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The Effect of Growth Medium Vitamin Supplements on the Pathways of Glucose Catabolism and the Virulence of Staphylococci

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By

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LIFE

Charles Paul Bowling Jr. was born in Chicago, Illinois, on April 11, 1942. He resided in Chicago all of his life.

His major field in high school was a scientific-pre-engineering division. In June of 1960 he graduated from Mendel Catholic High School.

He entered St. Joseph's College, Rensselaer, Indiana, in the fall of 1960. After receiving his Bachelor of Science Degree in Biology in 1964 he was admitted to the Graduate School of Loyola University in Hines, Illinois, to begin his studies towards a Master's Degree in Microbiology.

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STATEMENT OF THE PROBLEM

The unique characteristics of Staphylococcus aureus have practically placed it on a separate pedestal when related to all other pathogenic bacteria. This has resulted in many investigations and more literature has been printed concerning Staphylococcus aureus than any other single organism (Shinefield and Ribble, 1965). Staphylococci are the microbes responsible for boils and carbuncles although they are also often associated with septicemia and pneumonia. Although many antibiotics are now available for the purpose of prevention and treatment of bacterial infections many hospital patients still die every year from Staphylococcus aureus infections (Ferrer and MacLeod, 1960; Minchew and Cluff, 1961). Among the reasons for these fatalities is the fact that staphylococci possess an amazing ability to yield antibiotic resistant mutants (Zinsser, 1965). Other factors contributing to their association with hospital acquired infections are the increasing number of diagnostic and therapeutic procedures employed in hospitals which facilitate the introduction of staphylococci into patients, and the high rate of human carriers who make sterile surgical operations almost an impossibility (Elek, 1959; Shinefield and Ribble, 1965).

The virulence of staphylococci for a given host is associated with a number of different biological properties rather than with

any single substance produced by the organism. In general, the action of coagulase, alpha-hemolysin, and leukocidin are considered as the most important staphylococcal products associated with staphylococcal virulence (Shinefield and Ribble, 1965). Yet certain staphylococcal mutants not possessing either bound or free coagulase are just as virulent as their "wild types" (Li and Kapral, 1962). Also no alpha toxin production has been found in about 4% of the staphylococci isolated from lesions (Lack and Wailling, 1954), and at least 25% of pathogenic strains of Staphylococcus aureus from human lesions have been found to be very weak in leukocidin production (Panton and Valentine, 1932).

There are a number of findings in the literature which suggest that virulent and avirulent strains of staphylococci differ somewhat in their metabolic activity. For example, the biochemical activity of strains isolated from pus and sputum are greater than those isolated from the nasopharynx of normal individuals (Krynski et al., 1962). Ivler (1965) studied the comparative metabolism of coagulase positive and coagulase negative staphylococci. He found that resting cell suspensions of "virulent" coagulase positive strains yielded a higher Q_{O_2} and utilized some of the free amino acids in the pool as a primary source for endogenous respiration, whereas "avirulent" coagulase negative strains had a relatively low Q_{O_2} and did not use the free amino acid pool for endogenous respiration. However all coagulase negative strains associated with endocarditis were not the same. Twelve relatively rare strains associated with this disease were found to

have biochemical properties comparable to coagulase positive strains. Evans (1948) compared the growth requirements of a large number of coagulase positive and negative staphylococci. In addition to finding that nicotinic acid and thiamine were both required as essential growth factors for staphylococci, he discovered that biotin also was needed by the coagulase negative strains. Also it was shown that different levels of acid phosphatase are produced by particular phage groups, with strains of the "80/81 complex" producing the highest levels and coagulase negative strains the lowest levels (Blumenthal and Pan, 1963). Acid phosphatase can potentially exert a strong influence on the course of metabolic reactions in the cell (Kuo and Blumenthal, 1961), although this has never been proven.

Even from this limited review it appears that no single factor can be attributed as the sole agent responsible for staphylococcal virulence. The capacity of a strain to induce infection is derived from the sum total of all properties at its command (Blair, 1962).

The primary aim of this thesis is to investigate the effect that alterations in the glucose catabolism of certain Staphylococcus aureus strains have on the virulence of these strains in the testes of mice. Such a study is possible because of the recent findings that the pathways can be altered by adding vitamin supplements to the culture medium (Montiel and Blumenthal, 1965) as discussed in the next chapter.

The measure of staphylococcal virulence depends on the host

and the route of infection. In this work virulence was analyzed by the technique of mouse testis inoculations (Blumenthal, 1964). Nine days after the inoculations the testes were removed for a macroscopic virulence grading. Since the degree of testicular involvement can easily be observed by this method, it has more sensitivity than those measures of virulence based solely upon a lethal or non-lethal grading.

History

As glucose occupies a key position in the metabolism of living organisms the degradation of this sugar and subsequent diversion of the metabolic intermediates into energy-yielding reactions by terminal respiratory processes is quite important to the cell's economy. Several pathways for both glucose catabolism and terminal respiration are available to the cell.

Staphylococcus aureus, in common with many other microorganisms, metabolizes glucose, aerobically or anaerobically, by both the Embden-Meyerhof (E-M) and hexose monophosphate (HMP) pathways (Strasters and Winkler, 1963; Pan and Blumenthal, 1962). Under anaerobic conditions the pyruvate formed glycolytically is converted to lactate. However, under aerobic conditions the tricarboxylic acid cycle functions and glucose is almost completely oxidized to CO_2 and H_2O (Powelson, Wilson, and Burris, 1947; Stedman and Kravitz, 1955; Elek, 1959). No evidence for the operation of the Entner-Doudoroff pathway of glucose catabolism has been obtained in staphylococci (Strasters and Winkler, 1963).

In the first chapter the idea of promoting an alteration in the pathways of glucose catabolism with vitamins was mentioned. Kligler, Grossowicz, and Bergner (1943), showed that nicotinic acid was essential for glucose metabolism by Staphylococcus aureus but that thiamine also had an effect. They suggested that

nicotinic acid was involved in glycolysis while thiamine was needed for the aerobic breakdown of pyruvate formed via glycolysis. Smyth (1940), also demonstrated that thiamine was involved in the tricarboxylic acid cycle (TCA). According to Hughes (1954), nicotinic acid was converted to nicotinamide by Staphylococcus aureus, which then formed diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN).

With the ready availability of differently labeled ^{14}C-glucose it became possible to devise quantitative procedures of the analysis of the pathways glucose traverses in the intact cell. The procedure used in these studies is based on yields of $^{14}CO_2$ from glucose-1-^{14}C and -6-^{14}C and will be described in a later section. In addition to estimating the percentage of glucose utilized via the E-M and HMP pathways, the yield of $^{14}CO_2$ from glucose-6-^{14}C also estimates the relative extent of operation of the TCA cycle (Blumenthal, 1965). This is due to the fact that C-6 of glucose traversing the E-M or HMP pathways does not appear as CO_2 until it has passed through an operative TCA cycle several times.

Using these techniques Montiel and Blumenthal (1965) demonstrated that the addition of nicotinic acid to Trypticase growth medium (2 mcg/ml) markedly stimulated the HMP pathway and reduced the E-M pathway for glucose utilization while thiamine stimulated the TCA cycle. Since it had been recognized earlier that the addition of glucose to a growth medium suppressed the operation of the TCA cycle (Collins and Lascelles, 1962; Strasters and Winkler, 1963) glucose was omitted from the medium in these experiments.

Thus the operation of the HMP and TCA cycles could be independently turned "on" or "off" to different degrees. More recently it has been observed that the TCA cycle will not become operative unless there is a small amount of nicotinic acid present (Montiel and Blumenthal, unpublished results).

The effects of vitamin additions can not be completely explained on a coenzyme basis since both DPN or TPN are formed from nicotinic acid in staphylococci. In the HMP cycle TPN is a necessary cofactor for two reactions although DPN is also needed. However, in the E-M pathway only DPN is used as a coenzyme. Thiamine is also involved in more than one catabolic pathway as its pyrophosphate. In the HMP pathway it acts as a coenzyme in the transketolase reaction while in the TCA cycle, thiamine pyrophosphate is required in reactions involving the entrance of pyruvate into the TCA cycle.

MATERIALS AND METHODS

A.) Mouse Virulence Assay

Housing of Mice: In order to determine if the virulence of various strains of Staphylococcus aureus had been altered by the addition of vitamins to the growth media, the mouse testis virulence assay (Blumenthal, 1964) was adopted. Male Swiss albino mice were used with an average weight of about 25 g. They were obtained from Abrams Small Stock Breeders in Chicago. Once the mice had been inoculated for a particular experiment, they were placed in small plastic cages in groups of ten mice per cage. Originally these mice which had been divided into the plastic cages for the experiment were maintained in the animal room from the time of inoculation until they were killed. However, it was found that during the fall months there were too many temperature fluctuations in the animal room. In later experiments, the mice were placed in groups of ten per plastic cage and the cages were stored in a vacant laboratory having a relatively constant temperature of 75° F. The mice received Rockland Mouse and Rat pellets and tap water ad libitum.

