A STUDY OF GRAM REACTION REVERSALS IN GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

DISSERTATION

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by

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INTRODUCTION

The advance of bacteriology has been closely allied with the development of staining techniques. The application of staining methods has been the basis for taxonomic studies which make it possible to divide all organisms into two classes, gram positive and gram negative. In clinical practice, the gram staining technique occupies an important place in diagnosis and identification of etiological agents.

Since the publication of the gram staining procedure in 1883, the literature directed to the explanation of the gram staining mechanism, the environmental conditions surrounding changes in the gram reaction and the various gram staining procedures has been voluminous.

At least three major theories have been proposed to explain the gram staining reaction. These are the structural theory, the permeability theory, and the isoelectric theory. In addition, for reasons not yet wholly apparent, many workers have found it possible to draw striking relationships between the gram reactions and the physiological activities of various organisms. From the work to date it is evident that the difference between gram positive and gram negative bacteria is more of a quantitative than qualitative nature.
Adequate as previous investigations may have appeared, many deficiencies are now apparent. Specifically, these have appeared in the definition of the exact environmental conditions which produce a given gram reaction under a given cultural condition. Considerable evidence now points to the fact that the cultural history may influence the gram reaction of micro-organisms.

The change from the gram positive to the gram negative state is very common in old cultures and can be produced at will under controlled conditions with such agents as bile salts and ribonucleases. The reverse change from the gram negative to the gram positive state has been reported only a few times. The exact conditions leading to this change have not been well defined. A chance observation by the author showed that some gram negative bacteria grown in potassium iodide broth appeared gram positive when stained by the Kopeloff and Beerman modification of the gram staining procedure. It was further observed that the mordanting step could be omitted from the staining procedure with no change in altered gram reaction.

These observations posed some interesting problems and it seemed possible that an investigation of the phenomenon might give some insight into the mechanism of the gram reaction.
THE PROBLEM

The problem under investigation was to determine the mechanism involved in the reversion of the gram reaction of gram negative bacteria which had been grown in a medium containing potassium iodide. It was felt that such an investigation would give some further insight into the mechanism of the gram reaction.

HYPOTHESES

To answer the questions posed by the problem, five hypotheses were formulated for test. They are as follows:

1. There is no difference in the amount of crystal-violet up-take by bacteria grown in nutrient broth and bacteria grown in nutrient broth with potassium iodide added.

2. There is no relationship between the amount of bacterial growth and the tendency to concentrate iodide.

3. There is no difference in surface charge on bacteria grown in nutrient broth and on bacteria grown in nutrient broth with potassium iodide added.

4. There is no difference in the antigenicity of bacteria grown in nutrient broth and bacteria grown in nutrient broth with potassium iodide added.

During the progress of this study an incidental hypothesis was formulated.
5. There is no difference in crystal violet up-take between gram negative bacteria when grown in nutrient broth with added potassium iodide and gram positive bacteria rendered gram negative by ribonuclease, trypsin, lipoid solvents, sodium taurocholate treatment or ultraviolet irradiation followed by treatment in buffered potassium iodide.
REVIEW OF LITERATURE

Previous studies in the literature, relevant to the investigation herein described, may be divided into four categories: (1) the historical development of staining, (2) the gram stain and its development, (3) the theories of the gram stain, and (4) the environmental influences on the stability of the gram reaction.

History of Staining

The use of stains in microscopic preparations is so common today that all beginning students in the biological sciences have some contact with their utilization. However, this is only a recent development. In historical reviews of staining Baker (1945) and Lewis (1942) both reported that the first application in microscopic studies was made by Leevenhoek. He used a wine solution of saffron to differentiate fat cells. This work was reported to the Royal Society in 1714.

The general use of stains in biology did not become a routine procedure until the 1840's. The application of the natural dyes such as carmine and indigo was well known in the textile industry, but their use with microscopic preparations did not become common until about 1850. Although the aniline dyes were synthesized
earlier, they were not available for biological use until 1856 (Conn 1946).

About 1876 Weigert introduced into bacteriological work the staining methods which were already well established in pathological techniques. In 1876 he described before the Silesian Society of National Culture a staining procedure with which he was probably the first to demonstrate micrococci in tissue sections (Conn 1933).

The Gram Stain

The gram stain procedure was developed as a method to demonstrate pneumococci in lung sections. It was originated in Friedlander's laboratory, and it was Friedlander, in 1883, who first reported on the value of the method. Later, Gram outlined his work and published it in 1884. Gram failed to recognize the value of his procedure in that he did not use the staining technique as a taxonomic tool, but employed it principally for differentiating bacteria in tissues. Roux (1886) is generally credited with first using the gram staining method as an aid in bacteriological diagnosis. He demonstrated the gonococci in the leukocytes.

Basically, the procedure for the gram stain has not changed since it was first published. The same four steps are followed today as in 1884. However, aniline-gentian-
violet has largely been surplanted by crystal-violet which is obtainable in a purer form. Atkins (1920) recognized the chemical instability of the gentian-violet stain and proposed a new method for making up the dye. Lugol's iodine is still employed with some methods, but Gram's iodine is now in general use. Probably more controversy has arisen over the third step in the procedure, decolorization, than any other. Modern laboratory workers generally employ ethyl alcohol. Either absolute or 95 per cent alcohol is utilized; these may be diluted with acetone to speed the rate of decolorization.

Although Gram's original method gave good results, it has undergone many important changes which give more reliable information. At the culmination of the work of a committee of the Society of American Bacteriologists (Conn et. al. 1919), two revised procedures were proposed. These procedures were suggested to correct an oversight in Gram's original publication where no mention was made concerning the time of application of the various reagents.

Hucker and Conn (1923) surveyed nineteen different gram staining methods. From these they selected the methods of Atkins and Hucker as being the best for general application and as giving the most consistent results. They also recommended that two different staining procedures be used, one serving as a check on the other.
The Theories of the Gram Stain

Although the gram stain has been a standard procedure for many years, one deficiency is inherent in all of the gram staining methods. The mechanism of the reaction is not fully known. As with most staining procedures, no one explanation seems to answer all of the questions. There are three theories proposed to explain the mechanism of the gram reaction: (1) the structural or morphological theory, (2) the permeability theory and (3) the isoelectric theory.

The Structural or Morphological Theory. The theories based on a structural or morphological interpretation propose that a gram-positive context surrounds gram positive organisms. This cortex is absent from gram negative organisms. The cytoplasm of all bacteria is postulated to be gram negative. This theory has been supported mainly by Gutstein (1925) and Churchman (1927, 1929).

Using a tannic acid mordanting method, Gutstein was able to demonstrate on gram positive bacteria an ectoplasmic layer which was not present on gram negative bacteria. He introduced the term "gram-fast" to describe the gram positive layer. He also observed the effects of various chemicals and the mechanical disruption of the cell membranes on the gram reaction. He believed that the "gram-fast" character of gram positive bacteria was
due to the behavior of their cell cortex and was dependent on its integrity.

Churchman (1927) in a study with Bacillus anthracis confirmed essentially all of Gutstein's work. By treating B. anthracis with acriviiolet, Churchman found that a gram positive to gram negative conversion took place. This was also accompanied by a decrease in cell "calibre" and weight. He thought this indicated an anatomical structure as the basis of the gram reaction. From his observations Churchman classified all bacteria into three groups: (1) those developing a complete cortex (gram positive), (2) those failing to develop a cortex (gram negative), and (3) those developing an incomplete rudimentary cortex (gram variable).

Considerable impetus for the "ectoplasmic layer theory" resulted from an early investigation by Deussen (1918). Using acid and base hydrolysis, he showed that nucleoproteins in the cortex were in some way responsible for the gram reaction. The nucleoproteins of the cortex appeared to be the site of the dye fixation.

Additional evidence for the "ectoplasmic theory" was presented by Henry and Stacey (1943) and Bartholomew and Umbreit (1944). Using impure bile salts and later crystalline ribonuclease, they were able to show that magnesium ribonucleate removed from gram positive bacteria
was involved in the gram positive state. They were able, using the converted gram negative cells, to replate the extracted magnesium ribonucleate back onto the gram negative medulla on the condition that the cells were maintained in a reduced state. This made the cells appear gram positive again. This evidence also indicated that a single chemical entity was closely connected with the gram positive state.

Knaysi (1951) placed little credence in the cortex theory. He believed that Churchman had made a misinterpretation concerning the structural difference between gram positive and gram negative bacteria. He demonstrated that the dissolution of the cortex by acriviolet is not general among the gram positive bacteria. In the genus Bacillus, Knaysi (1938) found the cytoplasm to be weakly positive. When stained with the Burke modification of the gram stain, the cytoplasm was gram negative. He reported that the cytoplasmic membrane which corresponds to Churchman's cortex was the most strongly positive part of the cell.

The observation of Bartholomew and Umbreit concerning the reduction in size of the gram positive cells after treatment with ribonuclease is explained by Knaysi (1945) as due to an artifact caused by the kind of fixative employed. He reported that no reduction in size was
evident after the enzyme treatment if the cells were mounted and observed in water.

The Permeability Theory. The theories based on a membrane permeability interpretation postulate that the cell membrane of gram positive bacteria is permeable to dye and to iodine, but not to the dye-iodine complex formed inside of the cell.

One of the first supporters of the permeability theory was Brudny (1908). He postulated that the primary difference between the gram positive and gram negative bacteria was the permeability of the cell to the iodine mordant. The iodine entered the gram positive bacteria and did not enter the gram negative bacteria. He stated that the ease of decolorization of the gram negative bacteria was due to the formation of the iodine-dye complex at the cell surface of the gram negative bacteria.

Burke and Barnes (1929) thought that iodine was a precipitating agent. They believed that the dye-iodine precipitate was insoluble in water and soluble in the decolorizer. The precipitate must pass through the cell wall of the gram positive organisms less readily than through the gram negative organisms. These authors showed that any factor which altered the cell wall or the dye-iodine precipitate might affect the gram reaction. They believed that the protoplasm of the cell played no
part in the gram reaction, and that the bacteria appeared gram positive, gram negative or gram variable depending upon the permeability of the cell wall.

