were observed in the air, in front of persons speaking loudly. Since that time the constant occurrence of Streptococcus salivarius types in human mouths, normal or pathological, has been repeatedly demonstrated. Streptococcus brevis of Gordon (1904) was found by Holte (1914) to be the most abundant form of salivary organism and he employed its occurrence as an index of buccal contamination of air. "Acid-forming" streptococci were found by Winslow and Sanjiyan (1924) on human tongues and recently contaminated fomites. Their observations that these streptococci also were found on objects not recently contaminated led them to conclude there were definite limits to the categorical use of the streptococcus as an indicator of recent salivary pollution. Recovery of streptococci from places not recently contaminated is understandable in view of recent reports by Buchbinder and Phelps (1941) wherein Streptococcus viridans was shown to survive for at least 24 hours on fomites in the dark.

The occurrence of alpha streptococci in air has been investigated quite thoroughly, most recently by Torrey and Lake (1941) and Buchbinder and co-workers (1938). Torrey and Lake were able to correlate rise in numbers of alpha streptococci in department store air with a peak number of employee-disabling colds. When the number of colds fell off, the number of streptococci recoveries fell off. Buchbinder, et al (1938) also studied the incidence of streptococci in air from public places, by means of the Well's air centrifuge and sheep's blood agar. Two thousand strains of alpha streptococci were isolated, of which a large percentage were found to be Streptococcus salivarius (Andrews and Horder) Sherman.

A streptococcus similar to Streptococcus salivarius (Andrews and Horder) was reported isolated from cow feces and mouths by Ayers and Mudge (1923). The organism yielded all typical Streptococcus salivarius fermentation reactions, did not produce ammonia from peptone nor hydrolyze sodium hippurate. Apparently this was the Streptococcus bovis of Sherman, who has reported marked similarity between the two (Sherman, Stark and Stafford, 1938). Colebrook, et al (1935) reported isolating alpha streptococci from hands of persons during an investigation into the vectors of puerperal fever transmission. They were not classified rigidly, but were tentatively placed in Streptococcus viridans of oral origin. Dick (1939) recovered Streptococcus salivarius (Andrews & Horder) from 100% of the lips of normal persons and concluded that their deposition on glassware during drinking processes was highly probable. Later (Dick and Hucker, 1940) this was shown to be true in all of 100 examinations made. Thirty-nine percent of 100 eating establishments examined showed the streptococci on drinking glasses after washing. Organisms of this general type have been isolated from bacterial endocarditis, rheumatism and tributary lesions (Holman, 1916).

The ecology of the "typical" mucoid strain of Streptococcus salivarius (Andrews and Horder) Sherman has been neglected. Mandia and co-workers (1942) reported recovering it from trickling filter beds. Rose and Georgi (1941) found it in human mouths and on human lips, unwashed and washed glasses, but not on fomites connected with restaurants. Niven, et al (1942) using an azide medium to inhibit other intestinal organisms, recovered it from feces of 15 out of 18 persons examined. It was found occurring in numbers ranging from 1200 to 129 million per gram of feces, while in 5 of the samples it comprised 50% of the streptococci which grew.

Walter (1942) using the same medium was unable to demonstrate its presence on restaurant ware, due to overgrowth by Proteus and coliform bacteria.

Isolation of streptococci by means of selective media: According to Levine (1925), Japha, in 1842 was the first to note that microorganisms reduced selenium. It was not until 1890, however, that Chabrie and Lapicque showed that 0.2% selenious acid prevented putrefaction of bouillion. Scheuerlen (1900) and later Klett (1900) further studied the bactericidal effects of tellurite and selenite on bacteria and molds. Gosio (1904) studied the use of tellurite and selenite in bacterial media as an indicator of microbial growth.

Apparently the first report of the value of potassium tellurite in selective media was that of Smith (1914) and it is noteworthy to mention that he suggested its use for the isolation of streptococci, which grew in sheep serum-peptone-tellurate agar as "minute discrete colonies". His suggestion, however, was not further investigated since it was not, as far as could be ascertained, until 1932 that a definite suggestion was again made that potassium tellurite be employed for the isolation of streptococci.

Potassium tellurate was used as an inhibitory agent in a selective medium designed for the isolation of Eberthella typhosa by Browning, et al (1914-15). The etiology and control of diphtheria began occupying the public health mind at about this time, and many media involving potassium tellurite were designed for the isolation and typing of the tellurite-resistant Corynebacterium diphtheriae. Occasionally in reports on the development and use of these media, one encounters mention of streptococci. Wood (1921) noted that streptococci did not reduce telluric acid in his medium and grew as greyish-white, small (less than 1 mm.), very flat, and

very transluscent colonies. Douglas (1922) reported similar observations, adding that black spots appear in the center after 24 hours. The use of potassium tellurite in a differential medium for isolating diphtheria bacilli was thoroughly investigated by Gilbert and Humphreys (1926). Colony characteristics on a tellurite medium had been used as criteria for the presumptive presence of Corynebacterium diphtheriae. It was felt necessary to investigate the growth characteristics of a variety of other organisms which might interfere. Sixty species were tested and their growth noted. It is worthwhile to mention here that Pneumococci Types I, II, and II, Streptococcus hemolyticus, Staphylococcus aureus, Micrococcus tetragenus, Lactobacillus acidophilus, Escherichia coli, Corynebacterium diphtheriae, diphtheroids, Hemophilus influenzae, Proteus vulgaris, Proteus X19, and Saccharomyces cerevisiae were among the organisms slightly or not at all inhibited.

Allison and Ayling (1929) had noted an overgrowth of tellurite diphtheriae plates by swarming Proteus species and by staphylococci. Addition of 1:2,000 copper sulphate limited proteus to minute colonies and inhibited staphylococci and streptococci entirely. However, a "gram-positive diplococcus" did appear on throat cultures. On a tellurite chocolate blood agar developed by Anderson, et al (1931) for the isolation and typing of Corynebacterium diphtheriae, some streptococci appeared subsequently as fine black colonies with green haloes. Yeasts also grew, as did "three forms of coccus," diphtheroids, and staphylococci. McGuinan, et al (1936) again noted that Streptococcus hemolyticus grows as a "minute, flat, black, glistening, circular colony with entire edges" on a tellurite medium used for diagnosis of diphtheria, while Glass (1937) reported that concentrations of 0.04% potassium tellurite or over markedly reduces the number of streptococci coming through on diphtheric swab cultures.

Fleming (1932) investigated the inhibitory effect of potassium tellurite on 20 species of bacteria. His suggestion that this salt be used in a solid medium for the isolation of streptococci is the first one we have been able to discover since that of Smith (1914). This can be explained by two facts: (a) The blood agar technique of Brown (1919) was found highly successful for the simultaneous isolation and partial characterization of streptococci from pathological sources, and (b) potassium tellurite was being used predominantly for the isolation and characterization of diphtheria organisms. A thorough survey of the literature concerning selective culture media was made fairly recently by Garrod (1933), preliminary to an investigation into the possibilities of a selective medium for the enrichment and subsequent isolation of Streptococcus pyogenes from pathological matter containing relatively numerous contaminating organisms. Streptococcus pyogenes was found to be almost entirely outgrown by Staphylococcus aureus and Escherichia coli in ordinary enrichment cultures from throats. Addition of glucose and body fluids improved the ratio relatively little. Salicin was found uniformly favorable to the streptococci and its effect was enhanced by serum or blood. A trypsin broth, pH 7.4, containing 1% glucose and 20% hydrocele fluid seemed to favor alpha or non-hemolytic streptococci from throat swabs. The relative effect of many inhibitory salts and dyes on three streptococci, two staphylococci and Escherichia coli was determined by observing the maximum concentration of the inhibitor allowing growth and the minimum concentration inhibiting growth. His results, in part, follow:

TABLE III

Limiting Concentrations of Tellurite

Bacterial species	Maximum conc. allowing growth, in percent	Minimum conc. inhibiting growth, in percent
Strep. pyogenes	0.05	0.01
Strep. salivarius	0.025	0.05
Strep. fecalis	0.1	Not determined
Staph. aureus	0.1	Not determined
Staph. albus	0.1	Not determined
Esch. coli	0.003	0.006

TABLE IV

Limiting Concentrations of Gentian Violet.

Bacterial species	Average lowest inhibitory dilution in broth	in serum broth
Strep. fecalis	1:58,000	1:46,000
Strep. salivarius	1:180,000	1:60,000
Strep. pyogenes	1:220,000	1:96,000
Pneumococcus	—	1:105,000
Staph. aureus	1:1,239,000	1:787,000
Staph. albus	1:1,366,000	1:1,113,000

On the basis of the above results, the medium finally selected for clinical use had the following composition:

Peptone broth pH 7.4	
Potassium tellurite	0.05% (1:2,000)
Gentian violet	0.0002% (1:500,000)
Sheep's serum	5.0%

In a study of tonsilar swabs using the above medium as an enrichment culture followed by plating in blood agar, Garrod reported that Streptococcus salivarius was often found to occur more numerously than Streptococcus pyogenes. He concluded that the medium developed lacked many of the qualities of a satisfactory medium for selectively isolating Streptococcus pyogenes.

Perry and Petran (1939) streaked throat swabs on tellurite medium slants as a preliminary step in the isolation and identification of throat streptococci. Fleming and Young (1940) studied the resistance of fecal bacteria to tellurite, suggesting a concentration of potassium tellurite of from 1:5,000 to 1:800,000 as effective in inhibiting gram-negative organisms in the isolation of fecal streptococci. The fecal streptococci are notably resistant to high concentrations of tellurite (Garrod, 1933). Bornstein (1940) confirmed Fleming's work and further noted that the Enterococci and Streptococcus equinus of Sherman (1937) grew on 1% glucose medium with 1:1,000 potassium tellurite, whereas the lactic streptococci and other streptococci tested did not.

Enterococci on tellurite medium grow typically as small, discrete, solid, black colonies. Rose and Georgi (1941) isolated Streptococcus salivarius from restaurant glasses on a medium containing sucrose, potassium tellurite and crystal violet. A recent report by Kaspar, et al (1942) indicates that potassium selenite may be applicable in culturing pneumococci.

Although the bactericidal nature of sodium azide was first reported by Loew (1891), its use in selective media for the isolation of gram-positive bacteria has been investigated only recently.

A paper by Hartmann (1936) was the earliest citation we could discover on the use of sodium azide as a selective bacteriostatic agent for use in culture media. Edwards (1938) studied the effect of sodium azide on the growth of small inocula of Streptococcus mastitis in the presence of large inocula of Escherichia coli. It was found that a medium with a pH of 6.8 allowed good growth of the streptococcus from inocula as small as 31-135 cells, while large inocula of the coliform organism were eliminated entirely. An enrichment medium was developed which would allow detection

of small inocula of streptococci where ordinary plating methods in blood agar would not work. Bryan and his co-workers (1939, 1941) suggested inclusion of sodium azide in samples being incubated for the microscopic test for mastitis, in order to eliminate gram-negative contaminants. Foley (1942) reported increased sensitivity of a modified Hotis test for mastitis when a solution containing sodium azide and crystal violet were added prior to incubation. A modified Edward's enrichment broth was also suggested containing sodium azide and crystal violet.

A fecal streptococcus count of raw v. effluent sewage has been suggested by Mallmann (1940, and Mallmann, et al (1941) as a measure of the efficiency of sewage disposal plants in the removal of pathogens. For this purpose an enrichment broth containing 1:5,000 sodium azide (1940) or nutrient agar containing 1:10,000 sodium azide (1941) was suggested. Niven, et al (1942) used sodium azide in a selective medium for the isolation of Streptococcus salivarius from human fecal matter. On this medium, "typical" Streptococcus salivarius (Andrews and Horder) Sherman grew as large mucoid colonies, while other fecal streptococci produced small colonies. Escherichia coli was partially to completely inhibited. Snyder and Lichstein (1940) found that 0.01% sodium azide in blood agar would prevent swarming of proteus species and allow isolation of streptococci from known mixtures. Later (Snyder and Lichstein, 1941 and Lichstein and Snyder, 1941), a more thorough investigation of the inhibition of Proteus and isolation of streptococci was reported. Alterations in the moisture content of the medium was accomplished by the use of unglazed porcelain covers, increased agar content (5%) and excess quantities of NaCl (6%). Spreading of Proteus was inhibited in each, but isolation of beta streptococci was prevented by dessication of the medium or because the beta-hemolytic streptococci were salt sensitive. Addition of blood to the medium caused renewal of Proteus spreading. Streptococcus viridans, being insensitive to 6% sodium chloride, could be isolated on salt media. The viridans streptococci referred to here

must be the fecal type since all others are sensitive to that concentration of salt. (Sherman, 1937). Lactic acid, alcohol, sodium azide and potassium tellurite were employed in selective media. Only sodium azide and ethyl alcohol were effective, but alcohol caused hemolysis of blood, obviating its use in blood agar.

Stiles and Chapman (1942) reported a Bacto tryptone agar medium containing sodium azide and crystal violet as effective in eliminating proteus spreaders and Escherichia coli from mixed cultures of these and streptococci. Nasal isolations were simplified by this medium on which diphtheroids and staphylococci did not grow. Later,(Personal communication) methylene violet was added to lower the toxicity of crystal violet toward streptococci. The only report wherein sodium azide was used in restaurant sanitation studies was that of Walter (1942) who employed the medium of Niven, et al (1942) in a study of streptococci occurring on restaurant glassware. His results were negative due to overgrowth of the medium by coliforms and yeasts.

Other means for detecting streptococci in mixed cultures have been reported. Winslow and Sanjiyan (1924) employed a lactose-litmus agar in detecting the presence of acid-forming streptococci in air, in respiratory passages and on fomites. Krumwiede and Kuttner (1938) described a 5% sheep blood medium as selective for the isolation of beta-hemolytic streptococci from throat cultures. Sheep blood is inhibitory to Hemophilus hemolyticus and Hemophilus influenzae. Folpmers (1940) used caffeine in a lactose enrichment medium for streptococci. Dick (1939) studied the occurrence of Streptococcus salivarius by culturing lip and glass swabs in enrichment broth followed by plating on 8% veal-infusion agar. Later, (Dick and Hucker, 1940) crystal violet was added to the medium to inhibit the growth of other cocci which might be present in saliva and on lips. The salivary

streptococci were able to grow well in a concentration of 1:400,000 crystal violet.

No reports on the use of sodium azide in enrichment media have been found in the literature nor have there been reports on the occurrence of "typical" strains of Streptococcus salivarius other than that of Rose and Georgi (1941).

METHODS AND MATERIALS

Culture media

Stock broth: The medium commonly used for culturing test organisms in broth prior to inoculation was that suggested by Hutner (1936) and had the following constitution:

Beef extract	3 grams
Peptonized milk	5 grams
Tryptone	1 gram
Yeast extract	5 grams
Glucose	2 grams
Calcium carbonate	excess
of dipotassium phosphate	3 grams
Distilled water	1000 ml.

It was unnecessary to adjust the pH of this medium, the pH falling consistently between 7.0 and 7.2. It was discovered early that the presence of calcium carbonate interfered in the detection of growth of certain "atypical" strains of Streptococcus salivarius, and dipotassium phosphate was therefore substituted as the buffering agent.

Basal substrate for selective media: The basal medium used in all selective media, with the exception of those noted in certain special experiments, was that of Sefford, et al (1937) and had been used in previous work. (Rose and Georgi, 1941) Its constitution is as follows:

Beef extract	3 grams
Proteose peptone	5 grams
Yeast extract	5 grams
Glucose	1 gram
Sucrose, glucose, or inulin	10 grams
Dipotassium phosphate	3 grams
Agar (if present)	15 grams
Distilled water	1000 ml.

The original broth medium as proposed by Safford, et al (1937) contained an excess of calcium carbonate as a buffer, but we consistently added 0.3% dipotassium phosphate in its place except where specifically noted.

Special experimental media: Two special substrates were employed in some of the experiments. Their composition is as follows:

A. Medium of Niven, et al (1942)

Sucrose	50 grams
Tryptone	10 grams
Yeast extract	5 grams
Dipotassium phosphate	3 grams
Agar	15 grams
Distilled water	1000 ml.

The pH of this medium was usually around 7.0. If not, it was adjusted to 7.0 prior to sterilization except in special experiments as noted.

B. Medium of Chapman (1942) modified:

Bacto tryptose agar	46 grams
Sucrose	10 grams
Sterile defibrinated horse blood	50 ml.
Distilled water to	1000 ml.

The pH of this medium was adjusted to 7.0 prior to sterilization.

Difco dehydrated products were used consistently for the nitrogen sources. Carbohydrate sources were Coleman and Bell or Mallinkrodt C. P. products. All solid media were prepared in Erlenmeyer flasks and sterilized at 15 lbs. for 15 to 20 minutes and cooled to 50°C. Sterile

inhibitory agents were added aseptically and plates poured directly from the flask.

Preparation of inhibitory agents: Crystal violet was prepared in 1% aqueous solution and allowed to stand two to three weeks prior to use. At the end of this time the solution was essentially sterile and was handled with aseptic precautions thereafter. Potassium tellurite (Eimer and Amend or Coleman and Bell) was prepared in 1% aqueous solution, passed through filter paper, and sterilized by filtration through a Berkefeld "W" bacteriological filter. The resulting solution was stored in sterile bottles and handled with aseptic precauti ons. The concentration of potassium tellurite in these solutions was determined by the method of Treadwell and Hall (1928) and was found to be 0.899% and 0.924% for the Eimer and Amend and Coleman and Bell products respectively. Sodium azide was prepared in 1% aqueous solution and sterilized in the same manner as the potassium tellurite. Both the tellurite and azide solutions were found to be stable for at least two to three months.

