THE BACTERIAL FLORA OF ENAMEL SLIP.

by

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Submitted to the Department of Bacteriology and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts.

Approved by:

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Grateful acknowledgment is hereby made to Dr. Noble P. Sherwood, Head of the Department of Bacteriology, for suggesting this problem for study and for his guidance and constant inspiration throughout the work; to Mr. R. D. Cooke, chief Chemist for the Columbian Enameling & Stamping Company, Terre Haute, Indiana, who kindly furnished the material for investigation.

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INTRODUCTION.

While engaged upon some chemical research relative to the manufacture of enamel Mr. R. D. Cooke, Fellow at the Mellon Institute and chemist for the Columbian Enameling and Stamping Company observed the production of gas in enamel slip water. This was brought to the attention of Dr. E. W. Tillotson, Assistant Director of the Mellon Institute and referred by him for solution to Dr. H. P. Sherwood, Head of the Department of Bacteriology of the University of Kansas. After carrying out a few preliminary experiments, he turned the biological aspect of this problem over to me for investigation.

Mr. Cooke had observed that the production of gas in enamel slip water proceeds differently in the presence of various metallic ions of the iron family when clays from different sources are used. In addition it might be said that in his work all reactions occured at room temperature.

The source of the microorganisms occurring in enamel slip is undoubtedly the clay used. As above mentioned, Mr. Cooke observed that there was a variation in reaction toward metallic ions when different lots of clay were employed. Another feature which lends itself to the belief that the normal habitat of these organisms is clays, is the chemical composition of the enamel slip.

The following data concerning the composition and treatment of the enamel slip at the factory from which the material for investigation was obtained was kindly furnished by Mr. Cooke.

"An enamel frit is made by melting a mixture of feldspar, borax, silica, sodium carbonate, sodium nitrate, fluorspar and cryolite at about 2200°F. When melted it is run into water, which coals it and causes it to break up into small particles. This frit is similar to glass and is insoluble except for a small amount of soda which dissolves from the surface.

"Next 1000 pounds of frit, 50 pounds of clay and 300 pounds of water are ground in a ball mill, until the frit is reduced to impalpable fineness. This is the enamel slip and is about the consistency of thick cream. On standing several days the solids settle somewhat, leaving more or less water at the top .....

It is slightly alkaline from the soda dissolved from the frit mentioned above and contains humus and other similar organic material from the clay which gives it the brown color. There is also a small amount of borax and a trace of sodium fluoride present, both derived from the frit."

A medium of the above composition would not be favorable for the growth and development of many kinds of bacteria. We can therefore say that enamel slip exerts a selective action upon bacterial types. This selective action may manifest itself in two ways:

1. The chemical composition of enamel slip does not include the organic substances and nitrogenous bodies necessary for growth and development of many bacteria.

2. There is probably some germicidal action due to the alkaline reaction of the solution and the presence of borates and fluorides (13).

Having thus seen that the bacterial flora of enamel slip is intimately related to that of clay, let us next consider what bearing this may have on the problem.

The bacteriology of clay is as yet largely an unsolved problem. The reason for this probably lies in the fact that agriculturally, clay is an unproductive soil and there has been no immediate need for a knowledge of the bacteria present.

The role of bacteria in clay, from the standpoint of its application to the arts and industries has not been touched upon.

The physical and chemical composition of clay renders it unsuitable as a medium for growth and multiplication of most forms of bacteria. A knowledge of the conditions existing in natural clays might furnish a clue to the prediction of the types of organisms which might there be encountered.

Clay is essentially a compound of aluminum silicate. A variety of other compounds are present and according to Bourry(1), calcium carbonate, iron oxides and iron sulphides are commonly present. The organic matter contained in clays according to this author may have three sources:

1-Infiltration of soils.

2-Deposition of clay in an estuary or marsh rich in vegetable matter.

3-Mixture with bituminous rocks.

Natural clays are relatively poor in nitrogenous compounds. Presence of nitrogen would probably indicate an infiltration of soluble nitrogen compounds from the soil or their association with the organic matter. Phosphates and sulphates are also present in varying quantities and other compounds of minor importance.

The substance itself is in a state of fine division generally, although there is a great variation in the size of the particles. Around the clay particle or filling the spaces between them is water. This water holds in solution those compounds which are utilized by bacteria present. (2) The paucity of assimilable organic and nitrogenous matter would be a factor in limiting the types of microorganisms.

Anaerobic conditions prevail. This would naturally lead to a further limitation of bacterial types.

Temperature relations are important. According to Lipman(2) clays can be classed as late soils or soils in which microbial activity is delayed in spring. This he attributes to the water content and the high specific heat of water. Such soils would warm up slowly and the range of temperature would not be as extreme as in the more aerable surface soils.

Lastly the reaction of the clay must be taken into account, for this would assuredly be a factor of importance in the "selective action" of clays on bacterial types. Clay soils are usually alkaline due to the decomposition of silicates(2) and to the presence of feldspar and mica. (1)

Such being the environmental conditions occurring in natural clays, the logical method of procedure in isolating any organism from such a soil, would be to duplicate in so far as possible the natural conditions.

PURPOSE.

The purpose of the present investigation is threefold:

1-To find whether or not the production of gas in enamel slip water is due to bacteria present.

2-If so, to make a bacteriological study of enamel slip with a view of determining the specific organism or organisms responsible.

3-To carry out morphological and physiological studies of the specific organism with a view of obtaining information that might answer the following questions:

a) Has the organism been previously described in literature?

b) What factors are necessary for gas production?

In answering the first and second of the above questions it is proposed to

1-Devise a medium upon which the isolation of the organisms can be accomplished.

2-To reinoculate the bacteria in pure culture into sterile enamel slip water, in order to find the specific bacterial cell responsible for gas production.

