1955

A Physiological Study of Micrococcus pyogenes var. aureus

Delmer H. Koehn

A PHYSIOLOGICAL STUDY OF MICROCOCCUS PYOGENES VAR. AUREUS

by

Delmer H. Koehn

Submitted to the Graduate Faculty

of

South Dakota State College

of Agricultural and Mechanic Arts

in Partial Fulfillment of the Requirement for

the Degree of Master of Science

July, 1955

A PHYSIOLOGICAL STUDY OF MICROCOCCUS PYOGENES VAR. AUREUS

By
Delmer H. Koehn

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

ACKNOWLEDGEMENT

I wish to extend my sincere appreciation to Dr. E. C. Berry, Professor of Bacteriology, South Dakota State College, for his helpful suggestions and valuable guidance during the course of this investigation, to Dr. O. E. Olson and Mr. E. I. Whitehead for their advice during the preparation of this paper, and to Miss Marjorie Thom for the typing of this manuscript.

Table of Contents

List of Figures

INTRODUCTION

Micrococcus pyogenes var. aureus (Rosenbach) Zoph has been known to man for some time, for it was back in 1878 that Koch noted the presence of micrococci in pus from wounds. (2, 7)

In all probability these organisms were Micrococcus pyogenes var. aureus or their very closely related type species Micrococcus pyogenes var. albus. It is very interesting to note the relatively small amount of attention paid to this genus after the early taxonomic work was completed, and it was for this reason that this study was undertaken.

In the fall of 1953 cases of tonsillitis, laryngitis, or sore throats were quite prevalent on the campus of South Dakota State College. The Health Service on the campus co-operated by providing fresh material. This material was obtained by swabbing the patient's (student's) throat, nasopharynx, and tonsillar crypts. The swabs were prepared by twisting a small piece of absorbent cotton on the end of a six inch wood applicator. Two of these swabs were placed in a six inch test tube and the tube was then cotton plugged and autoclaved. One swab was used for the patient's throat and the other for the nasopharynx.

In the laboratory each swab was streaked on Nutrient, Chapman Stone, and Blood Agar plates (6) and incubated at 37° C. for 12 to 18 hours. Immediately after streaking the plates, direct slides were prepared by rolling the swab on a clean slide.

These were then gram stained and examined under oil immersion. Direct microscopic examination revealed that the predominating organism was a gram positive micrococcus .8 to 1.0 microns in diameter, appearing singly and in small clumps. Other organisms present to a lesser degree included short-chain streptococci and diphtheroids.

After 12 to 18 hours of incubation the plates were examined, and it was easily discernible that the predominating organisms were micrococci. These micrococci were actively hemolytic, producing a clear, well defined zone of beta hemolysis on the blood agar plate. They were also most abundant on the nutrient agar plate and here grew with a somewhat yellowish to golden sheen. Since Chapman Stone is a selective medium for micrococci, they grew well, exhibiting a definite golden pigment. Therefore, from the isolation and growth characteristic studies, it was concluded that in all probability this organism was responsible for the small endemic outbreak of "sore throat" on this campus.

Appropriate biochemical tests and taxonomic procedures were applied to this organism to properly classify it as Micrococcus pyogenes var. aureus (Rosenbach) Zoph (2). The cultures obtained from seven patients were not mixed but were carried on separate nutrient agar and Chapman Stone slants and in Kracke Blood Culture Medium (6). The organism used for subsequent investigational purposes was the author's own particular isolate. Since all seven strains isolated had very similar characteristics, no particular reason or provocation can be given for picking this one from the rest, outside of a probable hypochondrial tendency on the part of the investigator. This also aided in making the work a little more stimulating.

LITERATURE

Koch (7) in 1878 first observed micrococci in pus from wounds, but it remained for Pasteur (7) in 1880 to cultivate them in a liquid medium. Ogston (7) at about the same time noted that they were constantly present in acute and chronic abscesses and were grown by him in eggs. He also noted that these organisms were pathogenic to guinea pigs and mice. Rosenbach (7) in 1884 obtained pure cultures on solid media and divided the genus into two species--Staphylococcus pyogenes var. aureus and Staphylococcus pyogenes var. albus. Staphylo is a Greek term denoting a cluster of grapes and coccus, a spherical bacterium. Pyogenes, also derived from the Greek, means pus-producing, while aureus and albus from Latin indicate golden and white, respectively (24).

Until recently these cocci were classified as a separate genus, Staphylococcus, of the family Micrococceae, but now have been assigned to the genus Micrococcus (7) in the same family, of which the principal pathogenic representative is Micrococcus pyogenes var. aureus. Since the term staphylococcus has been in use for approximately sixty years it is still widely used by microbiologists, although the term micrococcus is rapidly gaining popular favor. It is for this reason that the term micrococcus will be used throughout this dissertation.

That Micrococcus aureus is responsible for many of the woes of man is evidenced by its almost constant occurrence in many of his chief ailments. It is present in a variety of

clinical and pathological forms and is usually characterized by suppuration, ranging from mild localized pustules, such as sore throat, to fulminating, rapidly fatal, septicemia. It also causes a goodly share of furuncles, carbuncles, osteomyelitis, post-operative infection, pneumonia, meningitis, and suppurative infections of the pleural, peritoneal, and synovial cavities, to name only a few.

One of its prominent roles is that of being one of the most prevalent organisms found in common bacterial food poisoning. A characteristic feature of micrococcal food poisoning is that the symptoms generally appear in one to four hours after the food is eaten in contrast to a period of 12 to 24 hours or more which is typical of poisoning caused by Clostridium botulinum (5). The enterotoxin produced is heat stable and quite potent as has been demonstrated by inoculating 2 to 3 ml. of the filtrate intraperitoneally into a kitten weighing 350-700 grams. Within five to thirty minutes the kitten will usually show lassitude and weakness with severe vomiting, which may be followed by death an hour or two later (20). Since a close correlation exists between enterotoxin-producing micrococci and those producing staphylocoagulase, the test for the latter was performed on the particular coccus under investigation and it was found to be positive, thereby classifying it as a pathogen.

Micrococcus pyogenes var. aureus produces several other significant toxins. These toxins have the ability to lyse red blood corpuscles, kill leucocytes, lyse fibrin clots, and

coagulate plasma. It is for this reason that this bacterium is so highly pathogenic once it makes its entrance into the blood stream and sets up a focal point of infection.

The pyogenic micrococci are almost invariably hemolytic and on initial isolation on blood agar the colonies will be surrounded by a clearly defined zone of β-hemolysis. This hemolytic activity is also present in cell free filtrates (8) and has been found to consist of several distinct hemolysins or staphylolysins, namely α and β lysins. These two lysins are quite readily differentiable in that the α lysin acts on both sheep and rabbit erythrocytes at 37° C., while the β lysin acts on sheep, but not rabbit erythrocytes and is a hot-cold lysin (for example, following a preliminary warm incubation, hemolysis occurs when the cells are chilled). These two staphylolysins are sometimes referred to as the α and β toxins, or toxoids if they have been treated with formalin. The α lysin is also a leucocidin in that it will kill the white corpuscles but will rarely attack human red corpuscles (10). The β hemolysin usually attacks human red corpuscles and therefore human blood can quite readily be used for culture work and as a primary test for pathogenicity.

Since many hemolytic micrococci also liquify gelatin, it was thought that a correlation might exist between those liquifying gelatin and those being pathogenic, thereby giving a relatively simple test for pathogenicity. However, this is not the case, as was brought out by work done by Richou and Gerbeaux (18).

White and Pickett (23) describe a relatively simple phosphatase test for detection of potentially pathogenic strains and claim a close correlation between it and the coagulase test.

The term, "spreading factor", is often associated with Micrococcus pyogenes var. aureus and it is this form of bacterial invasiveness that enhances its chances of gaining entrance to the body, especially through minor skin abrasions. The enzyme hyaluronidase is responsible for this phenomenon and once secreted by this bacterium, it tends to hydrolyze the polysaccharide, hyaluronic acid (17). Hyaluronic acid is sometimes referred to as the "ground substance" present in connective tissue and tends to bind it together. Because hyaluronidase then will loosen the binding material of the connective tissue, the extent and rate of diffusion of Micrococcus pyogenes var. aureus will be increased many times once it gains entrance to the body.