Stock cultures

The stock cultures used in these experiments with their experimental numbers, classification, and source are listed in the following table:

Classification	Experimental number	Source
Streptococcus salivarius	S25D	Dr. J. M. Sherman
" "	S20B	Dr. J. M. Sherman
" "	83	Restaurant glass
" "	80	Restaurant glass
" "	102	Restaurant glass
" "	3	Restaurant glass
" "	208	Restaurant glass
" "	S44A	Dr. J. M. Sherman
" "	6	Restaurant glass

Classification	Experimental number	Source
Streptococcus liquefaciens		University of Nebraska
" "	16	Restaurant glass
Staphylococcus aureus	F.D.A.	University of Nebraska
Escherichia coli		University of Nebraska
" "	E2	University of Nebraska
" "	E3	University of Nebraska
Aerobacter aerogenes		University of Nebraska
" "	A3	Iowa State University
" "	A6	Michigan State University
Proteus vulgaris	Hovy	University of Nebraska
Proteus morganii		University of Nebraska
Proteus species		Restaurant glass
Salmonella typhimurium		University of Nebraska
Slow lactose fermenter #1		University of Nebraska
Saccharomyces cerevisiae		University of Nebraska
Saccharomyces ellipsoideus		University of Nebraska
Torula lactosa		University of Nebraska
Bacillus subtilis		University of Nebraska

Special attention is called to the following: Streptococcus salivarius S25D, S20B, #80, #83, and #102 are "typical" strains (Niven, et al 1941) forming mucoid colonies on sucrose-containing agar. The nature of Streptococcus salivarius #3 is still in question. Although it has been consistently demonstrated by us to be present in the human mouth, it apparently has some of the characteristics of Streptococcus bovis (Sherman, 1937). It ferments glucose, maltose, and lactose, but not mannitol, inulin or glycerol. It produces a very typical hard, crystalline appearing colony on sucrose medium, suggesting that it produces an insoluble levan similar to that of Streptococcus bovis (Niven, et al, 1941). However, it markedly differs from this organisms in several respects, viz: it fails to ferment salicin and produces a final pH in glucose broth of 4.8 to 4.9 instead of the reported 4.0 to 4.4 of Streptococcus bovis. Whereas this latter organism may or may not curdle milk, this bacterium does. Some strains have been isolated which are short-chain types fermenting salicin and may be Streptococcus bovis varieties. An example is Strain #208.

Cultures S44A and #6 are "atypical" strains of Streptococcus

salivarius. The latter has been consistently demonstrated in abundance in the human mouth, on washed and unwashed glasses, and in wash water. This strain is characteristic of the "atypical" forms reported in this paper. It and other similar strains are gram-positive diplococci sometimes occurring in short chains and have been found to produce an alpha to alpha prime hemolysis on blood agar, the colonies bearing marked resemblance to pneumococcus blood agar colonies. In broth it grows with faint diffuse turbidity followed by sedimentation and clearing of the supernatant. This again is characteristic of the pneumococci, as is the production by these strains of demonstrable capsules when grown in milk cultures. It produces weak acid in litmus milk and a final pH in glucose broth of 5.4. It ferments salicin consistently but may or may not ferment lactose and glycerol. Variations in lactose fermentation seem to occur during strain variations which have been observed. Bile solubility tests, Neufeld reactions, mouse pathogenicity, and inulin fermentations were run on cultures of #6 and similar organisms in an attempt to link them with the pneumococci, but all tests were inconclusive. Another odd characteristic is its ability to survive 60°C for 30 minutes which has been consistently demonstrated in all strains. These properties indicate the organism is apparently a form of oral streptococcus and, consequently, we have chosen to call it "atypical" Streptococcus salivarius.

The culture labeled Proteus species was brought to our attention by its repeated occurrence on plates of tellurite-crystal violet selective media streaked with swabs from drinking glasses. It forms large, spreading, mucoid colonies only when grown on a sucrose medium containing tellurite. Its occurrence caused quite a bit of trouble in the isolation of oral streptococci. On isolation and purification, it was found to be a gram-

negative to gram-variable rod producing acid and gas from glucose, maltose, sucrose, galactose, mannitol, and fructose but not lactose, inulin, or glycerol. It utilizes sodium citrate as the sole source of carbon, produces no indol, acetyl methyl carbinol, or hydrogen sulfide and only slowly acidifies litmus milk. Methyl red tests are positive and nitrites and ammonia are formed from nitrates and 4% peptone water. Its characteristics fit none of those listed by Bergey, et al (1939) for species of Proteus, consequently it was listed simply as Proteus species.

FIELD TECHNIQUES

In field studies, two sampling procedures were used. The first technique, used primarily when testing the effectiveness of enrichment media in isolating streptococci, was a modification of that of Fellers, et al (1936). Tightly wound cotton swabs were suspended through rolled cotton plugs in a test tube containing a small amount of water or moistened filter paper. These were sterilized at 15 lbs. for 15 minutes. A sampling kit consisted of a special cardboard carton containing space for 40 tubes. Twentyswabs and twenty tubes of enrichment broth were carried on each trip, thus furnishing enough material to test two restaurants. The glass to be tested was swabbed vigorously around the outside and inside of the rim with a swab previously slightly moistened in the sterile water. The cotton plug from the enrichment tube was removed and the swab plus its plug inserted. After vigorous agitation to suspend the organisms, these enrichment tubes were returned to the laboratory and incubated.

The final field experiments were carried out in conjunction with a general restaurant sanitary survey being conducted at that time. The sampling procedure was that of Hucker, et al (1942) in which the swab was suspended in 5 ml. of sterile water to be used for plating purposes. For the purpose of this experiment, one ml. of the suspension water plus the swab served as

inoculum for the enrichment broths. In this manner the percentage recovery was probably lessened somewhat, but the procedure fitted in well with the general restaurant sanitation survey technique under investigation.

EXPERIMENTAL PROCEDURE

Study of Inhibitory Effect of Selective Agar Media on Bacterial Species.

Dick and Hucker (1941) reported that Streptococcus salivarius occurred normally on all human lips and were deposited in all cases during the drinking process. In a previous report on the development of a selective medium for the isolation of "typical" Streptococcus salivarius (Rose and Georgi, 1941) these bacteria were isolated from only 45% of the unwashed and 10% of the washed glasses tested. It was felt at that time that the results obtained were not indicative of the true occurrence of these organisms on restaurant ware, and that possibly the medium, as reported, was too inhibitory to allow growth from the relatively small inocula expected from washed glasses. That this hypothesis might be true was born out by field surveys conducted with the selective medium. Decker (personal communication) was unable to demonstrate the presence of Streptococcus salivarius on washed glasses. A survey of taverns and restaurants in a national defense area by the Nebraska State Department of Public Health also failed to demonstrate the occurrence of these organisms using the technique suggested (Unpublished data). A review of the inhibitory power of the medium suggested by us in comparison with other selective media was therefore undertaken.

The media employed were as follows:

A. Niven, et al. (1942) basal substrate plus 0.02% sodium azide. (Hereafter called NSS)

B. Ross and Georgi (1941) basal substrate plus 0.03% potassium tellurite and 1:500,000 crystal violet. (Hereafter called RG)

C. The RG basal substrate plus 0.03% potassium tellurite.

D. Modified Chapman's (1942) basal substrate plus 0.02% sodium azide. (Hereafter called CH)

E. The RG basal substrate with 0.02% sodium azide and 0.03% potassium tellurite.

A number of organisms representative of the types expected to occur on washed glasses were chosen and cultured in the stock broth for 12-24 hours prior to streaking on the selective media. Each medium was prepared with and without the inhibitory agents. One loopful of stock broth cultures was streaked across the surface of both control and test media and the relative growth noted. In this manner the effect of the variations in substrate and inhibitory agents could be compared. The pH of the substrates was varied by adding sterile N/1 NaOH aseptically to the media and adjusting electrometrically to pH 7.0 or 7.3 in an attempt to determine the effect of reaction on inhibitory power. All media were incubated at 37° C for 72 hours prior to recording the results, it having been determined before that this length of time was necessary to assure a maximum and typical growth response.

A preliminary comparison was made between the growth of seven cultures on the KSS medium at pH 7.0 and 7.3 and the RG medium at pH 7.3. The results indicated that both media at pH 7.3 might be inhibitory to streptococci. This observation was more or less confirmed in a second experiment which was limited to a study of the KSS medium at pH 7.0 and 7.3 but extended to include a greater number of bacterial species. It will be noted in Table V that the gram-negative organisms and the yeasts were more effectively inhibited at pH 7.0, although in one instance (S20B) the typical

TABLE V

Growth of Fourteen Bacterial Species on The NNS Medium at Two Hydrogen Ion Concentrations

Bacterial species	pH 7.0		pH 7.3	
	Plain	NaN_3	Plain	NaN_3
Salmonella typhimurium	++	-	++	+
Slow Lactose Fermenter #1	++	±	++	++
Proteus morganii	++	+	++	++
Proteus vulgaris (Novy)	++	+	++	++
Staphylococcus aureus (F.D.A.)	++	++	++	++
Aerobacter aerogenes	++	-	++	-
Saccharomyces ellipsoideus	-	-	-	-
Saccharomyces cerevisiae	++	±	++	++
Torula lactosa	±	-	±	-
Streptococcus salivarius (S25D)	++	++	++	++
Streptococcus salivarius (S20B)	++	++*	++	++*
Streptococcus salivarius (#83)	++	++	++	++
Streptococcus liquefaciens	++	++	++	++
Proteus species	++	+	++	+**

* Gum formation inhibited.

** Tendency to form mucoid colonies and spread at this pH.

gum formation by this streptococcus was impaired. The spreading of Proteus species which tended to form mucoid colonies at pH 7.3 was effectively checked when a pH of 7.0 was used.

To obtain further data on the inhibitory power of medium RG, fourteen bacterial cultures were grown on the basal substrate, on the basal substrate plus 0.03% potassium tellurite only, and on the medium as reported. The pH was varied in each instance and the growth was reported in relation to that occurring on the RG basal medium as a control. The results are presented in Table VI. With potassium tellurite as the sole inhibitory agent, yeasts and some gram-negative organisms were able to grow to a slight extent at both pH 7.0 and 7.3. Two strains of "typical" Streptococcus salivarius were markedly inhibited by the RG medium at both pH 7.0 and 7.3 whereas, in the absence of crystal violet, growth almost equaled that on the control media. However, the Proteus species were observed to grow abundantly in both inhibitory media and at either pH, while Proteus vulgaris grew uninhibited in the RG medium at pH 7.3. These results seemed to indicate that the RG medium, either as first reported or without crystal violet, inhibited all organisms which might occur on glassware.

All of the selective media were tested simultaneously to obviate errors arising from the age or viability of the inocula. An attempt was made to judge the relative growth more closely. The pH of the media used was maintained at that originally reported, viz.

RG	pH 7.4
MSS	pH 7.0
CH	pH 7.0

The RG basal plus 0.03% potassium tellurite and RG basal plus 0.03% potassium tellurite and 0.02% sodium azide were adjusted to pH 7.0 since the

TABLE VI

Growth of Fourteen Bacterial Species on the RG Basal Medium at Two Hydrogen Ion Concentrations and Containing Potassium Tellurite and a Combination of Potassium Tellurite and Crystal Violet

Bacterial species	Plain		K_2TeO_3		K_2TeO_3 & Cr. violet	
	pH 7.0	pH 7.3	pH 7.0	pH 7.3	pH 7.0	pH 7.3
Sal. typhimurium	++	++	-	-	-	-
S. L. F. #1	++	++	-	-	-	-
Proteus morganii	++	++	±	-	+	+
Proteus vulgaris	++	++	+	+	+±	++
Staph. aureus F.D.A	++	++	±	+	-	-
Aero. aerogenes	++	++	-	-	-	-
Sacch. ellipsoideus	±	±	±	±	-	-
Sacch. cerevisiae	++	++	+	±	-	-
S. salivarius(S25D)	++	++	++	++	++	++
S. salivarius(S20B)	++	++	+±*	+±*	-	±
S. salivarius(#83)	++	++	+±*	+±*	±	+
S. liquefaciens	++	++	++	++	++	++
Proteus species	++	++	++	++	++	++

* There was a tendency for gum formation to be inhibited on this medium.

foregoing experiments had seemed to indicate, at least in the media containing sodium azide, that these inhibitory agents were more effective at a reaction approaching neutrality. The results of this experiment appear in Table VII and are also presented in graphic form in Figures 1 and 2. Figure 3 presents a comparison between the growth response of microorganisms other than "typical" Streptococcus salivarius and that of the "typical" strains used. Figure 2 presents essentially the same comparison with the exception that growth responses of all streptococci as against all other test organisms is included. A review of the data shows that all of the media investigated, with the exception of RG basal plus tellurite and azide, lack some property which would make them an ideal selective substrate. The inability of RG, NSS, and RG basal plus tellurite to suppress the growth on "contaminating" organisms such as Proteus, bacilli, and yeasts was again confirmed. The inhibitory power of RG medium toward the streptococci was reemphasized. The CH medium was found to be ineffective since all organisms used, with the exception of Torula lactosa, grew even in the presence of the sodium azide. The RG basal plus sodium azide and tellurite seemed to be effective in eliminating or hindering the growth of all but the "typical" streptococci and Streptococcus liquefaciens.

An experiment was carried out with this medium in an attempt to determine how much inoculum was needed to initiate growth on its surface. One/tenth cc. inocula from the original broth, 1:1,000, and 1:10,000 dilutions of twenty-four hour old cultures of S25D and #83 were streaked on the surface of agar plates. After incubation for two days the growth response was recorded as it appears in Table VIII. The data indicates that this medium was inhibitory to even relatively large inocula of "typical" Streptococcus salivarius. At the same time, eight strains of "typical"

TABLE VII

Growth of Micro-organisms on Media Designed for Selective Isolation of "Typical" Streptococcus salivarius

Bacterial species	Growth on Selective Media*				
	I**	II	III	IV	V
Escherichia coli	-	-	++	-	+
Aero. aerogenes	±	±	±	-	±
Sal. typhimurium	-	-	+±	-	+
Slow lact. ferm. #1	-	-	+	-	+±
Proteus morganii	+	+	+++	-	++
Proteus vulgaris	+++	+++	+++	-	++
Proteus species	++++	++++	++	+	++
Staph. aureus	-	+++	+++	++±	+++
Bacillus subtilis	±	+++	+++	-	++
Sacch. cerevisiae	+	++	+++	+±	++
Torula lactosa	+	+	+	+	-
Strep. liquefaciens	++	++++	++++	+++	++++
Strep. saliv. #6	++	++	+++	++	++++
Strep. saliv. #S25D	++	++++	++++	++++	++++
Strep. saliv. #83	++	++++	++++	++++	++++

* Results recorded on comparative basis with growth on control equal to ++++

** I = The RG basal medium plus K_2TeO_3(0.03%) and Crystal violet (1:500,000)
II = The RG basal medium plus K_2TeO_3(0.03%)
III = The MNS medium (0.02% NaN_3)
IV = The RG basal medium plus K_2TeO_3(0.03%) and NaN_3(0.02%)
V = Modified Chapman's medium (0.02% NaN_3)

GROWTH RESPONSES ON FIVE SELECTIVE MEDIA

FIG. 1

TYPICAL *S. salivarius* COMPARED WITH OTHER BACTERIAL SPECIES

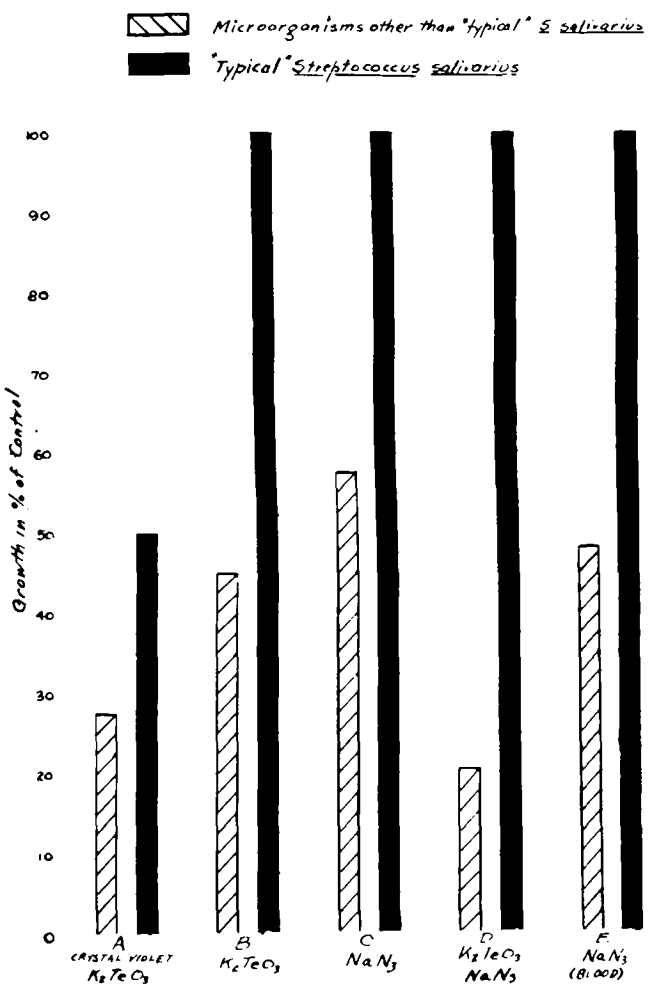


FIG. 2

TYPICAL *S. salivarius* COMPARED WITH ALL STREPTOCOCCI AND OTHER BACTERIAL SPECIES

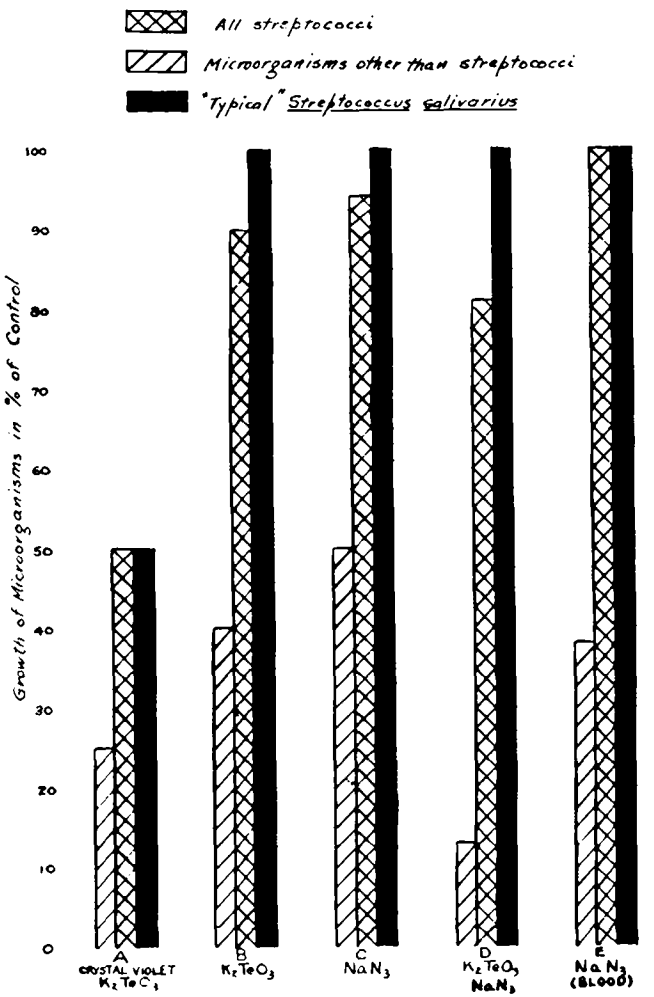


TABLE VIII

Effect of Inoculum Size on Initiation of Growth on the Surface of Azide-Tellurite Agar

Strain No.	Dilution of inoculum		
	Direct	1:1,000	1:10,000
S250	+	+	-
#63	-	-	-
Probable number of cells in inoculum	10,000,000	10,000	1,000

Streptococcus salivarius, two "atypical" strains and two strains of Streptococcus liquefaciens were inoculated on the surface of agar plates in order to determine the type of individual colony formation appearing on this medium. The results indicated that "typical" mucoid colony growth as previously observed by Rose and Georgi, (1941) was suppressed in all instances, the growth being limited to minute colony formation. It became apparent that none of the media tested were adequate for the purpose intended.

