

**THIS BOOK
CONTAINS
NUMEROUS
PAGES WITH
COPY LINES
RUNNING
THROUGH
THEM. THIS IS
AS RECEIVED
FROM
CUSTOMER.**

THE EFFECT OF BIOTIN DEFICIENCY ON SOME
PROPERTIES OF STAPHYLOCOCCUS AUREUS ISO-
LATES FROM MAN AND ANIMALS

by

JAMES DOSU ADEKEYE

D.V.M., Ahmadu Bello University, 1972

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Infectious Diseases

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1975

Approved by:



Major Professor

LD
2668
T4
1975
A34
C.2
Document

TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	2
MATERIALS AND METHODS	15
Coagulase Test	15
Fibrinolysin Test	16
Phosphatase Test	16
Antibiotic Susceptibility Test	17
Determination of Antibiotic Minimum Inhibitory Concentrations	18
Quantitative Phosphatase Test	20
Bacteriophage Typing	20
RESULTS AND DISCUSSION	22
SUMMARY AND CONCLUSION	34
ACKNOWLEDGEMENT	35
LITERATURE CITED	37

INTRODUCTION

Staphylococcus aureus (S. aureus) is widespread in nature and produces many forms of disease in man and animals. Diseases range from superficial skin infections and boils to osteomyelitis, mastitis in cows, and occasionally a severe form of pneumonia in man.

In animals, S. aureus occurs as a commensal of body surfaces in many species and lesions produced, though probably less frequent, are similar in character to those in man.

The universal interest in controlling and eradicating the organism can best be achieved through a more complete understanding of factors controlling its virulence and pathogenicity. However, there are two major difficulties in the identification of bacterial virulence factors. The first of these, except in some rare cases, is that virulence is determined not by one factor, but by several, and loss of any single factor can result in partial or complete loss of virulence. Secondly, virulence for a given animal is accurately determined only in vivo. In vitro conditions may result in loss of virulence factors or acquisition of factors that do not exist in vivo.

However, differentiation of potentially pathogenic strains from evidently saprophytic staphylococci is possible. This has led to the association of certain characteristics with pathogenicity; particularly surface antigens, hemolysins and other enzymes among which are fibrinolysin, phosphatase, and coagulase. Antibiotic resistance and phage susceptibility have also been studied. Although these properties

may be related to pathogenicity they are not confirmatory. Until more reliable factors are found these characteristics may continue to serve as a basis for separating potential pathogenic from saprophytic staphylococci.

Hugo et al. in (1973) found that growth of S. aureus under conditions of biotin limitation caused a decrease in total lipid content, small reductions in growth rate, oxygen uptake, and protein content, accompanied by a slight increase in teichoic acid. They also found that lipid-depleted cells were less resistant to a number of anti-bacterial drugs and that other factors associated with drug interaction such as drug uptake, and leakage of cytoplasmic contents were affected.

In the present investigation, the effects of biotin limitation on certain biochemical activities including coagulase, phosphatase and fibrinolysin formation and antibiotic and phage susceptibility of S. aureus isolates from man and some domesticated animals were studied.

Literature Review

Cruickshank, 1937; Cowan, 1938; Fairbrother, 1940 regarded coagulation of plasma as the most useful single test to identify S. aureus. Serological tests have been used for the same purpose. Precipitin tests were used by Julianelle and Wieghard, 1935 and Cowan, 1938.

In 1940 Christie and Keogh of the Commonwealth Serum Laboratories, Melbourne, studied the physiological and serological characteristics of S. aureus of human origin and concluded that coagulase production was the simplest test for pathogenicity. No other test, they said, was completely reliable.

Burns and Holtman (1960) examined 200 strains of staphylococci in one study, and 500 strains in another, and concluded that regardless of enzymatic or toxic activity of the organisms in vitro, there was no single, reliable, characteristic by which they could adequately assess virulence. Correlation between coagulase and deoxyribonuclease was higher among strains from suppurative lesions and lower among strains from other sources; consequently, they regarded combination of these two tests as better evidence of virulence, as did Jeffries and Wailes (1961) and Osowieki (1962). Zak, (1967), tested a number of strains for the quantitative production of extracellular and intracellular metabolites and concluded that no single test could be regarded as a criterion for positive identification of potentially pathogenic staphylococci.

Attempts were made to identify other enzymes associated with coagulase production in order to establish a meaningful correlation with pathogenicity. Andreoni (1960) isolated staphylococci from purulent exudates and found a correlation between presence of egg-yolk factor, coagulase activity, and virulence. Jeljaszewicz (1960) compared enzymatic activities of coagulase-positive and coagulase-negative strains and concluded that free and bound coagulase, along with phosphatase, occurred frequently in potentially pathogenic staphylococci. This was confirmed by Kedzia et al. (1966) and Kocur and Martinec (1967).

Willis et al. (1964) found that proteolysis and fibrinolysis did not correlate as well with pathogenicity of staphylococci as did coagulase, deoxyribonuclease, and phosphatase production; and suggested that the last three parameters be used as indicators of pathogenicity.

Vogelsang, et al. (1962a) suggested that, hyaluronidase and phosphatase along with coagulase were important and should be considered as part of the total picture of pathogenicity. Schmidt (1965a) correlated pathogenic staphylococci with their ability to form hyaluronidase and deoxyribonuclease.

Victor et al. (1969) found that hyaluronidase and coagulase production were correlated, and suggested use of a hyaluronidase test as a measurement of pathogenicity.

McCabe (1966), using embryonated eggs as a test system, could not establish a direct relationship between virulence and coagulase, gelatinase, or lipase production but did find that fibrinolysin activity correlated well.

Earlier Madison (1935-6), Fisher (1936), Neter (1937) suggested that fibrinolysis accompanied coagulase production and was characteristic of pathogenic staphylococci of human origin. In 1941 Christie and Wilson, experimenting on staphylococcal fibrinolysis, concluded that if strains of human origin only are considered, clearing of fibrinogen-agar is an indication of pathogenicity.

From the foregoing conclusions reached by different authors, attempting to attribute virulence to any single factor portrayed a mass of conflicting data. Up till now, no definitive correlation between quantitative production of enzymes and clinical virulence of staphylococci has been established.

Burns and Holtman (1960), suggested that the prototype of a virulent staphylococcus was a strain isolated from a typical pyogenic lesion and that fibrinolysin, coagulase and phosphatase production

were more often than not, associated with virulence.

Staphylocoagulase was first identified in 1903 when Loeb showed that Staphylococcus pyogenes caused coagulation of goose plasma in vitro. He suggested that an enzyme was formed in cultures which could coagulate blood in vivo.

Elek (1959), Jejaszowicz (1958) and Drummond and Tager (1965) reviewed staphylocoagulase from 1882-1965. The results reported by hundreds of papers contributed to the development of the present day method whereby coagulase production can be used to identify pathogenic staphylococci.

To elucidate the biologic nature, mode of action, and contribution toward pathogenicity, many authors have purified the enzyme. Tager (1948) purified staphylocoagulase some 300 fold, using cycles of alcohol precipitation and ammonium sulphate saturation. Duthie and Haughton (1958) precipitated culture supernatant fluid with cadmium sulfate, followed by dialysis and several cycles of ammonium sulfate fractionation.

In 1960, Murray and Gohdes used cadmium sulfate and ammonium sulfate precipitation to prepare a crude enzyme. Other but similar methods were used by other workers among whom were Blobel, et al. 1960, Zolli and San Clemente (1963), Hitokoto (1965). Based on the results of purification procedures a tremendous divergence in regard to homogeneity and activity of the endpoint has been reported. There was evidence that staphylocoagulase exists in multiple molecular forms. Rammelkamp, et al. 1950 identified three coagulases which could be differentiated immunologically. Five to eight staphylocoagulases were characterized by starch-gel electrophoresis. Miale, et al. 1963 said

that whether isoenzymes were produced or coagulases derived from different staphylococcal strains differed antigenically and physiologically needed to be examined.

With purification of staphylocoagulase, its nature, factors necessary for its action and its mode of action have been elucidated by numerous workers. In 1954a, Duthie introduced the term "bound coagulase" for a factor considered to be responsible for the clumping associated with the slide coagulase test. He found that "bound coagulase" was antigenically different from free coagulase. The clumping factor was believed to act directly on fibrinogen, whereas free coagulase required accessory factors.

Smith and Hale (1944) provided evidence that a plasma co-factor or accessory factor was necessary for the action of coagulase. This substance called activator by these authors was labeled coagulase reacting factor by Tager (1948). In 1959 Elek reviewed the discovery, nature, distribution, interaction and relationship of accessory factor to thrombin and coagulase. By 1963, Drummond and Tager noted that coagulase--coagulase reacting factor complex resulted in thrombin formation. They also found that the coagulase reacting factor accelerated the clotting ability of coagulase but clotting occurred in its absence. In 1968, Jeljaszewicz suggested that the mechanism of action of staphylocoagulase involved fibrinogen and prothrombin as substrates for the staphylocoagulase factor in vivo.

