SOME EFFECTS OF *IN VITRO* INDUCED PENICILLIN RESISTANCE ON *MICROCOCCUS PYOGENES VAR. AUREUS*

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SOME EFFECTS OF *IN VITRO* INDUCED PENICILLIN RESISTANCE ON *MICROCOCCUS PYOGENES VAR. AUREUS*

by

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To My Wife

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INTRODUCTION

Resistance of microorganisms to penicillin is of importance to the clinician, the geneticist and the biochemist.

Except for penicillin resistant micrococcci, the development of resistant microorganisms in the course of treatment of bacterial infections with penicillin has been rare. The steady increase in these forms among the micrococcci has, however, definitely limited the usefulness of penicillin in the treatment of micrococcal diseases. In an effort to define the nature of resistance to penicillin many investigators have successfully produced resistant forms by repeated subculture in increasing quantities of the drug. Authors have reported on the characteristics of these resistant variants, often with conflicting data. For example, there is lack of agreement as to the pathogenicity, coagulase production and biochemical and gram stain reactions of micrococci made resistant *in vitro*. Further, because some resistant micrococci have been reported coagulase negative, nonhemolytic and poorly pigmented, it has been assumed that they are no longer pathogenic. True, this is indicative of avirulence but proof lies in pathogenicity tests on suitable laboratory animals with comparable numbers of the sensitive and resistant bacteria. However, the information gained by a study of resistance produced in culture may not be entirely applicable to micrococci which have acquired resistance during treatment of a patient with penicillin.

This is because the characteristics of *in vitro* produced resistant strains may not be the same as those of strains which have acquired resistance *in vivo*.

Resistance of bacteria to penicillin is important to the geneticist. Geneticists are concerned with the origin of bacterial resistance and the changes produced in bacterial populations by penicillin. Biochemists use cultures of bacteria which are resistant to penicillin to derive information about intermediate steps in metabolism and mode of action of drugs.

The objectives of this study were to investigate some of the changes produced in *Micrococcus pyogenes var. aureus* by repeated subculture in penicillin. Particular emphasis has been placed on changes in virulence because the literature is not in agreement as to whether resistant cultures remain virulent or become avirulent.

HISTORICAL SURVEY

Pasteur and Joubert (1877) made what was perhaps the first recorded observation of antagonism between microorganisms. They noted that broth cultures of Bacillus anthracis which became contaminated failed to grow.

While investigating staphylococci, Fleming (1929) noted that a mold growing as a contaminant on a plate caused the staphylococcus colonies to become transparent and then undergo lysis. When the mold was grown in a liquid medium the inhibiting substance was found to diffuse into the broth. Further, a broth filtrate, even when greatly diluted, would inhibit the growth of many pathogens. The substance was no more toxic for rabbits than ordinary broth. Later, the mold was identified as Penicillium notatum and Fleming called the inhibiting substance penicillin. Cutterbuck, Lovell and Raistrick (1932) attempted to extract penicillin from a purely synthetic medium on which they grew the mold. During the extraction penicillin was found to be unstable and further work was abandoned.

Penicillin was largely forgotten until 1938 when Florey and Chain at Oxford reexamined the possibilities of isolation and concentration of penicillin (Florey 1944). In the next two years the Oxford group succeeded in purifying, concentrating and testing bacterial spectra and tissue toxicity of penicillin. Proof of the efficacy of the antibiotic came when human patients

were successfully treated in the winter and spring of 1940-41. Thus, the Oxford group produced penicillin as a proved chemotherapeutic drug. The problem of producing penicillin in large quantities still remained. Due to the war, England was unable to provide facilities for large-scale production, so Florey and Heatley came to America. Here development of high-yielding strains of P. notatum and modification of the culture medium played an important part in volume production.

Once the new drug became available research was begun on its mode of action on the bacterial cell. Gardner (1940) studied the changes induced in bacteria by growth in concentrations of penicillin insufficient to cause complete inhibition. Enlargement, pleomorphism and imperfect fission were observed in micrococci, streptococci and gram positive bacilli. Swelling and sometimes bursting was noted in gram negative bacilli like Escherichia coli and some of the salmonella. In contrast to this, no morphological changes were seen in penicillin sensitive meningococci. Gardner thought that penicillin might cause incomplete fission leading to cellular enlargement and, in some cases, lysis. Hobby, Meyer and Chaffee (1942) subjected susceptible bacteria to penicillin and determined the number of surviving organisms at different time intervals. They found that a straight line was obtained if the log of the number of surviving organisms was plotted against time until approximately 99 per cent of the organisms were dead. The remaining one per cent were killed at a slower rate. Bigger (1944) found that the number of

organisms able to survive inhibitory concentrations of penicillin was increased if their rate of growth was slowed down or stopped. For example, penicillin in a concentration of 10 units per ml caused death of all micrococci in a culture at 37 C, allowed a few organisms to survive at room temperature, and caused no reduction in the number of viable bacteria at 4 C. In similar experiments it was found that micrococci, placed in broth diluted 1:800 with distilled water or subjected to bacteriostatic concentrations of boric acid, showed higher survival rates in inhibitory concentrations of penicillin than micrococci grown in plain broth. Bigger stated that these 'persisters' were cocci with no greater resistance than normal organisms but that they happened to be in a non-dividing phase in which they were insusceptible to the action of penicillin.

Kirby (1944), by turbidimetric readings, found a period of rapid growth followed by a period of equally rapid lysis at a concentration of 0.1 unit of penicillin per ml. With concentrations of from 1.0 to 100 units per ml a period of slow growth was followed by slow lysis. This phenomenon occurred with each of 100 strains tested. Kirby attributed the more rapid lysis in small rather than large concentrations to greater initial growth in the former. Parker and Marsh (1946) and Eriksen (1946a) obtained the same initial growth as did Kirby when using small concentrations of penicillin but found the lethal effect to be immediate with large concentrations. Eriksen's technique was interesting. He added penicillin to a solid medium and inoculated

it with micrococci. By placing small squares of the medium on a slide and observing one area with a microscope it was possible to tell whether growth or lysis of the bacteria had occurred. These authors concluded that penicillin was active only against those bacteria which were dividing; that is, it was effective in stopping some mechanism which the cell needs for division. However, their results would also support the hypothesis that penicillin blocks some mechanism involved in food metabolism, because an active metabolism must precede growth and division.