Fortunately for man, Micrococcus pyogenes var. aureus is quite easily controlled by antibiotics developed within the last ten to fifteen years. It responds well to treatment with penicillin, aureomycin, terramycin, and bacitracin (12). Incidentally, it was in 1929 that a plate culture of micrococci was accidently contaminated by Penicillium notatum and a young scientist named Alexander Fleming (12) noted this bactericidal action. Since that time, chemotherapy with antibiotics has satisfactorily controlled most types of infection caused by this bacterium, and it is for this reason that research with Micrococcus pyogenes var. aureus has also been rather infrequent.

In the past several years more drug-resistant strains of Micrococcus pyogenes var. aureus have been reported (12), but it is presently thought that the reason that a particular strain has become resistant is because a large dose of the antibiotic was not initially administered. Penicillin resistant strains are commonly reported. This is probably due to the very common usage of low cost penicillin therapy by physicians. Usually switching to another drug, when a resistant strain is suspected will alleviate the malady. However, as demonstrated by Hartman (11), sulfonamides do not tend to have a marked bactericidal effect on Micrococcus pyogenes var. aureus, as sulfonamides usually are more effective against gram negative cocci (16). In general, chemotherapy of micrococcal bacteremia is strikingly successful, of localized infection in the form of abscesses less so, and of osteomyelitis not at all without adequate surgery. In the last two cases, however, it must be remembered that the microorganisms are partially or almost completely protected from the drug.

Since the classification of Micrococcus pyogenes var. aureus is largely accomplished by the use of biochemical tests employing the utilization or non-utilization of common carbohydrates, it was decided to make a study of the rate of utilization of these carbohydrates and any accessory growth substances that might be necessary for the propagation of this particular coccus.

PROCEDURE AND DISCUSSION

After isolation and identification the organism was carried for about six months on Chapman Stone media slants and in 200 ml. of Kracke blood culture medium stored at 10°C. This gave the organism a chance to stabilize in all its characteristics so that while the work was being carried on, it wouldn't gain or lose any characteristics. We have demonstrated that Micrococcus pyogenes var. aureus tends, many times, to lose its pigment rather easily, thereby making it increasingly difficult to distinguish it from the var. albus. Also when a change in pigmentation occurs, there is usually an accompanying loss or gain in some of its biochemical characteristics.

At intervals of about once a month, transfers were made to fresh media which were then incubated for 12 to 18 hours at 37° C. After six months an inoculating loop full of bacteria from each slant and flask was transferred to blood agar medium and to Chapman Stone medium. Biochemical tests were again made on the cocci exhibiting the typical golden sheen and hemolytic trait and checked against the original. It was observed that no significant changes had taken place except that it took a longer time to coagulate human blood plasma.

Micrococci producing coagulase are considered pathogenic and those not producing it are usually considered nonpathogenic. The exact mechanism of the reaction is not clearly understood, but it is thought, as pointed out by Tager and Hales (21), that a filterable substance, "coagulase", produced by the micrococci,

and a factor designated as "activator", normally present in man and some animals, interact to form the clot.

The test for coagulase was performed using both human plasma and a decalcified plasma[1] which is widely used in hospitals and clinics as prothrombin control. To both the human plasma and the commercial plasma, diluted 1:3 with physiological saline (1), (10), was added an inoculating loop full of bacteria from a nutrient agar slant. Controls were also set up using the 1:3 dilution but containing no bacteria. The tubes were then incubated at 37° C. Coagulation was checked at ten minute intervals and was found to be complete at thirty minutes. Therefore, it can be assumed that this was a pathogenic strain of micrococci, as most authorities agree that a bacterium may be deemed pathogenic if it will coagulate plasma in two to four hours. Schaub and Foley (19) report that a micrococcus coagulating plasma within eighteen hours may, in some cases, be deemed pathogenic.

Because a pathogenic strain of micrococcus was to be used for subsequent investigational purposes and to preclude a chance for infection, all laboratory equipment which came in contact with the coccus and cultures thereof were sterilized after use. The Warburg respirometer flasks were emptied and the effluent also sterilized. The flasks were then washed in gasoline for 12 to 18 hours, after which they were placed in a hot nitric-sulfuric acid solution for 30 to 45 minutes and then thoroughly rinsed with tap and distilled water.

---

1 Protrol supplied by Nickabocher Biologicals, Inc., Minneapolis, Minnesota.

Elementary optimum temperature determinations and biochemical tests were first determined, so as to have basis for subsequent work. The optimum growth temperature was determined by streaking the cocci on eight nutrient agar slants. Each of two slants were then placed in a refrigerator at 10° C., a room at 25° C., a walk-in incubator at 37° C., and a high temperature incubator at 45° C. These tubes were then allowed to incubate at these various temperatures for eighteen hours. On examination it was easily discernible that the cocci grew most luxuriantly at 37° C., although there was some growth at 25° C. and 45° C. The slants that had been placed in the refrigerator at 10° C. were negative. Although the growth at room temperature had not been as pronounced, the pigmentation seemed to be more clearly discernible, but when the cocci grown at 37° C. were placed in the refrigerator at 10° C. for a few hours they too had a definite golden sheen. Inasmuch as this particular strain had been isolated from a source whose temperature was approximately 37° C., it was not surprising to find the existing correlation as shown above. It was therefore decided to conduct the ensuing determinations at this temperature.

Thioglycollate broth medium with added indicator (methylene blue) and dextrose (6) was used to determine the cocci's respirative or fermentative ability. The medium was prepared and autoclaved and a straight inoculating needle of bacteria was stabbed into a tube of this thioglycollate broth medium. Examination after eighteen hours of incubation revealed a very heavy growth at the surface of the broth and also some light

and scattered growth following the path of inoculation to the bottom of the tube. This then would signify that this particular strain of Micrococcus pyogenes var. aureus was principally an aerobe with facultative anaerobic tendencies.

Nutrient gelatin was used to check the proteolytic activity of this micrococcus. The gelatin was inoculated and incubated and found to be completely liquified after twelve hours. High proteinase activity of this type is commonly found in Micrococcus pyogenes var. aureus and it, along with nitrate reduction, is one of its most prominent identification features.

For determining the ability of an organism to reduce nitrate to nitrite, tubes of medium prepared from Bacto-Nitrate Broth (6) were inoculated with pure culture of the micrococcus. The tubes were incubated at 37° C. for 12 to 24 hours. The medium was then tested for the presence of nitrite by adding a few drops each of sulfanilic acid and α-naphthylamine reagent solutions. A distinct pink to red color developed indicating the presence of nitrite resulting from the reduction of the original nitrate. Control tubes of uninoculated medium were also set up and these were negative.

Many bacteria produce indole as a by-product of bacterial metabolism, therefore the test for the presence or absence of indole production serves as a valuable aid in the classification of bacteria. Since indole production is dependent upon the presence of the tryptophane group in this medium, (9), (14), Bacto-Tryptone was used for this determination because it is rich in this type of nitrogen. Two solutions are necessary

for this test. Solution I consists of one gram p-dimethyamino-benzaldehyde in 95 ml. of ethyl alcohol (95%) and 20 ml. of concentrated hydrochloric acid. Solution II is a saturated aqueous solution of potassium persulfate. To about 10 ml. of an actively growing culture of Micrococcus pyogenes var. aureus, 5 ml. of Solution I was added and then 5 ml. of Solution II. This mixture was shaken well. A red color did not develop in five minutes thereby signifying that this organism did not produce indole.