3-To determine the source of gas and to devise a medium for the routine study of gas production.

Relative to the solution of the third problem it is proposed to

1-Carry out morphological studies including size, shape, capsule formation, sporulation, presence of involution forms, motility, Gram stain and general tinctorial reactions of the organisms.

2-To carry out physiological studies bearing upon, food requirements, production of extra-cellular enzymes, production of pigment, indol formation, hydrogen sulphide production, ammonia production, ability to attack and consume alcohols and carbohydrates, optimum temperature relations, optimum oxygen tension, acid limit of hydrogen-ion concentration and thermal death point.

3-To make a study of the source of gas produced in enamel slip and also to determine the factors necessary for its production.

E X P E R I M E N T A L

Two samples of enamel slip were obtained from the Columbian Enameling and Stamping Co., Terre Haute, Ind. These samples contained, in addition to the enamel slip water, 1.5% sodium acetate, 0.15% sodium nitrate and 0.15% aluminum chloride.(3)

One of the samples contained the organisms as they occur in the enamel slip; the other portion had been sterilized before shipping. This sterile lot was used as a medium for growing the organisms.

Preliminary Manipulations.

A series of small(hemolytic) fermentation tubes of the Durham type were set up. To each tube was added 1 cc. of the sterile slip. The tubes were then autoclaved at 10 lbs. pressure for 15 minutes. Inoculations were then made into five of the tubes using increasing amounts of unsterile slip. Incubation took place at room temperature.

Tube # 1 - 1 drop slip water	....	no gas, 24 hrs. Gas in 48 hrs.
" # 2 - 0.1 cc. " "		Gas in 24 hrs.
" # 3 - 0.5 cc. " "		" " " "
" #4 - 0.8 cc. " "		" " " "
" # 5 - 1.0 cc. " "		" " " "
" # 6 - Control		No gas.

This experiment shows that gas production could take place under conditions of the experiment. About 10 days had elapsed between the time of shipment from the factory and the beginning of this work. During this time the material had been stored in the ice-box for about five days. As will be noticed in the table, the smallest amount of slip inducing the formation of gas within 24 hrs. was 0.1 cc.

The above experiment was repeated using 0.1 cc of the slip in inoculating the tubes. When gas formation was well under way, another series of tubes were inoculated with material from the previous set. In this manner, the experiment was repeated four times.

The above results indicate strongly the fact that gas production in enamel slip water is of bacterial causation. For it will be seen that gas production does not occur in heated slip, whereas addition of a small amount of unheated slip to the sterile material, results in production of gas. In view of these facts an attempt was next made to isolate the specific organism concerned.

Isolation of Bacteria from Enamel Slip.

For this purpose a standard beef extract agar having a reaction of about Ph=7, was used; also an agar adjusted to Ph.=8.8 with Na_2CO_3 (2). This was done because the reaction of the slip water was found to be Ph.= 8.8.

Plates were made with each agar using varying quantities of slip, one tenth, one half and one cubic centimeter per plate. Control plates were also poured from each agar. The plates were incubated at room temperature.

No growth was visible on the plates made with neutral agar, even after several days incubation. In alkaline agar growth was abundant in 24 hrs. The colonies here are small, pin-point size, clear and when viewed obliquely in transmitted light appear iridescent. After 48 hours there was no great increase in size or any change in appearance. Examination with hand lens showed all the colonies to be the same apparently.

Several colonies were picked and transferred to alkaline agar slants, but at best growth was feeble. An attempt was therefore made to synthesize an agar more favorable to development.

Growth of Enamel slip bacteria on sodium nitrate agar and slip water agar.

1 cc. enamel slip was plated out in agar of the following composition.

Standard beef-extract agar	99 cc.
Sodium nitrate	0.1gram
Enamel slip water	1 cc.

II.

Standard beef extract agar	99 cc
$NaNO_3$	0.1 gram

The hydrogen-ion concentration was adjusted to Ph= 8.8, with sodium carbonate and the agar sterilized at 10 lbs. for 15 minutes. The media darkened somewhat during sterilization due to the sodium carbonate.

Growth in 24 hours was abundant and the colonies were slightly larger than in alkaline beef extract agar. In 48 hours they were about one millimeter in diameter. On further incubation they did not increase in size to any marked extent. There seemed to be no apparent difference in growth on either agar. The colonies on this agar are gray, raised, entire, and moist appearing. When touched with a needle, they show a distinct mucoid consistency, suggesting capsule formation.

After the first two days there occurred no great increase in size of the colonies, the average size being about one millimeter in diameter after seven days' incubation.

Several colonies were picked and streaked on alkaline nitrate agar plates. After incubation they appeared to be the same apparently as the original. A microscopic examination showed all of them to consist of rather long rod forms.

In about 10 days some of the colonies on the original plates began to show a red pigment formation. The majority of the colonies remained colorless or gray. This suggested the presence of an organism which slowly developed a pigment.

Microscopic examination of one of the red colonies, showed, however, the presence of two forms; one a long rod, the other much shorter. The longer organism resembled the bacteria producing the gray colonies. Transfers of the red colonies were made by streaking on nitrate agar plates. After 24 and 48 hours' incubation the resulting colonies appeared as gray, moist and apparently identical with the colorless colonies. In about 5 days pigmentation again occurred.

It seems therefore that two organisms were present in the enamel slip. One is a rather long rod type, the other is comparatively much shorter and produces a red pigment. The isolation of the pigment producing organism was accomplished after some time, due to the fact that, pigment formation was rather show and it was impossible to tell whether or not the right organism had been picked until pigmentation occurred, the colonies up to this time, having a remarkable resemblance to the non-pigment producing organism.

After growing this pigment producing species on culture media in the laboratory for three months, it has been found that coloration is more rapid and is to be seen after 24 hours on an alkaline nitrate agar.