Tager and Drummond (1970) compared the action of staphylocoagulase-thrombin system with the activities of snake venom. The only difference was the participation of prothrombin and coagulase reacting factor in the coagulase-thrombin system. In 1973, Zajdel,

et al. described staphylocoagulase as a protein produced extracellularly by pathogenic strains of staphylococci and in the presence of a plasma factor, prothrombin, staphylocoagulase transformed soluble fibrinogen into insoluble fibrin.

In 1951, Barber et al. observed that coagulase-negative strains of staphylococcus usually produced less phosphatase than did coagulase positive strains. This was also reported by Elek in 1959 who thought phosphatase was an intracellular enzyme. In 1963, Krynski et al. studied 3717 strains of coagulase positive and coagulase-negative staphylococci. Ninety-eight per cent of the coagulase-negative strains did not produce phosphatase whereas more than 99 per cent of coagulase-positive strains produced this enzyme.

Burns and Holtman 1960 postulated that virulent staphylococci were chemically more active than avirulent staphylococci, as did Ivler 1965.

Kedzia 1963 studied the quantitative phosphatase activity of 158 strains of coagulase positive staphylococci isolated from a variety of lesions and healthy carriers. Strains isolated from patients with clinical lesions had greater activity than those from asymptomatic carriers.

Bal (1965) studied 317 strains of staphylococci and found that 100% of the coagulase-positive strains produced phosphatase, as compared to 70% of coagulase-negative strains. When phosphatase tests were negative, coagulase tests were also negative.

The position of phosphatase production, in relation to pathogenicity of staphylococci, has remained hazy. Akator and Lebeder (1961) investigated the quantitative production of phosphatase activity by

100 coagulase-positive strains. Ninety per cent produced acid-phosphatase with 80-fold differences in activity and 52% produced alkaline-phosphatase with several hundred fold differences in activity. They were unable to correlate phosphatase activity and antibiotic resistance. Hence they concluded that phosphatase activity of coagulase-positive or coagulase-negative staphylococci should not be used as an index of pathogenicity. However, high phosphatase production was found among phage type 80/81 and group 1 strains regardless of source.

Blumenthal and Pan (1963) reported a correlation between resistance to mercuric ions and penicillin. Penicillin resistant strains tested belonged to phage group I, susceptible to phage 80--the group producing the greater quantity of acid phosphatase. Many coagulase-negative strains tested were also resistant to mercury-ions and produced small quantities of phosphatase.

Choudhuri and Chakrabarty (1969b) correlated phosphatase activity of coagulase-positive and coagulase-negative staphylococci, with phage typing. Phage group II showed 100% correlation with phosphatase activity. Eighty five per cent of coagulase and phosphatase-positive staphylococci were pathogenic as compared to 54% of coagulase-negative phosphatase-positive strains.

Elek (1959) suggested that coagulase and phosphatase activities of staphylococci may be a manifestation of a single protein. Inniss and San Clemente (1962), used ion exchange chromatography and starch, starch agar gel and paper electrophoresis and failed to separate acid-phosphatase from staphylocoagulase activity. They postulated that both coagulase and phosphatase were associated with a common protein carrier. San Clemente and Zolli (1963) using gel filtration were able to

separate coagulase and phosphatase. In 1967 Sawashige, using electron microscopy, found that acid-phosphatase activity was localized in the cytoplasm and cytoplasmic membrane. Also in 1967 Shah and Blobel found that coagulase-negative staphylococci had repressive alkaline phosphatase.

Reports by Madison (1935-6), Fisher (1936), Neter (1937) suggested that fibrinolysis accompanied coagulase production and was characteristic of pathogenic staphylococci of human origin.

In 1941, Christie and Wilson from the Commonwealth Serum Laboratories, Melbourne found that ninety-two of 99 coagulase-positive strains of human origin, but none of 42 coagulase-negative strains caused clearing of fibrinogen-agar. Four of 43 coagulase-positive strains of animal origin and one of 14 coagulase-negative strains caused clearing. They concluded that clearing of fibrinogen agar by human strains of staphylococci generally paralleled presence of coagulase and pathogenicity.

Christie, et al. (1946) reported that 95 per cent of 695 coagulase-positive cultures from man were fibrinolytic while only 9 per cent of 115 cultures from animals caused fibrinolysis. Elek (1959) found 81 per cent of staphylococcal isolates from human lesions and 38 per cent from animal infections produced fibrinolysis. Meyer (1967a) reported that the majority of coagulase positive staphylococci from animal species were nonfibrinolytic.

Abramson (1968) separated fibrinolysin from other enzymes by sequential ammonium sulfate fractionation of culture supernatant and found that fibrinolysin occurred in a purified fraction, at 80 per cent

saturation level.

Arvidson et al. (1973) in their work, "production, purification and partial characterization of staphylokinase", produced large quantities of purified fibrinolysin by isoelectric focusing. They concluded that fibrinolysin was relatively heat stable in solution at pH 6-8, but this decreased remarkably in dilute solutions. The enzyme was inactivated by adsorption to glass surfaces. They further described it as an extracellular protein which activated the proenzyme plasminogen to a proteolytic enzyme, plasmin. The later is active against fibrin and casein. The biological effects of fibrinolysin in in vitro and in vivo are still under investigation.

Elek (1959) indicated that production of fibrinolysin by coagulase-positive and coagulase-negative strains was associated with organisms isolated from infected as well as healthy individuals. Because of this finding, he believed that fibrinolysin importance as a pathogenic factor may have been overemphasized.

Use of antibiotics has substantially reduced mortality from staphylococcal infection. It has also led to the realization that some staphylococci developed resistant to certain antibiotics. It is the biochemical studies of antibiotic resistance that eventually revealed the mechanism of resistance.

Attempts have been made to correlate some biochemical properties of S. aureus with antibiotic resistance. Jessen et al. (1963) found that 81 per cent of the bacteremia strains and 65 per cent of those causing furunculosis in man were penicillin-resistant. Forty-eight per cent and 40 per cent respectively were resistant to streptomycin. More than half of the bacteremia strains were resistant to sulfonamides

and 18 per cent to tetracyclines. Resistance to penicillin, streptomycin and sulfonamides was found in 45 per cent of the bacteremia strains. The majority of the nasal strains were antibiotic sensitive. They also found that the resistant furunculosis strains belong to the large phage group consisting of types 80 and 52/52A/80.

Krynski et al. (1962) worked on the sensitivity to antibiotics and biochemical properties of S. aureus 52, 52A, 80, 81 complex, and concluded that ability to produce a tween 80 splitting enzyme and susceptibility to phage 81, as well as resistance to antibiotics were related.

Work done through the years by Skaggs and Nicol (1961); Wallace, et al. (1962); Moeller et al. (1963); Live (1972) led to the realization that both man and animals developed troublesome infections caused by antibiotic resistant staphylococci primarily of the 80/81 phage complex.

The sensitivity of staphylococcal cultures to phages has been used to supply information with respect to the relationship of isolates from different sources. Jessen et al. (1959) attempted to correlate the supposed pathogenic properties of S. aureus with phage type. They studied 446 strains isolated from different types of human infection. They found that phage types 80 and 52/52A/80 were responsible for a large proportion of the bacteremia cases. They also found that impetigo staphylococci were characterized by a high frequency of strains of phage type 71 and related types. This confirmed the observations of Spittlehouse (1955), Barrow (1955) and Parker (1958). This led them to believe that there might be some relation between phage type and the type of disease produced. They attempted to correlate mortality to

phage type but were unable to establish any correlation between a high mortality due to staphylococcal bacteremia and any single phage type.

Work done by Skaggs and Nicol (1961); and by many authors in late 1960's and early 1970's implicated phage type 80/81 as being associated with epidemic infections in man and cattle. Thus while phage susceptibility is best used to trace the sources of S. aureus, it might also be an index of pathogenicity.

Attempts have been made to show why coagulase, fibrinolysin, phosphatase, antimicrobial and phage susceptibility have been chosen in this work as indices of pathogenicity. Each of these aspects has at some time been suggested to be related to pathogenicity and then at a later date unequivocally found not to be related. It is possible that the "virulence factor" may be produced in vivo only and so not detectable by present day in vitro methods, or that all these factors mentioned and others not mentioned may synergistically combine to form a "shock" environment conducive to the type of pathogenicity related to staphylococcal disease. One should realize that though a single factor cannot always be correlated with pathogenicity, their significance need not be reduced. Each may constitute a link in a complex chain of many, presently undetermined, pathogenic factors that combine only in vivo to precipitate the present day staphylococcal phenomenon.

The universal interest in controlling and eradicating S. aureus has led to innumerable undertakings by many interested individuals to see these two aims achieved.

Many reports recognized biotin as a growth factor for micro-organisms including S. aureus, (Knight, 1935).