Ammonia production from peptone is also an aid in classification of the genus Micrococcus. Peptone consists of a mixture of the products of partial digestion of protein and includes many amino acids, polypeptides and proteoses, as well as other substances which result from the digestion of protein. The exact composition of peptone is not known and is quite variable. Numerous organisms, of which Micrococcus pyogenes var. aureus is one, will attack peptones, releasing ammonia in the process. Ammonia was simply detected by inserting a moistened strip of red litmus paper between the cotton plug and glass tube of an actively growing culture in peptone broth. The sense of smell might also be used here if desired since litmus may also be affected by volatile amines.

Litmus milk was also acidified and coagulated. Acidification is evidenced by the red color of the reduced litmus indicator and also the coagulation of the milk, which is undoubtedly due to the protein being carried past its iso-electric point. The strong acid condition exists because of the carbohydrate fermentation by Micrococcus pyogenes var. aureus with lactic acid as the chief end product.

The polysaccharide starch is not hydrolyzed, indicating that this bacterium needs simple sugars for its carbohydrate metabolism.

Fermentable carbohydrate determinations were used in fermentation studies and also cultural identification of the micrococcus under study. Phenol red broth base (Bacto) was used to supply the essential nutrients except the carbohydrates. This broth was prepared in culture tubes to which had been added an inverted fermentation tube to facilitate carbon dioxide release measurements. The culture tubes were then capped and autoclaved for 15 minutes at 15 pounds steam pressure. The desired carbohydrate was then added aseptically by the utilization of a prepared and sterilized Seitz filter. Enough of a freshly prepared carbohydrate solution was added to several ml. of the phenol red broth to give a final carbohydrate concentration of 6 to 8 per cent. The tubes were then inoculated with Micrococcus pyogenes var. aureus and incubated at 37° C. for 16 to 18 hours, after which time they were examined. It was found that glucose, lactose, sucrose, and mannose were all fermented without gas formation. Raffinose, salicin, and inulin were not fermented and consequently were void of gas. It could then be concluded that glucose, lactose, sucrose, and mannose would be ideal sugars to use in respiration rate studies.

After calibrating the Warburg (22) apparatus it was decided that, in an effort to standardize the actual Warburg procedure, a known amount of bacterial inoculum per respirometer flask should be used for each successive "run". Rather than visually

counting the bacteria every time, a densimetric type of counting procedure was investigated. Micrococcus pyogenes var. aureus was suspended in sterile, distilled water until the mixture took on a somewhat milky appearance. The cuvette containing the mixture was then placed in a Beckman quartz spectrophotometer and the maximum absorption peak obtained. A very definite absorption peak was noted at 525 mu and a somewhat smaller one at 600 mu (Figure 1). A Junior Coleman Colorimeter with a 525 mu filter was used throughout the entire investigation.

The cells were grown on Chapman Stone (6) agar slants and harvested after 12 to 16 hours of incubation at 37° C. Harvesting was carried out by washing the cells off the slant with several ml. of sterile, distilled water. The mixture was washed directly into a Coleman Colorimeter cuvette and brought down to the desired concentration by adding sterile, distilled water. The desired concentration and the ease with which the concentration could be arrived at was determined by several experimental Warburg "runs". This concentration, which was then used throughout the remainder of the research, had a transmittance percentage of 15 or an optical density of 0.825 as measured by the Junior Coleman Colorimeter.

A Petroff-Hausser counting chamber was used to visually count the bacteria per ml. at this concentration, the result being 1×10^{10} bacteria. Since only 0.3 ml. of inoculum was used in the respirometer flask and this diluted with 2.7 ml. of prepared substrate, only 3×10^{9} bacteria were present in the

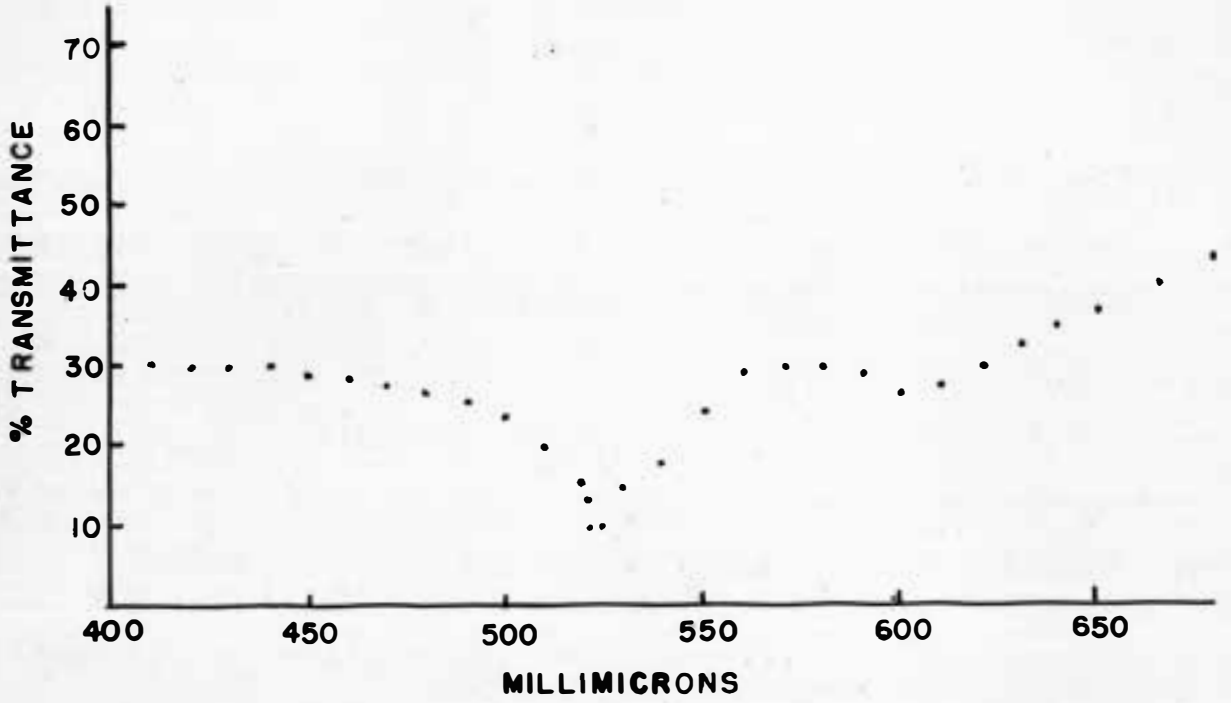

Figure 1. Optimum wave length using Micrococcus pyogenes var. aureus.

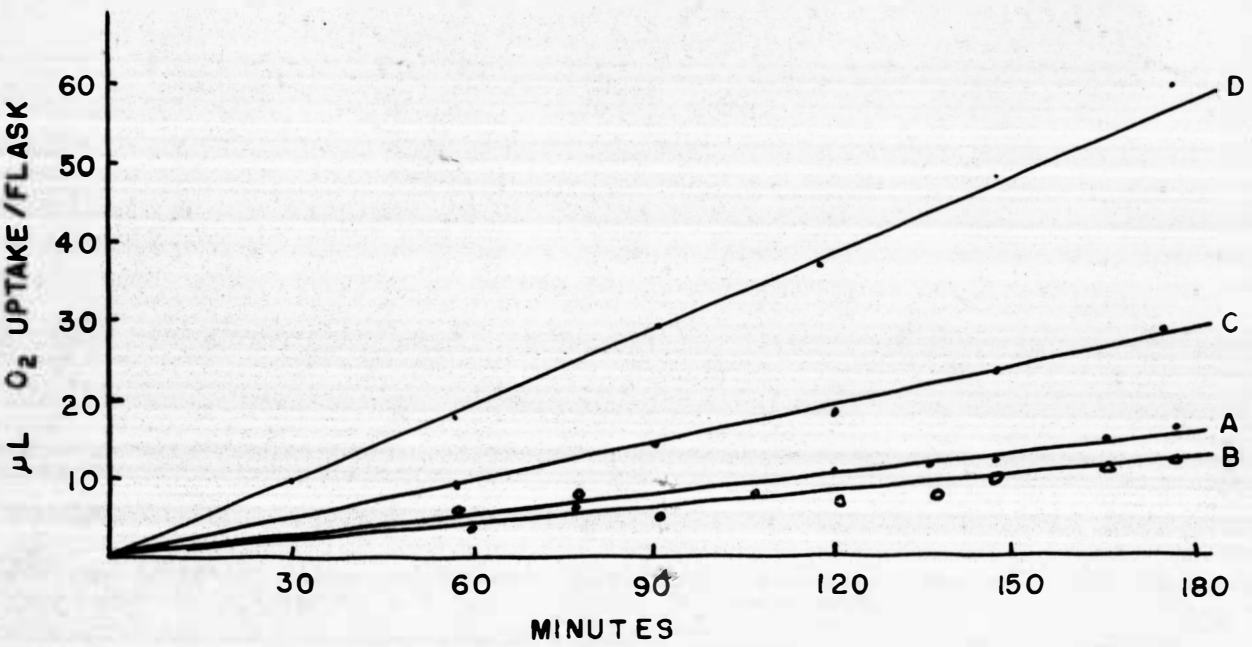

Figure 2. Oxygen uptake with basic substrate.