Evidence further confirming, in part, the above experiments was obtained by carrying out quantitative studies on the reduction of growth from small inocula. Three media were employed in these studies, viz., NSS, RG, and RG basal plus 0.03% potassium tellurite. Cultures of three "typical" strains were plated from 1:5 million or 1:10 million dilutions in quintuplet into the media without inhibitors as a control and into test media. After 48 to 72 hours incubation, counts were made and the inhibition recorded as percent reduction of numbers in test vs. control plates. From the results of a preliminary experiment with one strain of streptococcus, it was apparent that NSS medium at both pH 7.0 and 7.3 would allow growth from

minute inocula of Streptococcus salivarius while RG medium entirely suppressed growth. These preliminary results were confirmed in an expanded experiment, the results of which are presented in Table IX. The RG medium entirely eliminated inocula as great as 133 cells of S20B, while an inoculum of approximately 92 cells of S25D was unable to grow in the RG basal plus tellurite along. These results further indicate that the medium of Niven, et al (1942) is relatively non-inhibitory to small inocula of streptococci. This medium was not employed for field analysis, however, since Walter (1942) had used it in routine restaurant sanitation studied and had found isolation of streptococci impossible due to overgrowth of the

TABLE IX

Quantitative Effect of Inhibitory Media on Growth from Small Inocula of Streptococcus salivarius

Strain No.	pH	Media* A Plain	A NaN_3	B Plain	B K_2TeO_3 Crystal Violet	C Plain	C K_2TeO_3
#83	7.0	24**	29	41.2	0	3.8	0
	7.3	25	24	34	0	2.4	0
S25D	7.0	3.2	0.6	4	0	92.6	1.6
	7.3	2.2	0.8	2	0	3.2	0
S20B	7.0	69	43	133	0	34.8	0
	7.3	72	45	113.2	0	29.2	0

*Media
A. Niven, Smiley, and Sherman (1942) medium
B. The RG medium
C. The RG basal medium plus K_2TeO_3 (0.03%)

**Average number of colonies appearing on five plates.

plates by Proteus, coliforms, and yeasts. It became apparent that the use of a solid medium in the isolation of streptococci from washed glasses was attended by several difficulties, not the least of which was an inability on the part of the organisms desired to initiate growth from small inocula. When the inhibitory power of a selective medium was reduced, undesirable contaminants appeared. These facts seemed to indicate that an enrichment culture might be essential to assure a larger percentage of isolations. With this in mind, the following series of experiments were undertaken.

Development of Enrichment Medium

Tellurite crystal violet broth: An attempt was first made to employ the RG medium as a broth enrichment culture for inocula from washed glasses. Two restaurants, a luncheonette and a tavern were examined over a ten-day period. Ten glasses from each establishment on ten successive days were sampled according to the technique previously outlined. The swabs were immersed at once in the enrichment medium, agitated vigorously, and allowed to incubate for 48-72 hours at 37° C. Inocula from tubes showing growth, as evidenced by turbidity or reduction of tellurite, were streaked on the RG solid medium and allowed to incubate for 48 to 72 hours. Colony characteristics of those organisms growing were noted, and some were isolated for identification. The results are presented in Table X. A review of the data shows that of the 400 glasses tested, 15% carried "atypical" Streptococcus salivarius, while only 1.5% carried the "typical" strain. It was evident that an enrichment medium of this constitution might also be too inhibitory to the "typical" strains desired.

Effect of reducing conditions on azide inhibition: The possibilities of using sodium azide in the RG basal medium was explored. Other experiments

TABLE X

Recovery of Streptococci from Washed Glasses
Using a Tellurite-Crystal Violet Enrichment Medium

Restaurant Number	No. Glasses Tested	Percent Recovery of Streptococci "Typical" Strain	"Atypical" Strain	Other Streptococci
1	100	0	10	5
2	100	2	2	3
3	100	0	21	27
4	100	4	27	10
Total	400	1.5	15	11.3

(See Part II) had indicated that sodium azide was a more powerful inhibitor under reducing conditions. A series of experiments involving the addition of graduated amounts of cysteine were undertaken in an attempt to determine the concentration of cysteine which would render the reducing conditions most favorable to growth of Streptococcus salivarius, while inhibiting "contaminating" bacteria.

Growth was quantitatively determined as turbidity by means of a Coleman spectrophotometer at 650μ. For the purpose of large experiments, a series of 18x150 mm. culture tubes were selected which varied no more than 1% from a given standard tube containing the medium to be used. The RG basal medium at pH 7.2 plus 0.02% sodium azide was employed as the substrate with varying concentrations of cysteine. This medium was tubed 9.2 cc. per tube and sterilized. Fourteen to 16-hour old cultures were inoculated 0.1 ml. to each test medium in duplicate and the sub-cultures incubated for 24-48 hours at 37° C., prior to collecting turbidity data. In order to follow the redox conditions in the experiment, inoculated or

uninoculated controls containing methylene blue were carried simultaneously with test media. Control media, less the inhibitor, was inoculated in each instance and the inhibition was calculated as the percent reduction of growth as follows:

Where A = Total growth in control = 100 - observed opacity
B = Total growth in test media = 100 - observed opacity
x = Percent reduction

The percentage growth in test as compared to control media can be calculated from the ratio A/B = 100/x so that x = 100 - 100B/A

A review of the data from these experiments, representative results of which appear in Table XI, indicated, primarily, that the desired results, e.g. increased inhibition of gram-negative organisms by reduced potentials and concomitant non-inhibition of streptococci, could not be realized by the addition of cysteine to sodium azide broth or semi-solid agar cultures. Examination of the results for any one particular organism shows growth to be erratic with no constant gradation either in test or control media. In general, the results show that gram-negative organisms are inhibited 60 to 90%, while the cocci are inhibited but 15 to 40 percent. These percentages obtained in all media studied. It was concluded, therefore, that satisfactory routine results could be obtained by the use of sodium azide broth without added cysteine.

Effect of Inoculum Size on Initiation of Growth: Previous experience has indicated that information on the inhibitory power of a selective medium on small inocula was highly desirable. A series of experiments were undertaken to determine what effect dilution of inoculum had on initiation of growth in the azide enrichment broth.

Sixteen-hour old broth cultures of "typical" Streptococcus salivarius and other organisms likely to interfere with its growth in

TABLE XI

The Effect of Lowered Oxidation-Reduction Potentials on Bacterial Inhibition by Sodium Azide

Bacterial Species		Media * I			II			III			IV			V		
		Plain	NaN_3	% Red.	Plain	NaN_3	% Red.	Plain	NaN_3	% Red.	Plain	NaN_3	% Red.	Plain	NaN_3	% Red.
E. coli	K2	51**	99.5	99	31.5	72	59.9	24	74	65.8	24.5	76	68.2	39	75	59
E. coli	K3	51.5	97.5	94.8	27	81	74	23	82.5	77.3	25	81.5	75.4	40.5	75	58
A. aerogenes	A3	44.5	98.5	97.3	22.5	81	75.5	25	78.5	71.3	29	92	88.8	37.5	95.5	92.8
A. aerogenes	A6	31.5	95.5	93.4	8	67.5	64.7	10	45.5	39.4	13.5	75	71	20.5	82	87.3
Proteus vulgaris	Novy	59	98.5	96.3	38.5	69	50.4	49	76.5	53.9	47.5	91	82.9	67	88.5	65
Proteus species		41.5	97	94.9	17.5	63.5	55.8	22	74	66.7	22.5	72.5	64.5	33	77	65.7
Staph. aureus	F.D.A.	31.5	100	100	26	69.5	58.6	15	48.5	39.4	21	73.5	66.5	17.5	48.5	37.6
S. liquefaciens		25	38	17.3	23	30.5	9.7	13.5	24.5	12.7	15	26	13	19	28.5	11.8
S. liquefaciens	#16	15.5	42	31.6	12.5	22	11	12	20.5	9.8	14	19.5	6.4	16.5	23	7.8
S. salivarius	#6	95	98.5	30	38	57.5	32.5	10	40	33	62.5	73	28	15	82	78.8
S. salivarius	(S44A)	11.5	87.5	85.9	13	77.5	74	53	58	10.9	11	82.5	79.2	63.5	75	31.6
S. salivarius	(S25D)	10	91	90	14.5	31	19.3	40	63.5	39.2						
S. salivarius	(S20B)	12.5	61	55.4	17.5	30	15.2	26	49	31.1	18.5	69.5	62.6	26.5	63.5	50.4
S. salivarius	#80	8	31	25	8	21.5	14.7	7	14.5	8.1				9	53	48.4
S. salivarius	#83	11	94	9393	12	31	22.7				10	86.5	[illegible]	12	69	62.2
S. salivarius	# 3							13.5	71.5	67	18.5	84	80.4	17.5	88.5	86

*, ** See next page.

TABLE XI (con't)

* I = The RG basal medium(glucose) + 0.02% NaN_3. Cultured under anaerobic conditions.
II = The RG basal medium (glucose) + 0.02% NaN_3
III = The RG basal medium (glucose) + 0.02% NaN_3 and 0.05% cysteine
IV = The RG basal medium (glucose) + 0.02% NaN_3 + 0.1% cysteine and 0.05% agar.
V = The RG basal medium (glucose) + 0.02% NaN_3 and 0.15% cysteine

** Average turbidity reading. Lower readings = heavier growth.

enrichment culture were serially diluted in units of ten in sterile 90 ml. water blanks. One ml. aliquots from each dilution were inoculated into tubes of azide enrichment broth from each dilution, the final concentration of inoculum being 10x greater than the dilution from which it was inoculated. Growth in glucose and inulin-containing azide broths were compared with inhibitor-free controls. An inulin-containing substrate was included in view of the report by Niven, et al (1941), who correlated inulin fermentation with the characterization of their "typical" Streptococcus salivarius. Few organisms ferment this polysaccharide, and it was felt that its inclusion might aid in the isolation of the desired organism. Five tubes were inoculated in the case of "typical" strains while other organisms were inoculated in triplicate. All tubes were incubated at 37°C for 48 hours and readings taken at various times. The results are presented in Table XII.

"Typical" strains S25D and S20B were able to grow from inocula as dilute as 1:10 million when cultured in an azide medium containing glucose. Previous experiments (see page 40) indicated that similar dilutions of these organisms contained in the neighborhood of from 50 to 100 cells. In an inulin-containing substrate, however, growth of S25D was initiated in the 1:100 dilution and then only in the control tubes less sodium azide. An inoculum of approximately 500 to 1000 cells of S20B were required to initiate growth in an inulin-azide medium. Culture # 208, which is a "typical" Streptococcus salivarius forming a crystalline rather than a mucoid colony, and "atypical" strain #6 were able to initiate growth in either media from minute inocula. Escherichia coli and Proteus species were markedly inhibited in the higher dilutions in both selective media but Staphylococcus aureus and Streptococcus liquefaciens #16 were able to grow from small inocula.

In another experiment, the effect of inoculum size on growth of two "typical" strains in glucose-azide broth was reinvestigated. Dilutions

TABLE XII

The Effect of Inoculum Size on Initiation of Bacterial Growth in Azide Enrichment Media.

Bacterial Species		Medium	Dilution of Inoculum 1:100	1:1000	1:10T	1:100T	1:1M	1:10M	1:100M	1:1B
S. salivarius	S25D	A	++*	++	++	++	++	++		
		B	+	+	+	+	+	+		
		C	+	-	-	-	-	-		
		D	-	-	-	-	-	-		
S. salivarius	S20B	A	++	++	++	++	++	++		
		B	+	+	+	+	+	+		
		C	++	++	+	±	±	-		
		D	+	+	-	-	-	-		
S. salivarius	#6	A	++	++	++	++	++	++	++	±
		B	++	++	++	++	++	++	±	-
		C	+	+	+	+	+	+	+	±
		D	+	+	+	+	+	+	+	-
S. salivarius	#208	A	++	++	++	++	++	++	++	++
		B	+	+	+	+	+	+	+	±
		C	+	+	+	+	+	+	+	+
		D	+	+	+	+	+	+	+	+
E. coli	E2	A	++	++	++	++	++	++	++	++
		B	+	+	+	-	-	-	-	-
		C	++	++	++	++	++	++	++	++
		D	+	+	+	+	+	±	-	-
S. aureus	F.D.A.	A	++	++	++	++	++	++	++	++
		B	+	+	+	+	+	+	+	±
		C	++	++	++	++	++	++	++	++
		D	+	+	+	+	+	+	+	+

TABLE XII (cont.)

The Effect of Inoculum Size on Initiation
Of Bacterial Growth in Azide Enrichment Media

Bacterial Species	Medium	Dilution of Inoculum 1:100	1:1000	1:10T	1:100T	1:1M	1:10M	1:100M	1:1B
Proteus species	A	++	++	++	++	++	++	++	++
	B	+	+	+	+	±	-	-	-
	C	++	++	++	++	++	++	++	-
	D	+	+	+	+	+	-	-	-
S. liquefaciens #16	A	++	++	++	++	++	++	++	++
	B	++	++	++	++	++	++	++	++
	C	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+

• Average growth response in duplicate or quintuplet tubes.

were increased 100 fold over those of the previous experiment and turbidity data on triplicate tubes was also taken. The results presented in Table XIII indicate that growth in azide enrichment media can be initiated from extremely minute inocula. Data from previous experiments (see page 40) indicate that the inoculum in a 1:100 million dilution approaches 1 to 10 cells. It is apparent that the relatively small inocula from restaurant glassware might easily initiate growth in enrichment media of this nature.

It had been observed beforehand that the ratio of growth in the plain medium to growth in azide medium was a constant regardless of inoculum size. The turbidity data gathered confirms this observation. The ratio of growth in plain medium to growth in azide medium as determined by turbidity was plotted against dilution of inoculum. The resulting graph in Figure 3 indicates that this ratio is a constant, for all practical purposes, over a wide range of dilutions.

Field Studies

Isolation of Streptococcus salivarius from unwashed glasses: An experiment was undertaken to determine the effectiveness of this enrichment medium in the isolation of "typical" Streptococcus salivarius from the mixed populations occurring on unwashed restaurant glasses. Glasses from a campus restaurant were chosen at random, and, since this experiment was carried out in the summertime (July, 1942), these included water, iced tea and coffee, and milk glasses. Swabs were made in the usual manner and inoculated directly into the enrichment medium, agitated vigorously, and incubated for 24 to 48 hours at 37°C. At the end of the incubation period, which varied with the time required for growth to appear, a loopful of the culture was streaked onto the RG medium. The plates were then incubated for 72 hours and examined for colonies. "Typical" and "atypical" colonies were noted

TABLE XIII

Dilution of Inoculum as it Effect Initiation of Growth and Total Growth by Two Strains of *Streptococcus salivarius* in an Azide-Containing Medium

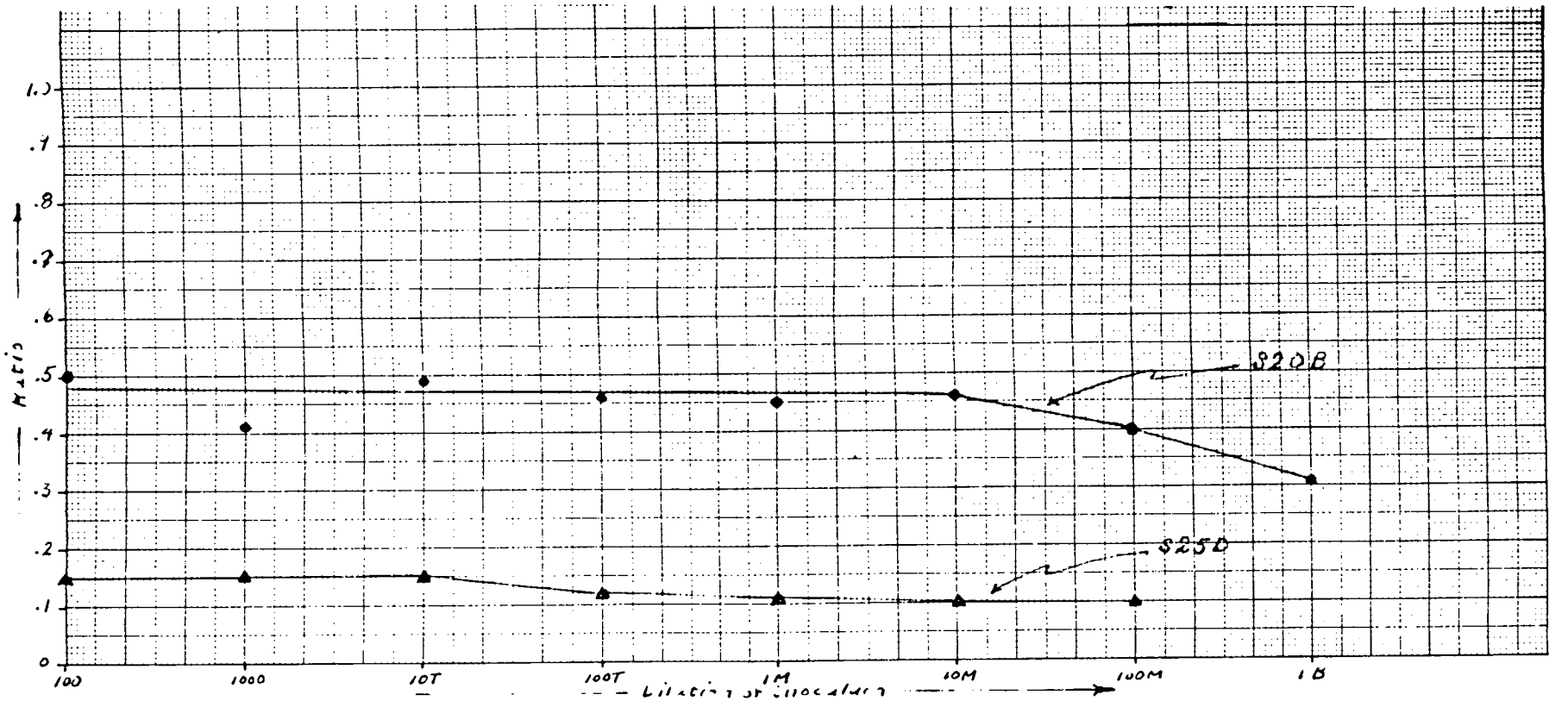
Culture No.	Medium	Dilution of Inoculum							
		1:100	1:1000	1:10T	1:100T	1:1M	1:10M	1:100M	1:1B
S20B	A*	17**	18	18	20	19	19	19	22
	B	34	44	37	44	42	41	47	71
S25D	A	7	7	7	7	7	8	7	7.5
	B	47	47	48	58	65	68	72	—

* Media same as in Table XII.