Bacteria able to grow in concentrations of penicillin greater than can be maintained in the human body are termed insensitive or resistant to the antibiotic. Strains of micrococci which are resistant to penicillin are occurring more and more frequently. Spink, Ferris and Vivino (1944) reported 12 per cent of 68 strains of coagulase positive micrococci to be resistant to between 0.4 and 0.8 unit of penicillin. Bondi and Dietz (1945) found that approximately 14 per cent of 115 recently isolated strains were able to withstand 0.45 unit of the antibiotic. The figure had risen to 21 per cent of 79 cultures as reported by Blair, Carr and Buchmann (1946). Their strains required from 0.3 to 50 units for inhibition. Roundtree, Barbour and Thompson (1951) ran sensitivity tests on staphylococci from patients at a hospital and reported 53 per cent resistant. Miyahara et al. (1953) reported the amazing total of 76 of 100 coagulase positive micrococci to be resistant. An even more striking example of the increase in resistant micrococci was that reported by Barber and

Rozwadowska-Dowzenko (1948). They found, in the same hospital, 14 per cent of the strains to be resistant to penicillin in 1946, 38 per cent in 1947, and 59 per cent in 1948.

In an attempt to define the nature of resistance numerous workers have developed bacteria in culture which are resistant to penicillin. The first of these were Abraham, Chain et al. (1941). By subculturing a strain of M. pyogenes var. aureus for 16 weeks in increasing quantities of penicillin they obtained a culture 1000 times more resistant than the parent strain. Rammelkamp and Maxon (1942) raised two strains of staphylococci to a 64 fold increase in penicillin resistance in eight weeks. Eriksen (1946b) obtained 12 strains of M. pyogenes var. aureus which showed a 500 fold increase in resistance. On solid media resistant cultures showed small colonies with poor pigmentation. Four of five subcultures on media without penicillin brought normal sized colonies into predominance.

Bellamy (1948), Bellamy and Klemik (1948), Klimek, Cavallito and Bailey (1948) and McVeigh and Hobby (1952) reported on the morphological and biochemical changes produced in M. pyogenes var. aureus by repeated subculture in a medium containing penicillin. All of these authors observed about the same changes in morphology. At first, exposure to penicillin resulted in an increase in size of the cells. After considerable resistance had been attained the cells became highly pleomorphic, varying from cocci to cocco-bacilli, rods and diphtheroid forms. As resistance increased the gram stain reaction became negative, the cells

became smaller and more uniform in size. Increased resistance was accompanied by a progressive loss in ability to ferment sugars, reduce nitrate and grow in high salt concentrations. For example, the resistant micrococci of Bellamy and Klimek (1948) were unable to grow in broth containing 6.5 per cent sodium chloride at 1000 times, unable to ferment sucrose and mannite at 4000 times, and unable to ferment lactose or reduce nitrates at 60,000 times the resistance of the parent sensitive strain. Bellamy (1948) and McVeigh and Hobdy (1952) found no decrease in resistance of their resistant micrococci on an enriched medium. However, highly resistant strains could be made much more sensitive by subculture on a deficient medium devised by Bellamy.

In contrast to the complete loss of ability of carbohydrate fermentation observed by the above investigators, Abraham, Chain et al. (1941), Spink, Ferris and Vivino (1944) and Blair, Carr and Buchman (1946) reported a decrease in the rate of fermentation. Since all authors, who have worked with organisms resistant to penicillin, have found a decreased rate of growth the fermentation reactions would be expected to proceed at a slower rate.

Authors also disagree on the coagulase reaction of micrococci made resistant in culture. Spink, Ferris and Vivino (1944), and Blair, Carr and Buchman (1946) found their resistant micrococci to be coagulase positive. Klimek, Cavallito and Bailey (1948), Suter and Vischer (1948) and McVeigh and Hobdy (1952) reported that in vitro produced resistant strains became coagulase negative.

Rake et al. (1944) were the first to report on virulence of pathogens made resistant in culture. They succeeded in developing resistance in a type III pneumococcus culture, a Streptococcus pyogenes culture and three cultures of M. pyogenes var. aureus. Over fifty transfers of a type I and a type II pneumococcus culture resulted in only a slight increase in resistance. Virulence for mice of the resistant cultures was reduced. Cultures, which consistently killed mice at a dilution of 10^{-7} before penicillin passage, killed only irregularly at a dilution of 10^{-1} after passage. The types I and II pneumococci, which had acquired little resistance, were only slightly less virulent.

Miller and Bohnhoff (1945) made seven strains of meningococci resistant to penicillin in vitro. Virulence was determined by inoculating mice intraperitoneally with mucin suspensions of meningococci. Six of the seven strains, which had acquired varying degrees of penicillin resistance up to five units per ml, lost very little or none of their original virulence for mice. The seventh strain which was able to grow in the presence of 14 units of penicillin remained highly virulent. However, when the strain had acquired resistance to 18 units per ml, virulence was lost and could not be restored by mouse passage.

Blair, Carr and Fuchman (1946) developed penicillin resistance in four strains of staphylococci by passage in a solid medium containing increasing quantities of the antibiotic. Virulence was determined by the intravenous injection in mice

weighing 16 grams of 0.5 ml of a suspension of an 18 hour slant culture, washed three times and resuspended in six ml of 0.85 per cent saline. Three of the four strains showed a considerable loss of virulence after the ninth subculture. The four strains were then maintained at room temperature and transferred to fresh medium every seven or eight weeks. Their resistance to penicillin and virulence for mice was determined at the end of the second and eighth month. At the end of the second month the three strains which had previously lost most of their virulence now became completely avirulent. The fourth strain remained virulent. Surprisingly enough the resistance of the strains increased considerably in the absence of penicillin. At the end of the eighth month all strains showed a loss in resistance to penicillin and the fourth strain became avirulent. Next, the four strains were subjected to serial daily transfers in broth without penicillin with the result that the sensitivity of three of the four strains was within the 'normal' range while one strain retained a considerable degree of resistance. Virulence for mice was again tested. Collectively, the four strains caused death in eight of 36 inoculated mice while at the begining of the experiment the parent sensitive cultures had killed 30 of 36 mice.