A. Nivens and Sherman medium
B. Johnson and Schwartz medium
C. Basic substrate
D. Basic substrate plus 0.25 per cent yeast extract

flask (1 billion/ml.). Per cent viability of the added cells was obtained by diluting 1.0 ml. of the inoculum in 100 ml. of sterile, distilled water in standard dilution bottles. This was then diluted 5 to 7 times and pour plates made using Chapman Stone and Tryptone Glucose Extract Agar (6). After 12 to 16 hours of incubation these were counted using a Quebec Colony Counter. Bacterial viability was ascertained to be about 80 per cent plus or minus 5 per cent.

A basic medium to which a known carbon source could be added was a prerequisite for further investigational procedure. A bio-assay medium suggested by Nivens and Sherman (15) was tried. Composition of this medium was 0.2 per cent KH_2PO_4, 0.2 per cent $MgSO_4 \cdot 7H_2O$, 0.0015 per cent $FeSO_4 \cdot 7H_2O$, 0.03 per cent $(NH_4)_2SO_4$, and 1.05 per cent carbon source (glucose).

Respiration, as measured with the Warburg apparatus utilizing this medium, was very poor. Omission of the ferric sulfate seemed to increase activity to some degree. Using a medium developed by Johnson and Schwartz (13), which consisted of 0.1 per cent $MgNH_4PO_4$, 0.08 per cent K_2HPO_4, 0.01 per cent $CuSO_4$, a trace of $FeCl_3$, a trace of KI, and 1.0 per cent carbon source (glucose), respiration was again determined and was found to be even poorer than that supported by the Nivens and Sherman medium. Therefore, it was decided to devise a synthetic medium that would satisfactorily support the growth of Micrococcus pyogenes var. aureus.

Koch had noted that micrococci were not inhibited by a 7.5 per cent concentration of sodium chloride in a solid medium.

Chapman (4) also reported that most bacteria other than micrococci were inhibited on such media and that the pathogenic micrococci grew more luxuriantly than did nonpathogenic strains. Since a liquid medium was to be used, a 5.5 per cent concentration of sodium chloride was selected for the medium.

Rather than use tryptone or peptone, which are not chemically defined media, it was decided to add 7.5 per cent ammonium sulfate as the nitrogen source. 0.5 per cent dipotassium phosphate was added to supply inorganic phosphate and also to buffer the medium at about a pH of 7.2 (3). The appropriate carbon source at a concentration of one per cent was used for initial experimentation with this medium. Oxygen uptake with this medium was about twice that of the Nivens and Sherman and Johnson and Schwartz media (Figure 2). It was apparent that, in order to increase the oxygen uptake, other growth substances were necessary, thus 0.25 per cent yeast extract (Bacto) was added and the oxygen uptake increased to about 270 microliters per three hours or about 90 microliters per hour. This was an increase of about three hundred per cent over the chemically defined medium previously mentioned.

This basic substrate or some slight variation thereof was used throughout the remainder of the investigation and hereafter will be referred to as "basic substrate". Percentages shown are those actually present in the respirometer flask after addition of all ingredients and diluents. Respirometer flasks were calibrated at 3.2 ml. This allowed 3.0 ml. for substrate and

bacterial inoculum and 0.2 ml. of 10 per cent potassium hydroxide to facilitate carbon dioxide absorption.

Using glucose as the carbon source, 0.05 per cent yeast extract and basic substrate, it was soon evident that the results being obtained were not reproducible or valid, since increasing or decreasing the carbohydrate concentration did not appreciably affect the oxygen consumption. Investigation revealed that in all probability the yeast extract concentration was too high, signifying that a base or minimum concentration would have to be obtained for the yeast extract. This minimum was arrived at by several Warburg "runs" utilizing the basic substrate and varying concentrations of yeast extract. These concentrations varied from 0.0007 per cent to 0.25 per cent. Controls containing the basic substrate but without yeast extract were also set up. Results ranged all the way from 4.0 ul. oxygen per hour (control) to 22 ul. per hour (Figure 3). Very little response was noted in the 0.0007 per cent flask as compared to the control, but the flasks having a concentration of 0.001, 0.003, and 0.007 per cent yeast extract were responsive ranging from 7 to 9 ul. per hour (Figure 3). The lower figure (0.001 per cent) was chosen for this minimum allowable amount of yeast extract. This amount of yeast extract was then included as one of the ingredients in the basic substrate for the remainder of the investigation.

The question of whether there was any significant change in respiratory rate, using washed or unwashed cells, arose. Cells were suspended in sterile, distilled water, agitated thoroughly,

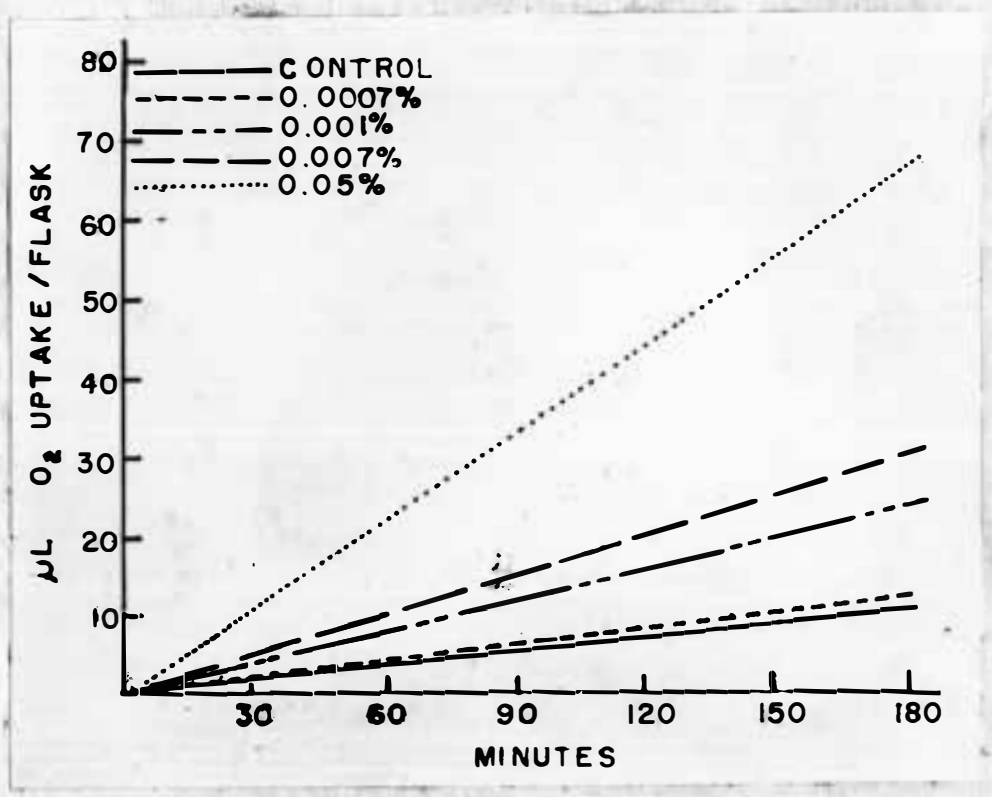

Figure 3. Run showing oxygen uptake employing varying concentrations of yeast extract.

and centrifuged. This procedure was repeated three times and the washed inoculum was then adjusted to the proper concentration employing the colorimetric method described previously. The basic substrate was used with varying amounts of yeast extract so as to compare it with the run employing unwashed cells and varying amounts of yeast extract, described previously. It was found that there was a very slight variation ranging from 1 to 3 ml. per hour, the washed cells respiring less. It was felt that this small variation was not significant. Therefore, cells were not washed for the remainder of the problem.