** Average turbidity reading of triplicate tubes.

FIG. 3.

EFFECT OF DILUTION OF INOCULUM ON QUANTITY OF GROWTH IN PLAIN MEDIA VS. AZIDE MEDIA. RATIO OF GROWTH BETWEEN MEDIA



and some were isolated for further study. Percent occurrence of all forms plus the total percent carrying any type of streptococci was calculated, the results appearing in Table XIV.

Attention is called to the fact that the "typical" Streptococcus salivarius forming a hard, crystalline colony was the predominant "typical" form isolated with inulin as an enrichment substrate, while the "mucoid" form was isolated more frequently with glucose as the substrate. A high percentage recovery of "atypical" forms occurred in any instance. These results correlate well with those previously reported (see page 47) on the ability of these strains to initiate growth from minute inocula in glucose- and inulin-azide enrichment broths.

Isolation of Streptococcus salivarius from washed glasses: In a survey of the occurrence of Streptococcus salivarius on washed restaurant glasses, forty restaurants, luncheonettes, drug stores, and taverns were examined, ten glasses in each establishment being sampled in the routine manner. When growth appeared in enrichment cultures, sub-cultures were made on the RG solid medium as before. Three enrichment media were employed in this study: (a) the RG basal medium (glucose as carbohydrate) plus 0.1% cysteine, 0.05% agar, and 0.02% sodium azide, (b) the RG basal medium (glucose as carbohydrate) plus sodium azide(0.02%), and (c) the RG basal medium (inulin as the carbohydrate) plus 0.02% sodium azide. The results obtained have been recorded as the incidence of two forms of "typical" Streptococcus salivarius and one form of "atypical" Streptococcus salivarius (a) in restaurants, (b) on washed glasses from these restaurants, and (c) in wash and rinse waters. A survey of the data in Table XV shows that "atypical" strains were recovered from 73 to 100% of the restaurants examined. Dishwashing procedures of 18.8 to 44.4% failed to remove "typical" strains from all of their glassware. When a glucose-containing substrate was used, "typical"

TABLE XIV

The Incidence of Oral Streptococci on Unwashed Glasses and the Effect of Carbohydrate Source in an Azide Enrichment Medium on their Percentage Isolation

Carbohydrate Source	No. of Samples	Unwashed Glasses Bearing Oral Streptococci										Percent bearing oral Streptococci
		"Typical" Strains* A.		B.		"Atypical" Strains		All three Strains		No Strep.		
		No.	%	No.	%	No.	%	No.	%	No.	%	
Glucose	30	17	53.3	10	33.3	14	46.6	23	76.6	0	0	100
"	14	6	42.8	3	21.4	11	78.6	0	0	0	0	100
Inulin	45	7	15.6	32	71.1	28	62.2	2	4.4	1	2.5	97.5
"	39	11	28.2	22	56.4	29	74.4	3	7.7	0	0	100

* Strain A = Mucoid colony-forming strain described by Rose and Georgi (1941) and Niven, et al (1941).

" B = Strain forming a hard, crystalline colony.

TABLE XV

Incidence of Oral Streptococci
Determined by Three Types of Enrichment Media

Enrichment medium *		No. Tested	"Typical" Strains**				"Atypical" Strain	
			A.		B.			
			No.	%	No.	%	No.	%
			In Restaurants					
A		15	5	33.3	0	0	11	73.3
B		9	4	44.4	0	0	9	100.0
C		16	3	18.8	9	56.3	14	87.5
			On Washed Glasses					
A		150	8	5.6	0	0	34	22.7
B		88	5	5.7	0	0	59	67.0
C		160	3	1.9	14	8.8	64	40.0
			In Wash and Rinse Waters					
A	Wash	13	2	15.4	0	0	7	53.8
	Rinse	6	0	0	0	0	1	16.7
B	Wash	7	3	43.0	0	0	3	43.0
	Rinse	3	0	0	0	0	3	100.0
C	Wash	9	0	0	0	0	5	55.6
	Rinse	5	0	0	0	0	3	60.0

* A = The RG basal broth (glucose as carbohydrate) + 0.1% cysteine and 0.05% agar, + NaN_3 (0.02%)
B = The RG basal broth (glucose as carbohydrate) + 0.02% NaN_3
C = The RG basal broth (Inulin as carbohydrate) + 0.02% NaN_3

** See Table XIV

strains were demonstrated in 5.7% of all the glasses examined, and "atypical" strains were isolated from 22.7 to 67.0%.

DISCUSSION

Gram-positive and gram-negative bacteria can be separated by virtue of their differing sensitivity to triphenyl methane dyes (Churchman, 1912) or inorganic salts (Eisenberg, 1918-1919). However, one encounters difficulty when attempting to apply this phenomenon to selective isolation of a specific bacterium. A medium designed to isolate oral streptococci from washed glasses must be inhibitory to the extent that coliforms, gram-positive spore-forming bacteria, and micrococci are eliminated but yet permit the growth of Streptococcus salivarius from small inocula. The difficulties involved have been emphasized by experiments which demonstrated that the selective medium developed by Rose and Georgi (1941) was inhibitory to small inocula of Streptococcus salivarius. Further studies showed that this organism grew uninhibited on an azide-containing agar, but the inhibitory power of this medium was sufficiently low to permit relatively good growth of other micro-organisms.

Adoption of an enrichment culture for the primary isolation of a specific organism is frequently desired. In a broth medium containing sodium azide, it was demonstrated that Streptococcus salivarius grew abundantly from minute inocula while coliforms and proteus species appeared to grow sparsely. It was further shown (Part II, page 76) that Streptococcus salivarius could outgrow large inocula of Escherichia coli when these two organisms were grown in mixed culture. These results were similar to ones obtained by Edwards (1938) and seem to indicate that routine use of this medium would assure isolation of Streptococcus salivarius from washed glasses.

It has been demonstrated that Streptococcus salivarius grows

characteristically on the surface of a tellurite-crystal violet-sucrose agar when the inoculum is sufficient to initiate growth. This substrate was employed as a confirmatory medium, therefore, in field experiments to demonstrate the occurrence of Streptococcus salivarius on unwashed and unwashed drinking glasses. Results, showing 47 to 79% recovery of "atypical" streptococci from unwashed glasses and 22.7 to 67% recovery from washed glasses, correlate well with the occurrence of Streptococcus salivarius on restaurant glasses as reported by Dick and Hucker (1940). The incidence of "atypical" strains is mentioned particularly since the streptococcus isolated by Dick and Hucker was an alpha-hemolytic form comparable to the "atypical" organism reported herein. Rose and Georgi, (1941) were the first to demonstrate the occurrence of "typical" Streptococcus salivarius on restaurant ware, and the experiments recorded in this thesis confirms that report.

Discussion of restaurant sanitation: In view of the lack of epidemiological evidence that eating utensils are vectors for a specific disease, one might question whether restaurant utensil sanitation is an important public health problem. It is difficult to answer that question with only experimental facts, yet it may be well to briefly review these facts in order to see what evidence there is for the belief that disease can be transmitted through improperly washed restaurant ware.

Bacterial count on restaurant ware, per se, is merely an indication of the care with which the article has been handled. Dishwater, towels, drainboards, exposure to air during storage, all may contribute to the final bacterial load of a glass, yet the organisms present are, in the main, of no sanitary significance. (Baveraux and Mallmann, 1936). Bacterial counts, therefore, cannot be used solely

as evidence that a glass is a disease vector.

Taylor (1921), Cumming (1919), Horwood and Pesare (1942), and others have repeatedly demonstrated the occurr ence of pathogenic organisms on eating utensils. Cumming (1920), using Serratia marcescens as a tracer organism, show ed that bacteria were transmitted from person to person through the medium of eating utensils and wash water. Dick and Hucker (1940) have demonstrated the presence of Streptococcus salivarius on 39% of the washed glasses they examined. Experimental evidence contained in this thesis demonstrates the occurrence of a streptococcus of unquestionable human origin on supposedly clean glasses from 19 to 45% of the restaurants examined. Homer N.Calver* has calculated the number of individual restaurant services in the United States and found them to be equivalent to one per person per day. On the basis of the 6% recovery of "typical" Streptococcus salivarius, this means that approximately five thousand people per day in Lincoln are drinking from glasses contaminated with the saliva of a previous user. The significance of these results are obvious. The evidence indicates that a perfect chain of disease transmission exists, making restaurant utensil sanitation an important public health problem.

SUMMARY

1. A selective medium containing potassium tellurite, or a combination of potassium tellurite and crystal violet, inhibits small inocula of "typical" strains of Streptococcus salivarius.
2. Selective media containing sodium azide do not inhibit small inocula of "typical" Streptococcus salivarius but permit the growth of other organisms likely to occur on restaurant glasses.
3. Minute inocula of oral streptococci can initiate growth in an enrich-

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* Ci ted by Horwood and Pesare (1942)

ment broth containing sodium azide.

4. The total final concentration of organisms per ml. in azide enrichment broth is constant regardless of the size of the inoculum.

5. Increased inhibition of "extraneous" micro-organisms with concomitant non-inhibition of streptococci cannot be realized by reducing the oxidation-reduction potential of enrichment broth with cysteine or cysteine plus 0.05% agar.

6. Oral streptococci can be demonstrated on unwashed and washed drinking glasses by means of a technique involving primary enrichment in azide-containing broth followed by streaking on tellurite-crystal violet-sucrose agar.

7. The role of drinking glasses in the transmission of disease is discussed.

PART II

The Effect of Sodium Azide on the Physiology of *Streptococcus salivarius*

Lockwood (1941) has reported that a technique involving application of biochemical methods to studies in vitro provides the only means for obtaining direct knowledge of the effect of sulfonamide drugs on bacterial nutrition. Also, referring to sulfonamide bacteriostasis, he stated ". . . investigators trained in bacteriological chemistry have brought forward explanations in chemical terms of certain phenomena which, without such work, would have remained in the realm of conjecture." This type of research he listed as one of the basic studies needed for an elucidation of problems involved in practical application of sulfonamide bacteriostasis. Such conclusions might well be extended to include any practical application of bacteriostatic agents. The development of a selective medium seems, therefore, to call for a study of the effect of the inhibitory agents employed on the physiology of micro-organisms.

LITERATURE SURVEY

Metabolism of streptococci: The metabolism of streptococci has been the object of extensive research. Particular attention has been directed toward carbohydrate utilization in view of the homofermentative nature of their glycolysis. Published reports have lacked specificity as to the exact organisms studied. This lack stimulated the recent work by Smith and Sherman (1942). They critically examined lactic acid production by streptococci of all classes and species, and reported a normal yield of approximately 90% lactic acid on the basis of glucose fermented. Twelve strains of Streptococcus salivarius produced an average of 93.6%. A single

strain studied, S30A, yielded 93.4% in growing cultures and 93.5% in "resting" cell fermentation, indicating that total lactic acid produced was essentially the same in "growing" as in "resting" cells.

The production of mucoid colonies by some coccoid forms when grown on sucrose substrate has been known to occur for some time, but only recently (Niven, et al. 1941) was this phenomenon reported as characteristic of the "typical" strains of Streptococcus salivarius. It produced an abundance of a soluble levan when grown on a well-buffered sucrose- or raffinose-containing medium. Over 40% of the fermented sucrose has been shown to go into polysaccharide synthesis. Inulin fermentation was correlated with levan production, although the organism did not produce the polysaccharide on this substrate. Neither are the following substrates sources for the levan: Glucose, fructose, galactose, maltose, glucose plus fructose, glucose plus fructose and galactose, fructose plus maltose, fructose plus lactose, or glucose plus inulin. No other strains of streptococci studied, including representative strains of all general classes, were able to produce a soluble polysaccharide, although Streptococcus bovis was observed to form an insoluble levan.

Enzyme systems in streptococci: With the exception of the report by Burnet (1927) that streptococci were among the "cyanide insensitive" bacteria, research on the specific enzyme systems functioning in streptococcal metabolism was almost non-existent prior to 1935. Fujita and Kodama (1935) reported on the oxidation of glucose by Streptococcus hemolyticus. Because it was found to lack cytochrome and was "cyanide insensitive" they concluded respiration was catalyzed by Waburg and Christian's yellow ferment.

Farrell (1935) carried out an extensive research into the enzyme systems of streptococci, employing twenty-two species from various groups as test organisms. Eighteen of the twenty-two species were found devoid of

catalase. Using the non-toxic indigo-tetrasulfonate redox indicator in a modified Thunberg technique, dehydrogenase activity on carbohydrates, sugar acids, and amino sugars was studied. Dehydrogenases were noted in all streptococci, activity being most apparent in the non-hemolytic species. Bromacetic and iodoacetic acids were found to poison glucose dehydrogenases while "several other respiratory or fermentative poisons" were ineffective. Seventy-one fatty, amino, dibasic, hydroxy, and keto acids were not dehydrogenated by the streptococci.

Although Yaoi and Tamiya (1935) reported the presence of cytochrome in Streptococcus erysipelatis, Farrell was unable to demonstratethis oxidase system in any of his cultures, including three strains of this organism. Indophenol oxidase, identified by Keilin (1929) as identical with Warburg's respiratory ferment, was not demonstrable by the Keilin technique, oxygen uptake studies, or by attempts to isolate it from lysozyme-ruptured cells. Using tincture of guaiac, o-tolidine, benzidine, and 2-7 diamino fluorene·HCl as test reagents, he found a peroxidase present in all of the seven species studied. However, repeated experiments showed this peroxidase to be iron-free and heat-stable, and, therefore, different from the horseradish peroxidase of Wieland and Sutter (1935) and others. On the basis of these results, Farrell postulated an enzyme system peculiar to the streptococci and involving dehydrogenases and a peroxidase.

Barron, et al (1938) studied further the oxidations produced by streptococci. The dehydrogenase studies of Farrell (1935) were confirmed and refuted, in part, by the Warburg manometric technique employed. Glucose dehydrogenase was found present but was inhibited by sodium fluoride in addition to iodoacetic acid. Since sodium fluoride inhibits splitting of phosphoglyceric acid and iodoacetic inhibits conversion of hexose diphosphate into triose phosphates (Embden and Deuticke, 1934), it was concluded that

glucose is first transformed into a triose phosphate via a hexose diphosphate before it is oxidised. The "cyanide insensitivity" of streptococci noted by Farrell and others was confirmed. Lactic acid was fermented as was pyruvic acid and glycerol.

It seems strange that a homofermentative lactic acid producer should actively ferment its own main end product. The reason may lie in the following data condensed from the paper of Barron and co-workers:

Substrate	Poisoned by Iodoacetic	NaF	HCN
Glucose	+	+	-
Glycerol	+	+	-
Pyruvate		+	+
Lactate	-		-

"Normal" fermentation, as exemplified by a proliferating culture fermentation of glucose and glycerol, is not inhibited by cyanide but is by iodoacetic acid and sodium fluoride. "Abnormal" fermentation, viz. lactate and pyruvate, show variations in enzyme poison sensitivity from the "normal". This abnormality may be a result of environmental conditions. These experiments were carried out in a Warburg manometer under agitation in the presence of oxygen whereas "normal" metabolism is anaerobic. The fact that acetic and formic acids were noted as the end products of these "abnormal" fermentations supports this view. Streptococci normally produce 90-95% lactic acid from glucose with small amounts of ethyl alcohol and carbon dioxide (Friedeman, 1939).

Nature of enzyme inhibition by sodium azide, an oxidase poison: Until 1933 no work had been done on the role of sodium azide as a cellular oxidation poison. Keilin (1933), working with tissue preparations, reported it inhibited cellular respiration, oxidation of cytochrome, and the indophenol reaction (oxidation by Warburg's respiration enzyme) in a manner similar to potassium cyanide. Keilin and Hartree (1934) showed sodium azide was an inhibitor of catalse. This observation was carried to the field of bacterio-

logy by Lichstein (1942) who reported catalase activity of Bacillus subtilis and Bacillus megatherium completely inhibited after six hours exposure to a 1:10,000 aqueous solution of sodium azide. Fifty percent reduction was noted in Corynebacterium diphtheriae and Staphylococcus aureus cultured after six hours exposure to 1:5,000 sodium azide. Further studies by Keilin on the catalase reaction (Keilin and Hartree, 1936) showed sodium azide formed a reversible complex with catalase which was still able to slowly decompose hydrogen peroxide. This was unlike potassium cyanide which united with the metal of the hematin nucleus in catalase, rendering it inactive. Catalase inhibition was, therefore, divided into two general types. Further differentiation was noted in studies on the xanthine oxidase reaction (Dixon and Keilin, 1936). This enzyme, which catalyzes oxidation of hypoxanthine to xanthine, is inactivated when incubated with hydrogen cyanide prior to addition of the substrate, i.e., cyanide combined with the enzyme. Sodium azide, on the other hand, inhibited the reaction only when incubated with the substrate prior to addition of the enzyme, i.e., sodium azide apparently combined with the substrate and not with the enzyme.