Suter and Vischer (1948) tested the virulence of cultures of micrococci made resistant to penicillin in vitro. They injected mice with 16 hour broth cultures of sensitive and resistant micrococci suspended in mucin. The penicillin resistant cultures

remained as virulent as the sensitive ones.

Matsui (1950) reported that streptococci made resistant *in vitro* became avirulent for mice.

Several authors have reported on pathogenic cocci which have acquired resistance *in vivo*. Rammelkamp and Maxon (1942) recovered four strains of penicillin resistant *M. pyogenes var. aureus* from fourteen patients treated with the antibiotic. Cultures of the four strains obtained before and after treatment disclosed increases of from 16 to 140 fold in resistance. Schmidt and Sesler (1943) developed two resistant strains of pneumococci by serial passage in mice treated with penicillin. The resistant variants appeared stable. Thirty serial passages of one strain in normal mice produced no loss of resistance. Blair, Carr and Buckman (1946) obtained resistant *M. pyogenes var. aureus* from nine of 41 patients undergoing penicillin treatment for chronic osteomyelitis. As with strains made resistant in culture these resistant forms showed a tendency toward loss of pigment and retardation of metabolic processes. The coagulase reaction was unchanged. No loss of resistance occurred over a period of from five to sixteen months. Six of the nine strains showed no change in virulence for mice when compared with the parent sensitive strain. The other three showed an appreciable loss of virulence. North and Christie (1946) obtained three isolates of *M. pyogenes var. aureus* at weekly intervals from a leg wound in a patient being treated with penicillin. The first isolate required 0.6 unit per ml for inhibition, the second 2.5 units per

ml, and the third 10 units per ml. The three isolates were believed to be of the same strain because all belonged to the same phage type and all were culturally identical. Mice were inoculated with a broth culture of the resistant micrococci diluted in saline to contain four billion organisms. Twenty-five thousand units of penicillin failed to protect a group of mice injected with the culture resistant to 10 units of penicillin. The same dose of penicillin protected all mice injected with the culture requiring 0.6 unit for inhibition.

Some organisms have been shown to be able to destroy penicillin. This is due to the elaboration of an enzyme called penicillinase. Abraham and Chain (1940) first demonstrated its presence in Escherichia coli and Micrococcus lysodeikticus. A crushed extract of the cells was capable of destroying penicillin as evidenced by the growth of a sensitive micrococcus in the presence of enzyme-treated penicillin. Penicillinase producers have been found widely distributed in nature. Woodruff and Foster (1944) found an intracellular enzymatic substance destructive to penicillin in cultures of spore-forming bacteria, other bacteria, actinomycetes, fungi and yeasts. They found no correlation between the resistance of a microorganism to penicillin and its ability to produce penicillinase. Bondi and Dietz (1944a) found the enzyme among coliforms, aerobic-sporeformers and in the genus Shigella. In another publication (1944b) they reported no penicillinase activity in Salmonella, Pseudomonas or Brucella although organisms in these genera were not susceptible to the

action of penicillin. By subculturing M. pyogenes var. aureus, Salmonella typhosa and Proteus vulgaris in increasing quantities of penicillin, resistance could be increased but no penicillinase activity could be detected. The authors concluded that the inability of an organism to produce penicillinase is not necessarily a determining factor in its sensitivity to penicillin.

However, in the genus Micrococcus the ability to produce penicillinase is directly correlated with resistance acquired **in vivo**. Kirby (1944) used Harper's acetone-ether extraction method to demonstrate penicillinase activity of micrococci. Seven resistant strains isolated from patients produced the inactivator while seven sensitive strains showed no penicillinase activity. Bondi and Dietz (1945) tested 115 strains of micrococci 16 of which were resistant. Only the resistant strains produced the enzyme.

Apparently strains of micrococci made resistant **in vitro** do not produce penicillinase. Spink and Ferris (1945) and North and Christie (1946) found no penicillinase activity in micrococci made resistant by subculture in increasing quantities of penicillin.

CULTURES USED IN THIS STUDY

209P Received in lyophile from the Michigan Department of Health in 1953.

N Isolated from a lesion on the face of a man at the Department of Bacteriology and Public Health, Michigan State College in 1949. The strain was maintained in stock culture at room temperature with occasional transfers until used in this study.

C Isolated from the vagina of a mare during an examination at the Department of Bacteriology and Public Health, Michigan State College in 1954.

RF Isolated from a lesion on the arm of a student at the Department of Bacteriology and Public Health, Michigan State College in 1954.

The above organisms were typical of M. pyogenes var. aureus in every respect. All produced a golden-yellow pigment, were coagulase positive and hemolytic.

Throughout this study the letter S after the name of an organism such as N-S was used to designate a parent sensitive strain. Any number occurring after the name of a strain indicates that the strain has been cultured in the presence of penicillin and is resistant. The number indicates how many times more resistant the culture was than its parent sensitive strain.

MATERIALS AND METHODS

Sensitivity Tests And Development Of Resistant Cultures

Buffered crystalline penicillin G potassium obtained from E. R. Squibb and Sons was used throughout this study. The vials of penicillin were reconstituted with 0.85 per cent sterile saline, and stock solutions, also diluted with saline, were prepared from these. Such solutions were maintained under refrigeration and used for a period of no longer than four days. Penicillin now is a chemically defined, pure product. For this reason the quantity μg instead of units of penicillin was used throughout this study. One unit is equal to 0.667 μg.