Glucose, maltose, and lactose are three of the most commonly used carbohydrates in a bacteriological laboratory. Not only are they used for taxonomic reasons, but also for bacterial propagation and in the preparation of standard media. Heavy glucose concentrations in the form of a paste have been employed for centuries as a common bactericidal device for treatment of common skin eruptions caused by the genus Micrococcus. The respirometer study employing these common sugars was then initiated for two reasons: (1) to find the optimum sugar concentration and rate of respiration at this concentration, and (2) to ascertain the concentration at which the carbohydrate tends to have a bactericidal effect.

Basic substrate, employing 0.001 per cent yeast extract and varying concentrations of carbohydrates, was used. Two controls were also employed; one control contained inoculum and basic substrate devoid of yeast extract, while the other included

inoculum and basic substrate containing 0.001 per cent yeast extract. Sugar concentrations ranged from 1 to 30 per cent by weight. This procedure was followed for all three sugars and the results recorded.

It was found that glucose at a concentration of 1 to 10 per cent gave an oxygen uptake of about 13 microliters per hour, but the uptake fell off at a concentration of 15 per cent to 8 microliters per hour. This diminished rapidly to the respiratory level of the control at 20 per cent concentration (Figure 4). At a concentration of 30 per cent carbohydrate respiration fell below the control level. As depicted by figure 4 the optimum seemed to be about 1 to 10 per cent concentration of glucose, with a definite depressant effect at 15 per cent.

Maltose did not give as active a response as glucose. Its optimum concentration again was reached in the range of 1 to 10 per cent, here giving an oxygen uptake of 11 microliters per hour, and again, the oxygen uptake fell off rapidly at 15 per cent to 6 microliters per hour, which was below the 9 microliter per hour control level. Respiration, using maltose at 30 per cent concentration, was also below the control level (Figure 5).

When lactose was used as the carbohydrate source, it did not cause as pronounced a response as glucose, but seemed to be more efficient than maltose. The optimum level again was about 1 to 10 per cent with an uptake of 11 microliters per hour,

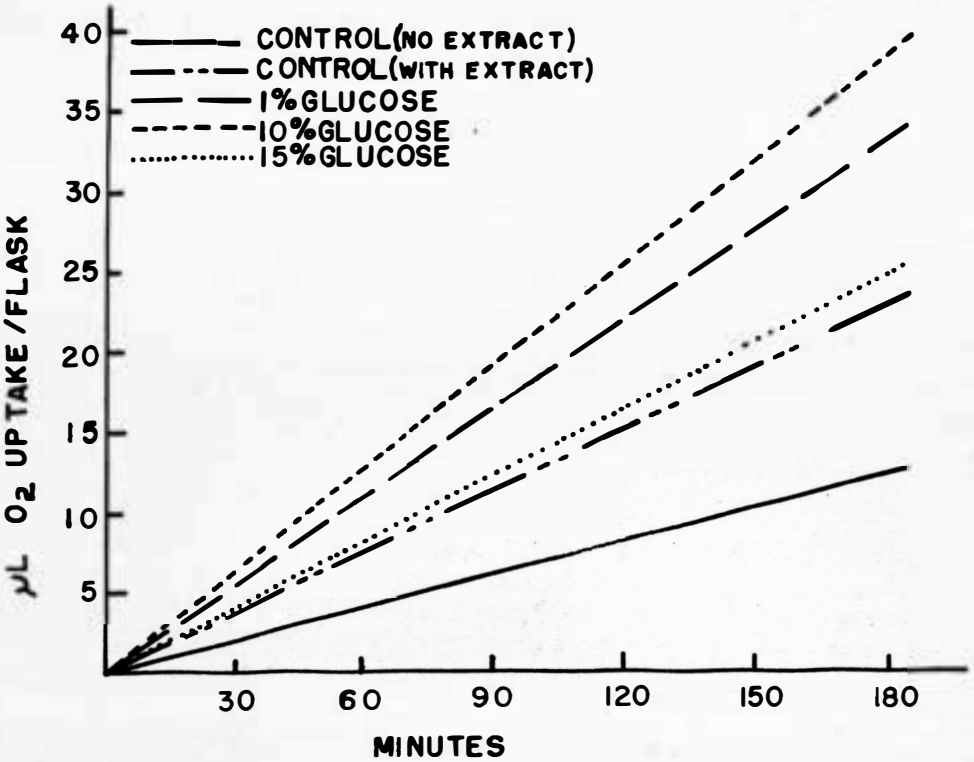

Figure 4. Run showing oxygen uptake using glucose.

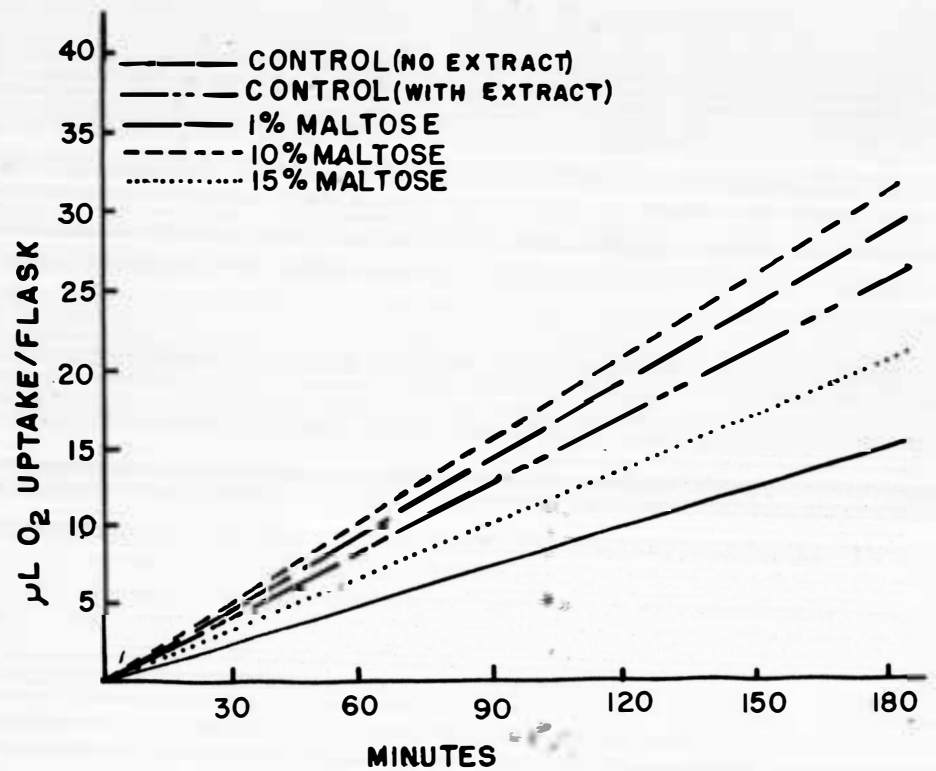

Figure 5. Run showing oxygen uptake using maltose.

falling off to 8 microliters per hour at a concentration of 15 per cent.

Reviewing the preceding results, the conclusion can then be drawn that, utilizing a certain chemically defined medium, glucose, maltose, and lactose all have about the same optimum concentration and also exhibit a depressant effect at about the same concentration. Respiratory rates, of course, were dependent on the sugar being utilized.