Briggs (1961), Lynen (1967) and Knappe (1970) reviewed the

general role of biotin in the metabolic functions of the cell. Lynen et al. (1959) unfolded the co-enzymic function of biotin in carboxylations and transcarboxylations. Martin and Vagelos (1962) fully documented the role of these reactions in the mechanism of biosynthesis of long chain fatty acids from acetyl co-enzyme. Friedman and Moat (1958) suggested that biotin may play a general role in nucleic acid metabolism and Ahmad et al. (1961) reported that biotin deficiency affected the synthesis of nucleic acids and proteins of Saccharomyces cerevisiae. A review of this literature by Hugo and Davidson (1973) suggested that growth in conditions of biotin limitation might reduce cell lipid levels. This led Hugo et al. (1973) to undertake some work with S. aureus. They found that under conditions of biotin limitation the major effect was on lipid content and composition of S. aureus. Total lipids fell by 20 per cent with an increase in the lysyl-phosphatidyl glycerol content of over 100 per cent. Other observable changes included small reductions in growth rate, oxygen uptake, and protein content, accompanied by a slight increase in teichoic acid content.

Hugo and Davidson (1973) concluded that lipid-depleted cells of S. aureus obtained by growth under conditions of biotin deficiency were less resistant to a number of antimicrobial drugs than normal cells. Other factors associated with drug cell interaction such as drug uptake and leakage of cytoplasmic constituents supported their viability data. They concluded that cellular changes could possibly be linked to an alteration in permability, due to an involvement of D-biotin in membrane fatty acid composition.

It was the objective of this work to investigate the effect that

such lipid-depletion had on the ability of S. aureus to produce coagulase, phosphatase, fibrinolysin, and its response to antimicrobial agents and bacterio-phages.

MATERIALS AND METHODS

Most of the staphylococcus strains had been preserved on agar slants and maintained at 4°C. Before testing, 1.5 ml of sterile nutrient broth was added to each agar slant. These were incubated for 24 hours at 37°C and were transferred to blood agar plates and incubated overnight at 37°C. Single colony isolates from these plates were used for the following tests.

Coagulase Test

Isolates from blood agar plates were subcultured overnight in nutrient broth and Biotin assay medium* plus 12 micrograms of biotin per liter [hereafter referred to as BDM (biotin-deficient medium)]. The later broth was prepared according to Hugo et al. (1973). All containers used for preparation and use of BDM were kept overnight in chromic acid and rinsed several times with distilled water. The medium was first boiled for three minutes before autoclaving 10 minutes at 15 lbs pressure at 121°C. Nutrient broth was autoclaved for 15 minutes under the same conditions of temperature and pressure.

With sterile dispo-pipettes, 5 drops (approx. .125 mls) of each of these overnight cultures were added to test-tubes (16 x 100 mm.) containing .5 mls of 1/5 concentrations of coagulase plasma,** human

*Difco Laboratories, Detroit, Mich.

**Difco Laboratories, Detroit, Mich.

and equine plasma. The tubes were incubated at 37°C, and examined for coagulase formation at 4, 6, 12 and 24 hours. A positive test was a solid coagulum that did not fragment when the tubes were tilted.

Fibrinolysin Test

In addition to the fibrinolysin agar as prepared by Christie and Wilson (1941), a modified fibrinolysin agar was prepared to satisfy the conditions of biotin limitations. Biotin assay medium* plus 12 micrograms biotin per liter was autoclaved at 121°C; 15 lbs pressure for 10 minutes, purified agar sterilized for 15 minutes at the same pressure and temperature was added to a final concentration at 1.5% carefully mixed and cooled at 57°C in water bath. Rabbit plasma was added (12ml/100ml), and the mixture maintained at this temperature for 10 minutes before being poured to plates. The plates were allowed to dry. Cultures grown overnight in test-tubes of nutrient broth and BDM were spot-inoculated into the two types of fibrinolysin agar. Inoculated plates were incubated overnight at 37°C and examined for clearing of the heat precipitated fibrinogen.

Phosphatase Test

Two types of phosphatase agar were prepared. Phenolphthalein diphosphate (0.1M) was prepared by adding 0.5 gram of phenolphthalein diphosphate to 100 mls of distilled water, in a beaker and dissolved at 50°C. The chemical was allowed to cool to room temperature and sterilized by filtration through a sterile selas O3 filter.

In addition to the phosphatase agar as recommended by King (1943),

*Difco Laboratory, Detroit, Mich.

a second type of phosphatase agar was prepared by adding 1 part of phenolphthalein diphosphate to 49 parts of a mixture of Biotin assay medium containing biotin 12 microgram/liter (sterilized for 10 minutes at 121°C, 15 lb pressure) and purified agar (1.5%) sterilized for 15 minutes. The two different kinds of agar plates were dried by incubation overnight at 37°C.

The modified BDM (agar) was streaked with those cultures which had been grown overnight in BDM while the standard phosphatase agar was streaked with the same organisms grown overnight in nutrient broth. The streaked plates were incubated overnight at 37°C.

Each plate was held over an open bottle of ammonium hydroxide for 2 minutes and colonies examined for a pink color change that indicated the presence of phosphatase. Parts of each plate where there was no growth were used as controls.

Antibiotic Susceptibility Test

Biotin deficient agar was prepared by adding 37.5 gms of Biotin assay medium (BAM) to 1000 mls distilled water in a 2 liter flask. The mixture was shaken and boiled for 3 minutes. Then 24 ug biotin was added and the mixture autoclaved for 10 minutes at 121°C, 15 lbs pressure, before being transferred to a water bath at 45°C.

A 3 per cent solution of purified agar was also prepared in a liter of distilled water and autoclaved for 15 minutes. It was cooled to 45°C and both batches of medium were carefully mixed giving a mixture of 2 liters of BAM containing 12 mg/liter of biotin. Forty-two mls of this mixture were poured to 150 x 15 mm sterile petri dishes and dried by incubating overnight at 37°C.

Plates of Mueller-Hinton agar were prepared in a similar manner. Each isolate was grown overnight at 37°C in 5 mls of Mueller-Hinton broth and in BDM. Cultures were well shaken and diluted with sterile distilled water until the turbidity was visually the same as a 1 per cent Ba So₄ solution.

If one of the paired cultures was less turbid than the Ba So₄ solution, the other member was diluted until the two cultures had the same turbidity.

Using sterile swabs, cultures grown in Mueller-Hinton broth were streaked evenly in 3 planes onto the surface of Mueller-Hinton agar. Surplus suspension was removed from the swab by rotation against the side of the tube before the plates were seeded.

Broth cultures from BDM were similarly seeded on the biotin-deficient agar. Seeded plates were allowed to dry for 3-5 minutes and antibiotic sensi-discs* were applied using 12 magazine 150 mm dispenser. The discs were pressed down on the agar with sterile forceps to ensure that there was contact between agar and the discs. The plates were incubated overnight after which zone diameters were measured in millimeters using the DIFCO antibacterial zone gauge.

The discs used and antibiotic concentrations were Ampicillin (10mg), Bactracin (10 units), Chloramphenicol (30mg), Erythromycin (15mg), Kanamycin (30mg), Methicillin (5mg), Neomycin (30mg), Penicillin-G (10 units), Streptomycin (10mg), Tetracycline (30mg).

Determination of Antibiotic Minimum Inhibitory Concentrations

To rule out the possibility that differences in antibiotic

*Difco Laboratories, Detroit, Mich.

diffusability influenced results obtained with the two different agars ten isolates, some of which were resistant, and others sensitive, to penicillin G, tetracycline, and streptomycin were selected. These strains were tested for minimum inhibitory concentrations of penicillin G, tetracycline and streptomycin using a tube dilution method.

Cultures of selected organisms were grown overnight in 2 mls of Mueller Hinton and BDM broths. There was no detectable difference in turbidity of the paired cultures.

Each culture was diluted in its sterile corresponding broth to an approximate 10^{-5} dilution.

The following dilutions were prepared in Mueller Hinton and BDM broths to a total volume of 5 ml/tube.

Penicillin.* (units/ml)

25; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39; 0.19; 0.097; .048; .024; 0.

Streptomycin.** (ug/ml)

100; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78; .39; .19; 0.097.

Tetracycline.*** (ug/ml)

80; 40; 20; 10; 5; 2.5; 1.25; 0.625; 0.302; 0.181; .0906; 0.

Tubes containing the antibiotic dilutions were inoculated with 1.5 ml of the diluted cultures and incubated 18 hours at 37°C. The minimum inhibitory concentration was recorded as the smallest amount of antibiotic completely inhibiting growth.

*Penicillin = Pfizerpen (Pfizer buffered potassium penicillin (z)).

**Streptomycin = Streptomycin sulfate (Pfizer).

***Tetracycline = Achromycin (Lederle, Tetracycline-Hcl).

Phosphatase Test

Phosphatase activities of normal and lipid depleted cells of S. aureus strains from different animals were quantitatively compared.

Cultures of selected strains were inoculated into 5 mls of Brain heart infusion (BHI) broth and BDM and incubated 18 hours at 37°C.

Each culture was well mixed and poured into a preweighed tube and centrifuged for 30 minutes at 2,000 RPM at 4°C. The supernatant was decanted and the test-tube and precipitate were weighed to determine wet weight of the culture.