Studies were also made by Keilin (1936) on the effect of sodium azide on hemoglobin compounds and on the reactions they produced. Sodium azide was found to form a red complex with methemoglobin with absorption maxima at 575.0 and 542.5 mm. as opposed to the 500.0 and 635.0 mm. maxima of methemoglobin itself. Further, sodium azide united with methemoglobin in approximately molecular proportions. That it combined with the iron of the methemoglobin molecule was doubted since its inhibition of the other ferric-containing compound of the hemoglobin complex, hematin, was not noted. A study was made into the effect of sodium azide on oxidations of cysteine by hematin. The oxidation, as measured by oxygen uptake, was found to be incomplete. Since sodium azide did not combine with hematin,

per se, the following mechanism was suggested: On shaking hematin with cysteine in air oxidation of cysteine occurred with the breakdown of some hematin, thus releasing iron. Sodium azide combined with the free iron and a catalytically less active iron-free "derived hematin" was formed which was unable to completely oxidize the cysteine present. This contention was borne out by the fact that alpha-alpha' dipyridyl produced the same effect and the iron was almost completely recovered as the iron-alpha-alpha' dipyridyl complex. Other systems were studied and the results are presented in the following table taken from Keilin's paper:

System	KCN	NaN_3
Respiration (oxidation uptake)	+	+
Cytochrome oxidase	+	+
Catechol oxidase	+	+
Catalase (Yeast and liver)	+	+
Peroxidase	+	+
Uricase	+	+
Amino acid oxidase	-	-
Xanthine oxidase	+	-
Methemoglobin (Fe^{+++})	+	+
Hemoglobin (Fe^{++})	-	-
Hematin (Fe^{+++})	+	-
Heme (Fe^{++})	+	-

In 1939, Keilin and Hartree (1939) announced the discovery of another cytochrome component in beef heart muscle. This was called "a_3" and was found to act as a cytochrome oxidase. They reported that sodium azide combined reversibly with the oxidised state of this enzyme system.

Using a "resting" cell technique, Cook and Stephenson (1928) demonstrated that microbial oxidation of fermentable carbohydrates resulted in the absorption of only approximately three-fourths of the oxygen required for complete oxidation of any given substrate. This could not be explained by the then existing theory that absorption of carbohydrates

(carbohydrate synthesis) was a property only of actively proliferating cells. Barker (1936) suggested the observed discrepancy might be explained by assuming polysaccharide synthesis also occurred in "resting" cells. His theory was further advanced by Clifton (1937) who found that sodium azide, among other inhibitors, could effectively block "carbohydrate synthesis" by Pseudomonas calco-aceticn, Escherichia coli, and Spirillum serpens. In the presence of sodium azide, "resting" cells of these organisms absorbed oxygen equivalent to that required for complete oxidation of the substrate to carbon dioxide and water. The observations made by Clifton have been extended and confirmed for bacteria by Clifton (1938) and Clifton and Logan (1939), and for yeasts by Winzler (1940), Pickett and Clifton (1941), and van Niel and Anderson (1941). In addition, van Niel and Anderson studied the oxidative assimilation of Streptococcus fecalis and found it absent. Their conclusion that the homofermentative lactic acid producers exhibited no oxidative assimilation is pertinent to this research.

That polysaccharide synthesis does occur along with fermentation was shown in the case of muscle and yeasts by Willstatter and Rohdewalde (1940) and for Escherichia coli by Leibowitz and Kupermintz (1942).

On the other hand, Burris and Wilson (1942) reported that increased oxidation of glucose, produced in Rhizobium trifolii "resting" cell cultures by action of 2-4 dinitrophenol (D.N.P.)(which has the same effect on oxidative assimilation as sodium azide, Clifton and Logan, 1939) could be induced either by addition of D.N.P. initially or after assimilation of glucose was complete. In other words, with the rhizobia, at least, it is unnecessary to postulate an inhibition of assimilation to explain the increased substrate oxidation. It seems likely, however, in view of the evidence for the assumption, that oxidative assimilation does

occur in "resting" cells, and that sodium azide can poison this synthesis in some manner.

METHODS AND MATERIALS

Culture media

Stock culture broth: The liquid culture medium consistently used to prepare inocula was that of Hutner (1938) with 0.3% dipotassium phosphate substituted for an excess of calcium carbonate.

Experimental media: The basal substrate for all experimental media was the Rose and Georgi (1941) basal medium reported in Part I. (see page 26) Glucose was employed as a substrate carbohydrate in 1.0% concentration, except where specifically noted. In a few experiments the phosphate buffer was eliminated to decrease titration difficulties. In some experiments the sterile inhibitory agent was added to the sterilized media, but in others, involving the effect of sodium azide on bacterial physiology, it was added prior to sterilization (Mallmann, 1941)

Glucose substrate for studies on lactic acid production: For studies of the effect of sodium azide on lactic acid production by "resting" cells of Streptococcus salivarius, the glucose substrate of Smith and Sherman (1942) was employed. Glucose, dipotassium phosphate, and potassium dihydrogen phosphate were dried in an oven at 105°C for 24 - 48 hours to render them anhydrous and preserved in a desiccator over anhydrous calcium chloride. The substrate had the following composition:

Glucose	2.5 grams
Dipotassium phosphate	5.0 grams
Potassium dihydrogen phosphate	2.5 grams
Distilled water	500 ml.

pH 7.0

The anhydrous ingredients were weighed on an analytical balance and added to a 500 ml. volumetric flask which was filled to the 500 ml. mark with

distilled water. Fifty ml. aliquots were transferred quantitatively into 100 ml. volumetric flasks by means of a volumetric pipette. The required quantity of sodium azide was added to one-half of the flasks and the media sterilized for 15 minutes at 15 lbs. pressure. The final solution in each flask contained approximately 250 mg. of glucose, with very little variation. This procedure was in accordance with that employed by Smith and Sherman (1942) who weighed their ingredients on a torsion balance and found the resulting solutions to vary but one to four miligrams from 250 mg. after sterilization.

Aseptic tubing: The apparatus employed in the aseptic tubing of media was that reported by West, et al (1942). The freshly prepared agar was tubed while hot and maintained liquid in a constant temperature water bath at 50°C until inoculated.

Stock cultures

The same micro-organisms were employed in this part of the research as in Part I. (see page 28)

"Resting" cell technique

In general, the procedure of Smith and Sherman (1942) was followed. Cultures S25D and S20B were carried through three successive transfers in stock broth medium, 5 ml. of the final transfer at 12 hours of age being inoculated into liquid media in centrifuge bottles for mass cell cultivation. Smith and Sherman (1942) employed a mass cell culture medium of the following composition:

Glucose	1 gram
Tryptose (Difco)	10 grams
Meat infusion	500 ml.
Tomato juice	25 ml.
Water to 1000 ml.	
pH 7.0	

We employed this medium in the first two experiments but found the final cell mass after centrifugation contained much tomato juice protein. A

"resting" cell fermentation implies enzyme activity in absence of growth. To prevent growth, cells must be washed free from proteinaceous material prior to inoculation in a protein-free substrate. The presence of tomato tissue renders it impossible, at least theoretically, to obtain "resting" cells. We employed our stock culture broth as a substrate and found the harvested cells to be as abundant and more active than those produced on Smith and Sherman's substrate.

After growing for 10 to 12 hours, the cells were centrifuged from the medium and washed once prior to suspending in sterile saline. The final suspension was used for inoculating the various substrates.

Determination of bacterial growth

In experiments involving changes in bacterial populations, a standard procedure was followed. One ml. aliquots were diluted to 1:100 in 90 ml. sterile water blanks. Further dilutions were made in increments of ten in 90 ml. blanks until the desired range was obtained. Each dilution was shaken 50 times and duplicate plates were inoculated with 1 ml. aliquots. Incubation was consistently for 48 hours at 37°C and counts were made with a Quebec colony counter and a hand tally.

Plating media: Escherichia coli was plated in the RG basal medium without carbohydrate to obviate gas formation. In the experiments involving growth in mixed cultures with Streptococcus salivarius, 1:100,000 crystal violet was added to inhibit the latter. (Garrod, 1933). Streptococcus salivarius was plated in the RG basal medium with sucrose as the carbohydrate substrate. With this sugar present, large, easily counted colonies of the streptococcus were formed. Minute colonies formed on other carbohydrate substrates rendered counting difficult. In mixed cultures with Escherichia coli, 0.02% sodium azide was added to inhibit the latter.

Chemical determinations

Hydrogen ion: Changes in pH were determined by means of a Cameron potentiometer.

Total acid: Ten ml. of culture media was titrated with sodium hydroxide to a phenolphthalien color standard at pH 8.4. The standard was prepared at each run by adding phenolphthalien and sodium hydroxide solution to an uninoculated control tube until the pH, as determined potentiometrically, was 8.4. In the experiments with Escherichia coli, 0.1 N NaOH was used, this being replaced later by 0.05 N NaOH.

Glucose: The procedure of Stiles, et al (1926) was employed in all reducing sugar determinations. The dilution schemes varied and they will be noted in the specific experiments. The effect of sodium azide, a reputed slow oxidizing agent (Wallmann, 1941), onglucose recoveries was determined by studying its effect on the analysis of known solutions of glucose. The effect was found to be negligible even in markedly high concentrations. The effect of incubation time on the auto-oxidation of glucose in uninoculated control flasks was studied by determining the glucose concentration in the RG basal medium with and without sodium azide over a period of 72 hours. Glucose concentration remained constant with incubation within titration error of the technique.

Lactic acid: The procedure of Troy and Sharp (1935) was used in all lactic acid determinations. This procedure involves the acid permanganate oxidation of lactic acid to acetaldehyde,which is distilled over and trapped in a bisulfite solution. The excess bisulfite is oxidized with iodine to a faint blue starch end-point, the bound sulfite released with sodium bicarbonate, and the released bisulfite titrated with standard iodine solution. The iodine solution was standardized before each experiment with

sodium thiosulfate which was in turn standardized each time with standard potassium iodate solution. Precautions were taken to prevent loss of acetaldehyde during the procedure by cooling the receiving flask in a running tap water bath at 10°-12°C during the oxidation and by immediate titration after completion of the oxidation.

Interfering substances are few. Troy and Sharp (1935) reported that acetone, formaldehyde, glycerol, 2-3 butylene glycol, and malic acid give high yields, none of which are present in streptococcal fermentation. Large quantities of ethyl alcohol or methyl alcohol also interfer. Monobasic aliphatic acids to not interfer, nor do citric, oxalic, succinic, or tartaric acids. Split protein products gave a higher yield than theoretical, obviating the use of our regular culture medium in lactic acid studies.

Standardization: Standard zinc lactate was prepared according to the method of Friedemann and Graeser (1933) and on analysis by dehydration and combustion was found to contain 33.51% ZnO by weight. The theoretical is 33.44%, resulting in an error of two parts per 1000, which is experimentally acceptable. An attempt was made to determine a known quantity of this zinc lactate in the RG experimental media. Results were consistently higher than theoretical, and apparently was due to split protein products in the protein precipitation filtrate. This obviated use of experimental media for determining effect of sodium azide on lactic acid production and resulted in the use of the "resting" cell technique previously described. Aqueous solutions of standard zinc lactate run through the complete determination showed percentage recovery of known amounts of lactic acid to be 94.5%. Hence, a 6.5% recovery factor was included in the calculations.

EXPERIMENTAL RESULTS

Effect of sodium azide on the growth, pH, total acid production and glucose utilization of micro-organisms: A review of the literature revealed

no experimental data on the effect of sodium azide on the physiological changes occurring during normal bacterial growth periods. It was felt that quantitative data on the effect of this oxidase poison on growth curves, acid production, and glucose utilization might furnish further information on the nature of its inhibitory power. The ability of sodium azide to retard cell multiplication of gram-negative bacteria is a well established observation. Its effect, however, on gram-positive organisms such as the streptococci is not so well established. In fact, Dr. J. M. Sherman (personal communication) expressed the belief that growth and biochemical activity of the streptococci is effected little if at all by this poison. The following series of experiments tend to disprove this belief by demonstrating that glucose utilization, acid production, and total, as well as rapidity of, streptococcal growth is markedly reduced in the presence of sodium azide.

Effect of Sodium azide on growth, pH, total acid, and glucose utilization: Two strains of "typical" Streptococcus salivarius were chosen as test cultures and were inoculated into stock broth 24 hours prior to the start of the experiment. Four 500 ml. Ehrlenmeyer flasks containing 250 ml. of the RG basal broth were prepared and 0.02% sodium azide was added to two of the flasks prior to sterilization. Two flasks were inoculated at zero time with 1 ml. of a broth culture of S25D and two flasks at zero time one-half hour later with S20B. At zero, 3, 6, 12, 26, and 50 hours, aliquots were withdrawn and total counts, pH, total acidity, and glucose determinations were made. The results of this experiment appear in Table XVI. These determinations were plotted against time and recorded in Figures 4, 5, 6, and 7. The data indicates that all of the physiological properties studied are markedly effected

by the addition of sodium azide.

Two other experiments of shorter duration were carried out in the same manner. The results obtained confirm those reported in the first experiment in all respects. The curves obtained were similar to those presented in Figures 4 to 7, hence it was concluded that these results were characteristic of azide inhibition of these strains of Streptococcus salivarius.

To understand better the nature of this inhibition, rates of chemical change per cell per hour were calculated from the recorded data by the equation of Buchanan and Fulmer (1930).

$$m = \frac{2.303\ S\ \log b/B}{t(b - B)}$$

Where m = Amount of change produced per organism per unit time.
S = Total amount of change in time "t".
B = Number of bacteria at beginning.
b = Number of bacteria at end of time "t".

The equation is based on the fact that enzyme activity, as evidence by chemical change, increases in ratio to the rate of cell division and, hence, the equation is valid only during a normal phase of logarithmic growth. The following time intervals, chosen from the various experiments, represent those in which logarithmic changes were attained. Therefore, the changes occurring during these time intervals were employed in the calculations.

Experiment No.	Organism	Time interval
1	S 25D	3 - 6 hours
1	S20B	0 - 3 hours
2	S25D	0 - 3 hours
2	S20B	0 - 3 hours
3	S 25D	3 - 6 hours
3	S20B	3 - 6 hours

The results in Table XVII show, in general, that the rate of chemical change per cell per hour is greater in the presence of sodium azide than in its absence. This apparent "stimulation" of enzymatic activity can be explained if it is assumed that cell division, per se, rather than carbohydrate

TABLE XVI

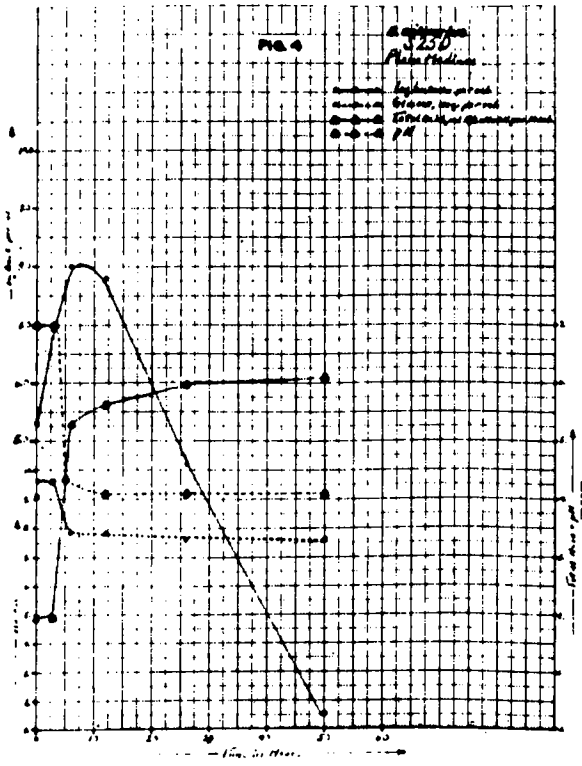
Change in Rate of Multiplication, Glucose Utilization, and Acid Production, as Determined by Hyndrogen-ion Concentration and Titrable Acidity, of two strains of Streptococcus salivarius when cultured in a Broth Medium Containing Sodium Azide

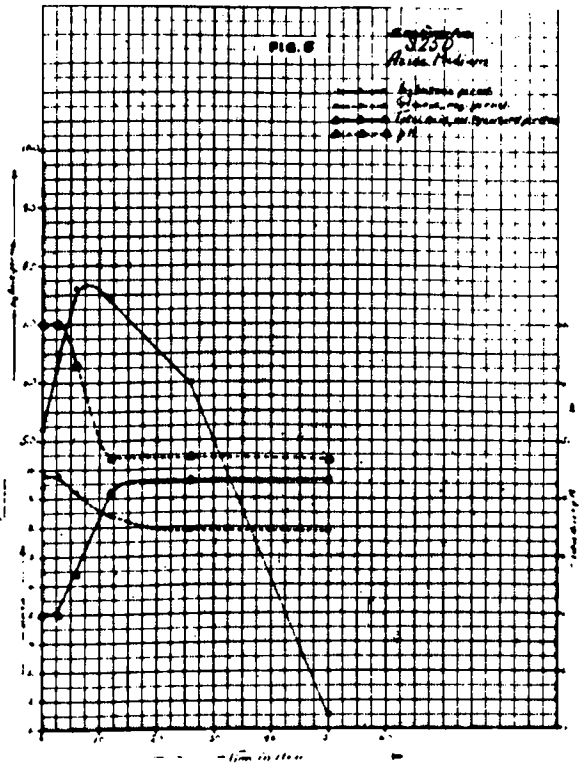
Strain No.	Culture Medium*	Determination**	TIME (in hours) 0	3	6	12	26	50
S25D	Plain	pH	7.0	7.0	4.35	4.1	4.1	4.1
		Total acidity	1.95	1.96	5.28	5.63	5.97	6.08
		Glucose	9.080	9.685	7.825	7.865	7.640	7.620
		Bacterial count	5.29776	6.93952	8.01284	7.81954	4.63347	<2.00000
	Sodium azide	pH	7.0	7.0	6.3	4.7	4.7	4.65
		Total acidity	2.06	2.05	2.70	4.12	4.31	4.34
		Glucose	9.440	9.795	9.215	8.400	7.910	7.930
		Bacterial count	5.21880	6.46240	7.63347	7.42160	6.00000	<2.00000
S20B	Plain	pH	7.0	5.85	4.80	4.40	4.30	4.20
		Total acidity	2.04	2.60	4.16	4.97	5.27	5.30
		Glucose	9.265	9.480	9.085	8.560	8.245	8.250
		Bacterial count	6.21219	7.65706	8.24055	7.61278	5.30103	2.00000
	Sodium azide	pH	7.0	6.5	5.4	4.85	4.80	4.80
		Total acidity	1.91	2.30	3.01	4.00	4.16	4.02
		Glucose	9.395	9.270	9.000	8.765	8.395	8.590
		Bacterial growth	6.26834	7.67210	7.67210	7.61805	6.04139	3.13033

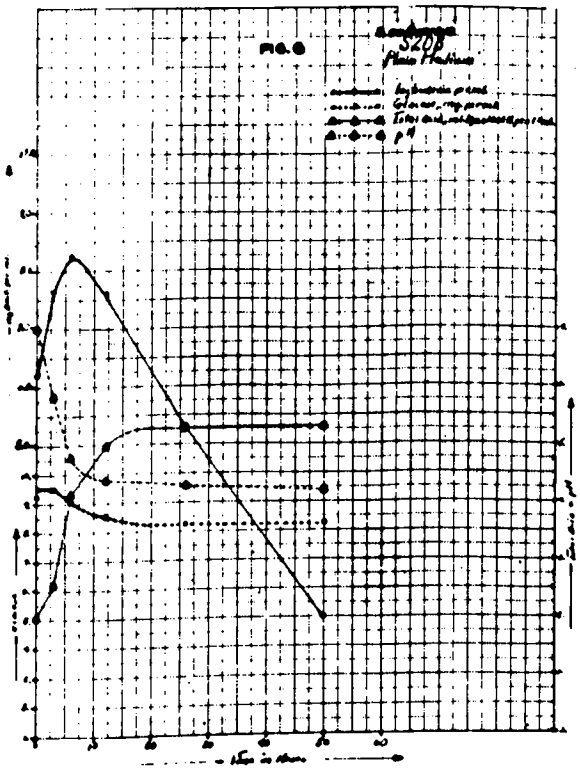
* The RG basal medium (glucose as carbohydrate) without added buffer.