Because most bacteriological media contain only 1 to 2 per cent, and at the most 10 per cent, of a given carbohydrate source already discussed, it is easily discernible that the bacteria must require some other substance, be it beef extract, yeast extract, tryptone, peptone, et cetera for respiration and growth. During the course of the investigation, a Hexavitamin capsule[1] originally intended for human consumption was finely ground and added to the basic substrate, at a concentration of 0.001 per cent. The basic substrate also contained 0.001 per cent yeast extract. This mixture gave an oxygen uptake of 36 microliters per hour (Figure 6) or an increase of about 262 per cent above the basic substrate-10 per cent glucose mixture. According to the specifications on the container, the vitamin capsule contained 5000 USP units Vitamin A, 400 USP units Vitamin D, 2 mgm. B_1, (thiamine hydrochloride), 3 mgm. B_2 (riboflavin), 75 mgm. C (ascorbic acid), and 20 mgm. nicotinamide.

---

1 Hexavitamins are distributed by Strong Cobb and Co., Cleveland, Ohio.

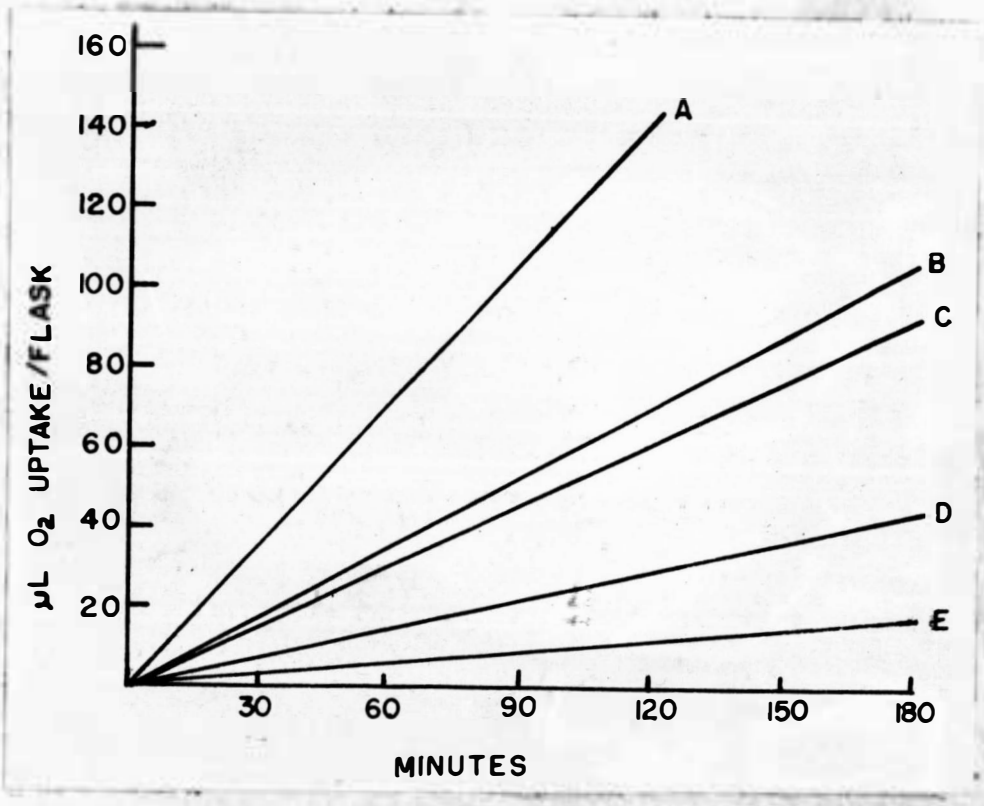

Figure 6. Run showing the effect of vitamins plus glucose.
A. Hexavitamin plus 1% glucose
B. Hexavitamin -- no glucose
C. Multivitamin plus 1% glucose
D. Multivitamin -- no glucose
E. Control (basic substrate plus 1% glucose)

A Multivitamin capsule[1] was also finely powdered and used in the basic substrate at a level of 0.001 per cent. This vitamin capsule, according to the label, contained the following amounts of vitamins: 6 mgm. B_1, 2 mgm. B_2, 300 mgm. B_6, 75 mgm. C., 10 mgm. nicotinamide, 750 mcgm. calcium pantothenate and excipients from hydrolyzed yeast. Respiration using this capsule was about 15 microliters of oxygen per hour (Figure 6). Adding 1.0 per cent glucose to the Hexavitamin and Multivitamin mixtures resulted in increases in oxygen uptake of 34 and 17 microliters respectively, the Hexavitamin increasing to 70 microliters per hour and the Multivitamin to 32 microliters per hour (Figure 6). Therefore, the inclusion of 1.0 per cent carbohydrate source increased respiration about 100 per cent.

In an effort to check individual vitamin response, a Warburg run was set up using available vitamins. The vitamins A, B_1, B_2, C, and nicotinic acid were made up to the concentration as represented by the Multivitamin capsule. These were individually added to separate respirometer flasks, which also contained basic substrate, inoculum, and 1 per cent glucose. Companion flasks containing the above mentioned ingredients but devoid of glucose were prepared, as were dual controls, containing basic substrate and inoculum. To one of these controls a 1 per cent glucose solution was added.

---

1 Multivitamins are manufactured by Vitamin Industries, Omaha, Nebraska.

Results indicated, as shown by figure 7, that no one vitamin gave any appreciable response. In fact, vitamin A seemed to be a depressant, the flask containing it having an oxygen uptake of only about 5 microliters per hour as compared with the control of 8 microliters per hour. Vitamin B_2 produced the most marked increase in respiration giving 12 microliters per hour as compared with B_1, C, and nicotinic acid which gave a respiratory rate of 11.5, 8.5, and 8.3, respectively. The flasks in which the glucose was omitted, except the flask containing Vitamin A, showed a lower respiratory rate as compared to those containing 1 per cent glucose. Differences between flasks were noted as follows: B_1, 1.7; B_2, 2.3; C, 0.6; and nicotinic acid, 4.3 microliters of oxygen per hour. Vitamin A plus glucose lowered the oxygen uptake 2.4 microliters per hour as compared to the flask not containing glucose.

Because no individual vitamin produced any noticeable response, an amino acid study was initiated. Fifteen amino acids were individually prepared in sterile, distilled water using a concentration of 0.001 per cent per respirometer flask. Basic substrate, 1 per cent glucose, and inoculum were also added. Dual controls, devoid of amino acids, but containing basic substrate, and inoculum were also prepared. One per cent glucose was also added to one of the controls. The results varied quite widely (Figure 8) ranging from 6 to 14 microliters of oxygen per hour. The amino acids giving the most noticeable response were cysteine and histidine. Leucine and lysine gave the least response and were below control level (Figure 8).