Growth of Stock Cultures: The stock cultures used in these experiments were maintained on 4% Trypticase Soy Agar slants (BBL) in a refrigerator, and were transferred to fresh slants every two

months. Cultures were grown for 24 hr at 37° C and then refrigerated. For the growth of staphylococci employed in the daily experiments, slants no more than one week old were used. To obtain these slants a loopful of staphylococci was transferred from the monthly stock cultures to fresh slants. These later slants were originally only Trypticase Soy Agar, but in more recent experiments 5% human or citrated fresh whole rabbit blood slants were used with Trypticase Soy Agar 4% as the base. The various strains of staphylococci were grown at 37° C in screw cap tubes.

Stock Culture Strains: The strains of staphylococci used are given in Table 1. The Towler strain was used for the majority of the virulence experiments.

Preparation of Liquid Media: Trypticase (BBL) or Vitamin-Free Casitone (Difco) were prepared in a concentration of 2% (W/V) in distilled water. The media had a pH of 7 after autoclaving for 15 min at 120° C. Prior to autoclaving, the media was dispensed into separate specially washed 250 ml Erlenmeyer flasks in aliquots of 100 ml per flask. These flasks were washed in boiling distilled water containing 2% (V/V) Fl-70 Concentrated Biodegradable Detergent (Fisher). The flasks were then rinsed at least three times in distilled water after cleaning. Once the medium was autoclaved and cooled, it was then inoculated with vitamins and bacteria.

Preparation of Vitamins: Nicotinic acid (Merck), thiamine-hydrochloride (Merck), and nicotinamide (Sigma) were the vitamins used.

To avoid any possibility of degradation by heat sterilization, all of the vitamin solutions were sterilized by filtration and added to the medium only after it had been autoclaved and cooled. The vitamins were prepared in distilled water in a stock concentration of 0.2 micrograms per milliliter (mcg/ml). When used for intraperitoneal injections, 0.9% saline replaced the distilled water. Once in solution a syringe was filled separately with each of the vitamins, a sterile Millipore "Swinnex" filter with a pore size of 0.45 microns was then placed on the tip of the Luer-lok syringe, and the vitamin solutions were filtered into separate sterile screw capped tubes. The sterile stock vitamin solutions were stored in the refrigerator for no longer than one month.

Syringes: The "Agla" micrometer syringes (Burroughs-Wellcome Co.) were used for inoculating the testes of the mice. The glass syringes were frequently sprayed with silicone Antifoam-A Spray (Dow-Corning) to maintain smooth movement and prevent air leaking around the plunger. The syringes were fitted with 27 gauge needles which were kept sharp and were specially slanted to about 30° from the horizontal plane to reduce even more any possible tissue damage resulting from these inoculations. The syringes and needles were individually sterilized by autoclaving them in 150 mm glass test tubes.

Preparation of Diluent: Saline (0.9%, W/V) and 0.2 M potassium phosphate buffer, pH 7.0, were prepared and autoclaved separately.

The buffer was added to the saline to give a final concentration of 5% V-V buffer. Then 0.33 ml of 30% bovine serum albumin (Nutritional Biochemical Corp., Cleveland, Ohio), was added to each 100 ml of buffered saline solution and this complete albumin-phosphate-saline (APS) was stored in the refrigerator.

Harvesting Cells: The bacterial cells were harvested after growth for 17 hr on a rotary shaker (New Brunswick Scientific Co.) at 37° C and 240 r.p.m. A sample (usually 10 ml) was removed from a given flask and pipetted into a capped sterile 50 ml plastic centrifuge tube. Each tube was then centrifuged in a cold room at 10,000 x g for 10 min in a Servall SS-1 high speed centrifuge. The supernatant fluid was then decanted and the cells were kept in the refrigerator until they were standardized and diluted. This was ordinarily for a period of less than one hour. The packed cells were resuspended in 5 ml of APS diluent with the aid of a 5 ml sterile pipet and mixed for one minute with a Vortex Jr. mixer. These diluted suspensions were maintained in the refrigerator in the centrifuge tubes until they were standardized.

Cell Standardization: Staphylococcal suspensions were standardized turbidimetrically using a Klett-Summerson Colorimeter with a 600 mμ red filter. Suspensions were generally standardized at 65 Klett units, using distilled water as a blank. Since the control cultures without added vitamins had a slightly lower percentage of viable cells than cultures with added vitamins, they were adjusted to 70 Klett units to compensate for this deficiency.

Dilutions: Each standardized staphylococcal suspension was then diluted serially to 10^7 to prepare the inoculum and to determine the viability by plate counts. To a sterile screw-capped culture tube containing 4.5 ml of APS was added 0.5 ml of the standardized cell suspension. This 10-fold dilution was mixed on the Vortex Jr. for 0.15 min and 0.5 ml was removed for the next dilution, etc. Sterile disposable 1 ml plastic serological pipets containing a cotton plug (Falcon Plastic No. 7056) were used for making the 0.5 ml transfers.

Viable Plate Count Method: Viable plate counts were performed with the 10^5, 10^6, and 10^7 dilutions of each dilution set. The Miles and Misra (1938) dropper method was used for this procedure. The end of a sterile 1 ml syringe was fitted with a rubber bulb instead of a syringe plunger to increase sensitivity of action. The entire assembly had previously been calibrated and found to deliver 55 drops per ml. The sterile syringe was boiled in distilled water to sterilize it between fillings of different dilutions. The hot syringe was inserted into the particular dilution tube to be counted, cooled by alternately drawing in and flushing out the culture three times, then some of the culture for the particular dilution was drawn into the syringe, the syringe was held vertically over a sterile petri plate containing sterile Trypticase-Soy agar and one drop was allowed to fall in a marked spot on the plate. The agar plates were dried overnight before use in a 37° C incubator. The 10^5, 10^6, and 10^7 dilutions were each added

in duplicate on the same petri plate. The plates were incubated 24 hr at 37° C before the colonies were counted.

Mouse Inoculation Procedure: Using the mouse testis virulence procedure (Blumenthal, 1964) previously mentioned, merely requires putting all of the afore stated procedures in a proper order. The appropriate strain of Staphylococcus aureus was inoculated from a fresh Trypticase Soy Agar slant or a rabbit blood agar slant into a 250 ml Erlenmeyer flask containing 100 ml of either 2% Vitamin-Free Casitone (Difco) or 2% Trypticase (BBL). This starter flask was placed on a rotary shaker for 5 hr. Each of several fresh 250 ml flasks containing the same media as the starter flask were inoculated with 1 ml of the culture. At this time, the filter-sterilized vitamin solutions were also added into the flasks, and the flasks were replaced on the incubator shaker for 17 hr.

After 17 hr 10 ml was removed from each flask and placed in separate capped, sterile centrifuge tubes and centrifuged for 10 min at 10,000 x g. The supernatant fluid was then decanted, and the cells were resuspended in 5 ml APS with the aid of the Vortex mixer for 1 min. Each cell suspension was then separately standardized as described earlier.

Following standardization each suspension was diluted 100-fold in APS using two 10-fold dilutions as previously described. The tubes containing the 10^2 dilution were placed in the refrigerator and were used for carrying out the dilutions to 10^7 later in the experiment. A group of ten male Swiss mice were individually

anesthetized in a jar containing ether, and a cap containing an ether saturated pad was placed over each mouse's nose to keep them quiet during the inoculation. Using the "Agla" micrometer syringe the mice were inoculated in each testis with 0.01 ml of one of the 10^1 dilutions of cells from a particular dilution set. Generally 10 mice per group were always used. The mice were placed in plastic cages, one group per cage, for nine days. They were then killed with ether, weighed, and the testes were excised and visually graded. They were then placed into test tubes containing a 1:4 dilution of 40% formaldehyde in saline. The number of testes containing any lesions within each group was recorded and the extent of the destructive lesion was graded as will be explained. The testes were preserved since microsectioning and viewing of the testes for damage of tissue had been considered but was not performed.

After the mice were inoculated, the 10^2 dilutions kept in the refrigerator were remixed for 0.15 min each on the Vortex mixer, and diluted to 10^7. Using the Miles-Misra (1938) dropper method, the 10^7, 10^6, add 10^5 cell dilutions of each series of dilutions were plated. They were counted as viable colony forming organisms the following day after incubating 37° C overnight. The testes inoculum was usually around 5×10^5 viable cells as assayed by this procedure.

Testes Grading System: The testes were first graded according to the number of testes infected to any degree in each group of mice.