Kaplan and Kaplan (1935) made the observation that the decolorizing power of alcohol was reduced when it contained iodine in solution. These investigators concluded that the degree of reduction in decolorizing power by a given concentration of iodine was obviously a measure of the permeability of the cell wall to iodine in alcoholic solution. Methyl alcohol, which decolorized most gram positive bacteria almost immediately, decolorized very slowly when as little as 0.01 per cent iodine was added. They reported that gram positive bacteria showed a lower permeability to iodine in alcoholic solution than gram negative bacteria. Because gram positive bacteria differ from one another in the degree of permeability, this observation was used to explain differences in gram positiveness. "The Gram Test, which really amounts to the slowing down of the dye extraction by the alcohol, may then be explained as due to a low permeability of the gram positive bacteria to dye."

Benians (1912) reported that the intact cell membrane exhibited a differential permeability which was the basis for the gram reaction. In gram positive bacteria, the alcohol was unable to remove the dye, but as
soon as the cells were injured or broken, the dye largely dissolved out. The smallest break in the cell wall was found to be sufficient to allow the cell to be decolorized. Benians presented good evidence that the iodine-dye-cell substance did not exist as such. The dye-iodine had no greater affinity for the bacterial tissue than the original dye. Benians postulated that the iodine treatment rendered the cell membrane of gram positive bacteria impermeable to alcohol.

In a later publication Benians (1920) divided all bacteria into three categories: (1) the regular gram positive cells in which a large compound molecular body, which in alcoholic solution does not pass easily out of the cell, is formed by the dye and iodine, (2) the regular gram negative bacteria in which the dye is only peripherally adsorbed, and (3) the group represented by the gonococcus in which the dye and iodine permeate the cell, but from which the stain is readily removed by the alcoholic decolorizer. Benians thought that the permeable gram negative bacteria in Group 3 had more in common with the gram positive bacteria than with the impermeable gram negative bacteria of Group 2. This latter observation has since been confirmed by many workers (Bartholomew and Mittwer 1952).
The Isoelectric Theory. The theories based on a physical-chemical interpretation are those which postulate a relationship between the isoelectric point of the cell protoplasm and its affinity for the primary stain. Gram positive bacteria appeared to have an isoelectric staining point at a lower pH value than the gram negative bacteria and therefore fix stain with a greater affinity and resist alcoholic decolorization longer.

The foremost supporters of the isoelectric theory have been Stearn and Stearn (1924, 1929, 1930). They associated gram positiveness with the unsaturated lipoproteins of an acid nature, explaining the increased dye uptake as due to the increased negative charge on the colloidal particles of the cell substance. They showed a difference in the isoelectric point between gram positive bacteria and gram negative bacteria. The application of iodine lowered the isoelectric point of the gram positive bacteria to a greater extent than in the gram negative bacteria. Stearn and Stearn demonstrated that increasing amounts of basic dyes were taken up as the pH increased over the isoelectric point, and that increased amounts of acid dyes were taken up as the pH decreased from the isoelectric point. They thought that the iodine produced a change in the bacterial substance itself. They found
the general behavior of the gram positive and gram negative bacteria was the same. The differences became apparent in the degree of lowering of the isoelectric point after the application of the iodine. One fault has appeared in their work, that of not using a counter-stain. In the opinion of Burke and Barnes (1929), they did not study the complete differentiation required in the gram reaction.

Modern Concepts of the Gram Stain

Although many significant experimental facts are known, as yet no one has been able to describe the gram staining mechanism with any definitude. It is generally conceded that the gram staining mechanism is more complex than once thought and does not depend on any one inherent factor in the bacterial cell.

Knaysi (1951) ascribed the greatest importance to a difference in chemical composition, particularly in the composition of the cytoplasmic membrane, giving permeability a secondary role. The difference was due to the type of nucleic acid which gram positive bacteria contain as compared with gram negative bacteria. The gram positive bacteria may contain a more acidic form of nucleic acid than the gram negative bacteria.

Bartholomew and Mittwer (1952) maintained that
permeability plays the primary role in determining the gram reaction. However, they stated that chemical composition and permeability are jointly involved in the gram differentiation. Specific entities such as nucleic acids, lipoids, and proteins have been implicated as responsible for the gram positive state. Extraction of these substances from the gram positive bacterial cell converts the cell to a gram negative state. Bartholomew and Mittwer believed the damage caused to the cell by these extractions probably affected a greater cell membrane permeability. The resulting damage to the cell membrane, rather than the loss of cell material, caused a change in the gram reaction.

Environmental Influences on the Stability of the Gram Reaction

No great amount of effort seems to have been expended in studying the effect of environmental factors influencing the stability of the gram reaction. What information that does exist is sparse, and much of the published data have not been confirmed.

Neide (1904), determining the speed of decolorization by alcohol, found that the kind of medium used for culturing influenced the time required for complete decolorization. He reported that it took 25 minutes longer
to decolorize *Bacillus tumerens* when grown in plain agar than when grown on dextrose agar. No explanation was offered as to why this happened. However, Stearn and Stearn (1924) observed that the formation of acid in the medium was a common cause of the gram positive to gram negative change.

The presence of large amounts of fermentable carbohydrate found in enriched media, which results in the production of acid, or the acid in pus may cause a gram stain reversal. Burke and Ashenfelter (1926) found the pH of the medium to be quite critical in the maintenance of the gram positive character. They recommended that the gram reaction should always be determined using cultures grown at the optimum pH range of the species.

Eisenberg (1910) reported that the cultural conditions had a marked effect on the gram reaction. Many gram negative species became gram positive when grown in high concentrations of fat, starch, or protein. He maintained that the gram positive bacteria thus obtained could be deprived of their gram positive character by pepsin treatment. However, Eisenberg used impure pepsin, and his work has not been confirmed. Stearn and Stearn (1924) reported that bacteria which synthesizes large amounts of lipoidal material seem to have a stronger gram positive character than other species.
The influence of osmotic pressure and surface tension has been shown by some workers to be important in the maintenance of the gram character. Beguet (1929), studying *Shigella dysenteriae*, reported that the inclusion of 20 per cent MgSO$_4$, 5 per cent NaCl, or 35 per cent glucose produced a gram negative to gram positive change. Likewise a decrease of osmotic pressure or surface tension produced a reversal from the gram positive to gram negative state. As a result of his work with surface active agents such as sodium sulforincinate, he reported the critical surface tension to be 40 dynes for the maintenance of the gram character. A complete loss of the gram positive reaction resulted when the surface tension fell lower than 32 dynes.

Frobisher (1926) also investigated the effect of various surface active agents and found that a low surface tension in the culture medium may be an important factor in determining the size of the individual cells, the rate and extent of cell division, and the cell grouping. With the pneumococci he observed an interesting sequence of events. When pneumococci were mixed with sodium oleate, the cells underwent a rapid disintegration, starting with a loss of ability to retain the gram stain, followed by a swelling and granulation, a loss of capsule, and ending
with complete dissolution.

Many workers have been successful in demonstrating that the amount and type of nutrients in the medium has an effect on the gram reaction of bacteria. Lasseur and Schmitt (1927) observed that enriched media produced some peculiar effects. They found that staphylococci grown on human blood agar underwent a change in gram reaction. Staphylococci, tetragenae, enterococci and sarcinae grown in glucose broth were observed to exhibit various degrees of gram positivity.

The effect of starving on bacteria was studied by Stearn and Stearn (1929). They observed that Bacillus cereus stored in distilled water tended to lose the gram positive character at 37 C, though not at ice-box temperatures. Absence of growth was given as a probable reason in the latter case. The results in the former case were ascribed to bacteria using up the acidic reserve food materials, such as the nucleo-proteins. This loss of acidic character caused the basic dyes to have affinity for the cells, resulting in a loss of gram positivity. Knaysi (1948) showed that spores of Bacillus mycoides developed into gram negative cells on a medium deficient in nitrogen. DeLey (1951), in a ramification of Knaysi's work, noted that the gram reaction of nitrogen deficient bacteria was uniformly gram positive, but the staining
was less intense. As deficiency progressed, one or more strongly gram positive bodies was seen in the weakly stained cytoplasm. He thought the bodies were of a nuclear nature. The decrease in gram positive substance seemed to run parallel to a decrease in the adaptive enzymes such as lactase. On the other hand, there was little or no decrease in the constitutive respiratory enzymes.

The influence of the age of the culture on the gram reaction has been demonstrated to be important by most authors. Hucker and Conn (1923), studying staphylococci of milk and human origin, observed that 48 hour cultures were more gram positive than younger cultures. Kopeloff and Cohen (1928) found that 24 hour cultures were more gram positive than 48 hour cultures. Lasseur and Schmitt (1927) pointed out that no generality can be stated as each species is different. However, they found that for each species there was a time when the gram positive character was most pronounced. Bartholomew and Mittwer (1952) agreed with Hucker and Conn (1923) that the true gram reaction might be obtained with more reliability if the stains were made at various times from 12 hour up to several days.
Reversion of the Gram Reaction

Many publications have appeared describing the reversion of the gram reaction. The reversion has generally been from the gram positive to the gram negative state although the opposite change, gram negative to gram positive, has been seldom reported. Most of this work has not been confirmed, and considerable disagreement exists. Neide (1904) observed that prolonged treatment with gentian-violet, iodine, or heat caused gram negative bacteria to stain as gram positive bacteria. Churchman (1927), on the other hand, showed that the addition of acriviolet, gentian-violet, or acriflavine to a suspension of Bacillus anthracis caused this organism to stain as a gram negative bacterium. The reversion took from 2 to 19 hours. The same results were observed with Bacillus subtilis, Bacillus megatherium, and Bacillus mycoides.

Many of the reported conversions have been due to faulty staining techniques or to changes taking place in the staining reagents. Such a case was reported by Shepp and Constable (1923). They reported that Lugol's iodine exposed to bright light or high temperature became acid. This caused gram positive bacteria to appear gram negative. To avoid this fault, they recommended that all reagents be stored in a cold, dark place. If acid formed in the
mordant, they suggested that sodium bicarbonate be added to neutralize the acid just prior to use.

Extractions of various substances from bacterial cells has been found to reverse the gram stain reaction. Jobling and Peterson (1914) reported a gram positive to gram negative change when the lipoids were removed from the cells. However, they observed that the complete extraction of the lipoids was difficult because of the high concentration of bound lipoids. The lipoids of gram positive bacteria were found to be extracted more slowly than those from negative bacteria when lipid solvents were used.