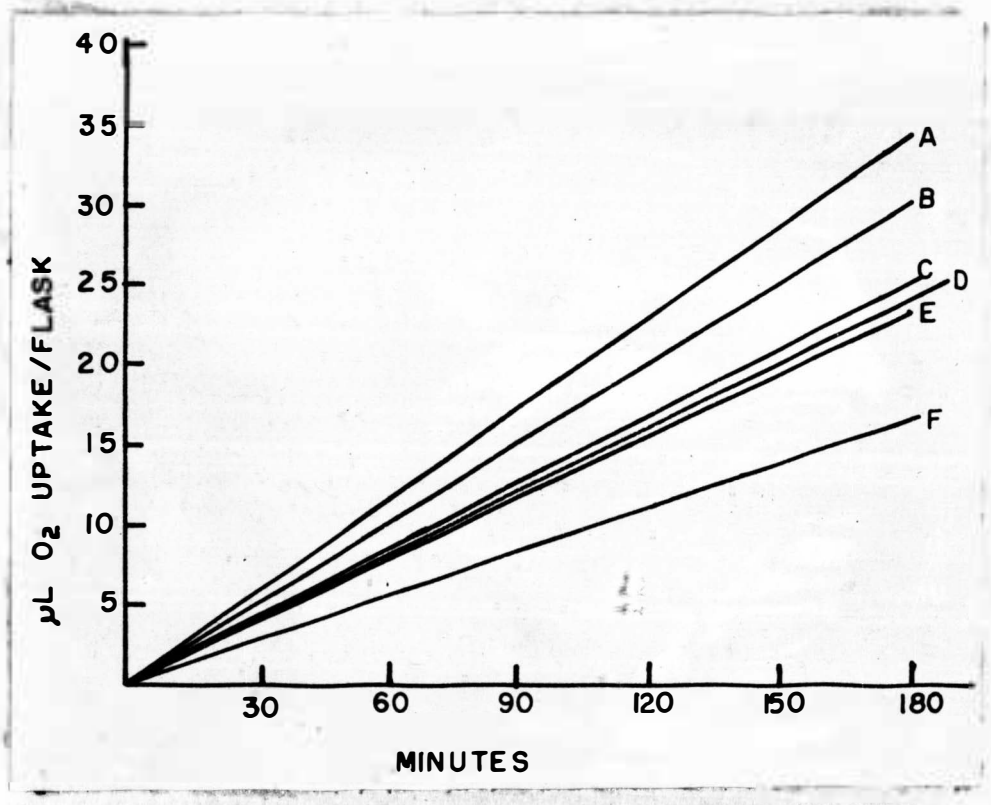

Figure 7. Oxygen uptake employing individual vitamins.

A. Vitamin B_2 plus 1% glucose
B. Vitamin B_1 plus 1% glucose
C. Vitamin C plus 1% glucose
D. Nicotinic acid plus 1% glucose
E. Control (basic substrate plus 1% glucose)
F. Vitamin A plus 1% glucose

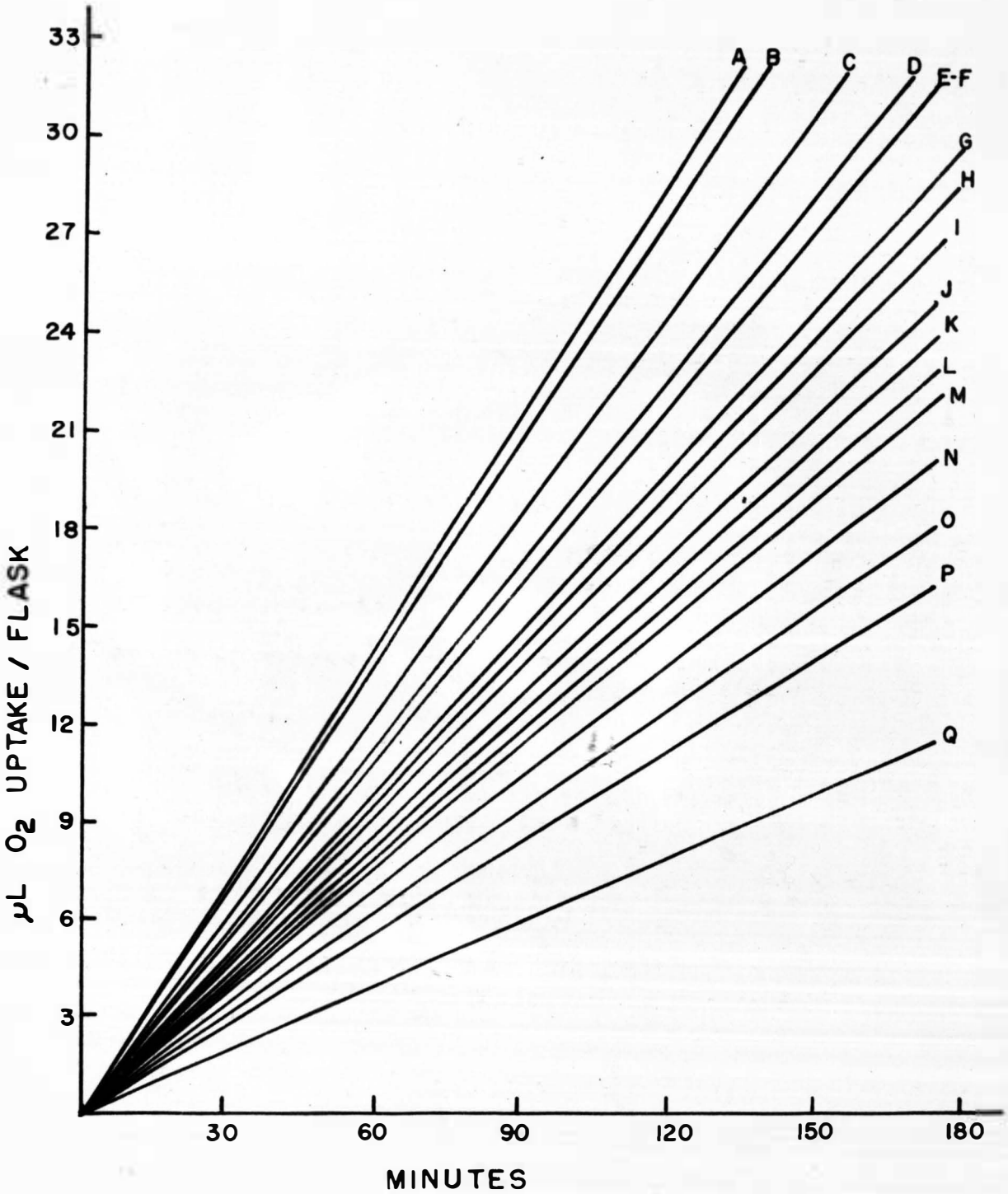

Figure 8. Oxygen uptake using individual amino acids.

A. Cysteine
B. Histidine
C. Serine
D. Glutamine
E. Valine
F. Glycine
G. Threonine
H. Tryptophane + 1% glucose
I. Tyrosine
J. Phenylalanine
K. Control + 1% glucose
L. Methionine
M. Arginine
N. Lysine
O. Tryptophane (no glucose)
P. Leucine
Q. Control (no glucose)

Tryptophane had been set up in two flasks, one containing 1 per cent glucose and the other devoid of glucose. It was found that glucose definitely increased respiration, in this case the increase being over 3 microliters of oxygen per hour. Whether this increase would hold true for all of the other amino acids is not known, as this procedure was carried out using only tryptophane.

The question arose whether amino acids or vitamins were limiting factors in cell respiration and which of the two, if removed from the substrate, would limit respiration to the greatest degree. A Multivitamin mixture as described previously and containing B_1, B_2, B_6, C. nicotinamide, calcium pantothenate and excipients from hydrolyzed yeast was prepared at a 0.001 per cent concentration. Another vitamin mixture containing thiamine, nicotinamide, and pyridoxine was also prepared at the above mentioned concentration. Basic substrate devoid of ammonium sulfate and glucose was also used. Glycine at 0.001 per cent was used for a simple carbon and nitrogen source and ammonium sulfate at a 7.5 per cent level for a nitrogen source. Seven amino acids were made up in a 0.001 per cent concentration, and the mixture labeled solution "A". These amino acids were chosen because they were readily accessible. Any other amino acids might have been used, since the problem was to determine whether a simple amino acid, such as glycine, would be as effective as the more intricate ones. This run was then carried

to completion as shown in Figure 9. As depicted by figure 9 the Multivitamin mixture plus glucose and solution "A" supported a respiration of 48 microliters of oxygen uptake per hour. Using ammonium sulfate, respiration dropped off considerably to 14 microliters per hour, but adding glycine increased it to 32 microliters per hour.

Using just Multivitamin plus glucose, respiration was 40 microliters per hour. The reason for this high rate is at the present time unexplainable.

The vitamin mixture containing thiamine, nicotinamide, and pyridoxine did not support the respiration (Figure 9) that the Multivitamin mixture did when used with ammonium sulfate, glycine, and solution "A", indicating that in all probability the lack of proper vitamin would tend to be more of a limiting factor than the lack of the proper amino acid.