The medium chosen for stock cultures, sensitivity tests and development of resistant cultures was Penassay broth, Difco. This medium does not inhibit the action of penicillin and provides the essential growth requirements of M. pyogenes var. aureus.

Before attempting to develop resistant strains it was necessary to determine the smallest quantity of penicillin which inhibited parent strains. The lowest concentration of penicillin which completely inhibits the growth of an organism will hereafter be referred to as the minimum inhibitory concentration (M.I.C.), after McVeigh and Hobdy (1952). Table I gives the amount of broth, and the amount of culture added to sterile four-inch culture tubes to produce the final concentration of penicillin shown.

Diluted culture used as the inoculum in sensitivity tests was prepared as follows. A 24 hour culture was standardized in a Cenco Industrial type 12 photometer using a red filter to give a transmission of 60 per cent. The standardized culture was then diluted 1:10 with Penassay broth. One-tenth ml of the diluted culture contained approximately 10.6×10^6 organisms as determined by direct microscopic counts.

TABLE I

METHOD USED FOR DETERMINING THE M.I.C. OF SENSITIVE STRAINS

Ml Penicillin from stock solution	Ml Broth Added	Ml Diluted Culture Added	Final Concentration Penicillin in μg/ml
0.02	1.9	0.1	0.01
0.04	1.9	0.1	0.02
0.06	1.8	0.1	0.03
0.08	1.8	0.1	0.04
0.10	1.8	0.1	0.05
0.12	1.8	0.1	0.06
0.14	1.8	0.1	0.07
0.16	1.7	0.1	0.08
0.18	1.7	0.1	0.09
0.20	1.7	0.1	0.10

The lowest concentration of the antibiotic in which no turbidity was evident at the end of 48 hours incubation at 37 C was considered the M.I.C. The M.I.C. of penicillin for resistant

cultures was determined in the same manner except that more concentrated stock solutions were used in order to obtain inhibition.

Preliminary observations revealed that cultures which had attained considerable resistance by repeated subculture on a medium containing penicillin grew very slowly. Bellamy and Klimek (1948) attributed the slow growth of such resistant cultures to their inability to grow anaerobically. It seemed reasonable that any method that would provide aeration would increase the rate of growth of resistant cultures. Shaking proved to be the needed stimulus. No studies of comparative turbidities of shaken and unshaken cultures were undertaken. However, the rate of growth of shaken cultures was at least doubled over unshaken ones for any given incubation period. Shaking cultures had no effect on resistance attained by organisms. Penicillin sensitivity tests on resistant cultures were performed in duplicate. One set was shaken at 37 C while the other set was placed in the 37 C incubator. The M.I.C. recorded at either 24 or 48 hours was invariably the same. Shaking was accomplished with a Burrell Model C12 shaker adjusted to move the culture tubes two cm in a vertical plane 180 times per minute. This movement agitated the cultures as vigorously as possible without wetting the plugs.

The method used for development of resistant cultures was the same as that used for testing the M.I.C. of cultures. The only differences were that the inoculum was not standardized or

diluted and shaking was employed. Results were recorded after 24 hours incubation at 37 C. The culture containing the highest concentration of penicillin in which good growth occurred was used to inoculate a new series. When a strain grew in broth containing 5, 10, 100, 1000 and 10,000 times more penicillin than was required to inhibit the sensitive parent strain it was subcultured at that concentration two or three times until maximum growth was attained. The M.I.C. of penicillin for that culture was then tested and the culture served as an inoculum for studies of virulence, coagulase production, pigment production, hemolytic activity, biochemical reactions, gram stain and morphology.

During frequent subculture any microorganism may undergo changes in virulence, biochemical reactions, etc. To control this variable the sensitive parent strain was subcultured on Penassay broth each time a subculture was made of the resistant strain. Any differences between sensitive and resistant strains could then be attributed to growth of the resistant strain in the presence of penicillin.

Preparation Of Standard Suspensions

Twenty-four hour cultures grown in Penassay broth were centrifuged in an International refrigerated Model PR-1 centrifuge equipped with an angle-head for one-half hour at 3500 rpm. Under these conditions the centrifuge developed a relative centrifugal force of 1670 times gravity. The supernatant broth was removed and the cells were washed three times by centrifugation in 0.85 per cent saline. The bowl of the centrifuge was

maintained at 43 F when the centrifuge was in operation.

To break up clumps of cells after centrifugation saline suspensions were placed in 250 ml Erlenmeyer flasks containing about 20 glass beads, six mm in diameter, and shaken in the Burrell shaker for one hour. Microscopic examination of shaken suspensions revealed that all large clumps were broken up but approximately 10 per cent of all bacteria were still in groups of two, three or four. For the purpose of clarity, saline suspensions of micrococci after washing and shaking, will hence-forth be referred to as the crude suspension.

A portion of the crude suspension was diluted with 0.85 per cent saline to give a reading of 60 per cent transmission in a Cenco Industrial type B2 photometer with a red filter. The standardized cell suspension was diluted 1:50 in saline and a portion used to fill a Petroff-Hausser bacteria counting chamber. Counting was best accomplished with an American Optical Co. microscope equipped with phase contrast, using the oil immersion objective and a magnification of 970 diameters. The bacteria in one-half of the 400 squares of the chamber were counted and this number was multiplied by five million to give the number of bacteria per ml of standardized cell suspension. Two counts were made on each suspension. From the results of the cell counts the number of organisms per ml contained in the crude cell suspension was calculated. These data are given in Table 2.

The number of viable micrococci contained in cell suspensions standardized to 60 per cent transmission on the photometer was

also calculated using drop plates. One-tenth ml of a 1:1 million dilution of a standardized cell suspension was pipetted in four equal portions on the surface of a tryptose agar plate. Colony counts were made after 18 hours incubation at 37 C. Drop plates were made in duplicate. The results are shown in Table 3.