The gram positive to gram negative conversion has been accomplished using several kinds of enzymes. The first mention of this conversion was by Avery and Cullen (1923). They did not add an enzyme to the culture but allowed pneumococci to undergo normal autolysis. Autolysis liberated an enzyme that depolymerized the ribonucleic acids of the cytoplasm; this resulted in the cells staining as gram negative bacteria.

Henry and Stacey (1943) and Bartholomew and Umbreit (1944), using purified ribonuclease, showed that the enzyme was able to convert most gram positive bacteria to the gram negative state. Fischer and Larose (1952) were able to draw similarities between the behavior of Bacillus cereus, which is originally gram positive, and the stain-
ing action of degraded wool. After *B. cereus* and wool were converted to the gram negative state, they found that it was possible to revert them to the gram positive state by an extended treatment in crystal-violet.

The role of desoxyribonucleic acid substrate in the gram staining reaction was emphasized by the work of Baker and Bloom (1948). They showed that *Escherichia coli* could be made gram positive by plating the cells with a concentrated solution of desoxyribonucleic acid. It would appear, however, that the change induced was not of a permanent nature, and that the substance responsible for the gram positiveness could be eluted off the cells with distilled water but not with physiological saline.

The most recent development in the gram stain reversion was made by Bartholomew and Mittwer (1952). By means of ultra-violet irradiation, they found it possible to convert fixed smears of most gram positive species to the gram negative state. The speed of the change of the gram reaction was shown to be influenced by the method of fixation.

It was observed that osmium tetraoxide fixation caused the reversion of the gram reaction to occur much faster. The sequence of events taking place in the cells under ultra-violet irradiation was shown to be very similar to that reported by DeLey (1951), who studied
the effect of nitrogen deficiency on the gram reaction.

Relation of Electrophoretic Velocity To the Gram Reaction

With the manufacture of better cataphoretic cells, especially those described by Northrop and Kunitz (1925), considerable work has been done to determine the electrophoretic mobility of bacteria. Buggs and Green (1935) tried to differentiate toxogenic and nontoxogenic strains of diphtheria by studying the difference in their average electrophoretic mobilities. When the average mobility of toxogenic strains was compared with the average mobility of non-toxogenic strains, they showed that the mobility of the former was 19.8 per cent slower than the mobility of the latter. In a later work Buggs and Green (1935a) investigated the age of a culture in relation to its electrophoretic mobility. They reported no difference in the mobilities of 6 hour and 10 day cultures of Escherichia coli and Staphylococcus aureus. They noted that treatment which extracted a part of the surface constituents or altered their nature was found to change the electrophoretic mobility.

Using electrophoretic mobility data an attempt to differentiate the coli-form groups was made by Dozois (1936). He showed that the mobility rate was constant.
for each species, but that there was a wide range of mobilities between strains of the same species. Since a strain of one species may have the same electrophoretic mobility as a strain of another species, and, since the average mobility of one genus may be near that of another genus, it was not possible to make a differentiation in the coli-form group.

Pedlow and Lissee (1936) reported the electrophoretic mobility of *Escherichia coli* grown in peptone broth containing CaCl₂ increased to a constant value. They also reported that the age of the culture, up to 28 hours, had little influence on the mobility rate.

The effect of dissociative changes on the electrophoretic mobility was studied by Stearns and Roepke (1941). They found that electrophoretic measurements may not show all the changes that occur during bacterial dissociation. However, the observations of the electrokinetic potential with respect to a given medium may offer a refined method for studying bacterial dissociation.

Little work has been done to determine the relationship between electrophoretic mobility and the gram reaction. In an early study of the effect of pH on the electrophoretic mobility, Winslow and Upton (1926) found no apparent relationship existed between the gram reaction and the electrophoretic mobility. Burke and
Gibson (1933) reported that gram positive and gram negative cells may show a similar electrophoretic migration curve. Both kinds of cells were found to carry a negative charge and to move toward the positive pole. The charge could be changed by the addition of acid or base. The surface charge on gram negative cells could be reversed without reversing the gram reaction. On the other hand, the gram reaction of gram positive cells could be reversed without reversing the charge. From their observations they concluded that the gram reaction was not correlated with the electric charge at the surface of the bacterial cell.

Knaysi (1951) stated that since electrophoretic mobility is a surface property and the gram reaction is a property of the protoplasm, no relationship between the two is to be expected.
MATERIALS AND METHODS

Cultures

The organisms used in this study were obtained from the Department of Bacteriology, The Ohio State University, and are listed below:

2. *Pseudomonas aeruginosa*, OSU 349.
5. *Bacillus mycoides*, OSU 23.

Culture Media

Throughout this study all stock cultures were carried on nutrient agar (Difco). After incubation they were stored at 4°C. Nutrient broth (Difco) was used as the medium when normal morphology and staining reactions were studied. The above mentioned media were modified in some experiments by the addition of potassium iodide, ranging from 1 to 70 mg per ml of medium.

Preparation of Cultures

The influence of growth in potassium iodide, as compared with growth in nutrient broth, was studied by inoculating the organisms into tubes of medium containing
30 mg of KI per ml. Morphological changes and the gram staining reactions were determined to observe the effect on changing the gram negative organisms to gram positive in the 24 hour cultures. Hereafter the organisms grown in KI will be referred to as E. coli (KI), P. aeruginosa (KI) and P. vulgaris (KI).

Growth Studies

Growth rate studies were conducted on E. coli, P. aeruginosa and P. vulgaris. Nutrient broth and nutrient broth containing 30 mg per ml of KI were used. The amount of growth was determined by making turbidity readings at regular intervals with the Evelyn Photoelectric Colorimeter using a 660 mp filter. From the data obtained growth curves were plotted. The determinations of pH were performed with a Fischer Titrimeter which was standardized with a phosphate buffer at pH 7.0. From the data obtained pH curves were plotted.

Standard Crystal Violet Curve

Since photometric measurements of colored substances are convenient and accurate, this method was used to determine the amount of crystal violet fixed by the above bacteria. To obtain maximum sensitivity for the colorimetric work the adsorption spectrum of crystal violet
was determined. From this information and from actual trials in the Evelyn Photoelectric Colorimeter, the most suitable wavelength of light was calculated. The crystal violet, Certification No. 32, used in this work was manufactured by the National Aniline Company. The dye content was 91 per cent.

The stock solution of crystal violet, a 0.01 per cent solution, was made up in phosphate buffer at pH 7.0. It was stored in the dark in an air-tight bottle. This solution remained stable for at least 3 months. The solutions for the dye fixation experiments, containing 0.006 mg of crystal violet per ml, were made up in buffer just prior to use.

A standard curve was plotted from data obtained by determining the percentage of transmission of light for dye solutions containing from 0.01 mg to 0.002 mg crystal violet per ml of solution. This standard curve was later used to calculate the amount of dye fixed by normal cells as compared to cells grown in nutrient broth containing KI. Examination of this curve showed that the maximum sensitivity for detecting small changes in dye concentration was between 0.006 mg and 0.0005 mg dye per ml of solution. Therefore, dye fixation experiments were done with a crystal violet solution containing 0.006 mg dye per ml.
Crystal Violet Fixation

In order to obtain detectable fixation of the dye heavy cell suspensions of the test organisms were required. These suspensions were obtained from growth in nutrient broth and nutrient broth containing 30 mg KI per ml of medium. The bacteria were grown in 300 ml quantities of broth at 37°C for 36 hours. The cells were collected by centrifugation at 5000 rpm for 30 minutes and washed twice in pH 7.0 buffer prior to standardization and storage. All suspensions were adjusted to 50 per cent light transmission using the Evelyn Photoelectric Colorimeter and a 620 mp filter. Phosphate buffer at pH 7.0 was used as the diluent. These same suspensions were later used to produce immune sera to determine if any change was affected in the antigenic mosaic.

In order to determine the amount of dye fixed by the test organisms, 6 ml of dye solution containing 0.01 mg of dye per ml, 3 ml of buffer and 1 ml of the bacterial suspension were added to calibrated colorimeter tubes. Control tubes were prepared containing 0.006 mg of dye per ml. This concentration of crystal violet gave 23 per cent transmission of light using a 620 mp filter. Control tubes of buffer were used to adjust the colorimeter to 100 per cent transmission. The colorimeter tubes containing the dye and cell suspensions were incubated
for 24 hours at 37 C to obtain maximum dye fixation. The cells were then separated from the dye solution by centrifugation at 5000 rpm for 25 minutes. The supernatant was decanted into previously standardized colorimeter tubes, and the percentage of light transmission determined. By interpolation on the standard curve the amount of crystal violet in the supernatant was determined. The amount of crystal violet fixed by the cells was calculated by subtracting the amount of dye remaining in the supernatant from the amount in the original solution.

Iodine Determination

In order to ascertain if *E. coli*, *P. aeruginosa* and *P. vulgaris* had the ability to fix iodine, it was necessary to have a method for the determination of iodine in bacterial cells. Connor's (1949) method for the determination of blood iodine was used. In the above method bound and free iodine are determined separately. As total iodine was desired in this work, the part of the procedure separating the bound and free iodine was eliminated. The amount of iodine was determined colorimetrically. A standard curve was plotted from data obtained using known amounts of iodine. The iodine content of the cells was determined by interpolation on the standard curve. (See Fig. 1).
To prove that the iodine present in the cells came from the iodine added to the medium and not from the iodine which might have been present in the medium as a contamination, control determinations were made on nutrient both and on cells that had been grown in nutrient broth.

Staining Methods

Throughout this work a modification of the Kopeloff and Beerman (1922) gram staining procedure was used. This method was as follows:

1. Stirling's crystal-violet was applied for 1 minute to fixed smears.

2. Kopeloff and Beerman's iodine mordant was applied for 2 minutes.

3. Decolorization was accomplished by using a mixture of ethyl alcohol and acetone, 70 per cent alcohol and 30 per cent acetone.

4. The counterstain, 0.25 per cent aqueous safranine, was applied for 20-30 seconds.

A variation of the above method was used to stain the gram negative bacteria that had been grown in nutrient broth containing potassium iodide. The only change was the omission of the iodine mordant. After the extraction of the various cellular substances, the cell wall was stained following the method of Knaysi (1930, 1940).