The precipitate was resuspended in a buffer [sodium acetate-acetic acid (pH 5.2), containing .001M. magnesium chloride] and was sonicated for 6 minutes cooled in ice to prevent overheating. Phosphatase activity of each culture was determined quantitatively on a Coleman Hitachi 101 at 410 nm, using paranitrophenol phosphate as the substrate. As sonicated cell suspensions were turbid, a separate blank was prepared using 1.0 ml buffered substrate to which was added 0.2 ml of distilled water. This mixture was incubated for thirty minutes, then 9.7 ml of 0.02 N. sodium hydroxide and 0.2 ml of the sonicated cell suspension were added. The spectrophotometer was set for 100 per cent T. using this blank.

Bacteriophage Typing

S. aureus typing bacteriophages (phages) and their propagating strains (PS) were obtained from Dr. J. M. Williams, Director of Bacteriology, Philips Roxane Incorporated, St. Joseph, Missouri.

Twenty phages representing the four serological groups and the

five lytic groups, were used for the experiment. They were 29, 52, 52A, 79 and 80 in group I; 3A, 3C, 55, and 71 in group II; 6, 42E, 47, 53, 54, 75, 77, and 83 in group III; 42D in group IV; and 187 and 81 in group miscellaneous.

These were obtained in sufficient concentrations for determination of Routine Test Dilutions (RTDs).

Phage RTDs were determined using PS bearing the same number, i.e., phage 3A RTD was determined on PS 3A, etc. Thus, PS 3A, PS 3C, PS 6, PS 29, PS 42D, PS 43E, PS 47, PS 52, PS 52A/79, PS 53, PS 54, PS 55, PS 71, PS 75, PS 77, PS 79, PS 80, PS 81, PS 83A, and PS 187 were used for phage typing as recommended by the International Reference Center. S. aureus isolates from humans and animals were grown in both biotin limited conditions (BAM + 12 micrograms/L. biotin broth, and then transferred to BAM + 12 micrograms/L. biotin agar) and Trypticase-soy broth and agar.

RESULTS AND DISCUSSION

Coagulase Production

The results of coagulase tests conducted in rabbit, human and equine plasma are presented in Table 1.

TABLE 1. A Comparison of Coagulase Activity of Isolates of *Staphylococcus aureus* from Man and Other Animals in Rabbit, Human, and Equine Plasma, when Grown in Nutrient Broth and a Biotin Deficient Medium (BAM+12)

ANIMAL ISOLATED FROM	NUMBER OF ISOLATES	NUMBER POSITIVE IN					
		RABBIT PLASMA		HUMAN PLASMA		EQUINE PLASMA	
		BAM+12	Nutrient	BAM+12	Nutrient	BAM+12	Nutrient
HUMAN	24	24	24	2	23	4	23
CANINE	23	23	23	6	21	16	22
BOVINE	14	14	14	2	14	2	14
EQUINE	11	11	11	3	8	4	10
OTHERS	12	12	12	3	10	2	10
TOTAL	84	84	84	16	76	28	79

Staphylocoagulase activity was most readily detected in rabbit plasma in cultures grown in both biotin-deficient and nutrient broth. When organisms were grown in biotin-deficient medium the ability to coagulate human and equine plasma was reduced. Coagulase activity of human strains was most affected by growth in biotin-deficient medium as shown in both human and equine plasma. Canine, bovine, and equine isolates and those from other animals were about equally affected.

The fact that staphylocoagulase was detected in all cultures grown in biotin-deficient medium, when tested with rabbit plasma, but was not demonstrated in human or equine plasma, suggested that among the effects of lipid depletion produced by growth in biotin-deficient medium (Hugo et al.) there is a reduction in the ability of some isolates of S. aureus to form coagulase. This reduced ability was not detected with rabbit plasma, but was obvious in human and equine plasma. It is possible that only organisms that normally produced large quantities of coagulase continued to do so in biotin-deficient medium and this activity was detectable in human and equine plasma.

Differences in coagulase activity in human and equine plasma of isolates from different animal sources after growth in biotin-deficient medium may be of epidemiologic importance. Strains isolated from a human source were most severely affected. It is possible that there are differences in the metabolic activity of isolates from different animals that influence the effect that biotin-deficiency has on ability to produce coagulase.

The effects that growth in a biotin-deficient medium had on fibrinolysin activity are summarized in Table 2.

No fibrinolysin activity was detectable in cultures grown in biotin-deficient agar.

The inability of many strains of S. aureus isolated from animals to produce fibrinolysin supports the findings of others (Christie et al., 1946; Elek, 1959; Marsalek, 1969 and Meyer, 1967). Fibrinolytic activity is generally a property of human strains of staphylococci but occurs occasionally in isolates from other animals. It is possible, particularly among canine strains used in this experiment, that some of

TABLE 2. A Comparison of Fibrinolysin Production of Isolates of Staphylococcus aureus from Man and Other Animals when Tested in Nutrient and Biotin-Deficient Agar

ANIMAL SOURCE	NUMBER OF ISOLATES	BAM+12 MICROGRAMS/LITER BIOTIN + FIBRINOGEN	NUTRIENT + FIBRINOGEN
HUMAN	15	0	11
CANINE	6	0	4
BOVINE	11	0	6
OTHERS	10	0	5
TOTAL	42	0	26

the fibrinolytic isolates from animals were originally of human origin.

The loss of fibrinolytic activity by strains from all animals when grown in biotin-deficient medium is an indication of reduced biochemical activity of the lipid-depleted organisms.

The results of the antibiotic sensitivity test by the disc method (Bauer et al., 1966) on Mueller-Hinton and biotin-deficient agar are presented in Table 3.

Inhibitory zones were larger in biotin-deficient agar with all antibiotics except neomycin.

Zones of inhibition produced by neomycin in biotin-deficient agar were generally smaller than in Mueller-Hinton agar. With all other antibiotics, the inhibitory zones were larger when the isolate was grown on biotin-deficient agar. The explanation for the neomycin phenomenon is not readily apparent. Neomycin acts by inducing abnormal protein synthesis as does kanamycin. However, inhibitory zones produced by kanamycin were larger on biotin-deficient agar. Therefore, the

TABLE 3. A Comparison of Antibiotic Susceptibility of Isolates of *Staphylococcus aureus* Tested on Mueller-Hinton Agar (MHA) and Biotin Deficient Agar (BDA)

MEAN ANTIBIOTIC INHIBITORY ZONES IN (MM)												
ANTIBIOTICS	HUMAN (9 strains)		CANINE (11 strains)		BOVINE (11 strains)		EQUINE (7 strains)		OTHERS (7 strains)			
	MHA	BDA	MHA	BDA	MHA	BDA	MHA	BDA	MHA	BDA	MHA	BDA
BACITRACIN	17.78 ⁺ 2.11	24.67 ⁺ 4.69	18.36 ⁺ 1.50	26.00 ⁺ 4.20	19.64 ⁺ 3.56	27.64 ⁺ 3.98	16.85 ⁺ 1.95	21.71 ⁺ 9.69	18.00 ⁺ 2.31	28.29 ⁺ 5.59		
CHLORAMPHENICOL	21.11 ⁺ 3.18	30.67 ⁺ 4.12	22.00 ⁺ 2.83	30.55 ⁺ 5.30	21.64 ⁺ 2.66	30.36 ⁺ 2.50	22.00 ⁺ 2.31	30.86 ⁺ 4.30	21.71 ⁺ 1.38	32.29 ⁺ 5.94		
ERYTHROMYCIN	22.67 ⁺ 3.01	33.67 ⁺ 6.12	22.50 ⁺ 2.98	30.25 ⁺ 5.60	22.38 ⁺ 2.39	34.25 ⁺ 3.92	22.57 ⁺ 2.51	32.57 ⁺ 3.78	21.00 ⁺ 3.46	29.00 ⁺ 11.49		
KANAMYCIN	19.56 ⁺ 2.96	24.56 ⁺ 5.03	21.64 ⁺ 6.10	26.18 ⁺ 6.10	20.73 ⁺ 4.41	24.73 ⁺ 5.82	21.43 ⁺ 4.58	24.29 ⁺ 4.39	16.29 ⁺ 2.14	23.43 ⁺ 5.26		
METHICILLIN	16.67 ⁺ 2.24	23.11 ⁺ 2.85	16.36 ⁺ 6.06	19.64 ⁺ 7.15	18.73 ⁺ 3.00	27.55 ⁺ 5.89	16.85 ⁺ 2.79	21.43 ⁺ 3.41	22.29 ⁺ 4.96	27.43 ⁺ 8.77		
NEOMYCIN	16.56 ⁺ 2.88	16.33 ⁺ 6.00	17.00 ⁺ 6.56	16.00 ⁺ 6.99	17.09 ⁺ 2.43	14.91 ⁺ 4.13	18.43 ⁺ 4.83	14.00 ⁺ 2.83	20.57 ⁺ 3.60	16.86 ⁺ 3.63		
PENICILLIN	23.63 ⁺ 10.00	31.88 ⁺ 15.10	23.09 ⁺ 12.97	30.55 ⁺ 13.77	26.45 ⁺ 11.74	36.91 ⁺ 12.85	24.29 ⁺ 16.67	34.86 ⁺ 18.18	28.86 ⁺ 15.61	39.14 ⁺ 18.33		
STREPTOMYCIN	10.67 ⁺ 6.40	15.67 ⁺ 9.80	17.82 ⁺ 3.52	20.91 ⁺ 6.22	15.55 ⁺ 4.32	20.55 ⁺ 6.14	15.14 ⁺ 5.40	18.86 ⁺ 5.98	14.29 ⁺ 6.78	17.71 ⁺ 9.05		