To demonstrate this further, a run was set up using a vitamin mixture containing thiamine, nicotinamide, and riboflavin in a concentration of 0.001 per cent. To the respirometer flask containing this mixture a 1 per cent glucose solution, solution "A" amino acids, and basic substrate were added. A 0.001 per cent concentration of glycine, a basic substrate, and a 1 per cent glucose solution were added to another flask containing the three vitamins mentioned above. The results of this run were essentially the same as those depicted by figure 9, although the over-all respiration rates were lower in this experiment with the flask containing the

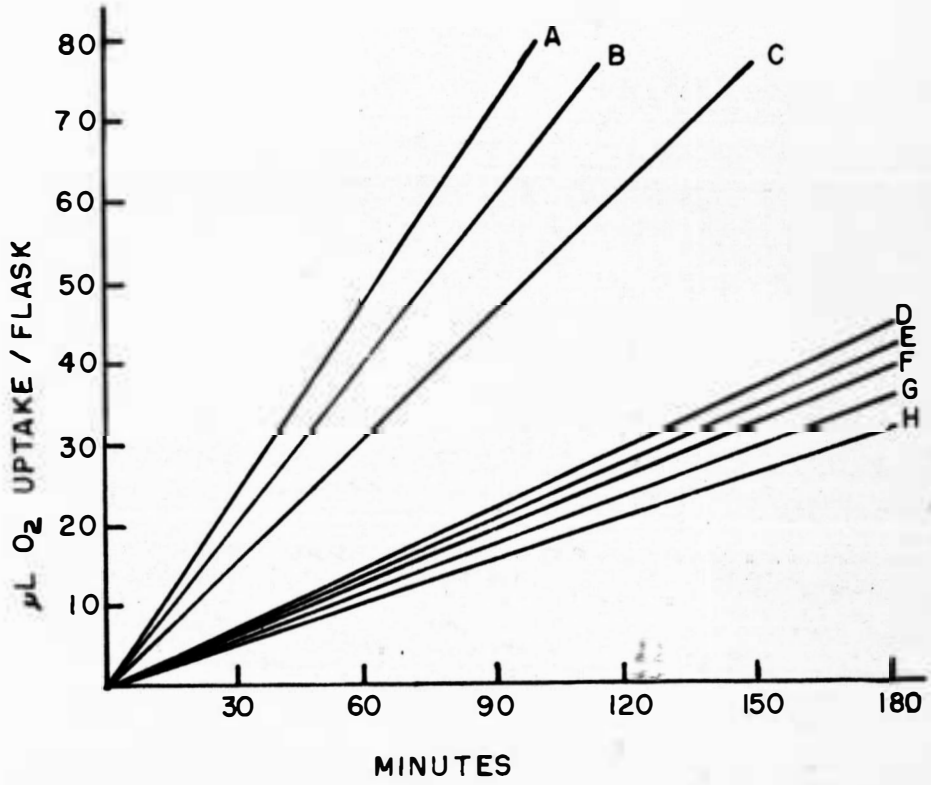

Figure 9. Oxygen uptake using amino acid-vitamin mixtures.

Multivitamin mixture plus

A. 1% glucose + solution "A"
B. 1% glucose
C. 1% glucose + glycine
E. 1% glucose + ammonium sulfate

Thiamine, nicotinamide, pyridoxine plus

D. 1% glucose + glycine
F. 1% glucose
G. 1% glucose + ammonium sulfate
H. 1% glucose + solution "A"

Solution "A" contains 0.001 per cent

Glycine	Methionine
Valine	Tyrosine
Leucine	Tryptophane
Glutamic acid	

solution "A" amino acid mixture having an uptake of 33 microliters per hour and the one containing the glycine respiring at 25 microliters per hour. This lower respiratory rate was probably due to the omission of an essential vitamin or some other growth substance, possibly not an amino acid.

SUMMARY

This problem concerned a particular isolate from a human source. After appropriate taxonomic and biochemical studies, this bacterium was identified as a pathogenic strain of Micrococcus pyogenes var. aureus (Rosenbach) Zoph.

A simple chemically defined medium was developed so that respiration studies using a Warburg respirometer could be carried out. This medium contained sodium chloride, ammonium sulfate, dipotassium phosphate, yeast extract, and the appropriate carbohydrate.

Respiration studies revealed that this micrococcus utilized glucose, maltose, and lactose most efficiently when their individual concentrations were in a range of 1 to 10 per cent. When the carbohydrate concentration was increased to 15 per cent, respiration decreased markedly. Further investigation revealed that growth substances other than those included in the basic substrate were necessary for optimum respiration. It was found that the vitamins, B_1, B_2, and nicotinic acid, produced the largest individual response in respiration. Amino acids studies were also included in this investigation and the conclusion was reached that even though some of the amino acids give a respiratory response they are not as essential as certain vitamins for maximum respiratory rate.

BIBLIOGRAPHY

1. Bray, W. E. Clinical Laboratory Methods. C. V. Mosby Company, St. Louis, 1944.

2. Breed, R. S., E. G. D. Murray, and A. P. Hitchens. Bergey's Manual of Determinative Bacteriology. The Williams and Wilkins Company, Baltimore, 1948.

3. Britton, H. T. Hydrogen Ions. Chapman and Hall Limited, London, 1929.

4. Chapman, G. H. The Significance of Sodium Chloride in Studies of Staphylococci. J. Bact. 50:201, 1945.

5. Dack, G. M. Food Poisoning. The University of Chicago Press, Chicago, 1943.

6. Difco Manual. Difco Laboratories, Detroit, 1953.

7. Dubos, Rene J. Bacterial and Mycotic Infections of Man. J. B. Lippincot Company, Philadelphia, 1952.

8. Etimne, Jairis. Purification of Staphylococcal Hemalysins with Trichloracetic Acid. Compt. Rend. Soc. Biol. 146:1298-1301. 1952. Chem. Abs., 47, No. 13:6491f. 1953.

9. France, R. L. Studies of Bacterium coli in Privately Owned Rural Water Supplies. J. Bact. 25:623. 1933.

10. Frobisher, M. Fundamentals of Microbiology. W. B. Saunders Company, Philadelphia, 1953.

11. Hartman, F. W. Local Application of Sulfonomides to Experimental Staphylococcus Infections. J. Bact. 47:259-63. 1944.

12. Herrel, W. Penicillin and Other Antibiotic Agents. W. B. Saunders Company, Philadelphia, 1945.

13. Johnson, F. and H. W. Schwartz. Carbohydrate Utilization by Hydrocarbon Bacteria. J. Bact. 47:373-75. 1944.

14. Manual of Methods for Pure Culture Study of Bacteria. No. 3, Sect. V. Society of American Bacteriologists. Geneva, New York, 1946.

15. Nivens, C. F. and J. M. Sherman. Bio-assay Media. J. Bact. 47:335-342. 1944.

16. Northey, E. The sulfonomides and Allied Compounds. Reinhold Publishing Company, New York, 1948.

17. Oginsky, E. and W. Umbreit. Bacterial Physiology. W. H. Freeman Company, San Francisco, 1954.

18. Richou, R. and C. L. Gerbeaux. Bacterial Enzymes. Compt. Rend. Soc. Biol. 146:1493-4. 1952. Chem. Abs. 47, No. 15, 7584h. 1953.

19. Schaub, I. G. and M. K. Foley. Diagnostic Bacteriology. C. V. Mosby Company, St. Louis, 1944.

20. Slanetz, L. W. Studies on Staphylococcal Toxins. J. Bact. 43:105-106. 1942.

21. Tager, M. and H. B. Hales. The Experimental Production of Antibiotics to Staphylocoagulase. J. Immunal. 60:475-485-1943.

22. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. Manometric Techniques and Tissue Metabolism. Burgess Publishing Company, Minneapolis, Minnesota. 1949.

23. White, M. L. and M. J. Pickett. A Rapid Phosphatase test for Micrococcus pyogenes var. aureus for Detection of Potentially Pathogenic Strains. Am. J. Clin. Pathol. 23:1181-3. 1953. Chem. Abs. 48, No.3:1475g. 1954.

24. Winter, G. Die Pilze. Rabenhorst's Kryptogamen flora. 2nd Edition, 1. 1880.