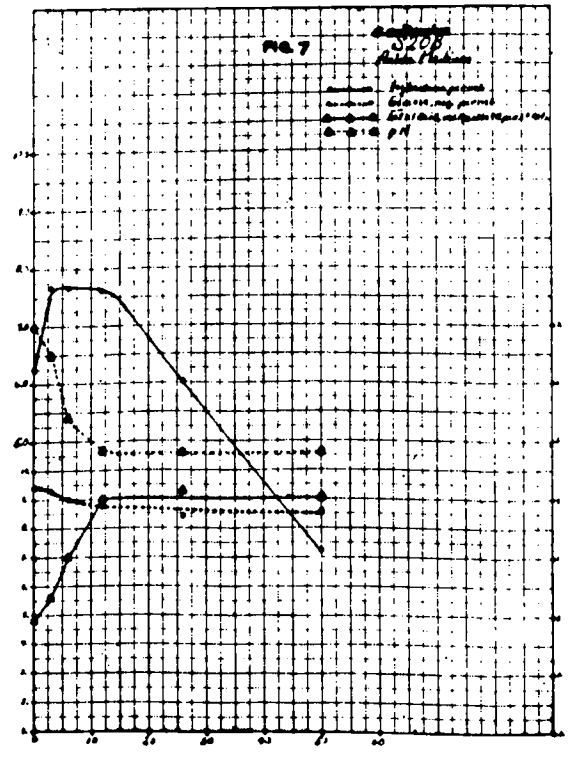
** Total acidity = Milliliters N/20 NaOH to neutralize 10 ml. culture medium.
Glucose = Milligrams per milliliter of culture medium.
Bacterial count = Number of bacteria per milliliter of culture medium.

CHANGE IN pH, TOTAL ACIDITY, NUMBER OF BACTERIAL CELLS AND GLUCOSE UTILIZED BY TWO STRAINS OF *S. salivarius* IN THE PRESENCE AND ABSENCE OF SODIUM AZIDE









enzymatic activity is the function inhibited. In other words, while cell division is retarded, the carbohydrate enzymatic activity of the cells already present continues unchecked. The effect on values in the Buchanan and Fulmer equation would be as follows: (a) "S" would increase in proportion to the number of cells already present and functioning in carbohydrate metabolism, (b) normal increase of the value log b/B would be reduced, but this would be offset by (c) the greater decrease of the value t(b - B) in the denominator. The net result would be an increase in "m", the chemical change per cell per unit time.

This hypothesis assumes that generation time is longer in azide than in plain media. The equation of Buchanan and Fulmer (1930) was used to calculate generation time in an attempt to confirm this assumption.

$$G.\ T. = \frac{t \log 2}{\log b/B}$$

where "t", "b", and "B" are the same as in the previous equation. Table XVII also shows generation times over the same increments of time used for calculating "m". In general, the calculations show generation time to be longer in azide than in plain media.

Experiments similar to the above have been carried out with two strains of Escherichia coli, E2 and E3. The results of these experiments appear in Table XVIII. When plotted against time, the curves shown in Figures 8 to 11 were obtained. The normal physiological activity of both strains became erratic and markedly inhibited in the presence of sodium azide. In the case of E3, all activity was halted and the cells seemed to die off at a slow but constant rate. The E2 cells apparently were able to recover after 25 hours and carry on normally, although with very much reduced carbohydrate metabolism. Another experiment with the E2 strain showed the same type of results. Recovery occurred at 14 hours and growth

TABLE XVII

Glucose Utilized and Acid Produced per Cell per Hour and Generation Time of *Streptococcus salivarius* in the Presence and Absence of Sodium Azide

Culture	Experiment No.	Culture Medium*	Mg. glucose Utilized/cell/ Hour	Acid Produced/cell/ Hour	Generation time in Minutes
S25D	1	Plain	1.748×10^{-8}	3.121×10^{-8}	46.9
		Sodium azide	1.312×10^{-8}	1.470×10^{-8}	45.9
	2	Plain	2.512×10^{-9}	2.878×10^{-9}	41.3
		Sodium azide	2.956×10^{-9}	2.150×10^{-9}	46.1
	3	Plain	5.835×10^{-9}	1.241×10^{-8}	50.0
		Sodium azide	1.197×10^{-8}	3.661×10^{-8}	51.6
S20B	1	Plain		1.211×10^{-8}	44.0
		Sodium azide		8.117×10^{-9}	44.3
	2	Plain		3.854×10^{-10}	38.5
		Sodium azide		6.924×10^{-9}	46.8
	3	Plain	3.974×10^{-9}	1.064×10^{-8}	46.5
		Sodium azide	9.674×10^{-9}	3.410×10^{-8}	54.4

* The RG basal broth medium.

TABLE XVIII

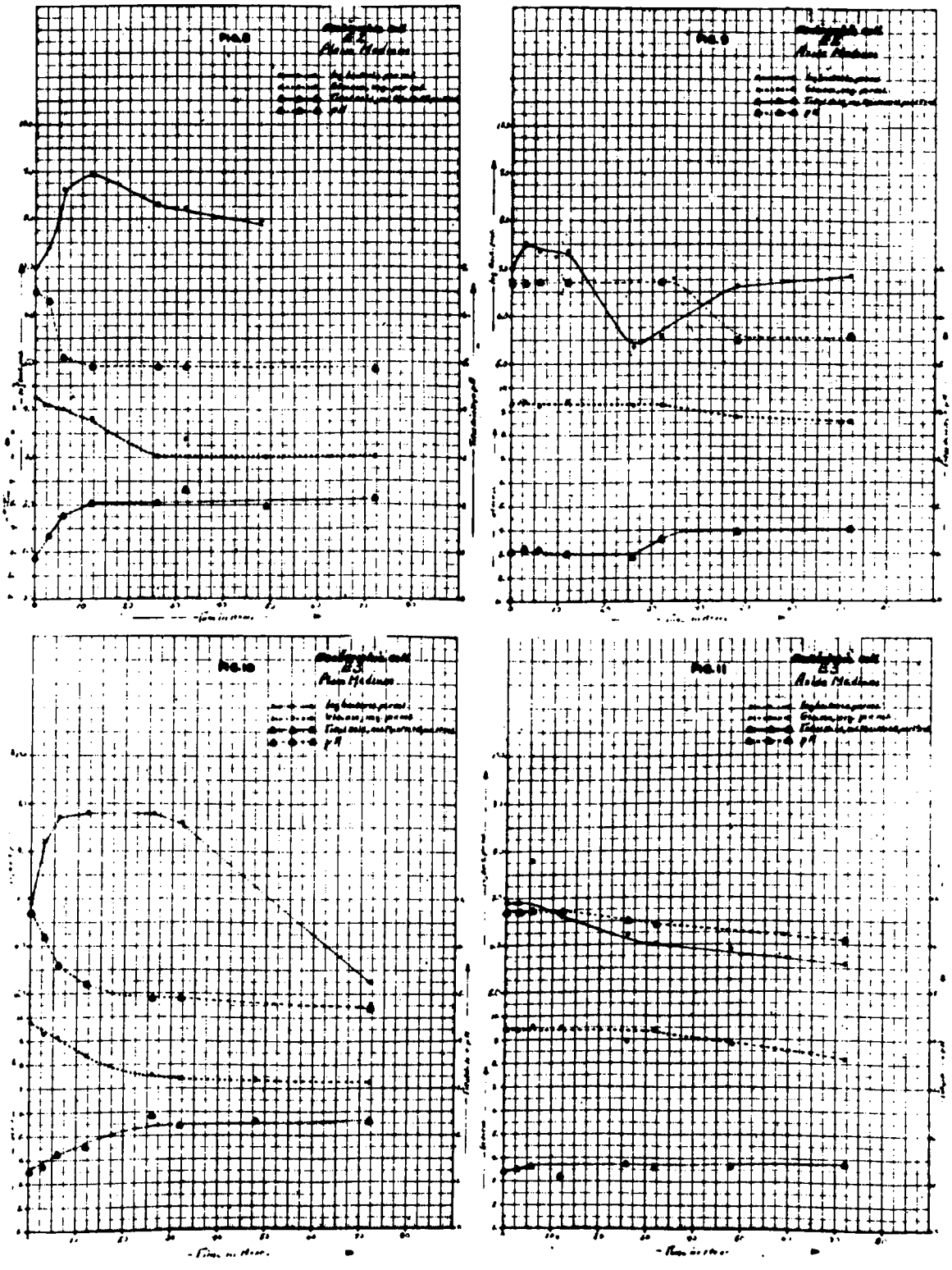
Change in Rate of Multiplication, Glucose Utilization, and Acid Production, as Determined by Hydrogen-ion Concentration and Titrable Acidity, of Two Strains of Escherichia coli when Cultured in a Broth Medium Containing Sodium Azide

Strain No.	Culture Medium*	Determination**	TIME (in hours) 0	3	6	12	26	32	48	72
E2	Plain	pH	6.7	6.3	5.1	4.9	4.9	4.9	4.9	4.8
		Total Acidity	0.84	1.35	1.78	2.09	2.09	2.34	1.93	2.14
		Glucose	9.455	9.205	9.015	8.640	7.045	7.785	7.030	7.030
		Bacterial count	6.99564	7.42325	8.63849	8.96343	8.32940	8.2442 8	7.9800	
	Sodium azide	pH	6.7	6.65	6.7	6.7		6.7	5.5	5.6
		Total acidity	1.07	1.28	1.17	1.02	0.92	1.31	1.47	1.52
		Glucose	9.260	9.475	9.225	9.410	9.260	9.260	8.750	8.590
		Bacterial count	6.99123	7.53275	7.35218	7.36173	5.35122	5.57921	6.62839	6.82607
E3	Plain	pH	6.7	6.2	5.6	5.2	4.9	4.9		4.7
		Total acidity	1.27	1.39	1.63	1.78	2.43	2.21	2.31	2.33
		Glucose	9.825	9.305	9.180	8.370	7.580	7.425	7.355	7.250
		Bacterial count	6.91116	8.20683	8.70757	8.83885	8.82607	8.60206		5.26717
	Sodium azide	pH	6.7	6.7	6.75	6.7	6.5	6.4		6.1
		Total acidity	1.21	1.28	1.36	1.09	1.39	1.29	1.35	1.38
		Glucose	9.435	9.390	9.640	9.475	8.890	9.380	8.765	8.210
		Bacterial count	6.83649	6.89763	7.82020	6.58883	6.22789	6.06070	5.91908	5.57403

* The EG basal medium (glucose as carbohydrate) with dipotassium phosphate as buffer.

** Total acidity = Milliliters N/10 NaOH to neutralize 10 ml. culture medium.
Glucose = Milligrams glucose per milliliter of culture medium.
Bacterial count = log number of bacteria per milliliter.

CHANGE IN pH, TOTAL ACIDITY, NUMBER OF BACTERIAL CELLS AND GLUCOSE UTILIZED BY TWO STRAINS OF *E. coli* IN THE PRESENCE AND ABSENCE OF SODIUM AZIDE



increased to a maximum at 32 hours, although carbohydrate metabolism was markedly inhibited.

Growth of Escherichia coli in mixed culture with Streptococcus salivarius: It was felt that Streptococcus salivarius might produce an anti-azide substance during the course of its metabolism so that azide-sensitive organisms might grow synergistically in its presence. This hypothesis was based on the following facts: (a) Sodium azide is a heavy-metal enzyme poison (Oppenheimer and Stern, 1939)(Keilin, 1936), (b) Fujita and Kodama (1935), Farrell (1935), and others have shown that streptococci are insensitive to cyanide and devoid of cytochrome, leading the former to postulate that respiration was catalyzed by Warburg and Christian's yellow enzyme, which is not effected by heavy-metal enzyme poisons, and (c) a statement by Oppenheimer and Stern (1939) that, according to the thermodynamic nature of enzyme catalysis, those systems lower in the thermodynamic scale, the dehydrogenases, could utilize almost any oxidase system available to complete their oxidation process. In other words, if the heavy-metal oxidase systems of Escherichia coli (the cytochromes, etc.) were blocked by azide, they might utilize the azide-insensitive mechanism of Streptococcus salivarius to complete their oxidation. During the process of the investigation, a paper by Henday (1942) appeared showing that sulfanilamide sensitive streptococci were able to grow relatively unchecked in the presence of sulfanilamide, if cell-free and enriched filtrates from sulfanilamide insensitive strains were used as a substrate.

Two experiments were undertaken. One ml. of 20 hour broth cultures of Escherichia coli and Streptococcus salivarius were inoculated individually into 500 ml. Erlenmeyer flasks containing 100 ml. of the

RG basal medium plus 0.02% sodium azide. A third flask was inoculated with one ml. of each culture. Plate counts were made in duplicate in the following media:

Culture	Plating medium
Escherichia coli	The RG basal (carbohydrate-free) + 1:100,000 crystal violet.
Streptococcus salivarius	The RG basal (Sucrose) + 0.02% NaN_3
The mixture	The RG basal (Sucrose) + 0.02% NaN_3 and The RG basal (carbohydrate-free) + 1:100,000 crystal violet.

Hydrogen-ion readings were taken and platings made at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 20, 24, (and 26) hours. After incubation at 37°C for 48 hours, counts were made with the aid of a Quebec colony counter and a hand tally. Glucose was employed in the Streptococcus salivarius plating medium on the first run, and the minute colonies resulting were impossible count accurately. The growth of Escherichia coli in the first experiment was confirmed in the next experiment, Table XIX, wherein the growth response of Streptococcus salivarius was also obtained. The results of the last experiment are shown graphically in Figures 12 and 13. Streptococcus salivarius was essentially unaffected by culturing with a large inoculum of Escherichia coli. The pH curve obtained from the mixed culture was almost identical with the one obtained from the pure culture of Streptococcus salivarius, indicating that acid production in the mixed culture was solely the result of the carbohydrate metabolism of the streptococcus.