TABLE 2

RESULTS OF DIRECT CELL COUNTS CALCULATED IN BACTERIA PER ML OF CELL SUSPENSION STANDARDIZED TO 60 PER CENT TRANSMISSION ON THE PHOTOMETER

Strain	Cell Count ($x10^8$)	Average
N-S	10.40 8.90	9.65×10^8
209P-S	10.60 8.50	9.55×10^8
C-S	9.80 10.00	9.93×10^8
RF-S	10.30 11.15	10.73×10^8

TABLE 3

RESULTS OF DROP PLATE COUNTS CALCULATED IN BACTERIA PER ML OF CELL SUSPENSION STANDARDIZED TO 60 PER CENT TRANSMISSION ON THE PHOTOMETER

Strain	Colony Count	Average per ml of Suspension
N-S	73 71	7.2×10^8
209P-S	60 74	6.7×10^8
C-S	61 75	6.8×10^8
RF-S	70 78	7.4×10^8

Virulence

Rabbits vary greatly in their susceptibility to M. progenes var. aureus. Julianelle (1944) inoculated 10 rabbits with 0.5 ml of a 16 hour broth culture of a highly virulent micrococcus. Of these, four became ill and recovered, three died of septicemia in 72 hours and the remaining three died after 12 days. In contrast to the results obtained by Julianelle, it was found possible to produce death in rabbits quite consistently if relatively large numbers of micrococci were injected.

Rabbits used for virulence tests on sensitive and resistant micrococci were approximately two months old and weighed between two and three kilograms. Before injection the rabbits were weighed to the nearest 10 grams. The smallest number of micrococci, per kilogram of rabbit weight, required to cause death of all injected animals in one to six days was considered the minimum lethal dose. The M.L.D. of each strain was determined seperately because strains differed markedly in virulence. The number of cells per ml contained in the crude cell suspension was calculated from the results of the direct cell counts. A rabbit could then be inoculated with any desired number of bacteria from the crude cell suspension. Rabbits were properly restrained and injected via the marginal ear vein with a syringe and a 21 gauge hypodermic needle. Observations of injected rabbits were made at least twice daily and the time of death to the nearest one-forth day was recorded.

The technique outlined above for preparing a standard inoculum and for testing virulence of sensitive cultures was followed in detail when testing resistant cultures. The results of M.L.D. determinations on sensitive strains are shown in Table 4.

Coagulase Production

Equal one-half ml quantities of fresh rabbit plasma and a 24 hour broth culture of the test organism were mixed in three inch tubes. The tubes were incubated in a 37 C water bath for 24 hours. Distinct coagulation of the plasma within three hours was considered positive.

Pigment Production and Hemolytic Activity

Staphylococcus medium number 110, Difco, was used to determine the pigment producing ability of both sensitive ans resistant cultures. Petri dishes containing the medium were streaked with the organisms and incubated at 37 C until colonies were visible. The medium was then incubated at room temperature for four days and the color of the colonies was recorded. When resistant organisms would no longer grow in 6.5 per cent sodium chloride the staphylococcus medium number 110 was prepared with 0.5 per cent sodium chloride instead of the 7.5 per cent which it normally contains.

Blood plates containing 5 per cent sterile defibrinated horse blood in a tryptose agar base were used to determine hemolytic activity of sensitive and resistant cultures. The plates were streaked in such a manner as to obtain isolated colonies and results were read after two days incubation at 37 C.

TABLE 4

M. L. D. DETERMINATIONS OF SENSITIVE PARENT STRAINS

Strain	No. of Bacteria Injected ($x10^9$) per Kg Rabbit Wt.	Wt. of Rabbit in Kg.	Time in Days to Death-D or Survival-S
N-S	1.50	2.35	2.75-D
	1.50	2.50	1.00-D
	0.75	2.20	5.75-D
	0.75	2.23	4.25-D
	0.38	2.80	6.25-D
	0.38	2.50	30.00-S
209P-S	4.50	2.10	1.00-D
	3.00	2.00	3.50-D
	3.00	2.30	4.00-D
	1.50	2.60	4.50-D
	0.75	2.90	30.00-S
C-S	4.50	2.95	0.50-D
	3.00	2.50	0.75-D
	1.50	2.60	1.00-D
	0.75	2.30	1.75-D
	0.75	2.22	2.00-D
	0.38	2.10	30.00-S
RF-S	4.50	2.15	1.00-D
	1.50	2.15	1.75-D
	0.75	2.63	1.50-D
	0.75	2.10	5.50-D
	0.38	2.33	13.00-D

Biochemical Reactions

Purple broth base, Difco, containing brom cresol purple as an indicator was used as a base medium for carbohydrate fermentation studies. The carbohydrate was added to the base medium to a one per cent concentration tubed in three ml quantities and autoclaved for 15 minutes at 121 C. Cultures were tested for their ability to ferment dextrose, lactose, maltose, mannite, sucrose and glycerol. The tubes were inoculated and incubated at 37 C for one week. The long period of incubation was necessary because resistant cultures often fermented the carbohydrates slowly.

To determine the ability of an organism to reduce nitrate to nitrite, tubes of Nitrate broth, Difco, were inoculated and incubated at 37 C for four days. The medium was tested for the presence of nitrite by adding a few drops each of sulfanilic acid and α-naphthyl-amine reagent solution. A distinct cherry red color indicated the presence of nitrite resulting from reduction of nitrate.

Gelatin liquefaction was determined on the staphylococcus medium number 110 used for chromogenic studies. The organism to be tested was streaked on a plate of the medium, incubated at 37 C until colonies were visible and then incubated at room temperature for four days. The plate was then flooded with a saturated solution of ammonium sulfate, placed in the 37 C incubator for 10 minutes and examined. Any clear zone around colonies indicated

gelatin liquefaction.

Ability of an organism to grow in the presence of 6.5 per cent sodium chloride was tested by inoculating a tube of Brain Heart Infusion broth, Difco, to which 6.5 per cent sodium chloride had been added, and incubating for one week. Any visible turbidity was considered positive.