The question next arises, which of these two organisms are responsible for gas production, or are both concerned in bringing this about?

To answer this question, inoculations of both organisms were made into sterile slip water. Incubation took place at room temperature. The results observed were that there occurred a production of gas in enamel slip water, inoculated with the non-pigmented form, whereas the inoculation of the red pigment producer into the same medium resulted in no gas formation. This does not however preclude the possibility for symbiotic action. Therefore the cultural characteristics of these two organisms has been studied in order to learn whether or not there is any such difference in physiological reactions as to indicate a possible symbiosis.

What is the source of the gas produced in enamel slip water?

To answer this question it might be well to again consider the composition of the slip. The most suggestive fact in this connection is the presence of nitrates. Other substances can of course, result in gas formation by the action of bacterial enzymes. However, in view of the fact that the source of these microorganisms is clay, and since soil is particularly abundant in denitrifying bacteria, it

would seem probable that the gas formed, comes as a result of the fermentative action on nitrates by bacteria, brought in with the clay. Moreover if this is the case, nitrates ought to favor the growth and development of these bacteria. That this is true, we have already seen. For, by the addition of small amounts of nitrates to an alkaline agar, growth of the organisms is greatly enhanced.

A stab was made into alkaline nitrate agar in a tube, also into plain alkalinized agar. Incubation at room temperature for two days resulted in gas production in the nitrate agar, splitting the media. The plain agar gave no evidence of gas formation. This shows therefore, that the organism responsible for gas production in enamel slip is a denitrifyer

Denitrifying bacteria are quite widely distributed in nature, but occur most commonly in soil. This phase of bacteriology has been investigated to a considerable extent and a number of denitrifying bacteria have been described in bacteriological literature. A historical summary of the work which has been done along these lines will next be considered.

HISTORICAL.

The study of nitrate reduction and de-nitrification as a biological phenomena dates back to the year 1836. When Dubrunfaut (4) observed the production of nitric oxide in beet sugar molasses. This he explained as the action of lactic acid resulting from fermentation of the sugar, upon nitrates present. The nitric oxide thus formed was oxidized by the air to nitrogen peroxide with a characteristic brown appearance. Reiset, many years later explained the phenomenon as an ammonia oxidation. A true explanation, however, was not offered until the discovery of de-nitrifying bacteria.

Pelouze in 1857 (5) observed the liberation of nitrogen from nitrates in the presence of organic material. Schloesing (1868) reported nitrate reduction in urine containing nitrates, in sugar solutions containing nitrates and cheese and in tobacco extract.

Tacke, 1887, showed that soils to which sugars had been added and which contained large amounts of nitrates were productive of gas, consisting mainly of nitric oxide and nitrogen peroxide. (8)

Mensel was perhaps the earliest investigator to advance evidence of the biological cause of de-nitrification.

Not only did he observe bacteria in his cultures, but he also found that antiseptics were detrimental to the process. (8)

Goyon and Dupetit (1882) working with soils also showed the bacterial nature of the process of de-nitrification. They found that soils which had been sterilized or to which antiseptics such as chloroform had been added, could cause no de-nitrification or nitrate reduction. In 1886 these two investigators isolated two specific organisms, capable of de-nitrification. These they named Bact. denitrificansA(alpha) and Bact. denitricans B (beta). The bacterial nature of the process of nitrate destruction was thus verified and shown not to be caused by the interaction of acid products of fermentation with nitrates as had been advanced by earlier investigators.(8)

Up to the present time many species of de-nitrifying bacteria have been described. Attention has been directed largely to the de-nitrifying process rather than to a systematic classification of these organisms. In the study of de-nitrifications, four requirements, as a rule must be fulfilled: (9)

1-Presence of utilizable nitrogen compounds, generally nitrates or nitrites.

2-Presence of assimilable organic substances.

3-Partial to total exclusion of oxygen.

4-Presence of proper bacteria.

De-nitrifying processes according to Jensen can be divided into four groups(6).

1-Reduction of nitrates to nitrites and ammonia.

2-Reduction of nitrates and nitrites to oxides of nitrogen.

3-Reduction of nitrates and nitrites to free nitrogen.

4-Synthesis of organic nitrogenous bodies from nitrates(Sal peterassimilation)

5-Liberation of nitrogen by putrefactive processes.

Many bacteria are able to reduce nitrates to nitrites and even to ammonia without production of free nitrogen. Maassen found that of 109 different organisms which he tested, 85 were able to bring about the formation of nitrites from nitrates (9). This is an important feature when we consider the possibilities for symbiotic relationships in this respect. Burri and Stutzer described two organisms (11) to which they gave the name B. de-nitrificans I and B. de-nitrificans II, later renamed by Lehman and Newman, Bact. de-nitrificans and B. Stutzeri. These two organisms showed a remarkable difference. While B. Stutzeri could bring about the destruction of nitrate in pure culture, Bact. de-nitrificans required the presence of B. coli or B. typhosus to do this. The explanation given by Weisenberg (11) for this behavior, is that Bact. de-nitrificans can reduce nitrites but not nitrates, and other organisms such as B. coli makes this possible by reducing nitrates to nitrites.

The reduction of nitrates to oxides of nitrogen occurs occasionally although less frequently than the reduction of nitrates to free nitrogen (9). Gayon and Dupetit (x) termed this the process of "indirect de-nitrification." Nitrate destruction in such cases is more slow and generally requires several days for its completion. Complete exclusion of oxygen is not always necessary. Some bacteria have the ability to produce either nitrogen oxides or free nitrogen depending on conditions. Thus, Bact. de-nitrificans A (Alpha) isolated by Gayon and Dupetit was able to reduce nitrates to nitric oxide (NO) in the presence of asparagin. In the absence of asparagin only free nitrogen was evolved.