TABLE 3. (continued)

MEAN ANTIBIOTIC INHIBITORY ZONES IN (MM)											
ANTIBIOTICS	HUMAN (9 strains)		CANINE (11 strains)		BOVINE (11 strains)		EQUINE (7 strains)		OTHERS (7 strains)		
	MHA	BDA	MHA	BDA	MHA	BDA	MHA	BDA	MHA	BDA	
TETRACYCLINE	17.56 ⁺	28.67 ⁺	24.18 ⁺	35.09 ⁺	22.73 ⁺	33.09 ⁺	21.71 ⁺	32.00 ⁺	21.14 ⁺	36.29 ⁺	
	6.45	10.05	3.28	5.09	2.72	3.62	1.38	2.31	21.59	7.70	
AMPICILLIN	24.00 ⁺	32.81 ⁺	23.27 ⁺	32.36 ⁺	27.64 ⁺	37.40 ⁺	24.51 ⁺	31.86 ⁺	28.00 ⁺	37.71 ⁺	
	9.17	11.05	10.63	11.59	10.50	10.70	13.99	11.81	13.32	13.31	

explanation of this deviation from the usual pattern is probably not related to the mode of action of the antibiotic. Both neomycin and kanamycin are aminoglycosides so chemical composition of the antibiotic is not considered to be a significant factor.

Several of the antibiotics tested (penicillin, methicillin, ampicillin) inhibit cell wall synthesis while others (bacitracin) cause deterioration of cell membranes. As growth in biotin-deficient medium affects the cell membrane the larger zones of inhibition produced on this medium were understandable.

Other antibiotics (chloramphenicol, erythromycin, tetracycline) interfere with ribosome synthesis and therefore with protein synthesis while streptomycin acts by inducing abnormal protein synthesis. Because larger inhibitory zones were present on the biotin-deficient medium it suggests that there is some interrelationship between protein synthesis and growth in a biotin-deficient medium.

In order to confirm observations made on the variability of antibiotic sensitivity in biotin-deficient agar 10 isolates were selected and tested using a tube method. Results of this experiment are summarized in Table 4.

These observations (Table 4) confirmed those recorded by the Baur et al. (1966) method. There was a lower minimum inhibitory concentration of antibiotics in all cultures grown in biotin-deficient medium irrespective of source.

It is also apparent that higher concentrations of streptomycin and tetracycline were required to inhibit S. aureus of human origin as the MICs in Mueller-Hinton broth were greater than for strains from other animals. This supported the findings in table 3. The lower

TABLE 4. A Comparison of Antibiotic Minimum Inhibitory Concentration (MIC) for 10 Strains of Staphylococcus aureus of Animal and Human Source Grown in Mueller Hinton Broth (MHB) and Biotin Deficient Broth (BDB)

S. AUREUS STRAINS AND ORIGIN	*MINIMUM INHIBITORY CONCENTRATION					
	PENICILLIN		TETRACYCLINE		STREPTOMYCIN	
	MHB	BDB	MHB	BDB	MHB	BDB
1. (HUMAN)	6.25	.39	1.25	0.36	12.5	6.25
2. (HUMAN)	6.25	.049	2.5	0.18	6.25	3.13
3. (HUMAN)	12.5	1.56	2.5	0.18	25	6.25
4. (FELINE)	6.25	.39	0.36	0.18	0.39	0.39
5. (HUMAN)	12.5	.049	2.5	0.63	12.5	3.13
6. (EQUINE)	12.5	.049	0.36	0.18	0.39	0.20
7. (EQUINE)	12.5	.39	0.36	0.18	1.56	0.78
8. (BOVINE)	12.5	.78	0.36	0.18	0.39	0.20
9. (EQUINE)	12.5	.049	1.25	0.18	0.39	0.20
10. (CANINE)	25.0	1.56	2.5	0.18	25	0.39

*For penicillin in units, tetracycline and streptomycin in micrograms/ml.

minimum inhibitory concentrations in biotin-deficient medium once more supports the idea of Hugo et al. (1973), that under this condition there is less resistance to anti-microbial agents. Hugo et al. (1973) suggested that the lowering of resistance was due to increased drug uptake.

The phosphatase test result (Table 5) showed that the same cultures produced more phosphatase when grown in brain-heart infusion broth than in biotin-limited broth. This was true of all strains tested irrespective of the animal origin. This may be explained by

TABLE 5. A Comparison of Phosphatase Activity by Strains of *Staphylococcus aureus* Grown in Brain-Heart Infusion Broth and Biotin-Deficient Broth

SOURCE	NUMBER OF ISOLATES	PHOSPHATASE ACTIVITY/10MG WET WEIGHT	
		BRAIN-HEART INFUSION	BIOTIN-DEFICIENT
HUMAN	14	2.0 ± 1.1	0.7 ± 0.5
CANINE	14	2.3 ± 1.5	1.1 ± 0.8
BOVINE	10	1.7 ± 1.2	0.7 ± 0.5
EQUINE	13	1.8 ± 1.4	1.2 ± 1.7
OTHERS	3	1.9 ± 0.4	0.6 ± 0.2
TOTAL	54	1.9 ± 1.3	0.9 ± 1.0
RANGE		0.14 - 6.18	0.08 - 6.32

the possibility of reduced ability of the cultures to produce the enzyme under biotin-deficient condition, or as a result of the cell lipid depletion, with the consequential leakage of protein from the cell, the enzyme might have leaked into the broth. This could not be substantiated as attempts to detect the enzyme in the culture supernatant failed. Inability to detect the enzyme in the culture supernatant suggested that the enzyme may be intracellular as claimed by Elek 1959.

Staphylococcus isolates from human and canine sources produced larger quantities of phosphatase than strains from other animals when grown in brain-heart infusion but not much difference was evident under lipid-depleted conditions. It is also noteworthy that the range of phosphatase activity under biotin-deficient medium (0.08-6.32) was high as one equine strain produced phosphatase activity of

6.32/10mg wet weight in the biotin-deficient medium. All other cultures produced relatively low phosphatase activity with lipid depleted conditions.

When organisms were tested on phosphatase and modified phosphatase (BAM + 12 microgram/L. biotin, 1.5 per cent purified agar and phenolphthalein) agars, all were phosphatase positive but the pink color faded much earlier on cultures on the biotin deficient agar than from cultures on phosphatase agar. This may have resulted from decreased phosphatase production by organisms grown in the biotin-deficient condition.

The results of bacteriophage typing of normal and lipid-depleted cells of S. aureus are presented in Table 6. Phage susceptibility of lipid-depleted organisms and normal cells were essentially identical for the strains tested. However, normal cells tended to be more susceptible to phage 77 than lipid-depleted cells. It appears that loss of lipid from cells does not alter phage susceptibility of S. aureus irrespective of the source.

TABLE 6. Results of Phage Typing of Staphylococcus aureus Cultures Isolated from Man and Other Animals when Grown in Biotin-Deficient and Trypticase-Soy Agar

HUMAN ISOLATES		
CULTURE NUMBER	BIOTIN-DEFICIENT MEDIUM	TRYPTICASE MEDIUM
1	29 ⁺⁺ ; 79 ⁺⁺	77 ⁺⁺ ; 79 ⁺⁺
2	81 ⁺⁺	negative
3	29 ⁺⁺	29 ⁺⁺ ; 77 ⁺⁺
4	52 ⁺⁺ ; 52A ⁺⁺ ; 53 ⁺ ; 80 ⁺⁺	52 ⁺⁺ ; 52A ⁺⁺ ; 80 ⁺⁺ ; 43 ⁺
5	negative	negative
6	6 ⁺⁺ ; 29 ⁺ ; 47 ⁺⁺ ; 54 ⁺⁺ ; 75 ⁺⁺ ; 81 ⁺⁺ ; 83A ⁺⁺	6 ⁺⁺ ; 43E ⁺ ; 47 ⁺ ; 54 ⁺⁺ ; 75 ⁺⁺ ; 81 ⁺⁺ ; 83A ⁺⁺
7	54 ⁺⁺ ; 75 ⁺ ; 81 ⁺	54 ⁺ ; 81 ⁺
8	54 ⁺⁺ ; 75 ⁺ ; 81 ⁺⁺	54 ⁺⁺ ; 81 ⁺
9	75 ⁺⁺	77 ⁺⁺
10	77 ⁺⁺	negative
11	52 ⁺⁺ ; 52A ⁺⁺ ; 53 ⁺ ; 79 ⁺⁺ ; 80 ⁺⁺	29 ⁺⁺ ; 52 ⁺⁺ ; 52A ⁺⁺ ; 53 ⁺ ; 80 ⁺⁺ ; 81 ⁺⁺ ; 79 ⁺⁺
12	79 ⁺⁺	--
13	negative	77 ⁺⁺
13 cultures	negative	negative