A figure such as 10/20 means that 10 of the 20 testes showed some degree of abnormality. This fraction of 10/20 was converted into a percentage, i. e. 50%. The term "degree of infection" was another converted ratio. The visual morphological extent of infection for each testes was graded from 1-4, depending on the number of quarters of the testes with macroscopic pathological involvement, where 4 was considered to be a completely infected testes. The average number of quarters of damaged testes divided by four times the percentage of infected testes gave the value for the degree of infection:

$$\text{Degree of infection} = \frac{\%\ \text{testes infected}}{} \times \frac{\text{avg. no. of quarters infected}}{4}$$

Thus if all of the testes were infected to an extent of 4, the 50% value derived for the number of testes infected and the degree of infection would be the same. If only they averaged 2 quarters, then the number infected would be still 50% but the degree infected would be 25%.

Significance of Virulence Results: The "P values" presented in Tables 3, 5, and 6 were calculated according to the procedures of H. Batson (1961).

B.) Growth Curve Experiments

Turbidity Readings: Turbidities were measured with a Klett-Summerson Colorimeter provided with a 600 mμ filter. Distilled water was used for the blank, since it was found to be almost identical in optical density to the inoculated media at this wavelength, and did not undergo a color change as did stored sterile media blanks.

Nephelometer Flasks: The nephelometer flasks were 300 ml Pyrex Erlenmeyer flasks with a glass sidearm tube joined to the neck region of each flask in such a way that the tube extended outward at a 60° angle from the vertical centerline. This sidearm arrangement was merely a modification of standard available nephelometer flasks with the arm protruding in a different direction. When placed on the shaker with the sidearm in this position the 100 ml of media used in all of these experiments could not flow into the sidearm and cause a non-uniform environment. The turbidity measurements were performed rapidly in the Klett colorimeter so that the bacterial cells were exposed to an environmental change for only a brief period of time.

Preparation of Media: A starter culture flask was innculated with a loopful of the desired staphylococcal strain obtained from a fresh stock slant. The starter culture medium was either Trypticase (BBL) or Vitamin-Free Casitone (Difco) made at a con-

centration of 2% (W/V), dispensed into specially cleaned 250 ml Erlenmeyer flasks 100 ml per flask, and autoclaved. Once inoculated these flasks were incubated at 37° C on the rotary shaker overnight.

The following morning the starter cultures were removed from the shaker and 1 ml samples were used as an inoculum for 100 ml of the same medium in a nephelometer flask. Filter sterilized vitamin solutions were added to the nephelometer flasks when required at the time of inoculation. The flasks were placed on the rotary shaker at 37° C, and removed periodically for the turbidity measurements. The readings were usually terminated after about 25 hr.

Vitamin Content of Media: The vitamin assays presented below were obtained from the respective companies and converted to the 2% (W/V) concentrations used in this research. They represent the base vitamin content of the media before it was supplemented with additional filter sterilized vitamins as required for performing the experiments contained herein. Casitone (Difco) medium is included only as a comparison with the vitamin content of the Vitamin-Free Casitone.

Trypticase (BBL) - Typical Assay

Nicotinic Acid	0.16 mcg/ml
Thiamine	+ Not Given
Biotin	0.00166 mcg/ml

Vitamin-Free Casitone (Difco) - Our Lot Number

Nicotinic Acid	0.0132 mcg/ml
Thiamine	0.000132 mcg/ml
Biotin	? Not Known

Casitone (Difco) - Typical Assay

Nicotinic Acid.....	0.48 mcg/ml
Thiamine.....	0.01 mcg/ml
Biotin.....	0.006 mcg/ml

C.) Analyses of Glucose Catabolism

Culture Preparation: Duplicate flasks were usually inoculated with 1 ml of a 5 hr starter flask, as previously described, in order to obtain the larger cell quantities needed for the metabolic experiments. The desired vitamins were again added and these flasks were then incubated for 17 hr at 37° C on the rotary shaker. The bacteria were harvested by centrifugation at 10,000 x g for 10 min in the cold and washed twice with, and suspended in, cold 0.1 M potassium phosphate buffer, pH 7.0. Each suspension was then adjusted to the same turbidity; usually a 1:10 dilution of the cell suspension read 180 Klett units (about 3 mg dry wt/ml) if the cell yield was sufficient.

Metabolic Flask Inoculations: Employing a 1 ml long tip pipet 0.5 ml of 0.5 M glucose-1-^{14}C (about 60,000 counts/min) was added to the main compartment of each of two special 50 ml Erlenmeyer metabolic flasks. These metabolic flasks contained a center-well capable of accepting a small vial and keeping it isolated from the surrounding flask contents. In a similar manner, glucose-6-^{14}C was added to two additional flasks. One ml of 0.1 M pH 7.0 potassium phosphate buffer was then added to the main compartment of all flasks using a long tip 5 ml pipet. For each metabolic flask in the experiment two serological tubes were prepared each containing 2.5 ml of 10% (V/V) perchloric acid. These were used for the quantitative determination of glucose in the supernatant fluid em-

ploying an anthrone procedure (Seifter et al., 1950).

The appropriate standardized cell suspension was then added to the main compartment of each metabolic flask at 2 min intervals. During each time interval the glucose-bacterial mixture was swirled about 10 sec, and then 0.5 ml was removed, using a separate 1 ml long tip pipet for each flask, and added to one of the serological tubes containing perchloric acid for the zero hour glucose determinations. Then a glass vial containing folded Whatman No. 1 filter paper to increase CO_2 absorption was added with a forceps to the center-well vial holder. Each flask was immediately sealed with a serum stopper and placed in a Dubnoff metabolic shaker at 37° C. After all of the flasks had been started, a 1 ml syringe with a long 22 gauge needle was used to add 1 ml of a CO_2 absorbing solution into each center-well through the rubber serum stopper. This was a solution containing 1:2 (V/V) ethanolamine and ethylene glycol (Sol A). The flasks were allowed to incubate while shaking for 2-3 hr.

Liquid Scintillation Counting of $^{14}CO_2$: When the incubation period was completed the flasks were removed at timed intervals in the same order as they were put into the bath. As each flask was removed, the stopper was taken off, the center-well vials along with their contents (trapped CO_2, Sol A, and paper) were carefully placed in scintillation vials. At this time another 0.5 ml sample of the incubation fluid was removed and added to the second serological tube containing perchloric acid.

After all the scintillation vials had received their appropriate center-well vials, 1 ml of Sol A was then added to each vial. Then 10 ml of fluor (Sol B; 1:2:0.181 ethylene glycol-toluene-diphenyloxazole V/V/W), was added to each scintillation vial from a 500 ml dispensing flask fitted with a calibrated 10 ml delivery tube (Jeffay and Alvarez, 1961). A background blank scintillation vial was always included consisting of a non-incubated center-well vial with paper, 2 ml of Sol A, and 10 ml of Sol B. Plastic screw cap tops were then placed on the scintillation vials, they were well mixed, and placed in a Packard Tri-Carb liquid scintillation spectrometer, Model EX, for counting the amounts of $^{14}CO_2$ produced.

Determination of Glucose Utilization: Following removal of the bacterial cells by centrifugation, glucose was determined by an anthrone method (Seifter, et al., 1950). Standard curves employing 15-150 mcg of glucose were prepared each time.

Calculation of Glucose Catabolic Pathways: From the combined information of the amount of radioactive $^{14}CO_2$ present as either ^{14}C-1 or ^{14}C-6 metabolic by-products along with the quantity of glucose metabolized from the flasks containing ^{14}C-1 glucose vs ^{14}C-6 glucose then the percentage of glucose metabolism via the E-M pathway and the HMP was determined using the basic mathematical formulae devised by Wang et al. (1958) and as modified slightly by Blumenthal (1965).

The calculations are based on the following information. When glucose -1-^{14}C is metabolized via the HMP pathway, there is an early loss of glucose C-1 as $^{14}CO_2$ whereas glucose-6-^{14}C does not yield $^{14}CO_2$ directly through this pathway. On the other hand, when glucose-1- or -6-^{14}C is utilized via the E-M pathway there is a symmetrical cleavage of the intermediate fructose-1,6-diphosphate yielding two triose phosphate molecules equilibrated by triose phosphate isomerace, hence making glucose C-1 and -6 equivalent. Before either C-1 or C-6 will appear as CO_2, they must be oxidized via the TCA cycle. The fact that C-6 of glucose traversing the E-M or HMP pathways does not appear as CO_2 until it has passed through an operative TCA cycle several times can also be used to estimate the relative extent of operation of the TCA cycle.

RESULTS

A.) Mouse Inoculation Experiments

Testis Inoculum: It would be difficult to list the results of each individual experiment, since they number in the forties for the nicotinic acid and somewhat less for thiamine. If the testes inocula of staphylococci were averaged they would range around 5×10^5 viable organisms per testis. Inocula for individual experiments will deviate from this mean, but when related to the particular control inoculum there was generally little deviation.