The process of "direct de-nitrification" according to Gayon and Dupetit (x) results in the liberation of free nitrogen . This is the most common end product when a gas is formed by nitrates being broken. Jordan states that B. coli., B. typhosus, B. pyocyaneus and B. fluorescens have the ability to bring about a "direct" de-nitrification.

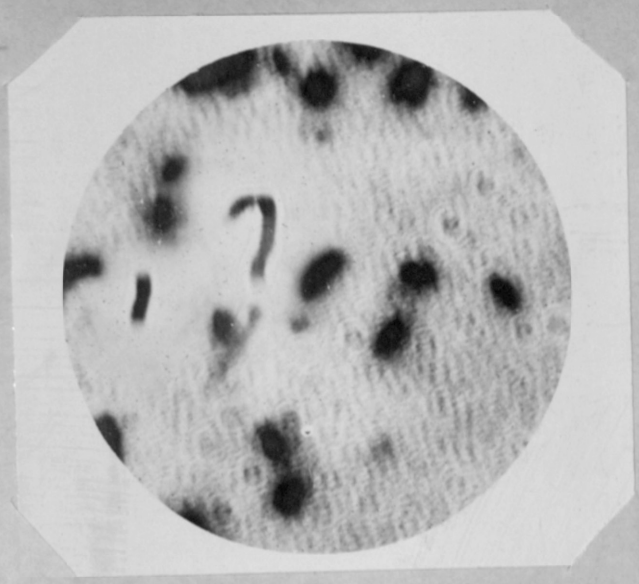
The reduction of nitrates and the combining of the nitrogen in organic form was repeated by Berthelot in 1888. Since that time various investigators have reported similar observations. Reliable data in this respect is lacking.(8)

The loss of nitrogen by putrefaction of organic nitrogen containing sugstances has been observed by many investigators. The exact nature of the process and the symbiotic relationships of various bacteria is still obscure. Ammonia production in urine and manure is common but the release of free nitrogen and the organisms concenned has not been investigated to any great extent.

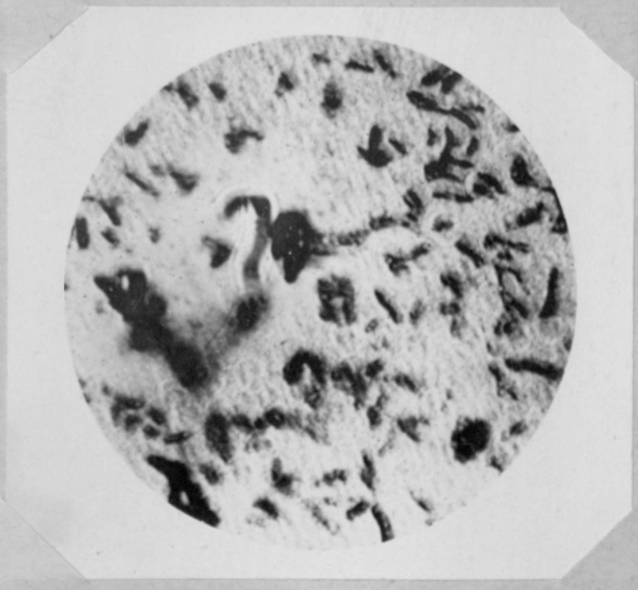
The carbon requirements of de-nitrifying bacteria are quite simple. The simplest case on record is probably that of a soil bacterium reported by Hiltner and Stormer (12) which was able to reduce nitrates with gas production, without utilizing any organic compounds. Beijerinck also reports the isolation of a sulphur bacterium, which reduces nitrates in the presence of carbonates without the consumption of carbon. He suggests the following explanation:

$$6KNO_3 + 5\,S + 2CaCO_3 \rightarrow 3K_2SO_4 + 2\,CaSO_4 + 2\,CO_2 + 3N_2 \quad (2)$$

Aside from these two instances, de-nitrification is dependent on the presence of assimilable organic compounds. This in turn limits itself largely to the salts of organic acids and carbohydrates. As a general thing, however, the carbohydrates are not utilized by these organisms, because carbohydrate fermentation gives rise to acids which are injurious and check the process.(8).



Denitrifying Bacteria, showing Capsules. (Hiss Capsule Stain.)



Denitrifying Bacteria.
Involution Forms.
(Carbol Fuchsin.)

The salts of organic acids on the other hand are readily used. Citric acid is commonly added to culture media in which these bacteria are to be grown.

Morphology and Physiology of de-nitrifying Organism present in Enamel Slip.

MORPHOLOGY.

The de-nitrifying bacterium present in enamel slip varies in size from 4 microns to 7 microns in length and 0.2 microns to 0.3 microns in breadth. (Fig). It stains easily with ordinary aniline dyes and is gram negative. A hanging drop suspension of a young culture shows no motility of the bacteria. Chain formation is sometimes to be found in liquid cultures. Old cultures contain involution forms. These have a varied appearance. (Fig.) The rods swell, become distorted and present a more or less "beaded" appearance due to vacuolated areas throughout.

Capsules are present (Fig) giving the culture a slimy consistency. This is especially marked in carbohydrate broth. No spores have been seen in stained preparations and heating a culture to 80° C for ten minutes results in sterilisation.

PHYSIOLOGY

The de-nitrifying bacterium grows best on an alkaline (Ph.= 8.8) nitrate agar. Addition of enamel slip to the

media did not seem to enhance its growth. The colonies vary in size, the average being about 1 mm. in diameter. They appear rather gray, moist, slimy and "string out" when touched with a needle. The colony is convex, entire and has a homogeneous composition. When young the colonies appear iridescent, viewed in oblique, transmitted light. Growth on alkaline beef extract agar is slow. Neutral agar used for growing most bacteria does not favor development of this type. On agar slant the growth is filiform. Stab growth in alkaline nitrate agar results in lively gas production within 24 hrs. splitting the agar.