TABLE 6. (continued)

CANINE ISOLATES			
CULTURE NUMBER	BIOTIN-DEFICIENT MEDIUM	TRYPTICASE MEDIUM	
1	79 ⁺⁺ ; 83A ⁺⁺	79 ⁺⁺ ; 83A ⁺⁺	
2	3A ⁺⁺	negative	
3	47 ⁺ ; 54 ⁺⁺⁺ ; 75 ⁺⁺ ; 77 ⁺⁺ ; 81 ⁺⁺ ; 83A ⁺⁺ ; 79 ⁻	42E ⁺ ; 54 ⁺⁺ ; 75 ⁺ ; 77 ⁺⁺ ; 81 ⁺⁺ ; 83A ⁺⁺	
4	negative	52 ⁺⁺ ; 80 ⁺⁺	
9 cultures	negative	negative	
BOVINE ISOLATES			
CULTURE NUMBER	BIOTIN-DEFICIENT MEDIUM	TRYPTICASE MEDIUM	
1	52 ⁺⁺ ; 52A ⁺⁺ ; 54 ⁺ ; 53 ⁺ ; 55 ⁺ ; 77 ⁺⁺ ; 79 ⁺⁺ ; 80 ⁺⁺ ; 81 ⁺⁺	77 ⁺⁺ ; 79 ⁺⁺ ; 80 ⁺⁺ ; 81 ⁺⁺ ; 52 ⁺⁺ ; 52A ⁺⁺ ; 54 ⁺	
2	42E ⁺⁺	42E ⁺⁺ ; 77 ⁻	
3	52 ⁺⁺ ; 52A ⁺⁺ ; 53 ⁺ ; 75 ⁺ ; 80 ⁺⁺ ; 81 ⁺⁺	29 ⁺⁺ ; 52 ⁺⁺ ; 52A ⁺⁺ ; 53 ⁺ ; 77 ⁺⁺ ; 80 ⁺⁺ ; 81 ⁻	
4	42E ⁺⁺ ; 47 ⁺⁺ ; 81 ⁺⁺ ; 54 ⁺⁺ ; 80 ⁺ ; 81 ⁺⁺ ; 75 ⁻	42E ⁺⁺ ; 81 ⁺⁺ ; 77 ⁺⁺	
5	negative	81 ⁺	
6	negative	42D ⁺ ; 71	
7	negative	42E ⁺⁺	
4 cultures	negative	negative	

TABLE 6. (continued)

EQUINE ISOLATES		
CULTURE NUMBER	BIOTIN-DEFICIENT MEDIUM	TRYPTICASE MEDIUM
1	81 ⁺⁺	6 ⁺ ; 54 ⁺ ; 75 ⁺ ; 81 ⁺
2	42E ⁺ ; 54 ⁺ ; 75 ⁺ ; 81 ⁺	42E ⁺ ; 54 ⁺ ; 77 ⁺⁺ ; 73A ⁺⁺
3	54 ⁺	42E ⁺ ; 54 ⁺ ; 81 ⁺
4	3A ⁺	3A ⁺
5	negative	77 ⁺⁺
6	negative	6 ⁺
5 cultures	negative	negative
ISOLATES FROM OTHER ANIMALS		
CULTURE NUMBER	BIOTIN-DEFICIENT MEDIUM	TRYPTICASE MEDIUM
1 (rabbit)	55 ⁺ ; 71 ⁺⁺	55 ⁺ ; 71 ⁺⁺
2 (ovine)	79 ⁺⁺ ; 83A ⁺	79 ⁺⁺ ; 83A ⁺⁺
3 (avian)	negative	negative
4 (feline)	negative	42D ⁺⁺
5 cultures	negative	negative

SUMMARY AND CONCLUSION

S. aureus strains isolated from man and animals were lipid-depleted by growth in biotin assay medium containing twelve micrograms of biotin per liter. The same organisms were grown concurrently in regular media and the two types of cultures were tested for the production of coagulase, phosphatase and fibrinolysin enzymes and also for response to various anti-microbial agents and bacteriophages.

Lipid-depleted organisms produced less phosphatase, less coagulase, no fibrinolysin and were more susceptible to anti-microbial agents than normal cells organisms, but had very similar bacteriophage activity.

ACKNOWLEDGEMENT

In accomplishing this piece of work, I sincerely thank KSU/AID and Ahmadu Bello University Nigeria for providing the money for the project and also for maintaining me in the U. S. during the course of this work. I am indeed very grateful. My sincere thanks go to my wife, Bimpe, our daughters Tope and Feyisike for sharing with me my joys, frustrations and achievements in the U. S. It was certainly a trying period for all, being away from our good loving family at home and for spending many hours without me, during the many hours I spent in the laboratory. The end justifies the means and I owe you all, a debt of gratitude.

I sincerely thank my major advisor, Professor Coles, my committee members--Dr. Iandolo, who also helped in ordering for bacteriophages at C. D. C. in Georgia, Dr. Kelly and Dr. Burroughs; for their good counsel and deep understanding of the academic problems that a foreign student may face. Your guidance has certainly given me a satisfying academic pursuance in the U. S.

My gratitudes also go to Dr. Bailie for his occasional advice on my research project and for his kindness in permitting me to use his laboratory freely. I am indeed grateful. My further thanks go to Mr. Bill Stowe for his kindness and expert technical advice. He certainly has contributed a lot to my knowledge.

Finally, I extend my sincere thanks to all the members of

faculty and staff of Infectious Diseases Department. Your warmth and friendliness to me is very reminiscent of the conditions in my home department in Nigeria. Please keep it up.

LITERATURE CITED

- Abramson, C., and H. Friedman. 1962. Extracellular enzyme and antigen patterns of staphylococci as related to phage type and antibiotic susceptibility. *Bacteriol. Proc.* 1964: 61.
- Abramson, C., and H. Friedman. 1967. Enzymatic activity of primary isolates of staphylococci in relation to antibiotic resistance and phage type. *J. Infect. Diseases* 117: 242-248.
- Abramson, D., and H. Friedman. 1967. Relation of antibiotic resistance to bacteriophage type and enzymatic activity of staphylococci. *Anti-microbial agents and Chemotherapy* 1966, pp. 244-351.
- Ahmad, F., A. H. Rose and N. K. Garg. 1961. Effect of biotin deficiency on the synthesis of nucleic acids and protein by *saccharomyces. cerevisiae*. *J. Gen. Microbiol.* 24: 69-80.
- Akator, A. K., and Z. I. Lebedev. 1961. On the quantitative relation between the phosphatase activity of staphylococci, their pathogenic properties and penicillin resistance. *Antibiotiki* 4: 363-368.
- Alami, S. Y., and F. C. Kelly. 1959. Demonstration of staphylococcal clumping factor and free coagulase in soft agar media. *J. Bacteriol.* 78: 539-544.
- Alami, S. Y., and G. J. Race. 1968. Factors affecting the demonstration of staphylococcal free coagulase and clumping factor. *J. Bacteriol.* 95: 1197-1198.
- Andreoni, O. 1960. Hemolytic properties and enzymatic activity on egg media (egg yolk reaction) of Staphylococcus aureus. *Boll. 1st Sieroterap. Milan*, 39: 429-432.
- Arvidson, S., R. Eriksson, T. Holme, R. Möllky, T. Wadstrom and O. Vesterberg. Production, purification and partial characterization of staphylokinase. (Contributions to Microbiology and Immunology, Vol. I. Staphylococci and Staphylococcal Infections, pp. 406-412 (Karger, Basel 1973).
- Baird, Parker, A. C. 1965. The classification of staphylococci and micrococci from world-wide sources. *J. Gen. Microbiol.* 38: 363-367.
- Bal, A. 1965. Phosphatase reaction of staphylococci isolated from ocular lesions. *J. Indian Med. Assoc.* 45: 490-491.