Reaction to Gram's Stain

The organisms to be stained were dried on a slide and fixed by heat. The slide was flooded with a one per cent aqueous solution of crystal violet and three or four drops of a five per cent solution of sodium bicarbonate were mixed with the dye. After allowing the preparation to stand two minutes, the slide was washed with water and flooded with Lugol's iodine solution. After one minute the slide was washed with water and blotted with tissue paper. The preparation was decolorized with acetone for about five seconds, washed and counterstained with a two per cent solution of safranin O for two minutes.

Morphological Variation

Unstained wet mounts of resistant strains prepared from a 24 hour Penassay broth culture were examined with the phase contrast microscope for evidence of pleomorphism caused by repeated subculture in penicillin. Mounts of the sensitive parent culture of each resistant strain were also made for the purpose of comparison. Unstained preparations examined under phase contrast were found to be superior to stained smears because the cells

appeared larger and were not altered by fixing and staining.

Measurement of the diameter of a representative sample of sensitive and resistant cultures was undertaken. A Filar ocular micrometer equipped with a moving scale was calibrated with the aid of a stage micrometer. Using the oil immersion objective and a total magnification of 970 diameters each division on the drum dial of the Filar micrometer was found to equal to 0.0909 microns. Twenty-five bacteria selected at random were measured. The diameter of the largest and the smallest bacterium measured as well as the average diameter of all 25 was recorded.

RESULTS

Development of Resistant Cultures and Sensitivity Tests

Figure 1 shows the pattern of development of resistance to penicillin G of the four strains of M. pyogenes var. aureus. Two of the strains, 209P and C, acquired resistance at a more rapid rate than did strains N and RF. After 50 daily transfers in broth containing penicillin strain 209P grew well in the presence of 200 μg while strain N grew in the presence of 40 μg of the antibiotic. After 42 transfers strain C became resistant to 200 μg while strain RF was able to grow in only 30 μg of penicillin.

The M.I.C. of penicillin for each resistant culture was tested at approximately each ten-fold increase in resistance except for the first variants isolated which were tested after a five-fold increase in resistance was attained. These results are shown in Table 5. The number of times the variant culture was more resistant than the parent strain was found by dividing the M.I.C. of penicillin for the resistant cultures by that of the parent strains. Resistant cultures will henceforth be identified by a number after the name of the strain which will indicate how many times more resistant it was than the parent strain.

Preparation of Standard Suspensions

It was expected that bacteria grown in broth containing penicillin would undergo changes in size and morphology. Because

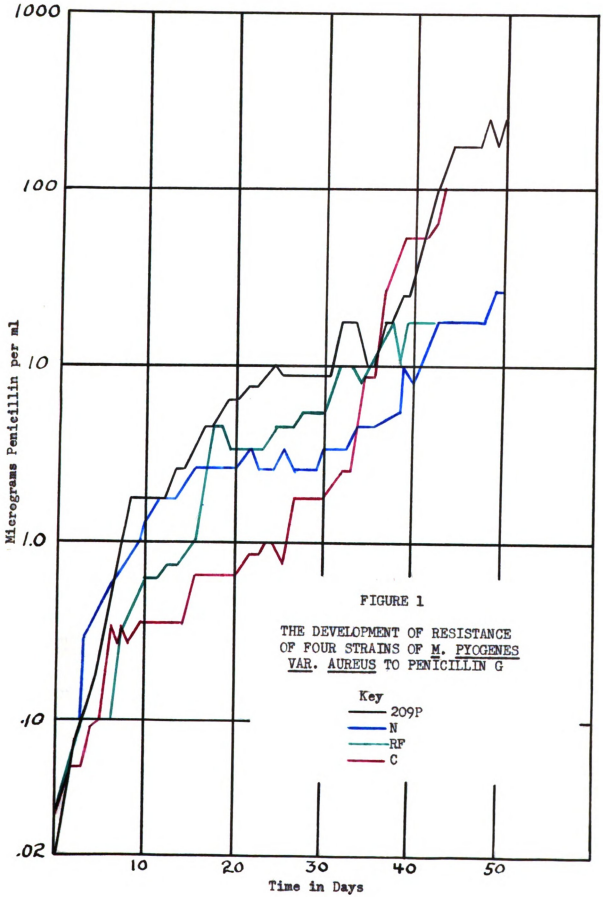

FIGURE 1

THE DEVELOPMENT OF RESISTANCE OF FOUR STRAINS OF M. PYOGENES VAR. AUREUS TO PENICILLIN G

TABLE 5

RESULTS OF M.I.C. DETERMINATIONS OF SENSITIVE AND RESISTANT CULTURES

Strain	M.I.C.	Number of Times More Resistant than Sensitive Parent Culture
209P-S	0.03	-
209P	0.15	13.3
	1.00	33.0
	6.00	200.0
	50.00	1666.7
	300.00	10,000.0
N-S	0.04	-
N	0.30	7.5
	0.90	22.0
	4.00	100.0
	50.00	1250.0
C-S	0.04	-
C	0.20	5.0
	2.00	50.0
	6.00	150.0
	50.00	1250.0
	300.00	7500.0
RF-S	0.04	-
RF	0.20	5.0
	3.00	50.0
	7.00	175.0
	40.00	1000.0

of these changes it seemed reasonable that a saline suspension of a resistant culture standardized to a reading of 60 per cent transmission would contain a number of bacteria significantly different from that of a similar standard suspension of the parent culture. For this reason suspensions of resistant organisms were standardized to a reading of 60 per cent transmission in the photometer and direct cell counts made of them. Contrary to what was expected there was no significant increase or decrease in the number of bacteria in such suspensions when compared to the number of organisms found in suspensions of sensitive micrococci. Results of direct cell counts on resistant organisms are shown in Table 6.