To determine what effect time of incubation had on the reversal of the gram reaction, KI tolerant strains
of **E. coli**, **P. aeruginosa** and **P. vulgaris** were inoculated into media containing KI in the following concentrations: 1 mg per ml, 2 mg per ml, 4 mg per ml, 6 mg per ml, 10 mg per ml, and 30 mg per ml. These cultures were incubated at 37 °C. Gram stains were made from the cultures at 24, 48 and 72 hours. Heat fixed smears were stained with Kopeloff and Beerman method and the modified method. Smears of **N. sicca** were employed to control the gram stain procedure.

**Methods of Fixation**

All smears were heat fixed in the usual manner with the exception of one half of the impression smears made from **B. mycoides**. These were fixed in osmium tetroxide vapors for three minutes.

**Nitrogen Determination of Normal Cells and Cells Grown in KI**

To determine what effect growth in KI had on the bacterial cell substance, nitrogen determinations were performed. These determinations were made using heavy suspensions of normal cells and cells grown in broth containing 30 mg KI per ml of medium. The bacteria were cultured in 300 ml quantities of broth. The cultures were incubated at 37 °C and harvested by centrifugation after 36 hour incubation. Pure cell suspensions
were washed three times in buffer at pH 7.0. The suspensions were maintained as concentrated as possible. The most dilute suspension gave a 10 per cent light transmission with a 620 mp filter. Therefore, all of the samples were diluted to obtain the same turbidity.

The actual nitrogen content of the cell suspensions was determined by the micro-kjeldahl method. Nitrogen determinations were performed in duplicate. One-half ml of the cell suspensions was used for each determination. Nitrogen was calculated on the basis of dry-weight of cells.

Photographic Procedures

All photographs in this study were taken with a IIc Leica camera with the Photo-copy attachment. The plain of focus of the ground glass screen was set 8 in from the microscope ocular. A light-tight cylinder was placed between the microscope and the Photo-copy attachment. Illumination was furnished by a Bausch and Lomb microscope lamp with a 200 watt projection bulb. Critical illumination was used. The microscope was equipped with a 97X achromatic objective and a 10X ocular.

The correct exposures were calculated after Plus X film had been standardized with light of various intensities. The intensity of the light was measured with
a Photovolt Meter (Model 200).

The exposure standardization of the film was performed in the following manner: A prepared slide having extreme ranges of color contrast was used as a specimen. The optical system and the light were adjusted to obtain the maximum light through the system. With maximum light and no filter the meter gave a reading of 30* at high ratio. With the system adjusted at a meter reading of 30, six exposures were taken on Kodak Plus X film. These exposures were 1/30, 1/40, 1/60, 1/00, 1/200 and 1/500 sec. The same procedure was followed with the light intensity reduced one half and the light intensity reduced one quarter.

The film was developed in Kodak Microdol and fixed in Kodak Acid Hardner Fix. Developing and fixing were done at 20 C. The photographic solutions were prepared just prior to use. The film was developed for 16 min and fixed for 15 min.

To determine the optimal exposure time at the three different light intensities, contact prints were made from the 35 mm film. The exposure time was 4 sec and the development time was 20 sec. Kodak D-72 was the developer used. The prints were fixed in Kodak F-5.

* Meter readings are given in arbitrary units on the galvanometer scale.
All prints were made on Kodak AZO II paper. An examination of the prints showed clearly which film exposure time was optimal. When the optimal exposure time was plotted against the meter reading, the following curve was obtained. (See Fig. 2).

At a meter reading of 30, the optimal exposure was 1/100 sec; at a meter reading of 15, the optimal exposure was 1/60 sec; and at a meter reading of 7.5, the optimal exposure was 1/30 sec.

From the above data the correct exposure for Kodachrome film was calculated. The ASA number of Plus X film is 32 for tungsten light, and for Kodachrome (Daylight type) the ASA number is 2.5. The optimal exposure for Kodachrome should therefore be 32/2.5 or approximately 13 times greater than the exposure time for Plus X film. Using a light source with a meter reading of 13, it was calculated from the graph that an exposure of 0.4 sec was optimal.

Immunological Procedures

Immunological methods were used to determine what changes had occurred in the antigenic mosaic as a result of growth in potassium iodide. Rabbits were used to produce immune sera to normal E. coli and to E. coli (KI). The inoculations were started with an intraperi-
Figure 2
Time-Light Relationship for Optimal Exposures with Plus-X Film

Light Meter Readings
toneal injection of 0.1 ml of a bacterial suspension containing approximately 10,000,000 cells per ml. Following the initial injection, the rabbits were given injections of 0.2, 0.3, 0.4, and 0.5 ml of the suspensions on the 3rd, 6th, 9th and 12th day respectively. The rabbits were bled five days after the last injection. Fifteen milliliters of serum were obtained for each of the above antigens. The sera were preserved in the deep freeze until used.

The titre of the adsorbed and unadsorbed immune sera was determined. The protocol given in Table I was followed. Agglutinations were incubated at 37°C for 2 hours. Readings were made immediately after the incubation and after an over-night period at 4°C.

Hereafter in the text *E. coli* antiserum will be referred to as Anti (N) and *E. coli* (KI) antiserum will be referred to as Anti (KI). The homologously adsorbed sera will be referred to as Homo. Ads. Anti (N) and Homo. Ads. Anti (KI). The heterologously adsorbed sera will be referred to as Hetero. Ads. Anti (N) and Hetero. Ads. Anti (KI).

The agglutinin titre of the different sera was determined by performing the following agglutination reactions:
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Dilution</td>
<td>1:5</td>
<td>1:10</td>
<td>1:20</td>
<td>1:40</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
<td>1:640</td>
<td>1:1280</td>
<td>1:2560</td>
<td>1:5120</td>
<td>Control</td>
</tr>
<tr>
<td>Amount of Serum in ml</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial Suspension in ml</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
<td>.1</td>
</tr>
<tr>
<td>Saline in ml</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
</tbody>
</table>
Adsorptions using the homologous systems were performed to determine what effect adsorption would have on the agglutinin titres. Two milliliters of serum were adsorbed with 0.1 ml of packed cells. This adsorption was repeated four times. The effect of the adsorption was determined by the following agglutination reactions:

1. **E. coli** with homo. Ads. Anti (N)
2. **E. coli** with homo. Ads. Anti (KI)
3. **E. coli** (KI) with homo. Ads. Anti (KI)
4. **E. coli** (KI) with homo. Ads. Anti (N)

The effect of heterologous adsorption was determined by adsorbing 1 1/2 ml of serum with the heterologous antigen. Six adsorptions were performed and 0.3 and 0.4 ml of packed cells were used each time. To get more complete removal of the antibodies the first and last adsorption were allowed to stand over-night in the cold room.

The following agglutination reactions were set up using the heterologously adsorbed sera:

1. Hetero. Ads. Anti (KI) with **E. coli** (KI) cells
2. Hetero. Ads. Anti (KI) with **P. aeruginosa** (KI) cells
3. Hetero. Ads. Anti (KI) with \textit{P. vulgaris} (KI) cells

4. Hetero. Ads. Anti (N) with \textit{E. coli} cells

5. Hetero. Ads. Anti (N) with \textit{E. coli} (KI) cells

Cataphoretic Velocity Determinations of \textit{E. coli} and \textit{E. coli} (KI)

In order to determine if a change in the electrical charge on the individual cells had occurred as a result of growth in potassium iodide, cataphoretic mobilities were calculated. A Northrop-Kunitz cell was used for this work. Before the cataphoretic determinations could be performed the equipment had to be standardized. Calibration of the apparatus included the following determinations:

1. Calculation of the inside depth of the cell to locate the stationary levels.

2. Measurement of the distance between the electrodes of the cell.

3. Determination of the ratio between the potential applied at the platinum electrodes of the electrolyte containers and the potential difference between the platinum electrodes of the cell.

The depth of the cell was determined by means of a microscope provided with a micrometer scale on the fine adjustment. The optical system consisted of a Bausch and Lomb microscope equipped with a 20X ocular and a 10X objective. A parabaloid condenser with a 6 v self-con-
tained light was used to obtain dark-field illumination. To find the inside top or bottom of the cell, a small amount of scouring powder in water was allowed to dry on these surfaces. By focusing on the particles of scouring powder the various levels were easily located. In order to insure greater safety in handling the fragile cell, it was mounted in a piece of 3/8 inch polystyrene. The ends of the cell were fastened to the polystyrene with melted carbo-wax. The cell was clamped to the stage of the microscope after being centered over the dark-field condenser. Due to variations of the different parts of the micrometer screw of the microscope, the depth measurements were made in the lower half of the scale.

The recorded depth of the cell at each point was the mean of a sufficient number of observations to minimize observational and manipulative errors. From these measurements the micrometer scale positions of two points were calculated. The first stationary level, 0.211 times the depth of the cell above the lower surface of the inside of the cell, and the second stationary level 0.789 times the depth of the cell below the upper inner surface of the cell were obtained. Determination of the stationary levels was made because of the phenomenon of electrosmosis in the cell.

From the average of 20 observations the depth of
the cell was calculated to be equivalent to 662 scale divisions. Using this figure, the position of the first stationary level was 662 x 0.211 or 131.2 scale divisions. The second stationary level was 662 x 0.789 or 490.7 scale divisions. Therefore, the first stationary level equaled one revolution of the micrometer screw plus 31.2 scale divisions from the bottom of the cell. The second stationary level equaled four revolutions plus 90.7 scale divisions from the bottom of the cell.

The distance between the tips of the platinum electrodes was found to be 36.5 mm. A standard micrometer disk was placed in the ocular to read migration in microns.

The ratio between the potential applied at the platinum electrodes and the potential difference between the platinum electrodes of the cell was determined by using a potentiometer circuit. The following schematic drawing and photograph show the electrical connections used in the calibration. (See Figs. 3 and 4).

A millivoltmeter having a range greater than the applied EMF from the dry cell was connected across the terminals of the platinum electrodes. The platinum electrodes of the cell were connected to the EMF posts of a potentiometer which was standardized against a standard cell. The mean of the potential difference between the platinum electrodes divided into the applied
Figure 3
Arrangement for Calibration of Micro-halocarbon Cell with Potentiometer
Figure 4 Arrangement for calibration of micro-cathaphoresis cell with potentiometer
EMF measured by the voltmeter was the ratio required.

Table II shows the results of this determination. From the data found in Table II, the mean ratio was calculated to be 16.12.