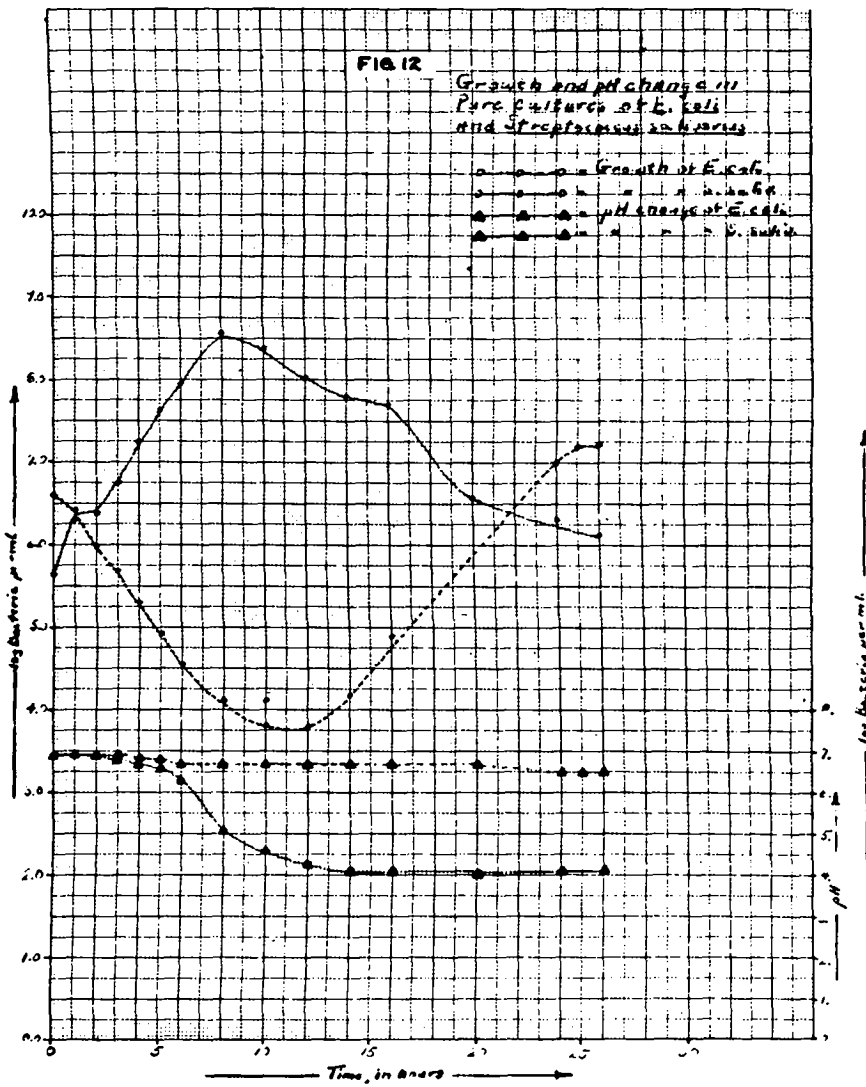
In both experiments, the inoculum of Escherichia coli was killed in 16 hours at a fairly constant rate, tending to disprove the hypothesis on which this experiment was based. Escherichia coli was not grown in sterile filtrates from Streptococcus salivarius broth cultures as a further test of the hypothesis. The growth curve of Escherichia coli

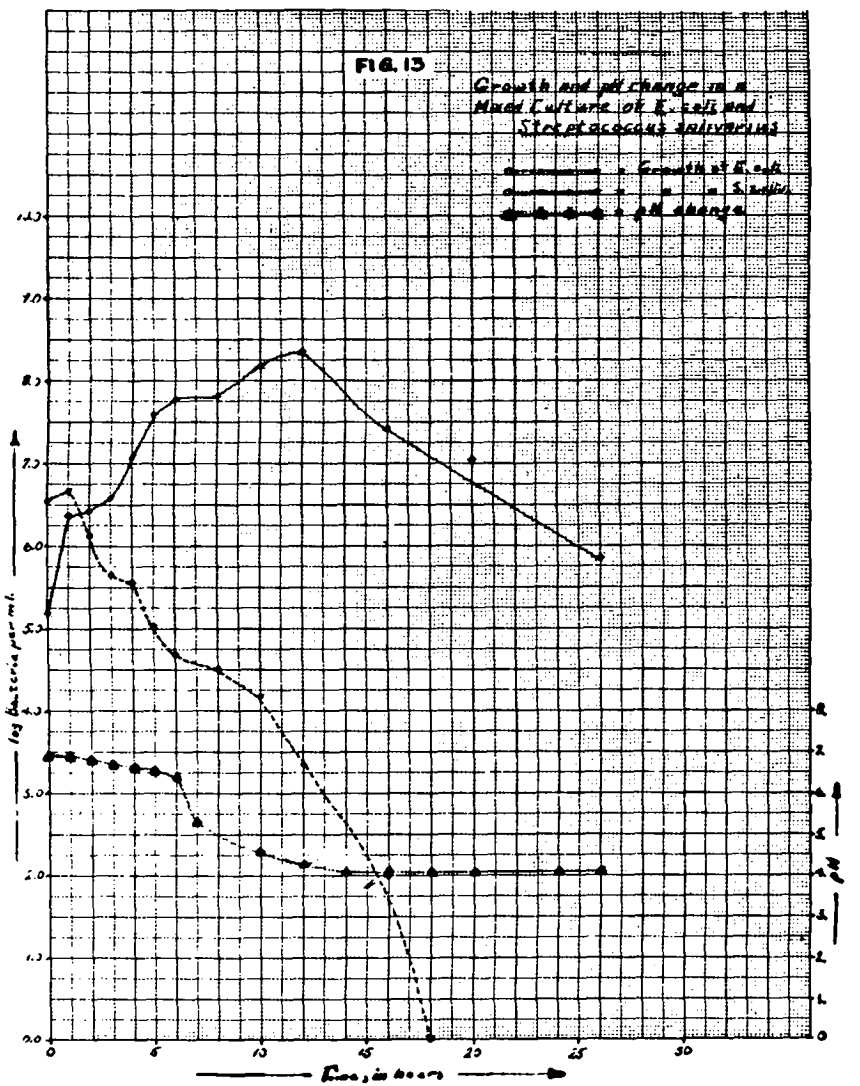
TABLE XIX

Growth and pH Changes Occurring in Pure and Mixed Cultures of Escherichia coli and Streptococcus salivarius in the Presence of Sodium Azide

Time (in hours)	Pure Cultures				Mixed Culture		
	E. coli		S. salivarius		Bacteria per ml.		pH
	Bact./ml.	pH	Bact./ml.	pH	E. coli	S. salivarius	
0	4,200,000	6.9	445,000	6.9	3,600,000	155,000	6.9
1	2,060,000	6.9	2,725,000	6.9	4,750,000	2,345,000	6.9
2	945,000	6.9	2,365,000	6.9	1,235,000	2,595,000	6.8
3	470,000	6.8	5,600,000	6.8	450,000	3,935,000	6.7
4	191,000	6.7	18,350,000	6.7	357,500	11,750,000	6.65
5	80,000	6.8	42,500,000	6.6	105,500	43,000,000	6.55
6	34,000	6.7	90,500,000	6.3	46,500	63,000,000	6.4
8	13,300	6.7	367,000,000	5.05	31,500	69,500,000	5.3
10	6,800	6.7	244,000,000	4.6	14,600	156,000,000	4.6
12	6,550	6.7	108,000,000	4.3	2,530	229,000,000	4.3
14	14,600	6.7	63,500,000	4.1	110		4.1
16	76,000	6.7	48,000,000	4.1	0	27,500,000	4.1
20		6.7	3,550,000	4.0	0	11,400,000	4.1
24	10,200,000	6.5	2,080,000	4.1	0		4.1
25	16,450,000	6.5					
26	18,100,000	6.5	1,335,000	4.1	0	670,000	4.1

CHANGES IN pH AND GROWTH OF PURE AND MIXED BACTERIAL CULTURES IN THE PRESENCE OF SODIUM AZIDE





in pure culture shows a decidedly prolonged lag phase. When growth does occur, metabolic activity, as evidenced by a fall in pH, is negligible. These results have a practical aspect, showint that heavy inocula of gram-negative organisms are not likely to outgrow streptococci in enrichment cultures designed for isolating the latter.

Effect of Sodium Azide on Lactic Acid Production by Streptococci

Using a "resting" cell technique, Smith and Sherman (1942) have shown that cultures of Streptococcus salivarius produce approximately 90% lactic acid on the basis of glucose fermented. Previous experiments (page 69) have indicated that sodium azide inhibits cell division, per se, and has little effect on carbohydrate metabolism. During "resting" cell fermentation, therefore, one would expect the same amount of lactic acid to be produced both in the presence and absence of sodium azide. The following experiments were undertaken to determine what effect, if any, sodium azide had on the homofermentative nature of Streptococcus salivarius.

Total lactic acid production: Employing the technique previously described, cells from four 120 ml. broth cultures of two strains of Streptococcus salivarius, S25D and S20B, were prepared in saline suspension. These cells were inoculated into 100 ml. volumetric flasks containing 50 ml. of glucose substrate (250 mg. glucose). The flasks were held at 37°C overnight to bring them to the optimum temperature for enzymatic activity, to obviate loss of activity by inoculation into cold media. At the end of 24 hours incubation at 37°C, the flasks were filled to the 100 ml. mark with distilled water, cooled to 25°C and again filled to the mark. After mixing well, 10 ml. of this mixture was removed volumetrically for glucose determination. The remainder was transferred quantitatively to a 250 ml. volumetric flask after adding 5 ml. of 20% sulfuric acid to halt

enzyme activity (Smith and Sherman, 1942).

Glucose determination: The 10 ml. sample was further diluted to 50 ml. during the protein precipitation procedure and two ml. duplicate aliquots of this dilution were taken for final analysis. Since the original 50 ml. of substrate had been diluted to 100 ml. before removal of the 10 ml. aliquot, residual glucose was calculated as follows:

Where x = Glucose determined in 2 ml. final aliquot,

Residual glucose = $x/2 \times 50 \times 10 = 250x$

Lactic acid determination: The 90 ml. of diluted substrate, after removal of 10 ml. for glucose determination, was diluted to 250 ml. during the cell protein precipitation procedure, and a 35 ml. aliquot of this was taken for final analysis. Hence,

Where L_o = Lactic acid determined in the 35 ml. aliquot,

$L_o / 35 \times 250 = Y$, (to correct for final dilution)

$Y + 0.1Y = Z$, (to correct for 10 ml. taken for glucose determination)

and Total lactic acid produced = $Z + 0.065Z$, (to correct for the recovery factor)

The results of the first experiment appear in Table XX. The percent lactic acid produced based on glucose fermented in plain and azide media by both organisms is essentially the same, the small variation noted being within experimental error of the method.

A second experiment was undertaken, varying from the first in the following instances: (a) Cells from 250 ml. broth culture were used as inoculum for each fermentation flask, (b) duplicate fermentations were made with each organism, using cells from broth medium of Smith and Sherman, (1942) and a modified Hutner's (1938) medium employed routinely as stock culture broth. The results appearing in Table XXI confirm the results of

TABLE XX

Total Production of Lactic Acid by "Resting" Cells of *Streptococcus salivarius* in the Presence and Absence of Sodium Azide

Strain No.	Fermentation Substrate	Mg. Glucose Utilized	Mg. Lactic Acid Produced	Percent Lactic on Basis of Glucose utilized
S20B	Plain	250	224.75	89.9
	Sodium azide	213	195.89	91.5
S25D	Plain	250	219.06	87.6
	Sodium azide	74	62.11	84.0

TABLE XXI

The Effect of Mass Cell Substrate and Sodium Azide on Lactic Acid Production by "Resting" Cells of Streptococcus salivarius

Strain No.	Medium for Mass Cell Growth	Fermentation Substrate	Mg. Glucose Utilized	Mg. Lactic Acid Produced	Percent Lactic on Basis of Glucose Utilized
S20B	Smith & Sherman	Plain	250	200.09	80.0
	Smith & Sherman	Sodium azide	250	191.36	76.5
	Hutner	Plain	250	206.76	82.7
	Hutner	Sodium azide	250	209.93	83.2
S25D	Smith & Sherman	Plain	250	193.77	77.5
	Smith & Sherman	Sodium azide	250	193.30	77.3
	Hutner	Plain	250	216.49	86.6
	Hutner	Sodium azide	236	200.68	85.0

the previous experiment, i.e., azide does not inhibit the percent lactic acid produced from glucose fermentation by two strains of "typical" Streptococcus salivarius. The cells cultured in modified Hutner's broth gave a consistently higher yield of lactic acid than did those cultured in Smith and Sherman medium. In all instances, in both this and the previous experiment, where a glucose residual occurs, the fermentation flask contained sodium azide. This is particularly true in the first experiment in which a smaller inoculum of cells was used in each flask.

Rate of Lactic Acid production: The above results do not correlate well with previous observations, e.g., they indicate that sodium azide slows enzyme activity as well as cell multiplication. An experiment was undertaken to detect whether such inhibition did occur. Culture S20B alone was used in this single experiment. After two serial transfers, twelve 250 ml. centrifuge bottles containing 150 ml. of Hutner's broth and 0.3% dipotassium phosphate, in place of excess calcium carbonate, were inoculated. After 10 hours incubation, the cells in each bottle were centrifuged, washed once with 10 ml. of 0.85% saline and then suspended in 5 ml. saline. After pooling and rendering the suspensions homogeneous by strong agitation, 10 ml. was inoculated into each of six fermentation flasks. All six contained 50 ml. of glucose substrate while three contained 0.02% sodium azide in addition. Initial glucose concentration was determined on two uninoculated flasks and was found to be 206.9 mg. in plain and 199.8 mg. in azide substrates. This average loss of 47 mg. was attributed to accidental extensive sterilization of this one set of media.

Inoculated flasks were incubated in individual sets for 5, 15, and 24 hours. At the designated time, five ml. of 20% sulfuric acid was added to each flask to halt enzyme activity, and the flasks were stored in

the refrigerator until the following day for analysis. Five ml. aliquots of each sample after final dilution were oxidized in the glucose determination instead of the two milliliters as before in order to reduce the error due to small samples. Lactic acid determination was the same as in the previous experiment. The results appear in Table XXII and in Figure 14. Although it is impractical to form a definite conclusion from but one experiment, the divergence in rates of glucose utilization and lactic acid production herein noted seems to indicate that sodium azide also has an effect on the carbohydrate enzyme systems of Streptococcus salivarius as well as on its cell division. When the logarithms of the milligrams of glucose utilized or lactic acid produced are plotted against time (Figure 14), this divergence is less pronounced than the data in Table XXII indicates. Apparently there is a break in rate of carbohydrate metabolism at 48 hours both in plain and azide media.

The Role of Medium Constituents in Sodium Azide Inhibition

The theory has been advanced that the effect of bacterial inhibitors in vitro is a result of combination between the inhibitor and some essential constituent of the medium. Keilin (1936) has found this to be true in the case of azide poisoning of the xanthine oxidase reaction. Our own experiments (page 48) showing a constant inhibition ratio regardless of inoculum size indicated there are factors other than enzyme poisoning which are functioning. It has been suggested that the nature of sodium azide inhibition might be similar to that of sulfanilamide and, therefore, susceptible to the counter-action of p-amino benzoic acid, a normal constituent of media containing yeast extract. An experiment was designed to determine what effect, if any, the constituents of the medium and p-amino benzoic acid might have on the inhibition ratio of sodium azide.

TABLE XXII

The Effect of Sodium Azide on Rate of Glucose Utilization and Lactic Acid Production by "Resting" Cells of Streptococcus salivarius S20B

Substrate	Determination	Time (in hours) 5	18	24
Plain	Glucose utilized	76.3 mg.	162.3 mg.	206.9 mg.
	Lactic acid produced	68.2 mg.	144.9 mg.	132.9 mg.
	Percent glucose to lactic acid	89.3 %	89.3 %	64.2 %
Sodium azide	Glucose utilized	42.3 mg.	95.9 mg.	93.3 mg.
	Lactic a cid produced	35.5 mg.	70.5 mg.	84.1 mg.
	Percent glucose to lactic acid	83.9 %	73.5 %	90.1 %

Twelve media were prepared with the RG basal medium (glucose as carbohydrate) as a universal substrate. The nature of this substrate was varied as follows:

a) No alteration. Inhibitor-free control.

b) With added sodium azide (0.02%)

c) Less yeast extract.

d) Less yeast extract but with added sodium azide (0.02%).

e) Less proteose peptone.

f) Less proteose peptone but with added sodium azide (0.02%).

g) Less beef extract.

h) Less beef extract but with added sodium azide (0.02%)

i) With added p-amino benzoic acid (0.003M)*

j) With added p-amino benzoic acid (0.003M) and sodium azide (0.02%).

k) Extracted with acid ether.

l) Extracted with acid ether and plus sodium azide (0.02%).

Two-hundredths percent sodium azide is equivalent to 0.0031 molar, and 0.003 molar p-amino benzoic acid was used to match this concentration. It has been reported that acid ether extraction will remove the anti-sulfanilamide factor. In this instance, the completed liquid medium at pH 1.6 was extracted four times with ether. This did not assure complete removal of the anti-azide factor, but it was felt that the reduction would be sufficient to become noticeable. All ingredients, with the exception of p-amino benzoic acid, were weighed with extreme care on a torsion balance and volumes of liquid were measured volumetrically. P-amino benzoic acid was weighed on an analytical balance. All media were adjusted to pH 7.2 to 7.3 electrometrically, 9.2 ml. added to each calibrated tube (see Part I, page 42), and sterilized at 15 lbs. for 15 minutes.

A broth culture of S20B was transferred twice prior to inoculation

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* Eastman Kodak

and one drop from the same culture was added to 5 tubes of each medium from a standard pipette. After 48 hours incubation at 37°C, turbidity was read on a Coleman spectrophotometer at 650mμ. Growth in each set of media was compared to a standard uninoculated control tube of that medium, thus eliminating errors due to variations in color with variations in constituents. The results of this experiment appear in Table XXIII and Figure 15. It appears that p-amino benzoic acid stimulates the growth of Streptococcus salivarius S20B but has no effect on preventing inhibition by sodium azide. Extraction with ether yields a substrate which stimulates growth but removes an anti-azide substance. Removal of yeast extract, protease peptone, and beef extract decreases growth, while the ratio of growth in the presence and absence of sodium azide remains relatively constant in all media.