In beef-extract broth these organisms grow producing at first a clouding of the fluid and later a mucoid sediment. Growth takes place in neutral broth but an alkaline broth quickens the growth.

INDOL PRODUCTION.

Indol production is a variable characteristic in bacteria. Many organisms have the ability to produce indol by cleavage of the tryptophane contained in peptones. Putrifying bacteria in general have this ability in marked degree. (15)

A culture in Dunham's peptone broth 7 days old, was tested for indol production according to methods recommended by various investigators as being most sensitive.

To 5 cc. of the culture in a test tube was added 1/2 cc. of a .02% sodium nitrate solution and the fluids mixed by shaking. 1 cc. of sulphuric acid wasthen run down the side of the inclined tube, as to form a layer on the bottom. Under these conditionc indol when present gives rise to a red color due to the formation of nitroso-indol (14) The test was negative.

The vanillin test for indol was tried as follows:-

To 5 cc. of the culture was added 5 drops of a 5% Vanillin in 95% alcohol, and 2 cc. Con. H_2SO_4. Indol when present gives an orange color insoluble in $CHCl_3$. This test was negative. (15)

Bayne, Jones and Zinninger (17) recommends the following modification of Erlich's test for indol as being sensitive to one part per million:

The culture is shaken with ether to dissolve any indol present and a few drops of reagent added.

Paradimethylaminobenzaldehyde	4 grams
95% alcohol - - - - - - - - -	369 cc
Concentrated hydrochloric acid	80 cc

In the presence of indol a rose color appears in the lower part of the ether. The results with this test were negative/.

250 cc of the culture were then distilled to about 1/8 volume and the distillate shaken with ether. (15) The ether extract was then tested for indol by the above method and also by the Vanillin test. Both tests were again negative.

It is to be concluded therefore that this organism

does not have the power to split tryptophane present in peptone with the formation of indol.

Hydrogen Sulphide Production.

Hydrogen sulphide is sometimes produced in broth cultures of bacteria due to decomposition of the sulphur compounds, notably cystine (19). In the present instance two methods were tried.

A strip of filter paper soaked in a saturated solution of lead acetate to which a few drops of glycerol had been added, was suspended above a broth culture in a tube and the mouth of the tube corked. (19). In this way any hydrogen sulphide evolved would blacken the bibulous paper. No blackening occurred during seven days' growth.

Lead acetate agar of the following composition was prepared:- (17)

Agar neutral	100 cc.
Peptone	2 grams

The agar was sterilized and cooled to 60° C. and 1 cc. of a 1% lead acetate solution added. The media was then put up in sterile tubes and slanted. Inoculation was made by streaking the surface and stabbing the butt and the tubes incubated at 29° C. for 3 weeks. There was no blackening of the agar.

Control tubes of the same agar inoculated with B. typhosus showed distinct blackening in 24 hours. These results indicate therefore, that no hydrogen sulphide is produced in destruction of peptones by this organism.

Ammonia production.

Many bacteria are able to produce ammonia when growing in media rich in proteins or peptones. This is a characteristic common to most putrifying bacteria.

Two media were used in testing for ammonia production. One was a standard beef extract broth used in routine work in the laboratory, the other consisted of the same medium plus 0.2 % sodium nitrate. 250 cc. of each medium was inoculated with the organism under investigation. After a week's growth at room temperature, the cultures were distilled. The distillates were diluted to five times their volume with ammonia free distilled water and a few drops of Nessler's reagent added to 5 cc. of the diluted fluid.

Plain broth inoculated--faint yellow.
" " sterile -- " "
Nitrate broth inoculated--deep yellow.
" " sterile--faint yellow.

These results show that in plain beef extract broth the denitrifying bacteria tested, produce no increase in the ammonia content; furthermore the addition of a small amount of sodium nitrate gives rise to a considerable production of ammonia.

Under these conditions it would seem that in the presence of peptones there occurs a reduction of nitrates to ammonia.

Fermentation of Carbohydrates.

This test was carried out using a 1% sugar agar to which 1% Androde indicator had been added. The media was tubed in 10 cc. quantities and slanted. Inoculation was made by surface streak and stab. The results obtained with various sugars are indicated in the following table:-

Dextrose -	No acid or gas in 3 weeks.
Lactose -	" " " " " " "
Saccharose-	" " " " " " "
Maltose -	" " " " " " "
Arabinose-	" " " " " " "
Raffinose-	" " " " " " "
Xylose -	" " " " " " "
Rhamnose -	" " " " " " "
Salacin -	" " " " " " "
Mannit -	" " " " " " "
Galactose-	" " " " " " "

Bacterial fermentation of carbohydrates results in the splitting of the sugar molecule with the production of acid and sometimes acid and gas are formed. In the present instance there was abundant growth but no acid or acid and gas production so that it is clear that this organism is inactive as far as sugars are concerned.

To substantiate these results an experiment was carried out, for the purpose of ascertaining whether or not there was any utilization of sugar. About a one per cent sugar solution was used. The media when sterilized was divided into two parts.

One part was inoculated, the other served as control. Dextrose, maltose and saccharose were used as type sugars. After one week's incubation at 29° C. titrations were made on both the inoculated medium and the control. In this way a difference in quantity of sugar would be indicative of sugar utilization. Titrations were carried out according to the method of Benedict. (15)

	Grams of Sugar Inoculated Media	Grams of sugar. Control.
Dextrose:		
1st trial	1.20 gms.	1.20 gms.
2nd trial	1.20 "	1.20 "
Maltose:		
1st trial	1.11 gms.	1.11 gms.
2nd "	1.11 "	1.11 "
Lactose		
1st trial	1.1492 gms.	1.1492 gms.
2nd "	1.1540 "	1.1492 "

The above results show that growth of the organism in maltose, saccharose and dextrose broth, results in no decrease in sugar content, and therefore, these sugars cannot serve as foods. This is in accord with results obtained by the various investigators mentioned elsewhere in this paper.