Nicotinic Acid Effects: In Table 3 there is listed a comparison of the number and degree of testes infected following inoculation with staphylococci grown on 2% Trypticase or Vitamin-Free Casitone versus the same values for those testes inoculated with staphylococci grown in the media fortified with total additional vitamin supplements of 1-4 mcg/ml nicotinic acid. In all cases the vitamins were present throughout the period of growth. The data were obtained merely by averaging the individual percentages as calculated previously (Table 2). It should be noted that for every individual percentage included in the nicotinic acid averaged data the corresponding percentages for the particular control were included in the control averaged data.

From the results of experiments with cells grown in the

Trypticase medium, it appears that the addition of nicotinic acid to this medium resulted in formation of staphylococci with a slightly enhanced 12% ability to infect mouse testes. However, the degree of infection increased even more significantly under these conditions to 19.7% over the control.

With the Vitamin-Free Casitone grown cells there was actually a slight decrease in both the number of infected testes and the degree of infection when nicotinic acid was added.

Thiamine Effects: The data for the effect of the addition of thiamine to the virulence of cells grown either on Trypticase or Vitamin-Free Casitone are presented in Tables 4 and 5. It should be noted that data are given for thiamine additions to the first lot of 2% Trypticase as well as the second. It was unfortunate that more experiments were not performed using this older batch (lot number) with thiamine, since a marked lessening of virulence seemed to occur with this vitamin. There was a 43% virulence reduction in the number of testes infected and the degree of infection with thiamine-supplemented Trypticase. When the second batch of Trypticase was used, there was about a 32% increase in both the number of testes infected and the degree of infection when the medium was supplemented with thiamine. With the vitamin-free media a 22% lowering of virulence, in both number and degree of testes infected, was noted in the presence of thiamine.

Influences of Vitamin Concentrations: The data presented in

Table 6 was an analysis of the effects of individual concentrations of specific vitamin additions to the media. Only the experiments in which the staphylococci were cultured in regular Trypticase were included in this analysis. Only thiamine concentrations of 1 mcg/ml displayed a virulence inhibition. However, since only this concentration was used when the old lot number of media was involved, it seems likely that the media was more responsible for this difference than the concentration. At a 4 mcg/ml thiamine concentration the virulence was increased about 35%. However, the number of experiments are not sufficient to make definite conclusions.

There appears to be some effect by nicotinic acid which is concentration dependent. Nicotinic acid added at a concentration of 1 or 4 mcg/ml had no effect upon the number of infected testes, but the degree of infection was increased insignificantly about 10% when compared with the controls. When 2 mcg/ml of nicotinic acid was present the number of infected testes increased about 27% over the controls while the degree of virulence increased 30%. These data suggest that there may be an appropriate combination of both nicotinic acid and thiamine that is capable of increasing staphylococcal virulence. This is dependent not only on the vitamin added, but also on the somewhat variable vitamin content of the different lots of this pancreatic digest of casein.

Virulence Influenced by Host's Vitamin Balance: Table 7 presents the results of two experiments measuring the effect of intraperi-

toneal administration of thiamine or nicotinic acid on the virulence of a standard dose of staphylococci for the mouse testis. Three groups of ten mice were inoculated with one of the following: 0.5 ml of 0.9% sodium chloride, 100 mcg of nicotinic acid in saline, or 100 mcg of thiamine in saline. The mice were given these inoculations intraperitoneally a total of six times in a seven day period. On the eighth day, all of the mice were given an intratesticular inoculation of Staphylococcus aureus strain Towler that had been grown in 2% Trypticase. The mice injected with nicotinic acid were found to have almost a 50% higher incidence and degree of infection than either those control mice injected with saline or those mice injected with thiamine. Since all mice were injected with the same cell suspension, these results suggest that the nutrition of the host, in this case altered by vitamin supplementation at a site other than the challenge site, can also affect the outcome of a challenge by a given number of staphylococci.

B.) Growth Curves

Purpose and Conditions: The main interest was to discover whether differences in growth rate might occur when one or more vitamins were added to the growth medium. These studies were of interest because of the findings (elsewhere in the thesis), that the carbohydrate metabolic pathways were modified considerably by the amount of nicotinic acid or thiamine in the medium and that these vitamins also had some effect on the virulence of staphylococci. Various strains of coagulase positive Staphylococcus aureus were used for these growth curves utilizing the procedures indicated in the section on Materials and Methods.

Growth of Staphylococci in Trypticase: Figure 1 presents the results of a typical general composite of the growth curves performed on several different coagulase positive strains. It can be seen that nicotinic acid and nicotinamide produce almost identical growth curves, with the nicotinamide generally capable of producing a slightly higher rate and extent of growth. This was probably due to the fact that nicotinic acid was converted to the amide form in metabolism, and the elimination of this reaction step with the presence of preformed nicotinamide reduced somewhat the generation time. Both vitamins appeared to produce a faster rate of cell multiplication in the log phase as compared to the control. It should be noted, however, that both control cultures and those grown in nicotinic acid or amide supplements reached the station-

ary phase at about the same time. This stationary phase was generally quite level in both instances.

The action of thiamine, on the other hand, was quite different from nicotinic acid and nicotinamide (Figure 1). Usually the addition of thiamine produced an initial log phase almost identical or lower than that produced by addition of nicotinic acid. However, with thiamine there never really was a true stationary phase in 24 hr. After 24 hr readings the difference in turbidity between flasks supplemented with thiamine and those supplemented with nicotinic acid was about 60 Klett units.

The Trypticase medium was chosen because it had a low nicotinic acid content of 0.16 mcg/ml. Although thiamine was present, its content was not listed in the assay (BBL). In certain experiments the effects of varying vitamin concentrations were tested. No significant differences in growth curves were found between bacteria grown in 1 mcg/ml or 4 mcg/ml of nicotinic acid. These findings were also applicable to the same variations in the media concentration of added thiamine.

Vitamin Supplemented Vitamin-Free Casitone: A typical growth curve is displayed in Figure 2 representing sixteen experiments using Vitamin-Free Casitone. This medium and Trypticase are both pancreatic digests of casein, but the "vitamin-free" medium contains about a 100-fold reduction in the content of nicotinic acid and thiamine. This typical growth curve obtained with thiamine added in amounts of 1 mcg/ml to 4 mcg/ml, produced no significant dif-

ference in growth rate over non-supplemented controls. The addition of nicotinic acid, on the other hand, produced a large increase in the growth rate in the mid-log phase causing the cells to reach a stationary phase roughly equivalent in turbidity to a control culture grown in regular Trypticase. Thus the content of nicotinic acid appears to be the growth limiting factor in this medium. The growth curve obtained was not significantly different when 1-4 mcg/ml of nicotinic acid were added .

Differences in Stock Culture Slants: Since the stock cultures were originally maintained on 4% Trypticase Soy agar slants, but later were grown on 5% human blood-4% Trypticase Soy agar slants in an attempt to increase the virulence of the strains, an effort was made to determine if this change caused any noticeable difference in the growth rate. For these experiments one starter culture originated from a Trypticase Soy Agar slant and a second from the culture grown on the blood agar slant. These cultures were then transferred to sidearm flasks containing vitamins and either 2% Trypticase or 2% Vitamin-Free Casitone. The results obtained showed no significant differences in the growth response of Towler strain under the conditions tested.

Balanced Supplemented Media: By this time other types of experiments had demonstrated that the relative concentrations of nicotinic acid and thiamine markedly affected the results obtained. Consequently, an experiment was performed to determine how the

levels of the two vitamins affected the growth rate. A starter culture of the Towler strain was grown in Vitamin-Free Casitone and used to inoculate three duplicate sets of nephelometer flasks: controls, flasks containing 2 mcg/ml nicotinic acid and 0.1 mcg/ml thiamine, and flasks containing 2 mcg/ml thiamine and 0.1 mcg/ml nicotinic acid. From these results, presented in Figure 3, it can be seen that when the proper proportions of nicotinic acid and thiamine were added to the medium excellent growth for this strain was obtained. The addition of high levels of nicotinic acid and low levels of thiamine appeared to result in somewhat better growth than did the addition of high levels of thiamine and low levels of nicotinic acid. However, by referring to Figures 2 and 3, it can be seen that the addition of thiamine alone did not stimulate staphylococcal growth in the Vitamin-Free Casitone. However, the addition of as little as 0.1 mcg/ml of nicotinic acid to the thiamine supplemented medium resulted in a profound stimulation of growth.

C.) Glucose Catabolism Analyses

Effectiveness of the Method: Since the main purpose in using vitamin supplements in the growth media was to alter the pathway of glucose catabolism to see if the virulence could be altered, it was necessary to test the effectiveness of this method. Since Staphylococcus aureus strain Towler was used for the majority of the virulence assays, this strain was also tested metabolically more than others. To make the experiments more complete and meaningful I have included some of the results obtained by Dr. F. Montiel (unpublished) whose general procedure for these experiments I followed completely. His data are averages of many more experiments than mine.