Table II

<table>
<thead>
<tr>
<th>Applied EMF</th>
<th>PD Between Platinum Electrodes</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.710</td>
<td>0.04440</td>
<td>16.1</td>
</tr>
<tr>
<td>0.700</td>
<td>0.0435</td>
<td>16.1</td>
</tr>
<tr>
<td>0.710</td>
<td>0.0435</td>
<td>16.3</td>
</tr>
<tr>
<td>0.694</td>
<td>0.0430</td>
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</tr>
<tr>
<td>0.740</td>
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</tr>
<tr>
<td>0.920</td>
<td>0.0575</td>
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</tr>
<tr>
<td>0.862</td>
<td>0.0527</td>
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</tr>
<tr>
<td>0.860</td>
<td>0.0540</td>
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</tr>
<tr>
<td>0.820</td>
<td>0.0512</td>
<td>16.0</td>
</tr>
<tr>
<td>0.570</td>
<td>0.0355</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Cataphoretic velocities were determined by filling the cell with a buffer suspension of the bacterial cells containing approximately 1 million organisms per ml. When the EMF was applied to the electrodes the velocities were observed by adjusting the microscope to the first stationary level and measuring the rate of migration of individual cells in microns per sec.

This was converted to \( \mu \) per sec per volt per cm by substitution in the following formula.
Gram Reaction Conversion of Bacillus mycoides

Twelve hour nutrient broth cultures of Bacillus mycoides were used for the study of the gram positive to gram negative conversion. The cells were collected by centrifugation and washed twice in pH 7.0 buffer before treatment with the various agents. The gram reaction conversion was produced by placing the cells in solutions of ribonuclease, trypsin, and sodium taurocholate. The effect of the above agents on the gram stain was studied by making impression smears of the treated cells. One drop of the cell suspension was placed on a water agar plate. Blocks were cut from the agar and the impression smears made from the agar blocks. The smears were heat fixed and stained with the Kopeloff and Beerman modification of the gram stain.

The ribonuclease used was prepared by The Armour and Company. It was employed in a concentration of 1 mg per ml of buffer. The trypsin was made by The Armour
and Company and was employed in a concentration of 10,000 units per ml. Five and ten per cent solutions of sodium taurocholate were also used for the conversion of the gram reaction. Two controls were performed with each of the converting substances. These were distilled water and N/10 HCl.

The effect of lipoid solvents on the gram stain stability was studied. The cultures were grown on nutrient agar plates for 12 hours. The petri dishes were then placed over Brewer anaerobic dishes containing the various solvents. Ether, acetone, carbon tetrachloride and ethyl alcohol were used. After various times of treatment, agar blocks were cut from the petri dishes and impression smears were made from the colonies on the agar blocks. The impression smears were heat fixed and stained by the Kopeloff and Beerman modification of the gram stain.

Ultraviolet irradiation was used to produce gram reaction conversions. A General Electric, germicidal lamp, with an output of 16 mw per cm² at 1 meter was used as a source of ultraviolet irradiation. The tube of the lamp was placed exactly 20 cm from the cells being irradiated. In one experiment with ultraviolet irradiation the cells were suspended in buffer and irradiated in a 1.5 mm layer contained in a flat coplin
A covered coplin jar containing the same number of cells served as a control. To obtain uniform irradiation, the jars were fastened to a Cenco Mechanical Shaker and agitated during the entire period of irradiation. A slow speed of agitation was employed. The liquid level of the jars was maintained by adding buffer to correct for the evaporation losses. Impression smears were made from water agar blocks to which one drop of the cell suspension was added at various times and stained by the Kopeloff and Beerman modification.

A second experiment was performed to determine the irradiation time necessary to effect a complete reversion of the gram reaction. Thirty-two impression smears were made from agar blocks. Each agar block contained a single colony of a 12 hour culture. One half of the slides were heat fixed and the other half were fixed in osmium tetraoxide vapors for three minutes. Gram stains and cell wall stains were made at various times of irradiation. One slide was used from each fixation method for each of the above staining methods. The cell wall stains were made to determine if any change occurred in cell diameter.

An attempt to reconvert the "gram negative" cells to the gram positive state was made after each of the conversion methods. The "gram negative" cells were
treated in a buffered solution containing 60 mg KI per ml.
The time of treatment ranged from 6 hours to 24 hours.
EXPERIMENTAL RESULTS

Growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* in potassium iodide

In the original observation it was evident the *E. coli*, *P. aeruginosa* and *P. vulgaris* underwent a gram reaction reversal when grown in various concentrations of potassium iodide. It was also noted that the cultures required a period of "training" before they would be grown in the final concentration of 30 mg of KI per ml of medium. Initially, the cultures were inoculated into nutrient broth containing 1 mg of KI per ml. After good growth occurred at this dilution, the cultures were transferred to broth containing 2 mg KI per ml. This process was repeated until the organisms grew well in a concentration of 10 mg KI per ml of medium. These "trained" strains were then transferred to a medium containing KI at a concentration of 30 mg per ml, and good growth was obtained. The "training" of *P. vulgaris* was more complete than that of *E. coli* and *P. aeruginosa* as the KI tolerant strains of *P. vulgaris* grew well in concentrations as high as 70 mg KI per ml of medium. The "trained" strains were maintained on nutrient agar plates and stored at 4°C. Transfers were made every 3-4 weeks. No change or loss of KI tolerance was noted in any subculture. Each strain pro-
duced heavy growth in broth containing 30 mg KI per ml after 24 hour incubation. The results of the gram reaction reversal are presented in the section devoted to photographic results. In addition to the gram staining reversal, other changes were noted in the above organisms. Differences in morphology between KI tolerant and normal strains were quite distinct. Photographs showing the morphological differences appear in the Appendix (Figures 1-6). When *P. vulgaris* was grown in KI, the cells were filamentous, assuming an actinomycete-like appearance. The cells of *E. coli* grown in KI were longer than the original strain and had a barred appearance. Cell division seemed to be retarded. *P. aeruginosa* was much larger when grown in KI than when grown in nutrient broth. Most cells appears in groups of two.

Differences in the rate of growth which might influence the uptake of iodine were determined in nutrient broth and nutrient broth containing 30 mg KI per ml. The growth curves were plotted through 56 hours. The results of this experiment are shown in Figures 5 and 6. As shown in Figure 6, there was better growth of *E. coli* in the presence of KI than in the absence of KI. The growth of *P. aeruginosa* was retarded by KI. *P. vulgaris* showed little difference during the early stages of growth. However, the maximum cell concentration was
greater when the cells were grown in the absence of iodide.

Because pH has been shown to influence the gram reaction, the pH of the medium was plotted over a period of five days growth. Figures 7 and 8 show the data obtained from these experiments. The pH of the culture was plotted against time. An examination of the figures shows that there is very little difference in pH curves of growth in nutrient broth and nutrient broth containing 30 mg KI per ml. The slight differences would not account for the changes in the gram reaction.

While studying the growth rates in potassium iodide, it was found that *P. aeruginosa* produced a slime. This was not evident in nutrient broth. Further work showed that other potassium compounds, potassium nitrate, potassium chloride, potassium acetate and potassium gluconate, produced the same effect.

Gram Reaction Reversal of *E. coli*, *P. aeruginosa* and *P. vulgaris*

The gram reaction reversal was studied by inoculating the KI tolerant strains of *E. coli*, *P. aeruginosa* and *P. vulgaris* into media containing 1, 2, 4, 6, 10, and 30 mg KI per ml. Heat fixed smears were made from the above cultures after 24, 48, and 72 hour incubation. The Kopeloff and Beerman and the modified staining methods
were used to stain the smears. *N. sicca* served as a control for the staining procedures. The results of this experiment are shown in Table III.

Table III

Influence of KI Concentration and the Age of The Culture on the Reversal of the Gram Reaction of *E. coli*, *P. aeruginosa* and *P. vulgaris*

<table>
<thead>
<tr>
<th>KI Concentration in mg per ml</th>
<th>Gram Stain Reaction After 2½ Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slight dye fixation. Most cells show bipolar staining</td>
</tr>
<tr>
<td>2</td>
<td>A few cells completely reversed. Most cells show bipolar staining.</td>
</tr>
<tr>
<td>4</td>
<td>Few cells show bipolar staining. Majority of cells stain light blue.</td>
</tr>
<tr>
<td>6</td>
<td>All cells stain blue.</td>
</tr>
<tr>
<td>10</td>
<td>All cells stain blue.</td>
</tr>
<tr>
<td>30</td>
<td>All cells stain dark blue.</td>
</tr>
</tbody>
</table>

Only the results of the 2½ hour cultures are given as the 48 and 72 hour cultures showed similar staining reactions. Likewise, only the data for *E. coli* are presented as the results for *P. aeruginosa* and *P. vulgaris* were the same.

The concentration in which the first complete gram reaction reversal occurred was 6 mg KI per ml. Reversal
was evident in 2¼ hours. No great change was observed after 2¼ hours.

In the previous experiment, time of incubation beyond 2¼ hours was found not to influence the staining reaction. A second experiment was performed to determine the minimal incubation time for the gram reaction reversal. Only three concentrations of KI were used, 2, 6, and 30 mg per ml of medium. The cultures were incubated at 37 C. Gram stains made from these cultures showed that a complete reversal of the gram reaction occurred in 12 hours in media containing 6 and 30 mg KI per ml. The results for E. coli, P. aeruginosa and P. vulgaris were similar.

Crystal Violet Fixation by Gram Negative Bacteria

The optimal wave length of light to be used in the Evelyn Colorimeter for detecting changes in crystal violet concentration was determined. These data were obtained with the Beckman Spectrophotometer. It was found that there were two wave lengths of maximum absorption, one at approximately 525 µ, the other at approximately 640 µ. To ascertain which of the above values was the better, trial runs were performed with the Evelyn Colorimeter. Three wavelengths of light were used in the colorimeter. The 420 µ filter was used as a control because this band
was not near the optimal values obtained with the spectrophotometer. The following table presents the data obtained. The readings are reported as per cent transmission of light.