The addition to sugar to media does not inhibit growth of the bacteria.

Growth in Gelatin.

Growth in standard nutrient gelatin results in no liquefaction in six weeks' time. Addition of 0.2% sodium nitrate to the media, gives rise to gas bubbles throughout, in about two days.

Milk

With respect to milk, the denitrifyer isolated, is inert. No acid or alkali is formed. There occurs no peptonization and no reduction of litmus.

Potato.

The organism does not grow on potatoe.

Starch Agar.

Grown on the standard starch agar and tested with Lugol's iodin for hydrolysis of the starch after seven days, indicates no diastasic action. (19).

Special Problems in Nitrate reduction.

The following series of experiments dealing more specifically with the denitrifying and reducing process, have been undertaken with a view of finding out some ~~this~~ of the requirements for nitrate metabolism. Throughout part of the work there has appeared consistently certain irregularities in behavior.

For example in a series of tubes arranged for the study of denitrification, all tubes containing the same media, and treated alike in every respect, when inoculated and incubated under the same conditions, there will occur a sproadic gas formation throughout the set. All the tubes may show growth and nitrite formation, yet some of them will show no signs of denitrification. The cause for this has not been found, although, precaution has been to maintain a chemical purity of the materials. It would seem however, that the difficulty lies in the production of gas from nitrites since nitrate reduction may proceed without gas formation. On the whole denitrification by this species is not very brisk, which suggests the "indirect" process of Gayon and Dupetit. Work along these lines is being continued at the present time.

The enamel slip water which was received for examination, contained as before mentioned an addition of 1.5% sodium acetate, 0.15% sodium nitrate and 0.15% aluminum chloride. The supply of enamel slip water being limited it was necessary to find a substitute medium. This was done by synthesizing a clay extract nitrate solution.

Clay	200 grams
Tap Water	1000 cc.

The mixture was well stirred and the clay allowed to settle out. The supernatant fluid remained slightly milky in appearance.

Clay extract	100 cc.
$NaNO_3$	0.2 gm.
$AlCl_3$	.15 "
$NaC_2H_3O_2$	1.5 "

The reaction was adjusted to Ph= 8.8 with Na_2CO_3 The solution was tubed in 1 cc. quantities in small Durham fermentation tubes and autoclaved at 10 lbs. pressure for 15 minutes. 0.1 cc. of unsterile enamel slip was then added and the tubes incubated at room temperature.

Tube # 1 - Gas in 24 hrs.
" # 2 - " " " "
" # 3 - " " " "
" # 4 - " " " "
" # 5 - " " " "
" # 6 - Control. Sterile.

The experiment was repeated, inoculating the tubes with a pure culture of the organism in place of using the enamel slip. This was done in order to rule out any possible effect which the 0.1 cc. of enamel slip might have. Gas production was just as vigorous as in the former instance. This would show therefore, that an extract of clay can be used instead of enamel slip and gas formation proceeds just as well in the absence of the slip.

In order to simplify the media still more and also to see whether or not gas production would occur in the absence of aluminum, the following media were synthesized.

1.

Clay extract -	100 cc.
$NaNO_3$ -	0.2 gm.
$NaC_2H_3O_2$ -	1.5 gm.

2.

Distilled water	100 cc.
$NaNO_3$	0.2 gm.
$NaC_2H_3O_2$	1.5 gm.

As in previous experiments the hydrogen-ion concentration was adjusted to Ph= 8.8 with sodium carbonate, the media tubed in fermentation tubes as before and autoclaved. Inoculation was made with pure culture. Incubation at room temperature resulted in gas production in both media.

These results indicate therefore, that the denitrifying species of bacteria in enamel slip, are able to bring about a denitrification of nitrates in alkaline solution in the presence of sodium acetate alone, aluminum not being necessary. Sodium acetate being a salt of an organic acid, the question arises; can othe. organic salts be utilized?

To answer this question, experiments were carried out using various organic compounds. Below is shown the general composition of the media:

$NaNO_3$ 0.2 gm.
Organic salt 1.5 gm.
Distilled water - 100 cc.

The reaction of the sterile medium was adjusted to Ph= 8.8. The following organic compounds have been tried:

Sodium potassium tartrate.

Sodium lactate.

Sodium Oleate.

Sodium Citrate

Sodium Carbonate.

Positive results were obtained with sodium citrate and with sodium carbonate. In solutions of the other organic salts no denitrification was observed.

Relation of temperature to nitrate reduction.

This work was begun during the summer of 1921. The temperature in the laboratory was quite constant for several days at a time. Incubation of cultures was allowed to take place at room temperature and denitrification proceeded at a constant rate, generally resulting in gas after 24 hours' incubation. Occasionally, however, following a rain, there would be a distinct drop in temperature. On such days cultures showed little activity and denitrification was delayed sometimes as long as 72 hours. Then again as the temperature rose, the time required for the process was shortened.

Such irregularities led to an attempt to determine in a general way the relation of temperature to nitrate destruction. The average laboratory temperature for several days was 29° C. The organism was grown in an alkaline solution of $NaNO_3$ and $NaC_2H_3O_2$. 100 cc. of the media was put up in Erlenmeyer flasks and sterilized. When cool the flasks were inoculated with a pure culture of the organism, and paraffin oil floated on the surface of the liquid to ensure anaerobic conditions. The flasks were then incubated at 37° C, 29° C, 25° C, and 20°C. Tests for nitrite formation were carried out each day on 1 cc. of the fluid, by adding to the tube, 5 drops each of

a. 5 N. acetic acid - 1000 cc.
   Sulphanilic acid - 8 gm.

b. 5 Nacetic acid - 1000 cc.
   Alpha amido Naphthalene 5 gm.