Glucose Catabolism in Non-supplemented Trypticase: Typical data from experiments in which the various strains of staphylococci listed had been grown in 2% Trypticase without any added vitamins are presented in Table 8. The coagulase-positive strains metabolized about two thirds of the glucose by the EM pathway and one third by the HMP. The coagulase-negative strain (Cook), seemed to have a somewhat more active TCA cycle than the coagulase-positive strains.

Catabolism in Vitamin Supplemented Trypticase: Table 9 contains the results of experiments showing what occurred to the glucose metabolism of staphylococci grown in this same 2% Trypticase

medium supplemented with vitamins. The glucose catabolism via the HMP pathway increased almost two-fold when nicotinic acid was added to the Trypticase medium while the relative increase exhibited in the amount of glucose metabolized via the TCA cycle (percentage yield from ^{14}C-6) was small. When thiamine was added to this growth medium in final concentrations of 1 to 4 mcg/ml, the HMP did not increase significantly (compared with the results in the previous table) whereas the activity of the TCA cycle was stimulated to a relatively high degree. Thus thiamine supplementation of the Trypticase medium yielded cells with a much higher degree of TCA cycle activity.

Vitamin Supplemented Vitamin-Free Casitone: Table 10 presents a larger combination of results related to the addition of vitamin supplements to various strains of staphylococci grown in 2% Vitamin-Free Casitone. The results indicate that in this medium cells grown on the minimal concentrations of thiamine and nicotinic acid which were present have a very low metabolic rate and a markedly decreased utilization of the HMP catabolic route. The addition of nicotinic acid to the medium increased the HMP about five-fold but still left the TCA cycle activity very low. The addition of thiamine to this medium produced only a very small increase in the TCA cycle activity and about a two-fold increase in the HMP.

Balanced Media Catabolism: These latter results with thiamine

present in 2-4 mcg/ml seemed very unusual since thiamine increased the TCA cycle activity when added to Staphylococcus aureus cultures grown in Trypticase media. It was postulated that a certain level of nicotinic acid must be present in order for thiamine to increase the TCA cycle activity, and that this level had not been attained with the Vitamin-Free Casitone medium. Then an experiment was performed in which 0.1 mcg/ml of nicotinic acid (total added concentration) was added along with the regular 2 mcg/ml added concentration of thiamine to the Vitamin-Free Casitone medium. The results of this experiment (Table 10) showed an intermediate level of glucose utilization via the HMP pathway but almost a six-fold increase in the level of TCA cycle activity.

In the last group of experiments in Table 10 the nicotinic acid concentration was increased ten-fold over the thiamine. That is to say, 1 mcg/ml of nicotinic acid was combined along with 0.1 mcg/ml thiamine in the same Vitamin-Free Casitone medium. The results of these two experiments indicated a somewhat lower HMP than was usually obtained with nicotinid acid, but an increased TCA cycle activity equal to that obtained when thiamine was present in a ten-fold higher concentration as previously discussed. These two experiments suggest that both thiamine and nicotinic acid are required for an active TCA cycle and that this cycle can function only minimally if either vitamin is not present in adequate amounts.

DISCUSSION

Elek (1959) has stated that "... the nature and role of the agents enabling staphylococci to survive and multiply in human tissues are ill understood. In all probability no single factor is of overriding importance. The virulence of staphylococci is due to a combination of factors, but their relative importance cannot be assessed until more is known about the nature of tissue reaction sterilizing a subclinical infection."

In the present study, an attempt was made to determine whether the relative degree of operation of the E-M pathway, the HMP pathway, and/or the TCA cycle by living staphylococcal suspensions was in any way associated with the virulence of individual staphylococcal strains in the mouse testis. Such a study became possible with the finding (Montiel and Blumenthal, 1965; Tables 8 to 10) that the relative degree of operation of these pathways could be modified in a major manner by controlling the amount of nicotinic acid and/or thiamine in the growth medium. The effect of nicotinic acid on the growth, metabolism, and virulence of staphylococci has been the most dramatic. When nicotinic acid was added to the Trypticase growth medium, which is a tryptic digest of casein without any additional carbon source added, the degree of utilization of glucose via the HMP pathway by the staphylococcal strains was stimulated about 30% with a concomitant decrease in

the utilization of the E-M pathway. Under these conditions, the HMP pathway replaced the E-M pathway as the major glucose pathway. The degree of activity of the TCA cycle, however, remained unchanged (Table 8). How did this physiological change effect the virulence of the staphylococci?

The results of a series of experiments involving a number of different strains indicated that with the addition of nicotinic acid there was a 12% increase in the number of testes infected when equal numbers of viable staphylococci, grown on control or nicotinic acid-supplemented Trypticase, were injected intratesticularly. Moreover, the average degree of pathology in the testes infected by the nicotinic acid supplemented staphylococci increased 20% over the control (Table 3).

The importance of the host as a "growth medium" was emphasized when a total of 600 mcg of nicotinic acid in physiological saline was given to a series of mice in six 100 mcg doses over a period of eight days preceeding the intratesticular inoculation of the mice and a control series receiving the physiological saline alone (Table 7). In these experiments, the mice receiving the nicotinic acid had an average of 38% more infected testes while the degree of infection rose to 47% more than the controls. There was a certain degree of specificity in this response since a parallel series with thiamine at the same time yielded only about a 15% increase over the control of both the number of testes infected and in the degree of infection. This is probably not a significant increase.

In these experiments, with nicotinic acid injections into the mouse, the results may not be due to nicotinic acid directly, since nicotinic acid is now known to have a number of physiological effects in higher organisms. For example, Bjorntorp (1965) reported that nicotinic acid decreased lipase activation by norepinephrine, thus, decreasing the fatty acid outflow from adipose tissue in the rat epididymal fat pad. Lee, Ellis, and Sigal (1961) working with rat epididymal adipose tissue found that nicotinic acid in vitro increased the concentration of reduced TPN in fat tissue, thus promoting reductive fatty acid syntheses. Jenkins (1965) showed that nicotinic acid in humans indirectly suppressed ketone body formation and these ketones in turn stimulated the pancreas.

When these experiments were first performed, the extent to which the level of the vitamin already in the Trypticase medium affected the physiological properties of the staphylococcal cells was not known. It was only after using Vitamin-Free Casitone, also a pancreatic digest of casein, as the growth medium that this became apparent. The casein used to make Vitamin-Free Casitone is extracted with alcohol to reduce, but not eliminate, the vitamin content. Using this medium, it was observed that the effect of thiamine in promoting the operation of the TCA cycle was only observed when small amounts of nicotinic acid were added (Table 10) and this effect was not observed when thiamine and nicotinic acid were present separately in a concentration of 1-2 mcg/ml. Although

the HMP pathway was stimulated by the addition of the vitamins separately, the TCA cycle was virtually non-operative. When one vitamin was added in a concentration of 1-2 mcg/ml and a second was present at a concentration of at least 0.1 mcg/ml then the usual alterations in metabolic pathways induced by vitamins in Trypticase also occurred in Vitamin-Free Casitone medium. These findings suggest the necessity of a nicotinic acid-thiamine threshold balance factor which is required for the operation of the TCA cycle.

The results of the initial experiments indicated that the addition of nicotinic acid to Trypticase medium resulted in stimulation of the HMP pathway and an increase in virulence, whereas the addition of thiamine resulted in an increase in the TCA and a decrease in the virulence (Tables 3 and 5). Many variables, some unexplained, then entered into these experiments. There were changes in the Trypticase medium lot number, switching to 5% blood agar stock slants, and temperature variations in the animal room. The metabolism of mouse testis is known to be very sensitive to temperature (Davis et al., 1966). Consequently any major temperature variations would affect the host parasite interaction in this tissue. In any case, nicotinic acid was capable of increasing especially the virulence degree using Trypticase, and this effect seemed optimal with an added concentration of 2 mcg/ml (Table 6).

From early research with thiamine it appeared that this vitamin caused a reduction in virulence. This reduction, about a

45% decrease in both the numbers of infected testes and their degree of infection, was limited only to the first lot number of Trypticase tried. These results have since not been possible to duplicate with newer lots of Trypticase. However, this same reduction in virulence was noted, about 23%, when Vitamin-Free Casitone was used (Table 5), even though the glucose metabolic studies showed that the TCA cycle was not increased in this medium (Table 10). It may be possible, nevertheless, that the potential to metabolize glucose via the TCA cycle is present and occurs once the bacteria infect the host. Although several strains have been tested in these studies the Towler striin was the predominant one used. It is premature at this time, however, to make a generalization that all coagulase-positive staphylococci will follow this pattern of virulence enhancement in the presence of nicotinic acid. There was no significant enhancement of virulence when the metabolism of coagulase-negative staphylococci (Cook), and Staphylococcus aureus strain 18Z, a relatively avirulent strain, were altered using vitamin additions in Trypticase. Again some of these studies may have to be repeated using the Vitamin-Free Casitone.