Table IV

Per Cent Transmission Obtained with Three Filters and Varying Concentrations of Crystal Violet

<table>
<thead>
<tr>
<th>Crystal Violet Concentration per ml of buffer</th>
<th>Percentage Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>420 μm</td>
</tr>
<tr>
<td>0.01 mg</td>
<td>87</td>
</tr>
<tr>
<td>0.008 mg</td>
<td>88</td>
</tr>
<tr>
<td>0.006 mg</td>
<td>91</td>
</tr>
<tr>
<td>0.004 mg</td>
<td>94</td>
</tr>
<tr>
<td>0.002 mg</td>
<td>96</td>
</tr>
<tr>
<td>Buffer control</td>
<td>100</td>
</tr>
</tbody>
</table>

An examination of Table IV shows that the 620 μm filter gave the greatest deviation over the range of dye concentrations. Therefore, the readings obtained with the 620 μm filter were used to prepare the standard crystal violet curve. (See Fig. 9). An examination of the standard curve shows that the greatest change in percentage of light transmission occurred between the values of 0.006 mg dye per ml of buffer and 0.0005 mg dye per ml of buffer. Therefore, all of the dye fixation experiments
Figure 9
Relation of Dye Concentration to Colorimeter Readings. 620 mp Filter was used.
were conducted using a starting dye concentration of 0.006 mg per ml. One ml of the standardized cell suspensions was added to the standard crystal violet solution to give a final dye concentration of 0.006 mg per ml of buffer.

After the cell suspensions were incubated in the standard crystal violet solution for 24 hours at 37 C, the suspensions were centrifuged and the amount of dye remaining in the supernatant was determined. The following table presents these data. Readings are recorded as per cent transmission of light.

Table V

Per Cent Transmission of Crystal Violet Solutions After Dye Fixation by Normal Cells and Cells Grown in a Medium Containing KI

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>P. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells</td>
<td>45</td>
<td>32</td>
<td>44</td>
</tr>
<tr>
<td>Cells grown in KI</td>
<td>50</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>Control tubes contain 0.006 mg dye per ml</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Buffer control tubes containing no dye</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Using the above data and the Standard Crystal Violet Curve (Figure 9), the amount of dye remaining in
the solutions was calculated. The following table gives this information.

Table VI

Crystal Violet Remaining in Solution After Dye Fixation by Normal Cells and Cells Grown In a Medium Containing KI

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>P. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells</td>
<td>0.00250</td>
<td>0.0039</td>
<td>0.00255</td>
</tr>
<tr>
<td>Cells grown in KI</td>
<td>0.00206</td>
<td>0.0033</td>
<td>0.00208</td>
</tr>
<tr>
<td>Difference</td>
<td>0.00044</td>
<td>0.0006</td>
<td>0.00047</td>
</tr>
</tbody>
</table>

In order to calculate the amount of crystal violet fixed by one gram dry-weight bacterial cells, the dry-weight of 1 ml of the standard cell suspensions was determined. One ml of the suspensions of E. coli and E. coli (KI) contained 0.0113 g and 0.0098 g respectively; one ml of the suspensions of P. aeruginosa and P. aeruginosa (KI) contained 0.0095 g and 0.0100 g respectively; and one ml of the suspensions of P. vulgaris and P. vulgaris (KI) contained 0.0087 g and 0.0087 g respectively. The following table presents the dye fixation data corrected to 1 g dry-weight of cells.
Table VII

Crystal Violet Fixation by Cells Grown in Nutrient Broth and Nutrient Broth Containing KI

Mg of Crystal Violet Fixed per g dry weight

<table>
<thead>
<tr>
<th></th>
<th>Mg of Crystal Violet Fixed per g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0.3097</td>
</tr>
<tr>
<td>E. coli (KI)</td>
<td>0.4002</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0.2209</td>
</tr>
<tr>
<td>P. aeruginosa (KI)</td>
<td>0.2700</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>0.3967</td>
</tr>
<tr>
<td>P. vulgaris (KI)</td>
<td>0.4508</td>
</tr>
</tbody>
</table>

Quantitative Determination of Iodine in the Bacterial Cells

The iodide content of the various cells was calculated to determine if E. coli, P. aeruginosa or P. vulgaris had the ability to concentrate iodide. The growth from 36 hour cultures was collected by centrifugation. Nutrient broth and nutrient broth with 30 mg KI per ml were used as culture media. The cell suspensions were washed three times before standardization and storage. Two ml of the standardized suspension were used for each determination. All iodine determinations were performed in duplicate. Control determination were made with nutrient broth and cells collected from nutrient broth. All readings were
taken after a 5 minute catalytic reactive period. The iodine content was determined by interpolation of the colorimetric readings on the standard iodine curve. The following table presents the data obtained from these determinations.

<table>
<thead>
<tr>
<th>Reactive Period</th>
<th>Per cent Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>E. coli</strong></td>
</tr>
<tr>
<td>1 min.</td>
<td>27</td>
</tr>
<tr>
<td>2 min.</td>
<td>28^3</td>
</tr>
<tr>
<td>3 min.</td>
<td>30^3</td>
</tr>
<tr>
<td>4 min.</td>
<td>32^3</td>
</tr>
<tr>
<td>5 min.</td>
<td>35^1</td>
</tr>
</tbody>
</table>

As the above determination is dependent upon a color reduction catalyzed by iodine, care had to be exercised in adding the correct amount of iodine to the reactive system, so that the data obtained at the 5 minute reactive period fell on the standard iodine curve. One-ninth of the iodine distillate was used for each determination. By using the standard curve and the values obtained after the 5 minute reactive period, the iodine content in the cells was: **E. coli** 0.120 μg; **P. aeruginosa**
0.234 µg; and *P. vulgaris* 0.485 µg. Total iodine was calculated for the 2 ml volumes used in each determination. *E. coli* contained 1.080 µg; *P. aeruginosa* contained 2.106 µg; and *P. vulgaris* contained 4.365 µg. The above data were converted to iodine per gram of dry cells. *E. coli* contained 55.08 µg; *P. aeruginosa* contained 120.042 µg; and *P. vulgaris* contained 435.500 µg. The values obtained for the control determinations were well within the range of values obtained with the reagent blank.

Photographic Comparison of the Staining Methods

The results of the Kopeloff and Beerman gram staining method and the modified method were recorded photographically. Kodachrome film was used to record the color differences. Photographs showing the gram reaction reversal are included in the Appendix (Figures 7-15).

The Kopeloff and Beerman method was employed to stain the normal cells grown in nutrient broth. Figures 7, 8 and 9 shows these results. The cells grown in KI were stained with the modified method and are shown in Figures 10, 11, and 12. Control smears to which *N. sicca* was added were stained by the modified method and are given in Figures 13, 14 and 15. Cells grown in KI and stained by the Kopeloff and Beerman method gave the same results as cells stained with the modified method.
Nitrogen Determination of Normal Cells and Cells Grown in KI

These determinations were performed with 1/2 ml of the various cell suspensions. The results were corrected to 1 g dry weight of cells and the percentage of nitrogen calculated. The following table presents the composite data.

Table IX

Nitrogen Content of Normal Cells and Cells Grown in a Medium Containing KI

<table>
<thead>
<tr>
<th>Mg Nitrogen per gram dry weight of bacterial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Normal cells</td>
</tr>
<tr>
<td>Cells grown in KI</td>
</tr>
</tbody>
</table>

Gram Reaction Reversals of *Bacillus mycoides*

The cells were collected by centrifugation from 30 ml of nutrient broth after 12 hour growth. The cells were washed in buffer twice before the ribonuclease was added. Ribonuclease was employed in a concentration of 1 mg per ml of buffer. The gram reaction was found to be reversed in 95 per cent of the cells after 36 hour treatment at 37°C. These "gram negative" cells could be made to stain gram
positively by a 6 hour treatment in KI at a concentration of 60 mg per ml of buffer. Photographs illustrating the above experiment are given in the Appendix (Figures 16-17). The cells which are shown were treated for 18 hours with ribonuclease.

Trypsin, in a concentration of 10,000 units per ml was found to induce a loss of gram positivity. This reversal was 90 per cent complete in 20 hours. Photographs showing the change are shown in the Appendix (Figures 18-19). It was also possible to reverse the loss of gram positivity by treating the cells for 6 hours in KI at a concentration of 60 mg per ml.

The gram stain reversal was also accomplished by treating cells of *B. mycoides* in 5 and 10 per cent sodium taurocholate. It required 10 hours to produce a complete gram reaction reversal with the 10 per cent sodium taurocholate. Figures 20-21 in the Appendix show these changes. Treatment in KI for 6 hours caused a reversion to the original gram positive state.

Four lipid solvents, carbon tetrachloride, absolute ethyl alcohol, acetone and ether, were used to produce gram reaction reversals. All of these solvents acted in a similar manner; however, ether produced the most distinct results. A complete loss of gram positivity was
observed after 11 hours treatment with ether. Figures 22-23 in the Appendix show the reversal of the gram reaction and the reconversion of KI treatment. This reconversion to the gram positive state is striking because the areas of localization of crystal violet are distinct. After KI treatment the cytoplasmic membrane retained more dye than the cytoplasm.

The action of ultraviolet irradiation on heat and osmium tetroxide fixed smears proved the most interesting of the gram positive to the gram negative conversions. The speed of reversal was influenced by the method of fixation. Using heat fixation, 50 per cent of the cells were still gram positive after 27 hours irradiation. With osmium tetroxide fixation, the gram reaction reversal was complete after 18 hours irradiation. Treatment of the irradiated cells in KI also produced different results from those observed with the other reverting agents. With the heat fixed smears, there was an increase of about 25 per cent in the number of gram positive cells after the KI treatment. With the osmium tetroxide fixed smears there was no change in the gram reaction after KI treatment.

Measurement of cells showed considerable difference in cell diameter between the heat fixed cells and the
osmium tetraoxide fixed cells. Before irradiation, cell wall stains showed a difference in cell diameter after fixation. The mean diameter for the heat fixed cells was 1.10 μ ± 0.07 μ; the mean diameter of the osmium tetraoxide fixed cells was 1.35 μ ± 0.1 μ. After 24 hours irradiation, the mean diameter of the heat fixed cells was 1.22 μ ± 0.09 μ; while the mean diameter of cells fixed in osmium tetraoxide was 1.51 μ ± 0.09 μ. Figures 24-25 in the Appendix show the loss of the gram positive character of cells fixed in osmium tetraoxide and the failure to regain the gram positive state after treatment in KI. Figures 26-27 in the Appendix show the difference in diameter of the cells fixed in osmium tetraoxide before and after irradiation.