The presence of nitrates is shown by the formation of a rose color, the intensity of the color being proportional to the amount of nitrite formed. Representing the intensity of color by / the following scheme represents the conditions of experiment:

37°	29°	25°	20°
/	////	///	//

After six days' incubation, qualitative tests for nitrate were carried out. The culture incubated at 29° showed no nitrate. The other cultures still showed the presence of nitrates. (20)

Growth on agar at the above temperature does not conform to nitrate reduction. If plates containing alkaline nitrate agar are inoculated and incubated under these conditions, growth is equally rapid as far as can be determined by examination, at 37°, 29° and 25°. At 20° it is markedly retarded.

Relation of oxygen to nitrate reduction.

For the purpose of this experiment two Erlenmeyer flasks each containing 100 cc. of sodium acetate, sodium nitrate media were inoculated with a pure culture of the organism. To one of the flasks was then added enough sterile paraffin oil to cover the surface. Two controls were used. They consisted of the same media in the same amount but were not inoculated. To one was added paraffin oil, in quantity sufficient to cover the surface. Incubation took place at 29°. The color test for nitrites was carried out on 1 cc. of the sample from each flask, every day and comparison made on basis of intensity of color developed.

	Growth	Nitrites.
Anaerobic culture	- Heavy	Strong.
Aerobic culture	- Slight	Very faint.
Anaerobic control	- None	None
Aerobic control	- "	"

This experiment shows, therefore, that it is essential that there be an exclusion of oxygen in order for nitrate reduction to take place at maximum velocity.

Thermal Death Point.

The committee on Identification of Species of the Society of American Bacteriologists recommends as technique for determination of the thermal death-point of bacteria, the exposure of the organism in nutrient broth for 10 minutes (19) (20) A broth culture of this organism, 24 hours old was used. 1 cc. of culture was exposed in hemolytic test-tubes at varying temperatures for 10 minutes. Exposure was commenced at 40° C, increasing the temperature two degrees each time up to 80° C. One half of the heated culture was then transferred to a tube of nutrient broth, the other half being plated out on alkaline nitrate agar. In this way it was found that:

a) At 55° C. for 10 minutes, there results no sterilization.

b) At 57° C. for ten minutes, results in sterilization.

c) Exposure at 80° C. for 10 minutes results in sterilization, showing that the organism is not a spore former.

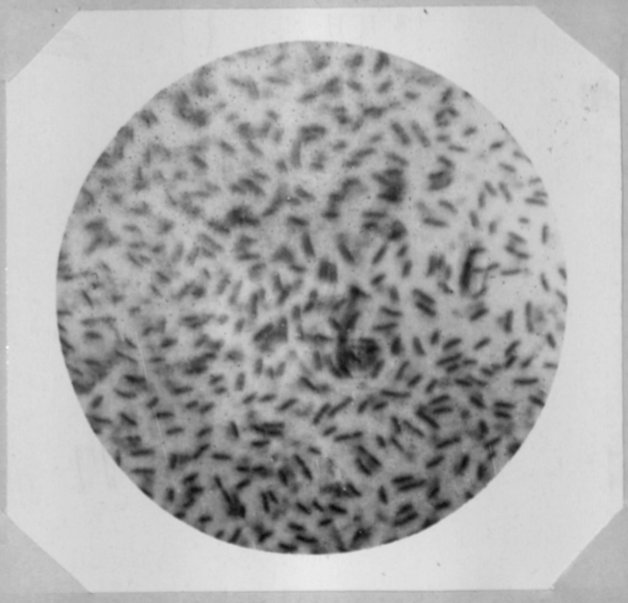
Acid limits for growth.

One of the most marked pecularities of the denitrifying species under investigation is its toleration of alkali. Bacteria as a rule require a hydrogen-ion concentration of about 1×10^{-7}. This organism is peculiar in that it grows best when the hydrogen-ion concentration approaches 1×10^{-9}.

To determine the acid limit of this species, a nutrient broth was used. The following hydrogen-ion concentrations were used:

Ph= 8.8	-	Growth in 24 hrs.
Ph= 8.4	-	" " " "
Ph=8.0	-	" " " "
Ph= 7.8	-	" " " "
Ph=7.6	-	" " " "
Ph= 7.4	-	" " " "
Ph= 7.2	-	" " " "
Ph=7.0	-	Slight growth in 24 hrs.
Ph= 6.8	-	" " " " "
Ph= 6.6	-	No growth
Ph= 6.4	-	" "
Ph=6.0	-	" "
Ph= 5.6	-	" "
Ph= 5.0	-	" "

Here we see that the acid limit for growth of this bacterium is very near the point of neutrality. This is suggested by the fact that the habitat of the organism, enamel slip water, has a reaction considerably on the alkaline side of neutrality or about Ph= 8.6.



Pigmented Bacteria from
Enamel Slip Water.
(Carbol Fuchsin.)

Is Ammonia Produced by Breaking down of Nitrates in the Presence of Acetates?

Destruction of nitrates by some bacteria goes through the intermediate step of ammonia formation with consequent reduction in hydrogen-ion concentration. In the present case as has been shown the addition of small amounts of nitrate to neutral broth results in ammonia production. It might seem therefore, that in a synthetic medium of sodium acetate and sodium nitrate ammonia would be formed. This is not the case as has been found by distilling a culture of the organism in this solution and testing for ammonia by Nessler's method. There is, however, a decrease in hydrogen-ion concentration in such a medium. This might partially be explained as a result of reduction of nitrates to nitrites; the former being the sodium salt of a relatively strong acid, the other the sodium salt of the unstable nitrous acid. Work is still in progress on this phase of the problem.