- Bal, A., B. R. Skula, and H. V. Nema. 1963. Hyaluronidase fibrinolysin activities of staphylococci. *Indian J. Med. Res.* 52: 545-548.
- Barber, M., and S. W. A. Kuper. 1951. Identification of staphylococcus pyogenes by the phosphatase reaction. *J. Pathol. Bacteriol.* 63: 65-68.
- Barber, M., B. L. Brocksbank, and S. W. A. Kuper. 1961. Staphylococcal phosphatase, glucuronidase and sulphatase. *J. Pathol. Bacteriol.* 63: 57-64.
- Barrow, G. I. Clinical and biological aspects of impetigo contagion. *J. Hyg. (Camb).* 53: 495, 1955.
- Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. Antibiotic susceptibility testing by a standardized single disk method. Technical bulletin of the registry of medical technologists. Vol. 36. No. 3, 1966. 493-496.
- Bhaskaran, C. S., and P. A. Jayakar. 1969. Biochemical activity of coagulase positive staphylococci isolated from cases and carriers. *Pathol. Microbiol.* 33: 39-43.
- Blair, J. E. 1962. What is a staphylococcus? *Bacteriol. Reviews*, 26: 375-381.
- Blobel, H. D., and D. T. Berman, and J. Simon. 1960. Purification of staphylococcal coagulase. *J. Bacteriol.* 79: 807-289.
- Blumenthal, H. J., and Y. Pan. 1963. Acid phosphatase production by mercuric ion--or penicillin resistant and sensitive staphylococcus aureus. *Proc. Soc. Exptl. Biol. Med.* 113: 322-326.
- Briggs, M. H. 1961. An evaluation of the metabolic status of biotin. *N. Z. J. Sci.* 4: 565-93.
- Burns, J., and D. F. Holtman. 1960. Biochemical properties of virulent and avirulent staphylococci. *Ann. N. Y. Acad. Sci.* 88: 1115-1124.
- Choudhuri, K. K., and A. M. Chakrabarty. 1969. Phosphatase activity of coagulase positive and coagulase negative staphylococci and their relation with pathogenicity. *Indian J. Exptl. Biol.* 7: 123-124.
- Christie, R. and E. V. Keogh. 1940. Physiological and serological characteristics of staphylococci of human origin. *Journal of Path.* Vol. LI. 189-197.
- Christie, R. and H. Wilson. 1941. A test of staphylococcal fibrinolysis. *Australian J. Exptl. Biol. Med. Sci.* 18. 329-332.
- Christie, R.; E. A. North and B. J. Parkin. 1946. Criteria of pathogenicity in staphylococci. *Australian J. Exptl. Biol. Med. Sci.*

24: 73-78.

- Cowan, S. T. and D. Lush. 1938. The classification of staphylococci by precipitation. *J. of Path.* Vol. XLVI, 31.
- Cruickshank, R. 1937. Staphylocoagulase. *J. Path. Bact.* 45: 295.
- Cruickshank Medical Microbiology ELBS Livingstone 1968.
- Davidson, F. M. 1960. Activation of plasminogen by staphylokinase. *Nature* 185: 626.
- Dlabac, V., and M. Rusinko. 1962. Contribution to practical diagnostic value of determinations of phosphatases in staphylococci. *Cesk. Epidemiol. Mikrobiol. Immunol.* 11: 87-90.
- Drummond, M. C. and Tager. 1959. Enzymatic activity of staphylocoagulase. I. Characterization of an esterase associated with purified preparations. *J. Bacteriol.* 78: 407-412.
- Drummond, M. C., and Tager. 1962a. Enzymatic activities associated with clotting and fibrinogen by staphylocoagulase and coagulase-reacting factor, and their inhibition by diisopropyl-fluorophosphate. *J. Bacteriol.* 83: 975-980.
- Drummond, M. C., and M. Tager. 1963. Fibrinogen clotting and fibrinopeptide formation by staphylocoagulase and the coagulase-reacting factor. *J. Bacteriol.* 85: 625-635.
- Duthie, E. S. 1954a. Evidence for two forms of staphylococcal coagulase. *J. Gen. Microbiol.* 10: 427-444.
- Duthie, E. S., and G. Haughton. 1958. Purification of free staphylococcal coagulase. *Biochem. J.* 70: 125-135.
- Duthie, E. S. and L. L. Lorenz. 1952. Staphylococcal coagulase: mode of action and antigenicity. *J. Gen. Microbiol.* 6: 95-107.
- Elek, S. D. 1959. *Staphylococcus pyogenes* and its relation to disease, E. and S. Livingstone, London.
- Fairbrother, R. W. and Miles. 1940. Coagulase production as a criterion for the classification of the staphylococci. *Journal of Path.* Vol. 1, 83.
- Finegold, S. M., and E. E. Sweeney. 1961. New selective and differential medium for coagulase positive staphylococci allowing rapid growth and strain differentiation. *J. Bacteriol.* 81: 636-641.
- Fisher, A. M. 1936. The fibrinolytic staphylococci. *Bull. Johns Hopkins Hosp.* 59: 415.

- Friedman, H. and A. G. Moat. 1958. A comparison of nutritional and genetic blocks in the synthesis of purines by yeasts, moulds and bacteria. *Archs Biochem. Biophysics*. 78: 146-156.
- Hajek, V., E. Marsalek, and I. Cerna. 1968. Plasma coagulation activity of staphylococci of various origin. *Cesk. Epidemiol. Mitrobiol. Immunol.* 17-1: 39-46.
- Henderson, A., and Brodi. 1963. Investigations on staphylococcal coagulase. *Brit. J. Exptl. Pathol.* 44: 624-528.
- Hitokoto, H. 1965. Studies on the purification of staphylococcal coagulase and its properties. *Japan J. Bacteriol.* 20: 627-633.
- Hugo, W. B. and J. R. Davidson. 1973. Effect of cell lipid depletion in *Staphylococcus aureus* upon its resistance to antimicrobial agents. *Microbios.* 8: 43-51; 63-72.
- Inniss, W. E., and C. L. San Clemente. 1962. Biochemical studies on staphycoagulase and an allied phosphatase activity. *J. Bacteriol.* 83: 941-947.
- Ivler, D. 1965. The staphylococci: ecologic perspectives. *Ann. N. Y. Acad. Sc.* 128: 1-456.
- Jay. O. Cohen. The staphylococci Wiley--interscience. 1972.
- Jeffries, C. D. and B. F. Wailes. 1961. Comparison of six physiological characteristics of staphylococci from laboratory specimens. *Am. J. Clin. Pathol.* 36: 114-118.
- Jeljaszewicz, J. 1960. Studies on staphylococcal coagulases. VI. appearance of various toxic and biochemical factors among coagulase negative and coagulase positive strains of staphylococci. *Med. Doswiadzalua Mikrobiol.* 12: 33-41.
- Jeljaszewicz, J. 1968. Mechanism of action of staphylocoagulase. *Proc. Congr. Hung. Soc. Microbiol.*, pp. 1-9.
- Jessen, O., V. Faber, K. Rosendal, and K. R. Eriksen. 1959. Some properties of *staphylococcus aureus*, possibly related to pathogenicity. *Acta Pathol. Microbiol. Scand.* 47: 327-335.
- Jessen, O., K. Rosendal, V. Faber, and K. Hove, K. R. Eriksen. 1963. Some properties of *staphylococcus aureus* possibly related to pathogenicity. *Acta Pathol. Microbiol. Scand.* 58: 85-98.
- Julianelle, L. A., and C. W. Wiegand. 1935. The immunological specificity of staphylococci. *J. Exp. Med.*, LXII: 11.
- Kato, Y., and G. Omori. 1959. Extraction of bound coagulase from staphylococcal cells. *Biken's J.* 2: 321-332.

- Kedzia, W. 1963. The phosphatase activity of coagulase positive *Staphylococcus aureus* strains isolated from patients and healthy persons. *J. Pathol. Bacteriol.* 85: 528-529.
- Kedzia, W., M. Musielak, B. Kedzia, H. Konair, and E. Pniewska. 1966. Enzymatic activity of coagulase positive *staphylococcus aureus* strains isolated from patients and healthy carriers. *J. Pathol. Microbiol.* 29: 307-323.
- Knappe, J. 1970. Mechanism of biotin action. *A. Rev. Biochem.* 39: 757-776.
- Kocur, M., and T. Martinec. 1967. Contribution to the characteristic properties of *staphylococcus aureus*. *Vnitrni Lekar.* 13, 9: 866.
- Kryniski, S., W. Kedzia, E. Becla and Z. Jacyno. 1972. Biochemical activity of *staphylococci* and *micrococci* isolated from various sources. *Pathol. Microbiol.* 26: 257-262.
- Live, I. 1972. Differentiation of *staphylococcus aureus* of human and of canine origins: coagulation of human and of canine plasma, fibrinolysin activity and serologic reaction. *Am. J. Vet. Res.* 33: 385-391.
- Loeb, L. 1903. The influence of certain bacteria on the coagulation of the blood. *J. Med. Res.* 10: 407-419.
- Lynen, F. 1967. The role of biotin dependent carboxylations in biosynthetic reactions. *Biochem. J.* 102: 381-400.
- Macbe, W. R. 1966. Studies of *staphylococcal* infection II. The role of bacteriophage type, antibiotic resistance, coagulase, and other exotoxins in infections in embryonated eggs. *Yale J. Biol. Med.* 38: 323-338.
- Madison, R. R. (1935-6). Fibrinolytic *staphylococci*. *Proc. Soc. exp. Biol.* 33: 209.
- Malveaux, F. J., and C. L. San Clemente. 1969a. *Staphylococcal* acid phosphatase: extensive purification and characterization of the loosely bound enzyme. *J. Bacteriol.* 97: 1209-1214.
- Malveaux, F. J., and C. L. San Clemente. 1969b. *Staphylococcal* acid phosphatase: preliminary physical and chemical characterization of the loosely bound enzyme. *J. Bacteriol.* 97: 1215-1219.
- Maria Zajdel, Z. Wegrzynowicz, J. Jeljaszewicz and G. Pulverer. Contributions to Microbiology and Immunology. Vol. 1. *Staphylococci and staphylococcal infections*, pp. 364-375 (Karger, Basel. 1973).
- Marsalek, E. and Hajak, V. (Olomouc). The classification of pathogenic *staphylococci*. Contributions to Microbiology and Immunology, Vol. 1. *Staphylococci and staphylococcal infections*, pp. 30-37 (Karger,

Basel 1973).