Antigenic Changes in Cells Grown In a Medium Containing KI

The following results were obtained when the titres of the immune sera were determined. Only the agglutinating titres were studied. E. coli serum is referred to as Anti (N), E. coli (KI) serum is referred to as Anti (KI).
Table X

Evaluation of Agglutinin Titres For the Homologous and the Heterologous Systems

<table>
<thead>
<tr>
<th>Reactive System</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. E. coli with Anti (N)</td>
<td>1:5120</td>
</tr>
<tr>
<td>2. E. coli with Anti (KI)</td>
<td>1:2560</td>
</tr>
<tr>
<td>3. E. coli (KI) with Anti (KI)</td>
<td>1:1280</td>
</tr>
<tr>
<td>4. E. coli (KI) with Anti (N)</td>
<td>1:160</td>
</tr>
</tbody>
</table>

The effect of homologous agglutinin adsorption was studied, and the results are shown in Table XI. Homologously adsorbed E. coli serum is represented by Homo. Ads. Anti (N). Homologously adsorbed E. coli (KI) serum is represented by Homo. Ads. Anti (KI).

Table XI

Effect of Homologous Adsorptions on Agglutinin Titres

<table>
<thead>
<tr>
<th>Reactive System</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. E. coli with Homo. Ads. Anti (N)</td>
<td>1:5120</td>
</tr>
<tr>
<td>2. E. coli with Homo. Ads. Anti (KI)</td>
<td>1:80</td>
</tr>
<tr>
<td>3. E. coli (KI) with Homo. Ads. Anti (KI)</td>
<td>1:320</td>
</tr>
<tr>
<td>4. E. coli (KI) with Homo. Ads. Anti (N)</td>
<td>1:2560</td>
</tr>
</tbody>
</table>

Heterologous adsorptions were performed and the effect on the agglutinin titres studied. The following table presents these data. Heterologously adsorbed E. coli serum is referred to as Hetero. Ads. Anti (N).
Heterologously adsorbed *E. coli* (KI) serum is referred to as Hetero. Ads. Anti (KI).

**Table XII**

**Effect of Heterologous Adsorptions on Agglutinin Titres**

<table>
<thead>
<tr>
<th>Reactive System</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hetero. Ads. Anti (KI) with <em>E. coli</em> (KI) cells</td>
<td>1:80</td>
</tr>
<tr>
<td>2. Hetero. Ads. Anti (KI) with <em>P. aeruginosa</em> (KI) cells</td>
<td>1:10</td>
</tr>
<tr>
<td>3. Hetero. Ads. Anti (KI) with <em>P. vulgaris</em> (KI) cells</td>
<td>1:10</td>
</tr>
<tr>
<td>4. Hetero. Ads. Anti (N) with <em>E. coli</em> cells</td>
<td>1:640</td>
</tr>
<tr>
<td>5. Hetero. Ads. Anti (N) with <em>E. coli</em> (KI) cells</td>
<td>1:40</td>
</tr>
</tbody>
</table>

**Cataphoretic Mobilities of Normal *E. coli* and *E. coli* (KI)**

To simplify all calculations the working potential was adjusted to 77 volts for each determination. The average results of 125 determinations of mobility, half taken at the first level and half at the second level, were as follows: *E. coli* 20.1 μ per sec and *E. coli* (KI) 30.3 μ per sec. This data were converted to μ per sec per volt per cm. The values were found to be 15.4 μ per sec per volt per cm and 23.2 μ per sec per volt per cm respectively.
DISCUSSION

The conversion of gram positive bacteria to the gram negative state has been reported numerous times in the literature, and the mechanisms for the change are fairly well understood. However, the reverse change, from the gram negative to the gram positive state, has been reported only a few times. The conversions occurred when gram negative bacteria were grown on rather unusual media. However, much of this work has not been confirmed by other investigators. The conversion described by Baker and Bloom (1948) with *E. coli* is entirely a function of the added layer of desoxyribonucleic acid. Bartholomew and Mittwer (1952) in their review of the gram stain state, "At the present time, it appears that there is no dependable way to convert gram negative cells to the gram positive state."

A method for the conversion of gram negative bacteria to the gram positive state has been described in this study and some of the reasons for this change investigated. It should be emphasized at the outset that the change observed appears to be the normal response of the gram negative cells to growth in a medium containing potassium iodide. The strains of gram negative bacteria which were grown in nutrient broth containing potassium iodide
were different in many respects from those grown in normal broth. The following differences were noted with *E. coli*, *P. aeruginosa* and *P. vulgaris* when these strains were grown in nutrient broth containing potassium iodide.

1. The bacteria stained gram positively.

2. The growth rates of the bacteria were altered.

3. The individual cell morphology was changed.

4. The bacteria concentrated iodine within the cell.

5. The antigenic composition of the cells was changed.

6. The cataphoretic mobilities of the cells were increased.

Exposure of fixed smears of *B. mycoides*, which had been converted to the "gram negative" state, to potassium iodide solutions produced varying degrees of reconversion to the gram positive state. If an analogy can be drawn between the two cases, the gram negative to gram positive change observed after growth in potassium iodide and the gram negative to gram positive change produced with *B. mycoides*, it would appear that the action of the potassium iodide is the same in each case. However, smears of *B. mycoides* fixed in osmium tetroxide and rendered gram negative by exposure to ultraviolet irradiation could not be converted to the gram positive state by treatment in potassium iodide.
If the gram reaction reversal is interpreted in terms of the mechanism described by Bartholomew and Mittwer (1952), three assumptions must be made. (1) The iodide of the medium was absorbed by the cells and reacted with the crystal violet to form a dye-iodide complex. (2) The iodide altered the permeability of the cell membrane so that the dye-iodide complex is not washed from the cells by the decolorizer. (3) The iodide in the medium acted in the same manner as the iodine mordant of the gram stain. Iodine determinations on cells grown in a medium containing potassium iodide showed that iodide was taken up by the cells. Immunological studies indicated that iodide was fixed to the cells and not merely adsorbed on the surface of the cells. It was further shown that the cells which had been grown in a medium containing potassium iodide fixed more crystal violet than the normal cells. Since increased amounts of dye and iodide were shown to occur in or on the cells grown in potassium iodide, the conditions for first assumption above have been fulfilled. The increased amount of dye-iodide complex formed may have been the reason for the gram reaction reversal. This inference is based on the law of mass action. On these grounds it is not necessary to assume any change in the permeability as in the second assumption above. Since
the time of decolorization was kept constant, all of the dye-iodide complex would not be removed from the cells containing large quantities of the complex. This would result in an apparent gram negative to gram positive change. The condition of the third assumption was fulfilled by using the modified staining method. With this method the growth in potassium iodide was assumed to have served the same function as the application of iodine in the gram staining procedure.

When the first gram reaction reversal was observed, it was localized at the ends of the cells giving them a bipolar appearance. According to Knaysi (1951) bacterial growth occurs at the ends of the cells, and the permeability of the cytoplasmic membrane at this site is greater than the permeability at the center of the cell. Knaysi further states that the high permeability during the active growth is probably due to an increase in pore width caused by turgor pressure on the cell and to the low lipoid content of the cytoplasmic membrane during this stage. In mature cultures the cell wall is under a much lower tension, and, in addition, the pores are partly clogged by insoluble products of metabolism. The increased iodide content was attained before and during the logarithmic phase of growth and was retained in the 24 hour cultures. This would result in more dye-iodide complex being formed.
during the gram staining procedure.

The increased crystal violet retention may better be explained on the basis of a chemical alteration caused by the iodide. The cataphoretic mobility determinations in this study showed that the cells obtained from broth containing potassium iodide had an increased negative charge. The mobility was increased approximately 50 percent over that of the normal cells. This study confirmed previous reports by many authors that bacteria carry a negative charge and that the amount of basic dye fixed by the cells is related to the charge on the cell. The evidence obtained from the cataphoretic determinations gives additional support to the work of Stearn and Stearn (1924, 1929, 1930). As the final pH observed in E. coli (KI) cultures, pH 8.3, was only slightly different from the final pH observed with E. coli (N), pH 8.5, the change in hydrogen ion concentration was not sufficient to cause the gram negative to gram positive change. The dissociative changes, occurring in cell ampholytes, which might effect the cataphoretic mobilities were negated by washing the cells in neutral buffer before the mobilities were determined. Neutral buffer was also used to suspend the cells in the cataphoretic cell. The altered mobility rates of the cells grown in potassium iodide are probable due to a more complete dissociation of the
acid groups of the nucleic acids and proteins. With the increase in the total negative charge on the cell, the positively charged cations of the basic dyes have a greater affinity for the cells.

Evidence for a chemical alteration in the cells after growth in potassium iodide was obtained by the immunological studies. The evidence indicates that the iodide caused a chemical alteration in the antigenic constituents of the cells. The antibody producing potential of E. coli (KI), as observed in two rabbits, was considerably reduced; the agglutinin titres obtained were lower than those produced with E. coli (N) cells. Evidence for a chemical alteration in the cells was also obtained from heterologous agglutinin adsorptions. These adsorptions showed that the antigenic character had undergone some change. E. coli (KI) did not remove all of the antibody formed against E. coli (N) since a titre of 1:640 remained for E. coli (N) after six adsorptions were performed.

It would appear that new determinants are formed on the cells which interfere with the adsorption of the antibody formed against the normal cells. A similar interference has been reported by Winn (1953) with trypsinized red blood cells. The adsorption interference may be due to a steric hinderance; the iodide determinant being too close to the normal determinant to allow adsorption.
The evidence obtained in this study, increased crystal violet fixation, antigenic alteration, iodide retention, and altered cataphoretic mobilities all point to the fact that the cells have undergone some chemical change during their growth in potassium iodide. It seems that gram reaction reversal may be the result of a strictly chemical alteration of the cell protoplasm.

No evidence was found that the "ectoplasmic or structural" theory would explain the gram negative to gram positive change. It was not possible to demonstrate a gram positive layer on the cells after growth in potassium iodide. The study of the gram reaction reversal of B. mycoides also failed to give any evidence in support of the "ectoplasmic" theory. Appreciable differences were not observed in cell diameter measurements made before and after the gram reaction reversal. However, an increase in cell diameter was found to occur when B. mycoides was irradiated with ultraviolet light. The increase in diameter was related to the method of fixation used for the cells. Osmium tetraoxide fixation was shown to cause a permanent gram reaction reversal and an increase of approximately 0.3 µ in cell diameter. Photographs illustrating the increase in cell diameter also show the disruptive action of the ultraviolet irradiation. It
is possible that the increase in cell diameter may have changed the cell membranes to the extent that the permeability of the cell was altered. If so, this would result in a rapid removal of the dye-iodide complex and explain the inability of the osmium tetraoxide fixed cells to be reconverted to the gram positive state.