Morphology and Cultural Characteristics of the Pigment-Producing Bacillus.

MORPHOLOGY.

This organism is a short rod form, varying from 1 micron to 3 microns in length and from 0.1 micron to 0.2 microns in breadth. (Fig.)

In stained preparations it resembles somewhat the colon bacillus. It stains readily with the ordinary aniline dyes and when treated with iodine according to Gram's method, it loses the stain (20). Examination of a 24 hour culture in hanging drop, shows no motility. No spores have been seen in stained smears and heating to 80° C for 10 minutes kills the organisms. Capsules have not been seen.

Cultural characteristics.

This organism grows well on alkaline nitrate agar. A red pigment is produced in 24 to 36 hours. The colonies are about 1 mm. in diameter, convex, entire, red and moist appearing. On alkaline agar slant its growth is filiform, moist and red. Growth in alkaline broth produces a uniform turbidity throughout at first, with sedimentation later.

No acid or alkali is produced in litmus milk and there is no peptonization or reducation.

Gelatin is not liquified in six weeks.

It produces no acid or gas from dextrose, maltose, saccharose, lactose, xylose, rhamnose, arabinose, raffinose, mannit or salacin. There is no utilization of dextrose, lactose or maltose. Starch is not hydrolyzed. Cellulose does not appear to be utilized.

Nitrates are not reduced in peptone, nitrate broth or in sodium acetate, sodium nitrate media.

Ammonia is produced in beef extract broth but no indol. Hydrogen sulphide formation does not occur.

Summary.

An attempt has been made in this investigation to show that the cause of gas production in enamel slip water is biological, due to a specific bacterial cell. The bacterial content of enamel slip water has been studied in order to find the specific organism involved. A medium which can be used for routine study of these microorganisms has been devised. In order to classify the bacteria isolated it has been necessary to carry out morphological studies of such a nature as to reveal any outstanding characteristics which might be of aid in identification. Physiological studies have been made for the same reason and also for the purpose of ascertaining whether or not there is a symbiotic relationship between the bacterial species which would favor gas production.

The organisms are markedly inert toward those substances generally attacked by bacteria. It has been found necessary therefore to depart somewhat from the ourtine methods of studying bacteria in order to determine some of the special physiological activities. This is confined largely to a study of nitrate and nitrite reduction. This has necessitated the use of synthetic media. Moreover the process of denitrification requires certain conditions for its completion. Attempts have been made to determine some of these requirements. This, of necessity must be done before the process itself can be studied. Lastly a search has been made of bactericlogical literature for a clue which might lead to the identification of these organisms.

Many problems have arisen in the course of the work, some of which have been answered. Many of these have not fallen within the scope of this work as at first planned, and therefore, have not been included. Other problems are still under investigation.

Conclusion.

That enamel slip is not sterile is evidenced by the isolation of two organisms from a smaple. Neither of these organisms are motile, neither produces spores. One is a non-capsulated, red-pigment producing rod form.

The other is a non-pigment forming denitrifying species. Both organisms are Gram negative.

That the source of these organisms is the clay used in the enamel manufacture. It is impossible for any bacteria to survive the temperature to which the frit is subjected, viz. 2200°F. Consequently the bacteria must be carried in by the subsequent addition of clay.

That the production of gas in enamel slip water is due to a specific species of a denitrifying bacterium.

That in the work which has been done so far, there is no suggestion of symbiotic relationship between these organisms. Culturally they parallel each other with respect to carbohydrate and protein metabolism. They are both markedly inert toward these substances. There occurs no fermentation or utilization of sugars, no diastasic action on starch. They cause no liquefaction of gelatin and no peptonization in milk. One causes a destruction of nitrates. The other seems to have no effect on these substances.

That aluminium is not necessary for growth or denitrification processes. On the other hand aluminium does not seem detrimental to growth.

That in an alkaline medium such as enamel slip one would expect to find organisms, with alkaline requirements. This is an outstanding feature, in comparison to most pathogens, that grow best in neutral media.

That inertness toward carbohydrates and proteins is a characteristic which is due to the fact that these substances are not present in the normal habitat of these organisms, so that these bacteria must be able to utilize compounds other than proteins and carboh drates. In this respect one might say that clay and enamel slip exerts a selective action on types of bacteria.

That growth of the denitrifyer on agar is rapid at 37° C., 29° C. and 25° C. and less rapid at 20° C, but nitrate reduction goes on best at 29° C, less rapidly at a higher or lower temperature.

That absolute anaerobic conditions favor nitrate destruction, whereas growth of the organism on agar is rapid in the presence of air. It is therefore an aerobe-facultative anaerobe. The significance of anaerobiosis in nitrate media, becomes apparent if we consider that exclusion of air probably results in nitrate reduction to provide the necessary oxygen.

That in the presence of acetates and probably organic salts in general there occurs first a reduction of nitrates to nitrites and then production of a nitrogenous gas without formation of ammonia.

That in the presence of peptones nitrates are reduced to nitrites and ammonia.

That an exposure of a culture in nutrient broth, to a temperature of 57° C. for 10 minutes results in sterilization. This is the thermal death-point of the denitrifying organism.

A search through bacteriological literature on classification of bacteria (23-27) has not resulted in the identification of these organisms. The red pigment forming bacterium has some characteristics in common with Bact. rodochroum Dyar and also Bact. salmonium Dyar (23). However, in the absence of complete characterization of these two organisms it is impossible to state whether it is identical with either of them, or not. According to the latest classification of Buchanan, this organism would be classed under the genus Erythrobacillus, Fortineau. The denitrifying organism has characteristics such as to not make it identical with any of the many denitrifying bacteria described in bacteriological literature. Just what its position may prove to be in this great group, is impossible to state at the present time.

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