Although relatively little is known about the biochemical aspects of microbial pathogenicity, there has been a lot of interest in the subject (Braun, 1960). Herzberg et al. (1965) recently found a correlation between the slow succinate metabolism and virulence in Salmonella typhimurium. Presumably here the rate

of operation of the TCA cycle is involved in some way with virulence. In a different type of host-parasite study, Otsuka et al. (1965) related the pathogenicity of 45 strains of Piricularia oryzae, the fungus that causes rice blast disease, and their vitamin dependence. A group of thiamine independent strains was weakly or not pathogenic while a group containing nicotinic acid and thiamine-dependent strains was strongly pathogenic. Although this study is not directly comparable to that described in this thesis, it emphasizes the fact that vitamins can have a profound effect on the host-parasite interrelationship. A more related study recently demonstrated that the diet and the strain of mouse used affected staphylococcal virulence (Nutini and Berbercle, 1965). Earlier studies on the influence of nutrition on host-parasite reactions emphasized that vitamin deficiencies generally made the host more susceptible to bacterial infections (Scrimshaw et al., 1959).

There exists good evidence that coagulase contributes to the pathogenicity of staphylococci (Yotis, 1964). In attempting to explain the results of the virulence alterations in Trypticase grown staphylococci, coagulase dilution tests were performed with several strains grown in this media with vitamin additions. Even when these organisms were diluted 10^4 no difference could be noted in clotting time or in the size of the clots no matter which vitamin additions were made to the staphylococci. No other tests have been attempted to determine if the production of coagulase, hyaluronidase, alpha toxin, leukocidin, or any other potential infection promoting agent had been affected by the vitamin meta-

bolic alteration in staphylococci. I feel that it is likely that alterations induced by the addition of vitamins must be influencing the production of at least one virulence factor although the idea remains to be proven.

Many interesting things also were discovered in performing growth curves. Culturing staphylococci in some labotatory media creates a relative "vitamin deficiency" in that either their particular media was low in vitamins (this is true for nicotinic acid in Trypticase but the thiamine concentration was not given), or they were destroyed in autoclaving. In any case, the addition of vitamins, especially thiamine, enhanced the growth rate very markedly. The reason for this is unknown, but comparing the growth curves on Trypticase with those in Vitamin-Free Casitone some ideas may be presented. This idea of a proper proportion between nicotinic acid and thiamine in the media to produce optimal growth is interesting. Thiamine is present in very low concentrations in the Vitamin-Free Casitone. When nicotinic acid is added growth is obtained which is higher than the Trypticase control cultures containing all the vitamins. Yet thiamine produces a higher growth rate in Trypticase media than does nicotinic acid. Thus, at least it can be said that nicotinic acid must be present in a larger threshold concentration than thiamine, since the growth rate of thiamine additions in Vitamin-Free media is increased only when a sufficient nicotinic acid quantity has been added. This idea was further substantiated in the $^{14}CO_2$ metabolic

studies on cultures grown in Vitamin-Free media, but unfortunately it can not be correlated with virulence effects using ideal concentrations of both vitamins.

Certainly there is a necessity then to perform more experiments employing additional strains of Staphylococcus aureus to determine if these findings are universal. Other studies must be performed on the effects of metabolic alterations in staphylococci in the production of their virulence factors, particularly in a completely synthetic medium. The present results do seem to substantiate the idea of the necessity of an optimal TCA-HMP proportion or at least an optimal nicotinic acid-thiamine proportion in order to produce an optimal virulence.

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TABLE 1

CHARACTERISTICS OF THE STAPHYLOCOCCAL STRAINS USED IN THIS RESEARCH

Strain	Coagulase Test	Phage Type	Virulence in Mice Testes[a]	Virulence Rating by Cloutier et al.[b]
Towler	+	53/77	3+	Not Done
H-18	+	Not Typable	3+	Not Done
3[c]	+	NG	3+	3+
23[c]	+	NG	2+	3+
10[c]	+	NG	2+	2+
20[c]	+	NG	2+	2+
52[c]	+	NG	1+	1+
54[c]	+	NG	1+	1+
182[c]	+	80/81	-	Not Done
Cook	-	None	-	Not Done
Woolworths	-	None	Not Done	Not Done

[a] 3+ = highly virulent
2+ = moderately virulent
1+ = weakly virulent
- = avirulent

[b] This rating was based on an assay of virulence using four different test routes, namely: I.C. (Mouse); I.P. (Mouse); I.M. (Mouse); and chicken embryo.

[c] Strains donated by Cloutier, Panisset, & Marois (1964)

NG = Not Given

TABLE 2

A SUMMARY OF INDIVIDUAL GROUPS OF VIRULENCE ASSAYS INVOLVING NICOTINIC ACID-SUPPLEMENTED MEDIA

Number of Testes Infected,[a] %	Degree of Testes Infection, %	Number of Testes Infected,[a] %	Degree of Testes Infection, %
Trypticase Control		Trypticase + Nicotinic Acid	
77.8	41.7	40.0	37.5
16.7	8.3	37.5	23.4
16.7	8.3	36.4	21.6
58.3	33.3	50.0	37.5
11.0	7.0	22.2	9.7
55.5	1.4	30.0	15.0
25.0	21.0	60.0	43.0
45.0	35.0	27.8	23.0
25.0	25.0	22.2	22.0
45.0	38.8	27.8	27.8
45.0	38.8	35.0	26.2
27.8	22.5	45.0	38.8
27.8	22.5	55.0	53.7
27.8	22.5	45.0	38.7
9.1	3.4	15.0	6.2
9.1	3.4	00.0	00.0
22.2	3.4	38.8	23.7
22.2	11.1	22.2	20.8
38.9	34.7	15.0	15.0
15.0	15.0	33.3	29.2
15.0	15.0	15.0	15.0
5.0	1.22	00.0	00.0
30.0	28.7	15.0	15.0
22.2	20.8	30.0	30.0
22.2	18.1	33.3	30.5
11.1	6.5	5.0	2.5
45.0	30.0	40.0	28.8
33.3	25.0	35.0	31.2
20.0	16.2	55.0	55.0
10.0	6.3	25.0	18.7
55.5	48.6	45.0	33.7

[a] Each percentage in this column represents approximately 20 testes.

TABLE 3

THE EFFECT OF NICOTINIC ACID SUPPLEMENTED GROWTH MEDIA ON THE VIRULENCE OF STAPHYLOCOCCI FOR MOUSE TESTES

Growth Medium[a]	Average Number of Testes Infected, %	% Difference from Control	P Value Significance[b]	Average Degree of Testes Infection, %	% Difference from Control	P Value Significance
Trypticase: 31 Experiments						
Control	27.1	12.1	0.25	20.0	19.7	0.05
Niacin	30.8			24.9		
Vitamin Free Casitone: 5 Experiments						
Control	32.7	6.2	NS	27.2	5.3	NS
Niacin	30.6			25.7		

[a]Total added niacin concentration of 1-4 mcg/ml

[b]NS = Not Significant

TABLE 4

A SUMMARY OF INDIVIDUAL GROUPS OF VIRULENCE ASSAYS INVOLVING THIAMINE-SUPPLEMENTED MEDIA

Number of Testes Infected[a], %	Degree of Testes Infection, %	Number of Testes Infected[a], %	Degree of Testes Infection, %
Trypticase (Lot 307602)		Trypticase (Lot 307602) + Thiamine	
65.0	53.7	38.9	29.2
45.0	35.0	27.8	23.0
25.0	25.0	10.0	7.5
Trypticase (Lot 508683)		Trypticase (Lot 508683) + Thiamine	
22.2	18.1	35.0	30.0
11.1	8.7	30.0	23.7
11.1	6.5	20.0	10.0
11.1	6.5	16.7	13.9
33.3	25.0	30.0	28.8
45.0	30.0	55.0	51.2
20.0	16.2	45.0	38.8
10.0	6.2	00.0	00.0
55.5	48.6	35.0	28.7
Vitamin-Free Casitone		Vitamin-Free Casitone + Thiamine	
35.0	28.7	15.0	11.2
20.0	13.8	11.1	8.3
25.0	18.7	16.6	11.1
33.3	29.1	40.0	37.5
50.0	45.8	44.4	36.1

[a] Each percentage in this column represents approximately 20 testes.