Recently, a possible explanation of the action of osmium tetraoxide has been published (Larose and Fischer 1953). They stated that the action of ultraviolet irradiation was to form \(-\text{SH}\) and \(-\text{SOH}\) groups from the cystine linkages of the proteins. These groups are oxidized by the osmium tetraoxide which hastens the gram positive to gram negative change. They believe that the oxidized cell protein would possess less affinity for the basic dyes than the reduced cell protein. The failure of potassium iodide treatment to convert the "gram negative" \(B.\ mycoides\) to the gram positive state suggests that the osmium tetraoxide occupies or alters the chemical groups which are acted upon by the iodide to cause the gram positive staining reaction.

The importance of the treatment in potassium iodide was shown by the conversion of the "gram negative" \(B.\ mycoides\), which had been treated in ribonuclease, trypsin, sodium taurocholate, and lipoid solvents, to the gram positive state. It has been reported by many authors
that the above reagents extract materials from gram positive cells which play an essential role in the gram reaction. Reconversions have been obtained only by treating the "gram negative" cells with highly polymerized nucleic acids. The evidence obtained in this study indicated that the materials removed from the cells did not play a role in the gram reaction reversal. It was possible by treatment with potassium iodide to reconver the "gram negative" cells to the gram positive state, if the cells were heat fixed. No reconversion was possible with osmium tetraoxide fixed cells as the action of osmium tetraoxide prevented a reconversion by potassium iodide treatment.

The differences in cell morphology may be caused by the high osmotic pressure of the medium containing potassium iodide. Since growth occurs for the most part at the ends in bacilli, the increased osmotic pressure would result in the elongation of the cells (Knaysi 1951). The appearance of the cells indicated that growth was not affected, but division was retarded, resulting in the formation of filamentous cells.

The micro-kjeldahl nitrogen determinations gave values which agreed well with the values reported by other authors. No appreciable differences were observed between the values obtained with normal cells and those obtained with cells grown in KI broth. There is no evi-
dent correlation between the amount of kjeldahl nitrogen in the bacterial cells and the amount of crystal violet fixed by the cells.

Slime formation has been reported by Haynes (1951) as a criterion for the classification of *Pseudomonas aeruginosa*. He used a medium containing potassium gluconate as the principal source of carbon and reported that the gluconate was responsible for the slime production. Results of this study indicate that the anion of the potassium compound is not the cause of the slime production. The slime production was due to the increased potassium in the medium, since potassium iodide, potassium nitrate, potassium chloride and potassium acetate produce the same results.

It has been known since the advent of the gram staining method that there are different degrees of gram positivity. At the present time no methods are available for the determination of these differences; as a result, quantitative gram stains are not done.

The method described in this study for the quantitative determination of dye-fixation might be adapted to this end. With standardization it would be possible to make quantitative gram stains. The sensitivity of the procedure was found to be such that a difference of ± 0.0002 mg of dye could be detected.
SUMMARY AND CONCLUSIONS

As a result of the experimental findings obtained in this study, the following hypotheses formulated in the introduction were rejected:

1. There is no difference in the amount of crystal-violet up-take by bacteria grown in nutrient broth and bacteria grown in nutrient broth with added potassium iodide.

2. There is no relationship between the amount of bacterial growth and the tendency to concentrate iodide.

3. There is no difference in surface charge on bacteria grown in nutrient broth and on bacteria grown in nutrient broth with potassium iodide added.

4. There is no difference in antigenicity between bacteria grown in nutrient broth and bacteria grown in nutrient broth with potassium iodide added.

5. There is no difference in crystal-violet up-take between gram-negative bacteria when grown in nutrient broth with added potassium iodide and gram positive bacteria rendered gram negative by ribonuclease, trypsin, lipoid solvents, sodium taurocholate treatment, or UV irradiation and incubated in buffered potassium iodide.

The last part of Hypothesis Five was not rejected as a result of the experimental evidence. Gram positive
B. mycoides which was fixed by osmium followed by UV irradiation producing "gram negative" cells could not be reverted to the gram positive state.

No quantitative data was obtained concerning the dye uptake of "gram negative" B. mycoides after treatment in potassium iodide.

By serial transfers in a medium containing increasing amounts of potassium iodide, it was possible to "train" E. coli, P. aeruginosa, and P. vulgaris to grow in a medium containing 30 mg KI per ml. The gram negative bacteria stained gram positively after growth in the potassium iodide using the Kopeloff and Beerman method with and without the iodine mordant. The gram reaction reversal was first evident in a medium containing 6 mg KI per ml after 18 hours incubation. The growth characteristics of the potassium iodide tolerant strains were different from the original strains. The potassium iodide tolerant strains fixed more crystal violet than the original strains. Morphological changes accompanied the gram reaction reversals. Iodide was concentrated in the cells of the potassium iodide tolerant strains. No difference in nitrogen content was found to exist between the potassium iodide tolerant strains and the normal strains. The cataphoretic mobilities of the gram-negative bacteria grown in potassium iodide were increased by
approximately 50 per cent over that of the original strains. Heat-fixed cells of *B. mycoides*, which were converted to the gram negative state by treatment with ribonuclease, trypsin, sodium taurocholate, lipid solvents, and ultraviolet irradiation were reverted to the gram positive state by treatment in potassium iodide. Osmium tetraoxide fixed cells of *B. mycoides* rendered gram negative by ultraviolet irradiation could not be reconverted to the gram positive state by treatment in potassium iodide. *P. aeruginosa* produced a slime when grown in a medium containing an excess of potassium ions.


37. Ibid., 1948 Preliminary observations on germination of the spores of Bacillus mycoides in a nitrogen-free medium and certain properties of the transparent cells. J. Bact., 52, 753-757.


50. Ibid., 1929 The variation in staining character of bacteria as related to the reserve food material within the organism. Stain Technol., 4, 105-109.


53. Winn, H. J. 1953 Personal communication.

APPENDIX
Fig. 1 *E. coli* grown in nutrient broth, stained by the Kopeloff and Beerman modification of the grams reaction.

Fig. 2 *E. coli* grown in nutrient broth, containing 30 mg KI per ml. Kopeloff and Beerman modification.

Fig. 3 *P. aeruginosa* grown in nutrient broth, stained by the Kopeloff and Beerman modification.

Fig. 4 *P. aeruginosa* grown in nutrient broth containing 30 mg KI per ml. Kopeloff and Beerman modification.

Fig. 5 *P. vulgaris* grown in nutrient broth, stained by the Kopeloff and Beerman modification.

Fig. 6 *P. vulgaris* grown in nutrient broth containing 30 mg KI per ml. Kopeloff and Beerman modification.
Fig. 7  *E. coli* grown in nutrient broth. Kopeloff and Beerman Modification

Fig. 8  *P. aeruginosa* grown in nutrient broth. Kopeloff and Beerman Modification

Fig. 9  *P. vulgaris* grown in nutrient broth. Kopeloff and Beerman Modification
Fig. 10 *E. coli* grown in nutrient broth with 30 mg KI per ml. Modified Staining Method

Fig. 11 *P. aeruginosa* grown in nutrient broth with 30 mg KI per ml. Modified Staining Method

Fig. 12 *P. vulgaris* grown in nutrient broth with 30 mg KI per ml. Modified Staining Method
Fig. 13  *E. coli* grown in nutrient broth with 30 mg KI per ml. *N. sicca* control organism. Modified staining method

Fig. 14  *P. aeruginosa* grown in nutrient broth with 30 mg KI per ml. *N. sicca* control organism. Modified staining method

Fig. 15  *P. vulgaris* grown in nutrient broth with 30 mg KI per ml. *N. sicca* control organism. Modified staining method
Fig. 16 B. mycoides after 18 hr. treatment in ribonuclease. Kopeloff and Beerman modification

Fig. 17 B. mycoides after 18 hr. treatment in ribonuclease followed by 6 hr. treatment in KI. Kopeloff and Beerman modification

Fig. 18 B. mycoides after 20 hr. treatment in trypsin. Kopeloff and Beerman modification

Fig. 19 B. mycoides after 20 hr. treatment in trypsin followed by 6 hr. treatment in KI. Kopeloff and Beerman modification

Fig. 20 B. mycoides after 10 hr. treatment in 10 percent sodium taurocholate. Kopeloff and Beerman modification

Fig. 21 B. mycoides after 10 hr. treatment in 10 percent sodium taurocholate followed by a 6 hr. treatment in KI. Kopeloff and Beerman modification
Fig. 22  *B. mycoides* after ether treatment for 11 hr. Kopeloff and Beerman modification

Fig. 23  *B. mycoides* after ether treatment for 11 hr followed by treatment in KI for 6 hr. Kopeloff and Beerman modification

Fig. 24  Osmium tetraoxide fixed *B. mycoides* after 18 hr. ultraviolet irradiation. Kopeloff and Beerman modification

Fig. 25  Osmium tetraoxide fixed *B. mycoides* after 18 hr. ultraviolet irradiation. Six hour treatment in KI. Kopeloff and Beerman modification

Fig. 26  Cell wall stain of Osmium tetraoxide fixed *B. mycoides* before ultraviolet irradiation. Knaysi cell wall stain

Fig. 27  Cell wall stain of osmium tetraoxide fixed *B. mycoides* after 25 hr. ultraviolet irradiation. Knaysi cell wall stain
AUTOBIOGRAPHY

I, Lewis John Griffith, was born in Shreve, Ohio, January 13, 1921. I received by secondary school education in the public schools of Shreve, Ohio. My undergraduate training was obtained at The Ohio State University, Columbus, Ohio, from which I received the degree Bachelor of Science in 1948. In 1950, I received the degree Master of Science from The Ohio State University. In the autumn of 1951, I received an appointment as Graduate Assistant in the Department of Bacteriology, The Ohio State University. I held this appointment while completing the residence requirements for the degree Doctor of Philosophy.