- Martin, D. B. and P. R. Vagelos. 1962. The mechanism of tricarboxylic acid cycle regulation of fatty acid synthesis. *J. Biol. Chem.* 237: 1787-92.
- Meyer, W. 1965. On the usefulness of the coagulase test with various types of plasma for the differentiation of strains of *staphylococcus aureus*. *Zentr. Bakteriell. Parasitenk. Orig.* 201: 331-338.
- Meyer, W. 1967a. Über die Branchbarkeit des Kristalviolett-Testes zur Differenzierung von *staphylococcus aureus*. *Stammen. Z. Med. Mikrobiol. Immunol.* 153: 158-168.
- Miale, J. B., A. R. Winningham, and J. W. Kent. 1963. Staphylococcal coagulases. *Nature.* 197: 392.
- Moeller, R. W., I. M. Smith, A. C. Shoemaker and R. A. Tjalma. 1963. Transfer of hospital staphylococci from man to animals. *J. Am. Vet. Med. Assoc.*
- Murray, M., and P. Gohdes. 1960. Purification of staphylococcal coagulase. *Biochem. Biophys. Acta* 40: 518-522.
- Neter, E. (1937). Fibrinolytic, anticoagulating and plasma clotting properties of staphylococci. *J. Bact.*, 34: 243.
- Noble, W. C. 1966. Virulence and biochemical characters of staphylococci. *J. Pathol. Bacteriol.* 91: 181-193.
- Oswiecki, H. 1962. Studies on staphylococcal desoxyribonuclease III. Interrelations of the staphylococcal synthesis of desoxyribonuclease, coagulase, and phosphatase. *Med. Doswiadezalna Mikrobiol.* 14: 193-197.
- Pan, Y., and H. J. Blumenthal. 1961. Correlation between acid phosphatase and coagulase production or phage type of *staphylococcus aureus*. *J. Bacteriol.* 82: 124-129.
- Parker, M. T. Some cultural characteristics of *staphylococcus aureus* strains from superficial skin infections. *J. Hyg. (Camb)*, 56: 238, 1958.
- Pulverer, G., and H. J. Entel. 1967. Physiological occurrence of coagulase positive staphylococci in animals. Demonstration of coagulase positive staphylococci in the nasopharynx of domestic and wild animals. *Zentr. Bakteriell. Parasitenk. Orig.* 202: 344-351.
- Rammelkamp, C. H. Jr., M. Hezebicks, and J. H. Dingle. 1950. Specific coagulase of *staphylococcus aureus*. *J. Exptl. Med.* 91: 295-307.
- Rische, H., Meyer, W., Tschäpe, H.; Voigt, W.; Ziomek, Dorothea, and Hummel, Ruth (Wernigerode). The taxonomy of *Staphylococcus aureus*.

- Contributions to Microbiology and Immunology, Vol. I. Karger, Basel. pp. 24-29. 1972.
- San Clemente, C. L. and Z. Zolli, Jr. 1963. Separation of phosphatase from staphylocoagulase preparations. *J. Bacteriol.* 86: 606-607.
- Sawashige, E. 1967. Electron microscopic studies on the enzymatic activities in the membrane system of staphylococcus aureus. *J. Electron Microscopy (Tokyo)* 16: 293-303.
- Schmidt, J. 1965a. Studies on staphylococci-hyaluronidase. VI. Investigations into connections between the ability to form hyaluronidase and other microbiological properties of staph. aureus. *Zentr. Bakteriolog. Parasitenk. Orig.* 197: 78-91.
- Smith, W., and J. H. Hale. 1944. Nature and mode of action of staphylococcus coagulase. *Brit. J. Exptl. Pathol.* 25: 101-109.
- Spittlehouse, K. E. 1955. Phage types of staphylococcus pyogenes isolated from impetigo and sycosis barbae. *Lancet.* 11: 378.
- Tager, M. and M. C. Drummond. 1965. Staphylocoagulase. *Ann. N. Y. Acad. Sci.* 128: 92-111.
- Tager, M. 1948. Concentration, partial purification, properties, and nature of staphylocoagulase. *Yale J. Biol. Med.* 20: 488-500.
- Tager, M. and M. C. Drummond. 1970. Implications of staphylocoagulase-thrombin and fibrinogen interaction. *Trans. Conf. Auspices Intern. Comm. Haemostasis and Thrombosis*, pp. 291-298.
- Tirunarayanan, M. O. 1968. Investigation on the enzymes and toxins of staphylococci. Study of phosphatase using p-nitrophenyl phosphate as substrate. *Acta. Pathol. Microbiol. Scand.* 74: 573-590.
- Victor, R., F. Lachica, K. F. Weiss, and R. H. Deibel. 1969. Relationships among coagulase, enterotoxin, and heat stable deoxyribonuclease production by Staphylococcus aureus. *Appl. Microbiol.* 18: 126-127.
- Vogelsang, T. M., A. Wormnes, and B. Ostervold. 1962a. Demonstration of staphylococcal enzymes by simple methods. *Acta. Pathol. Microbiol. Scand. Suppl.* 152: 313-314.
- Wallace, G. D., W. B. Quisenberry, and R. H. Tanimoto. 1962. Bacteriophage type 80/81. Staphylococcal infection in human beings associated with mastitis in dairy cattle. *Am. J. Public Health.* 52: 1309-1317.

- Wegrzynowicz, A., J. Jeljaszemicz, and B. Lipinski. 1969. Standardized procedure of determination of staphylocoagulase. App. Microbiol. 17: 556-558.
- Willis, A. T., S. I. Jacobs, and G. M. Goodburn. 1964. Pigment production, enzymatic activity and antibiotic sensitivity of staphylococci subdivision of pathogenic group. J. Pathol. Bacteriol. 87: 157-167.
- Zak, C., J. Jeljaszewicz and W. Kedzia. 1967. Metabolic and toxigenic activity of staphylococcus aureus isolated from blood and from healthy carriers. A comparative study. Zentr. Bakteriolog. Parasitenk. 204: 203-211.

THE EFFECT OF BIOTIN DEFICIENCY ON SOME
PROPERTIES OF STAPHYLOCOCCUS AUREUS ISO-
LATES FROM MAN AND ANIMALS

by

JAMES DOSU ADEKEYE

D.V.M., Ahmadu Bello University, 1972

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Infectious Diseases

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1975

Human and animal staphylococci release a number of active extracellular factors into their environment. These diffusable products, under defined conditions, are believed to have biologic effects on infected hosts. As a result of high correlation between production of these active substances and observed pathogenicity in man and animals, many attempts have been made to relate staphylococcal virulence to these factors.

Troublesome staphylococcal infections in both man and animals, coupled with the ubiquity of the organism and its resistance to many antibiotics, have caused an universal interest in controlling and eradicating it. These can only be achieved through a thorough understanding of factors controlling virulence and pathogenicity. In an attempt to contribute in this direction, this work was designed to investigate what effects lipid-depletion had on the ability of Staphylococcus aureus to produce coagulase, phosphatase, fibrinolysin and its response to anti-microbial agents and bacteriophages.

Staphylococcus aureus isolates from man and animals were lipid-depleted by growth in biotin assay medium containing 12 micrograms of biotin per liter. The same isolates were grown concurrently in media containing an adequate quantity of biotin and the two cultures were tested for the production of coagulase, phosphatase and fibrinolysin enzymes and for response to various anti-microbial agents and bacteriophages.

Lipid-depleted organisms produced less phosphatase, less coagulase, no fibrinolysin and were more susceptible to anti-microbial agents than normal organisms, but phage susceptibility was not greatly affected.

The reduced ability to produce coagulase by lipid-depleted organisms was more pronounced among human isolates than canine, bovine, or equine strains. Phosphatase production was more markedly reduced in human and canine strains.

The loss of fibrinolysin activity of lipid-depleted organisms was common to all isolates irrespective of source.

Susceptibility to various antibiotics was increased in the biotin deficient medium, except for neomycin. Lipid-depleted organisms were in fact less susceptible to neomycin than were normal cells. Lipid-depleted organisms recovered from human beings were more resistant to streptomycin and tetracycline than isolates from other animals. Lipid-depleted organisms were generally less susceptible to bacteriophage 77 than normal cells.