Virulence of Resistant Cultures

As the four strains of M. pyogenes var. aureus became resistance to penicillin there was a rapid and progressive loss in virulence for rabbits. Resistance cultures produced from strains C and RF failed to cause death in any injected rabbits, even when they were only five times as resistant as the parent strain and when five M.L.D. were inoculated. Culture Nx7.5 caused death when two and five M.L.D. were inoculated but not when 1 M.L.D. was used. Cultures of strain N more resistant than Nx7.5 failed to cause death in any injected rabbits. Resistant cultures produced from strain 209P maintained their virulence longer than those of other strains. Culture 209Px13.3 killed only the rabbit receiving 5 M.L.D., 209Px33 killed at 1, 5 and 10 M.L.D. and 209Px200 killed at 10 M.L.D. Cultures of strain 209P more resistant than 209Px-200 were avirulent. Factors other than the injected bacteria may

TABLE 6

RESULTS OF DIRECT CELL COUNTS ON RESISTANT CULTURES CALCULATED IN BACTERIA PER ML OF CELL SUSPENSION STANDARDIZED TO 60 PER CENT TRANSMISSION ON THE PHOTOMETER

Resistant Culture	Cell Count ($\times 10^8$)	Average
209Px13.3	8.85 9.80	9.33×10^8
209Px33	10.50 10.60	10.55×10^8
209Px200	11.20 10.95	11.08×10^8
209Px1667	8.95 8.00	8.48×10^8
209Px10,000	10.55 10.55	10.55×10^8
Nx7.5	8.95 10.00	9.48×10^8
Nx22	8.70 9.60	9.15×10^8
Nx100	8.95 7.75	8.35×10^8
Nx1250	9.00 9.30	9.15×10^8
Cx5	11.15 10.00	10.58×10^8
Cx50	8.50 10.00	9.25×10^8
Cx150	11.00 11.50	11.25×10^8
Cx1250	11.00 11.60	11.30×10^8
Cx7500	9.60 10.15	9.85×10^8
RFx5	11.05 10.60	10.83×10^8
RFx50	11.85 11.00	11.43×10^8
RFx175	10.50 11.50	11.00×10^8
RFx1000	9.80 10.90	10.35×10^8

have contributed to the death of the rabbit receiving one M.L.D. of 209Px33 since death occurred in only one day.

Each time the virulence of a resistant culture was tested the sensitive parent strain which had been subcultured a number of times equal to that of the resistant culture was used as a control. The only control culture which showed a loss of virulence was strain N-S. The N-S control on Nx22 required seven days to cause death of a rabbit while the N-S control on Nx100 survived. In the next series of rabbits injected with Nx1250 and N-S as a control it was necessary to double the number of micrococci contained in a M.L.D. in order for the N-S control to cause death in from one to six days. Table 7 shows the results of virulence tests.

Coagulase Production

The coagulase test was performed on all resistant cultures produced from the four sensitive parent strains. A coagulase negative microorganism and a tube containing undiluted plasma were used as controls. Throughout the experiment all sensitive parent and all resistant cultures were coagulase positive.

Pigment Production and Hemolytic Activity

Resistant strains showed little or no loss of pigment production. The pigment of all resistant cultures appeared identical to that of the sensitive parent strains.

As each strain became more resistant to penicillin its hemolytic activity was decreased. Two of the most resistant cultures,

TABLE 7

RESULTS OF VIRULENCE TESTS ON RESISTANT CULTURES

Culture	No. of Bacteria Injected ($x10^9$) per Kg Rabbit Wt.	No. of M.L.D.s Injected	Wt. of Rabbit in Kg	Time in Days to Death-D or Survival-S
209P-S control	3.00	1	2.14	2.00-D
209P x13.3	3.00	1	2.24	30.00-S
	6.00	2	2.70	30.00-S
	12.00	5	2.96	3.00-D
209P-S control	3.00	1	2.34	4.50-D
209P x33	3.00	1	2.82	1.00-D
	6.00	2	2.16	30.00-S
	15.00	5	2.35	11.00-D
	30.00	10	2.17	3.25-D
209P-S control	3.00	1	2.40	3.50-D
209P x200	30.00	10	2.35	8.00-D
209P-S control	3.00	1	2.52	2.50-D
209P x1667	30.00	10	2.35	30.00-S
209P-S control	3.00	1	2.30	3.00-D
209P x10,000	30.00	10	2.60	30.00-S
N-S control	0.75	1	2.38	4.50-D
Nx7.5	0.75	1	2.20	30.00-S

TABLE 7 (CONTINUED)

Culture	No. of Bacteria Injected ($x10^9$) per Kg Rabbit Wt.	No. of M.L.D.s Injected	Wt. of Rabbit in Kg	Time in Days to Death-D or Survival-S
Nx7.5	1.50	2	2.63	6.50-D
	3.75	5	3.16	1.50-D
N-S control	0.75	1	3.00	7.00-D
Nx22	0.75	1	3.00	30.00-S
	1.50	2	2.20	30.00-S
	3.75	5	2.40	30.00-S
	7.50	10	2.21	30.00-S
N-S control	0.75	1	2.41	30.00-S
Nx100	7.50	10	2.20	30.00-S
N-S control	1.50	1	2.48	3.50-D
Nx1250	15.00	10	2.50	30.00-S
C-S control	0.75	1	2.94	3.25-D
Cx5	0.75	1	2.25	30.00-S
	1.50	2	2.25	30.00-S
	3.75	5	2.65	30.00-S
C-S control	0.75	1	2.53	2.25-D
Cx50	0.75	1	2.10	30.00-S
	1.50	2	2.30	30.00-S
	3.75	5	2.21	30.00-S
C-S control	0.75	1	2.25	1.50-D

TABLE 7 (CONTINUED)