Effect of Oxidation-Reduction Potential on Sodium Azide Inhibition

Mallmann (1941) has reported that sodium azide is a slow oxidizing agent, and, as such, probably inhibits bacterial growth by exerting a "poising" action on the oxidation-reduction potential of the culture medium. If that is true, organisms will tend to grow better in its presence when redox potentials of the medium are lower than normal. It is generally known that an E_h gradient exists in agar tube media, giving rise to a zone phenomenon when methylene blue is present. (Williams, 1939, 1940, 1941). In media of low potential, the zone of oxidized methylene blue is shallow and confined to the surface. In media of higher potential, the zone extends deeper into the tube. The position of the methylene blue zone, therefore, is an approximate indication of the potential gradient existing in the medium. Also, the relation of bacterial growth to the zone of methylene blue reduction should

TABLE XXIII

The Effect of Medium Constituents, p-Amino Benzoic Acid, and Acid Ether Extraction on the Inhibition of Streptococcus salivarius by Sodium Azide

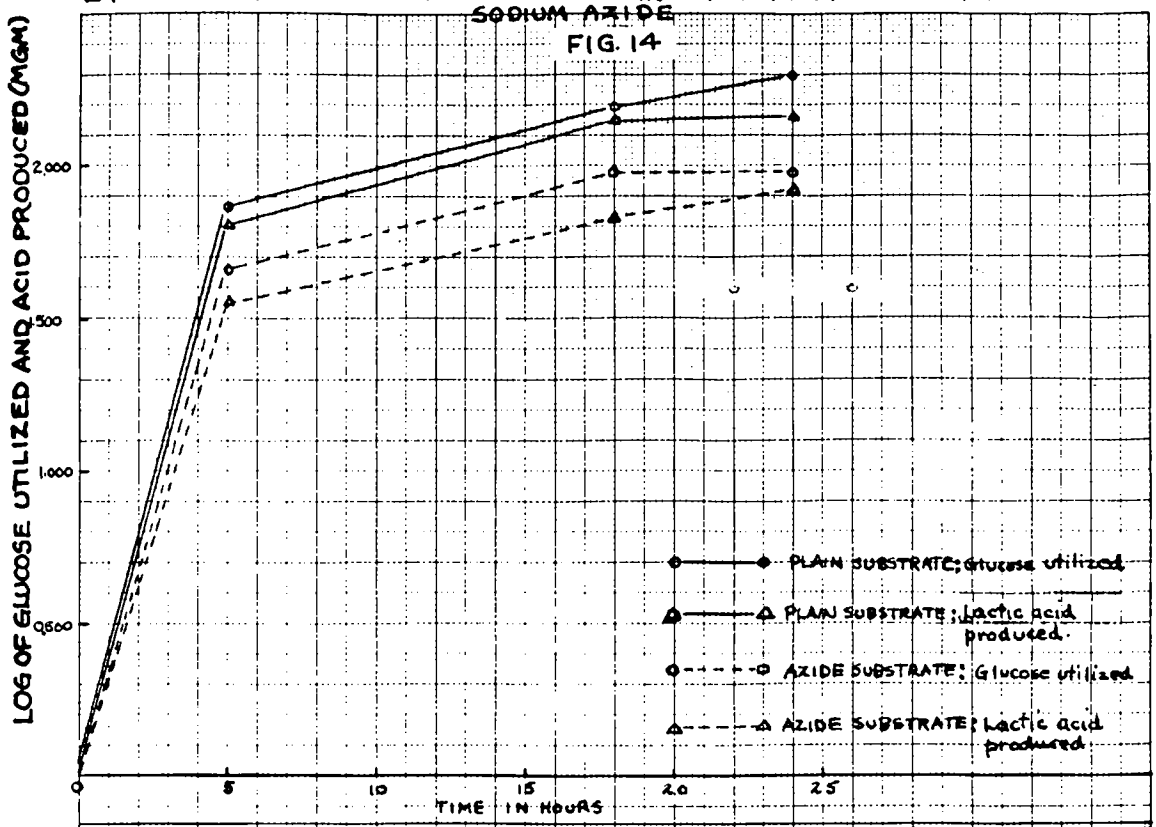
Media**	Total Growth*	
	Plain medium	Azide medium
I	86.4	68.4
II	81.4	59.0
III	75.2	53.4
IV	78.0	53.0
V	91.0	70.8
VI	90.2	54.8

* Calculated as 100 - observed average turbidity of 5 tubes.

** I = The RG medium without alteration.
II = The RG medium less yeast extract.
III = The RG medium less proteose peptone.
IV = The RG medium less beef extract.
V = The RG medium with added p-amino benzoic acid (0.003M).
VI = The RG medium after extraction with acid ether.

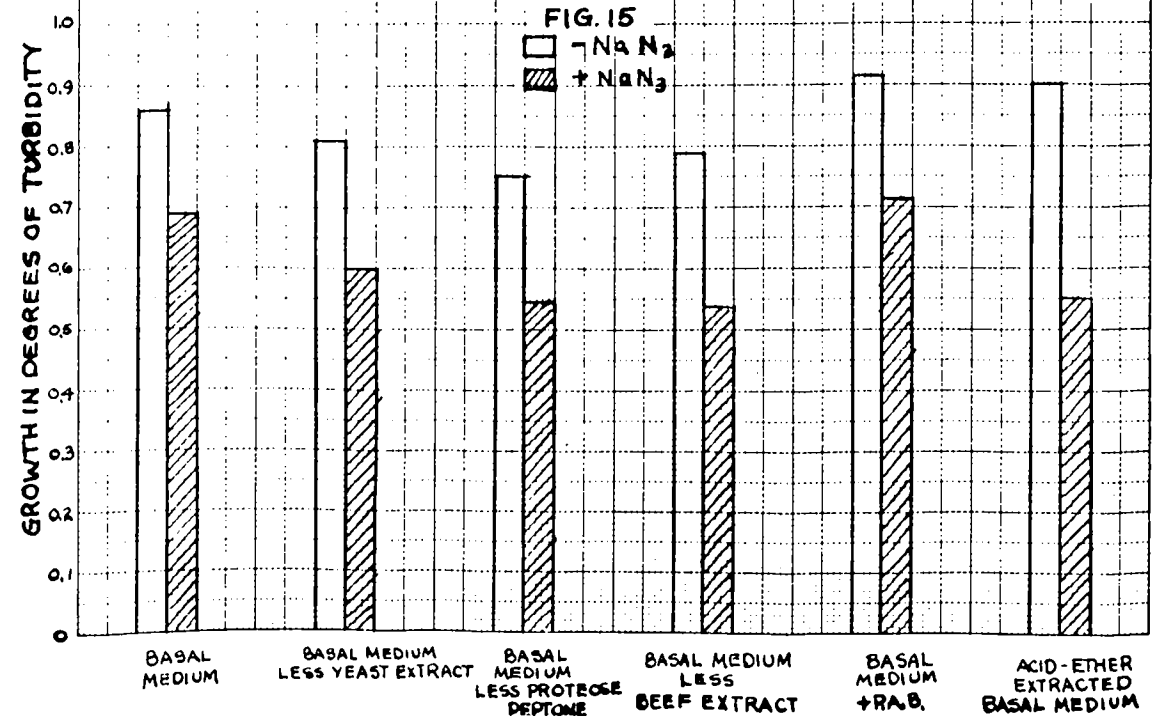
RATE OF GLUCOSE UTILIZATION AND LACTIC ACID PRODUCTION BY RESTING CELLS OF *S. salivarius* IN THE PRESENCE AND ABSENCE OF SODIUM AZIDE

FIG. 14



EFFECT OF MEDIA CONSTITUENTS ON *S. salivarius* IN THE PRESENCE AND ABSENCE OF SODIUM AZIDE

FIG. 15



give a rough estimate of the role which redox potential plays in the initiation of growth. Applying these phenomena to growth in a solid medium containing sodium azide, the effect of this substance on bacterial growth was studied.

The RG basal media, with and without sodium azide, were prepared in liter flasks and tubed aseptically, being maintained in liquid form at 50°C until time for inoculation. The potential was varied by adding (a) 1:10,000 potassium dichromate, (b) 0.05% cysteine, and (c) 0.1% sodium thioglycollate. Methylene blue, when present, was in a concentration of 1:1,000,000 or 1:200,000, depending upon the experiment. Twenty-four hour broth cultures of the test organisms were diluted 1:100 in sterile 90 ml. water blanks and one ml. aliquots of this suspensions were added as inocula. After the inocula were well dispersed by shaking, the tubes were placed in a cold water bath to solidify them rapidly. The cultures were incubated at 37°C for 48 hours to 72 hours before observations were made.

A preliminary experiment using 12 strains of bacteria in azide medium, less methylene blue, indicated that streptococci grew profusely throughout the tube while gram-negative species and Staphylococcus aureus were confined to the top one-half of the tube and, largely, at the surface.

This experiment was repeated in the presence of 1:200,000 methylene blue and observations of methylene blue reduction were taken every day for seven days. The presence and position of growth was recorded at its first appearance. Gram-negative organisms, which tend to produce highly reducing conditions, reduced methylene blue in plain media rapidly and grew throughout the tubes. In azide medium, however, growth was limited to the surface or the top one-half of the tube, while methylene blue reduction zones were the same as in uninoculated controls, viz., from 40

mm. below the surface extending to the bottom of the tube. Streptococci grew erratically. Apparently methylene blue in the concentration used was somewhat inhibitory to the streptococci since they were limited to surface growth. This observation is consistent with that of Farrell (1935) who reported methylene blue toxic to streptococci in certain concentrations.

The effect of reducing the potential by addition of 0.05% cysteine or 0.1% sodium thioglycollate was studied in another experiment, after decreasing the concentration of methylene blue to 1:1,000,000. Position of growth and methylene blue reduction was recorded daily for four days. The effect of reducing conditions was marked in azide media, growth of most organisms being limited to a narrow band at the surface where methylene blue existed in the oxidized state. Streptococcus liquefaciens, S25D, #6, and #101 were able to initiate small growth in the bottom of the tube after 2 - 3 days incubation.

The potential of the medium was poised at a higher E_h by the addition of potassium dichromate (1:10,000)(Mallmann, 1941). The results showed that, at higher oxidation-reduction potentials, cocci were able to grow throughout the tube. Gram-negative organisms were still effectively inhibited, although deep colonies were able to grow in two instances. (Cultures of Proteus species and Slow Lactose Fermenter #1)

These results have been compiled in Figure 16, wherein position of growth in the shake cultures has been compared with zones of oxidized methylene blue in media of various redox conditions. A correlation is seen between reducing conditions, as indicated by the depth of the zones of oxidized methylene blue, and position of growth. In media containing reducing substances, growth is limited to the surface. In media without poising agents, growth occurs deeper in the tube. In media containing an oxidising substance (potassium dichromate), growth extends even further into

THE EFFECT OF REDOX POTENTIAL ON BACTERIAL INHIBITION BY SODIUM AZIDE

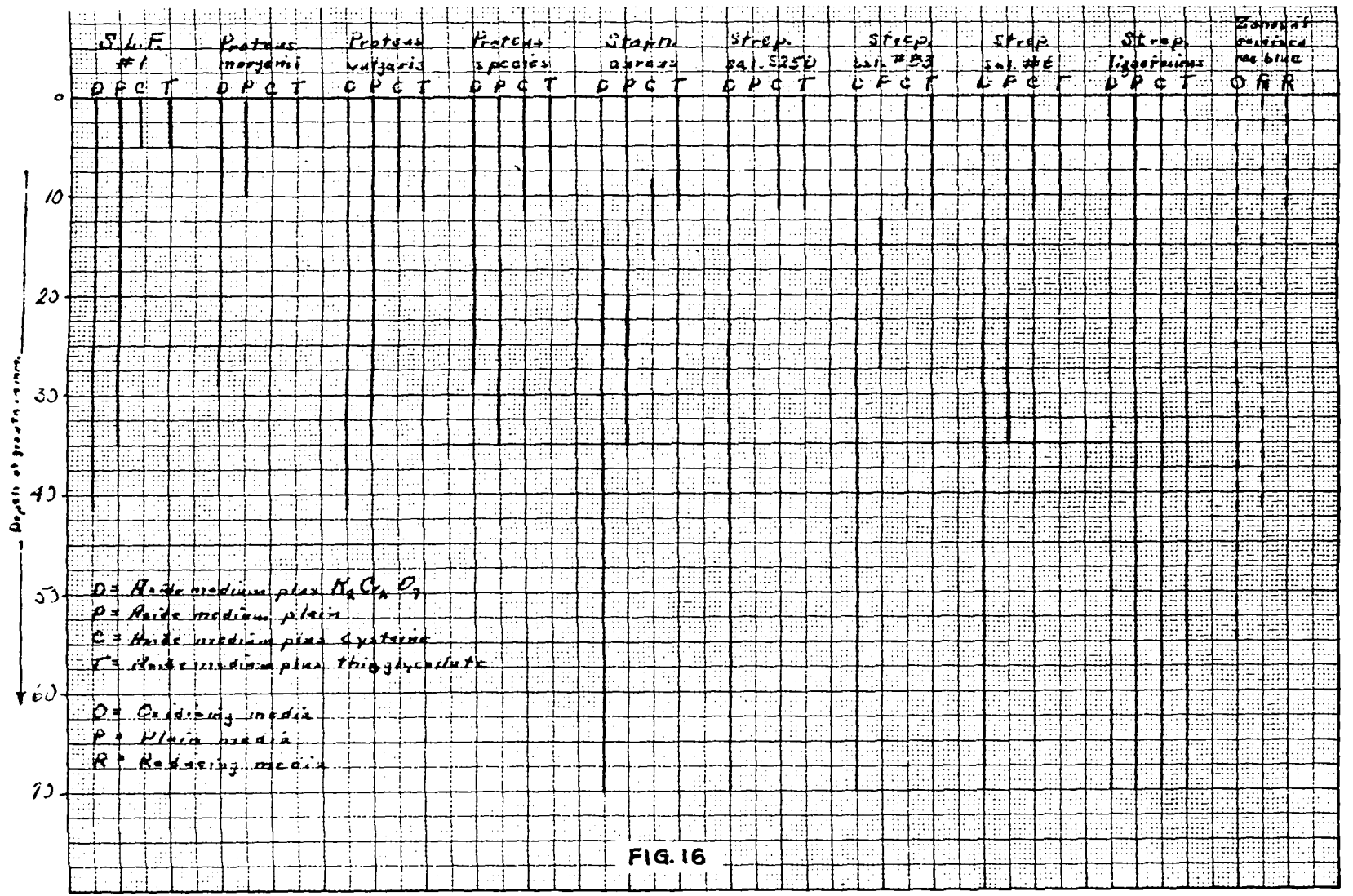


FIG. 16

the depth of the shake cultures. The redox conditions, per se, of the different media plays no part in the observed phenomena, since all species employed were able to grow abundantly throughout all the media in the absence of sodium azide. In general, these results show that sodium azide is more effective under reducing conditions than it is under oxidizing conditions. This seems to indicate that sodium azide does not act as a slow oxidizing agent as suggested by Wallmann (1940).

DISCUSSION

The results of the foregoing investigations have demonstrated that sodium azide alters the physiology of Streptococcus salivarius. When the rates of chemical change per cell per hour were calculated, it was observed that fermentation was apparently stimulated in the presence of sodium azide, although graphic presentation of the results seemed to indicate that the rate of glucose utilization and acid production by actively proliferating cells was reduced. An observed increase in generation times, however, led to the conclusion that the phenomenon could be correlated with an inhibition of cell division rather than to a stimulation of enzymatic activity.

"Resting" cells of Streptococcus salivarius produced the same amount of lactic acid, on the basis of glucose fermented, in both plain and azide-containing substrates, showing that the fermentation mechanism, as such, was not impaired. Other investigations on the rate of glucose utilization and lactic acid production by "resting" cells of this organism demonstrated that enzymatic activity was apparently decreased in the presence of sodium azide. Since the percent lactic acid formed on the basis of glucose fermented was the same in the presence and absence of this enzyme "poison", it can be concluded that sodium azide inhibited the fermentation at, or prior to, the conversion of substrate glucose into a

non-reducing substance. These results indicate that a streptococcal glycolytic mechanism, similar to that reported by Clifton (1937) and others for Escherichia coli may be inhibited. These investigators have postulated that approximately three-fourths of the carbohydrate substrate utilized by "resting" cells of Escherichia coli undergoes "oxidative assimilation", i.e., is synthesized into cellular polysaccharides. They have further demonstrated that this polysaccharide synthesis can be poisoned by sodium azide, while the other stages of fermentation proceed unaltered. The mode of action of sodium azide in blocking assimilatory processes is still obscure. It apparently inhibits respiration rather than fermentation. (Keilin, 1936) (Oppenheimer and Stern, 1939). The results presented in this thesis and those of Clifton (1937), who reported that Escherichia coli dehydrogenases were not effected by sodium azide, seem to confirm this hypothesis.

It is difficult to correlate the effect of oxidation-reduction potentials on sodium azide inhibition reported in these investigations with the above hypothesis. It was observed that marked inhibition occurred under conditions of low potential, which are favorable to fermentation, whereas under higher potentials favorable to respiration, growth appeared. It is possible that the reduced state of a reversible enzyme system required for the initiation of growth was selectively inhibited, similar to the "poisoning" of reduced cytochrome in living yeast cells reported by Keilin (1936). Under conditions of low potential, the bulk of such a reversible system would be in the reduced state and cell division would be effectively blocked. It is interesting to note that Chaix and Fromageot (1939) reported similar results with Propionibacterium pentoaceum when "poisoned" by sodium fluoride. Under anaerobic conditions, sodium fluoride inhibited decomposition of glucose and lactate,

while aerobic decomposition of these same compounds was not influenced.

Rose and Fox (1942) reported that bacteria could undergo a limited number of cell divisions in the presence of bacteriostatic quantities of a sulfonamide drug, regardless of the size of the inoculum. Results presented in Part I of this thesis showed the same phenomenon occurs with sodium azide. The occurrence of the same quantity of growth from inocula of approximately ten million or one thousand cells is difficult to rationalize with a hypothesis that sodium azide inhibition is solely a matter of enzyme inhibition. Rather, these results indicate that some essential constituent or constituents of the medium are made unavailable by the presence of sodium azide. By varying the nutrients in an azide-containing medium, it was possible to show that medium constituents had relatively little effect on the ratio of growth in plain media to growth in azide media. The presence of an anti-azide factor was demonstrated, however, by the marked increase in inhibitory power of azide in an acid-ether extracted medium. It appears probable that the constituents of the medium play a part in the inhibition ratio, but its nature remains obscure. Dixon and Keilin (1936) reported that sodium azide combined with the substrate and not the enzyme in the xanthine oxidase reaction. Whether or not a similar azide-substrate combination occurred in these experiments has not been determined.

SUMMARY

1. Decrease in rates of glucose utilization and production of acid was observed in actively proliferating cultures of Streptococcus salivarius growing in azide-containing broth.
2. "Resting" cells of Streptococcus salivarius ferment glucose to produce similar quantities of lactic acid, on the basis of glucose fermented, both in the presence and absence of sodium azide.

3. The rate of glucose utilization and lactic acid production by "resting" cells of Streptococcus salivarius is decreased by sodium azide.
4. Variations in the constituents of a broth culture medium affect the inhibitory power of sodium azide relatively little, but acid-ether extraction of the complete medium removes an anti-azide factor.
5. p-Amino benzoic acid stimulates the growth of Streptococcus salivarius but is not an anti-azide factor.
6. Sodium azide inhibits more effectively under conditions of low potential than under higher potentials.
7. Escherichia coli exhibits a prolonged lag phase and retarded fermentation when grown in pure cultures containing sodium azide. Large inocula of Escherichia coli are killed when grown in association with Streptococcus salivarius in azide broth.
8. The possible nature of sodium azide inhibition is discussed.

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