TABLE 5

THE EFFECT OF THIAMINE SUPPLEMENTED GROWTH MEDIA ON THE VIRULENCE OF STAPHYLOCOCCUS AUREUS FOR MOUSE TESTES

Growth Medium[a]	Average Number of Testes Infected, %	% Difference from Control	P Value	Average Degree of Testes Infection, %	% Difference from Control	P Value
Trypticase Lot 307602: 3 Experiments						
Control	45.0	43.2	0.02	37.9	47.5	0.02
Thiamine	25.6			19.9		
Trypticase Lot 508683: 9 Experiments						
Control	24.4	17.8	0.20	25.0	26.3	0.20
Thiamine	29.6			18.4		
Vitamin Free Casitone: 5 Experiments						
Control	32.7	22.2	0.20	27.2	23.5	0.20
Thiamine	25.4			20.8		

[a]Total added thiamine concentration of 1-4 mcg/ml

TABLE 6

THE EFFECT OF THE ADDITION OF DIFFERENT CONCENTRATIONS OF THIAMINE OR NICOTINIC ACID TO A TRYPTICASE MEDIUM ON THE VIRULENCE OF STAPHYLOCOCCI FOR MOUSE TESTIS

Vitamin Supplement[a]	Conc. added,[b] mcg/ml	Number of Testes Infected, %	Difference %	P[c] Value	Degree of Testes Infection %	Difference, %	P Value
NA	1.0	36.0	1.8	NS	26.8	12.5	.50
Cont	0(7)	35.4			23.5		
NA	2.0	37.1	26.8	.20	30.3	30.2	.20
Cont	0(8)	27.2			21.2		
NA	4.0	23.8	2.3	NS	21.3	9.1	.60
Cont	0(14)	24.4			19.3		
Thia	1.0	25.6	43.2	.02	19.9	47.5	.02
Cont	0(3)	45.0			37.9		
Thia	2.0	33.3	3.4	NS	25.8	0	NS
Cont	0(3)	32.2			25.8		
Thia	4.0	33.3	32.8	.10	29.5	40.0	.02
Cont	0(5)	22.3			17.7		

[a] NA = Nicotinic Acid
Thia = Thiamine-HCl
Cont = Control

[b] ( ) = number of experiments

[c] NS = Not significant or calculated

TABLE 7

ALTERATION IN THE TESTICULAR VIRULENCE OF STAPHYLOCOCCUS AUREUS TOWLER FOLLOWING INTRAPERITONEAL INJECTION OF NICOTINIC ACID (NA) OR THIAMINE (THIA)

Difference, %	Total Number of Testes Infected,[a] %	Content of Each of Six I.P. Inoculations mcg	Total Degree of Testes Infection %	Difference %
Experiment 1. (3.30×10^5 viable staphylococcal colony forming units per testis)				
	30.0	Control	27.5	
+ 45.5	55.0	100 mcg NA	50.0	+ 45.0
+ 22.7	38.8	100 mcg Thia	37.5	+ 26.3
Experiment 2. (6.60×10^5 viable staphylococcal colong forming units per testis)				
	38.9	Control	26.4	
+ 30.0	55.6	100 mcg NA	52.8	+ 50.0
- 3.6	37.5	100 mcg THIA	29.7	+ 11.2

[a] There are ten mice (twenty testes) in each group.

TABLE 8

PATHWAYS OF GLUCOSE CATABOLISM BY RESTING STAPHYLOCOCCI CULTURED IN 2% TRYPTICASE

	Percentage of			
Strain	^{14}C-1	^{14}C-6 (TCA)	HMP	E-M
Towler[a]	38.2	0.6	37.6	62.4
#20	36.2	0.3	35.9	64.1
#52	26.8	0.3	26.5	73.5
Cook[a]	54.4	4.7	49.6	50.4

[a]F. Montiel: Unpublished data.

Note: E-M = 100-HMP

TABLE 9

EFFECTS OF NICOTINIC ACID OR THIAMINE UPON THE PATHWAY OF GLUCOSE CATABOLISM BY *STAPHYLOCOCCUS AUREUS* CULTURED IN 2% TRYPTICASE

Strain	Percentage of				Conc. mcg/ml	Vitamin[b]
	^{14}C-1	^{14}C-6 (TCA)	HMP	E-M		
Towler[a]	66.5	0.7	65.7	34.3	1-4	NA
Towler	45.5	14.9	30.7	69.3	2	Thia
Towler[a]	30.6	11.7	18.8	81.2	1-4	Thia
Canadian 20	37.6	7.5	30.1	69.9	4	Thia
Canadian 52	22.4	6.3	16.1	83.9	2	Thia
Towler[a]	69.5	23.6	45.9	54.1	1 1	NA + Thia

[a]F. Montiel: Unpublished data.

[b]NA=Nicotinic Acid
Thia=Thiamine

Note: E-M = 100 - HMP

TABLE 10

GLUCOSE CATABOLIC PATHWAYS IN STAPHYLOCOCCUS AUREUS GROWN IN VITAMIN-SUPPLEMENTED 2% VITAMIN-FREE CASITONE

Strain	Percentage of				Conc. mcg/ml	Vitamin[b]
	^{14}C-1	^{14}C-6 (TCA)	HMP	E-M		
Towler[a]	10.6	3.4	7.1	92.9	0	Cont
Towler	15.3	4.2	11.0	89.0	0	Cont
#10	18.5	2.0	16.6	83.4	0	Cont
Towler	53.0	3.6	49.4	50.6	2	NA
Towler[a]	65.6	3.0	62.6	37.4	1-4	NA
#10	24.3	3.6	20.7	79.3	2	Thia
Towler[a]	13.2	3.7	9.4	90.6	1-4	Thia
Towler[a]	59.3	22.6	36.7	63.3	0.1 1	Thia NA
Towler[a]	46.4	22.6	23.7	76.3	0.1 1	NA Thia

[a] F. Montiel: Unpublished data

[b] Cont = media with no additional vitamins added

NA = Nicotinic acid

Thia = Thiamine

Note: E-M = 100 - HMP

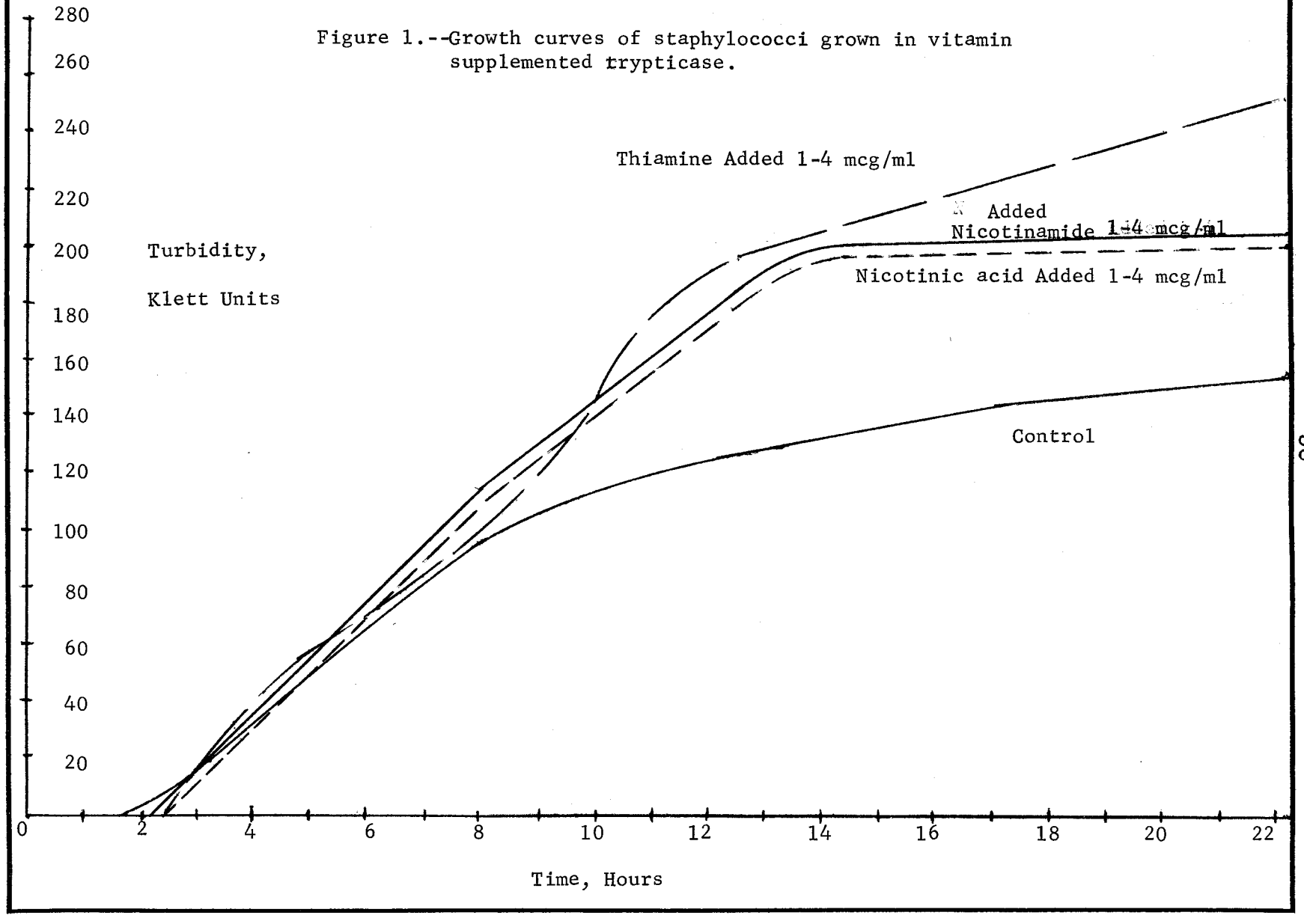


Figure 1.--Growth curves of staphylococci grown in vitamin supplemented trypticase.

Figure 2.--Growth curves of staphylococci grown in Vitamin-Free vitamin supplemented casitone.

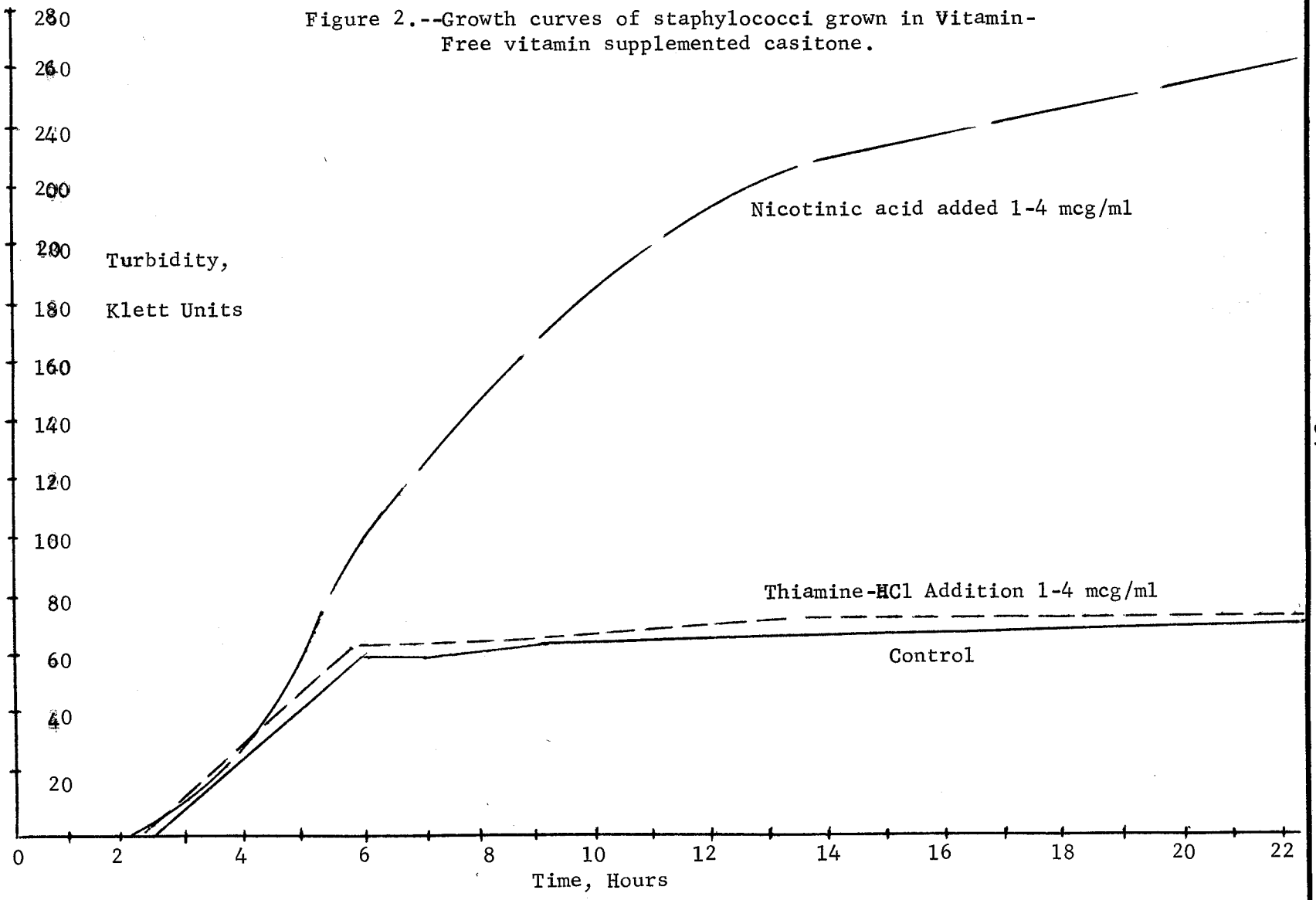
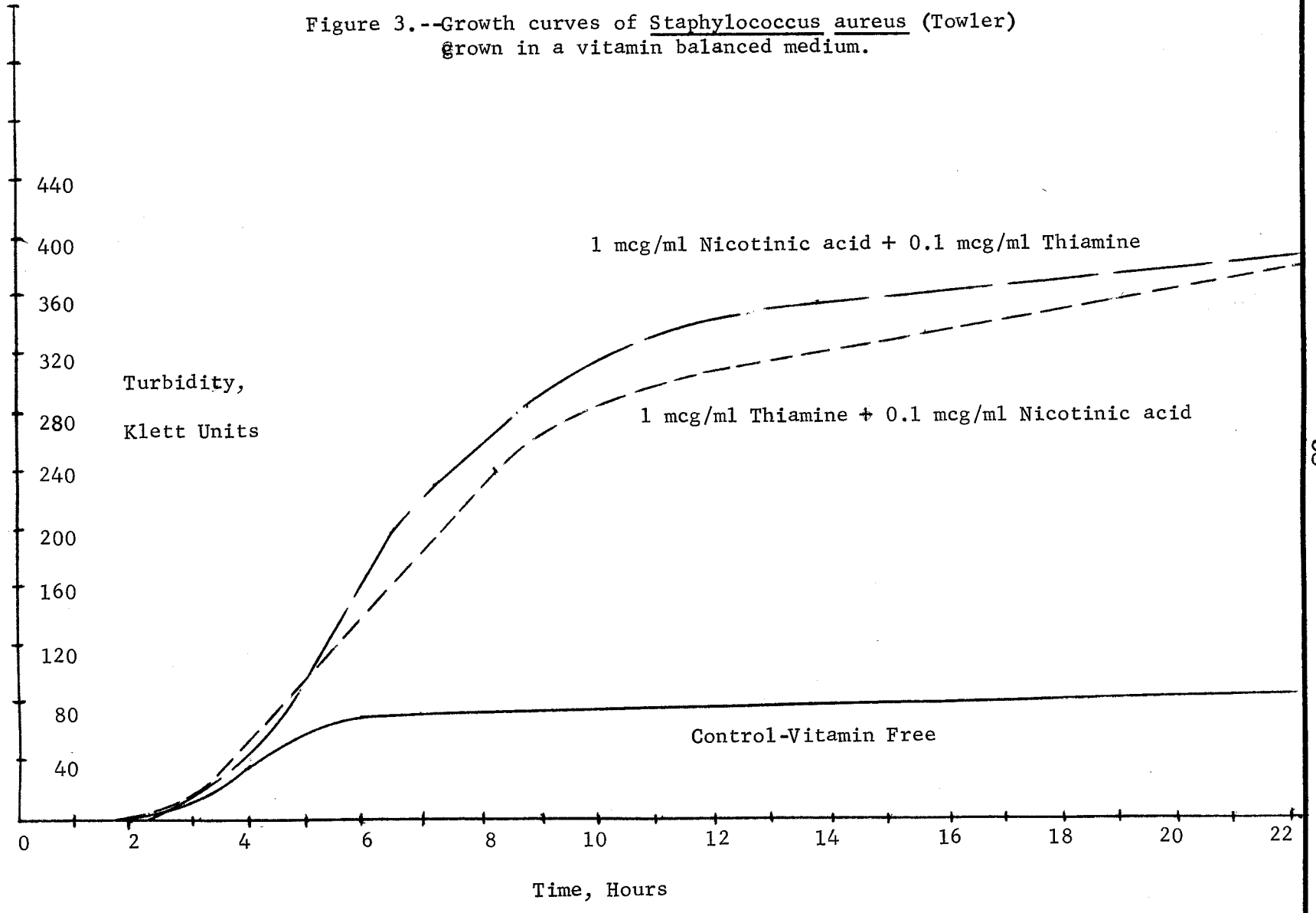


Figure 3.--Growth curves of Staphylococcus aureus (Towler) grown in a vitamin balanced medium.



APPROVAL SHEET

The thesis submitted by Charles P. Bowling Jr. has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 27, 1966

Date

Signature of Advisor