Culture	No. of Bacteria Injected ($x10^9$) per Kg Rabbit Wt.	No. of M.L.D.s Injected	Wt. of Rabbit in Kg	Time in Days to Death-D or Survival-S
Cx150	7.50	10	2.12	30.00-S
C-S control	0.75	1	2.30	2.00-D
Cx1250	7.50	10	2.65	30.00-S
C-S control	0.75	1	2.53	4.00-D
Cx7500	7.50	10	2.66	30.00-S
RF-S control	0.75	1	2.00	2.50-D
RFx5	0.75	1	2.40	30.00-S
	1.50	2	2.01	30.00-S
	3.75	5	2.44	30.00-S
RF-S control	0.75	1	2.89	1.75-D
RFx50	0.75	1	2.51	30.00-S
	1.50	2	2.30	30.00-S
	3.75	5	2.60	30.00-S
	7.50	10	2.37	30.00-S
RF-S control	0.75	1	2.50	2.25-D
RFx175	7.50	10	2.22	30.00-S
RF-S control	0.75	1	2.53	4.75-D
RFx1000	7.50	10	2.76	30.00-S

produced from strains 209P and C, became completely nonhemolytic. Resistant cultures developed from strains N and RF still retained some of their hemolytic activity at the end of this study but they did not reach the same degree of resistance as did strains 209P and C.

Resistant cultures produced from strains 209P, C, and RF showed increasingly smaller zones of clear hemolysis as resistance increased. Resistant cultures produced from strain N did not behave in this manner. The size of the zone of hemolysis remained about the same but as resistance developed hemolysis within the zone became less complete. On a blood plate the hemolytic zone of Nxl250 was just discernible.

Of the sensitive strains that were subcultured in broth each time a culture was made of a resistant strain only strain N-S showed a change in hemolytic activity. The zone of hemolysis it produced became less complete in a manner similar to that of the resistant cultures produced from strain N but not to the same degree.

Biochemical Reactions

The biochemical reactions of cultures resistant to penicillin are shown in Table 8. All resistant cultures fermented dextrose, lactose, maltose, mannite and sucrose. The ability of resistant cultures to ferment glycerol was lost when they became about 1000 times more resistant than the parent. When strains acquired resistance, growth in the presence of 6.5 per cent sodium chloride became scant and finally ceased altogether. The capacity of the

TABLE 8

RESULTS OF BIOCHEMICAL REACTIONS OF SENSITIVE AND RESISTANT STRAINS

Culture	Dext.	Lact.	Malt.	Mann.	Glyc.	6.5% NaCl	NO_3	Gelatin
209P-S	≠	≠	≠	≠	≠	≠	≠	≠ ≠
209Px13.3	≠	≠	≠	≠	≠	≠	≠	≠ ≠
209Px33	≠	≠	≠	≠	≠	≠	≠	≠ ≠
209Px200	≠	≠	≠	≠	≠	≠	≠	≠ ≠
209Px1667	≠	≠	≠	≠	-	-	≠	-
209Px10,000	≠	≠	≠	≠	-	-	≠	-
N-S	≠	≠	≠	≠	≠	≠	≠	≠ ≠
Nx7.5	≠	≠	≠	≠	≠	≠	≠	≠ ≠
Nx22	≠	≠	≠	≠	≠	≠	≠	≠
Nx100	≠	≠	≠	≠	≠	≠	≠	≠
Nx1250	≠	≠	≠	≠	-	-	≠	≠
C-S	≠	≠	≠	≠	≠	≠	≠	≠ ≠
Cx5	≠	≠	≠	≠	≠	≠	≠	≠ ≠
Cx50	≠	≠	≠	≠	≠	≠	≠	≠ ≠
Cx1250	≠	≠	≠	≠	-	-	≠	≠
Cx7500	≠	≠	≠	≠	-	-	≠	≠
RF-S	≠	≠	≠	≠	≠	≠	≠	≠ ≠ ≠ ≠
RFx5	≠	≠	≠	≠	≠	≠	≠	≠ ≠ ≠ ≠
RFx50	≠	≠	≠	≠	≠	≠	≠	≠ ≠ ≠ ≠
RFx175	≠	≠	≠	≠	≠	-	≠	≠ ≠ ≠ ≠
RFx1000	≠	≠	≠	≠	-	-	≠	≠ ≠ ≠

four strains to reduce nitrate to nitrite was unaffected by their becoming resistant to penicillin. Gelatin liquefaction of resistant cultures was reduced to some extent and cultures 209Px1667 and 209Px10,000 became gelatin negative.

Reaction to Gram's Stain

Burke's modification of the gram stain was used to stain preparations of sensitive and resistant cultures. Only cultures Cx7500 and 209Px10,000 showed a change in reaction to the stain. In culture Cx7500 about 25 per cent of the cells stained gram negative. Many of the gram positive cells seemed to retain the crystal violet sparingly. Gram stained slides of culture 209Px-10,000 revealed that approximately 75 per cent of the cells were negative while 25 per cent were positive. In this culture the individual cells appeared either distinctly gram positive or gram negative with no intermediate forms.

Morphological Variation

Wet mount preparations of resistant micrococci were examined for evidence of pleomorphism. In most wet mounts a small number of cells, usually less than one per cent, could be found which were not cocci. These forms varied from coccobacilli to short rods, diplococci and diphtheroid forms.

Penicillin also effected a change in the size of resistant cultures. These changes are shown in Table 9. As resistance to penicillin increased there was an increase in size of the cells in the culture. This continued until each culture became approximately 1000 times more resistant than its parent strain. At this

TABLE 9

RESULTS OF THE MEASUREMENT OF SIZE OF SENSITIVE AND RESISTANT CULTURES

Culture	Average Diameter in Microns	Diameter of Largest in Microns	Diameter of Smallest in Microns
209P-S	1.33	1.6	1.2
209Px13.3	1.34	1.6	0.8
209Px33	1.34	1.7	1.1
209Px200	1.63	1.9	1.2
209Px1667	1.63	1.9	1.1
209Px10,000	1.46	1.8	1.2
N-S	1.23	1.4	1.0
Nx7.5	1.15	1.4	0.9
Nx22	1.89	2.6	0.9
Nx100	1.83	2.1	1.1
Nx1250	1.33	1.5	1.2
C-S	1.28	1.4	1.1
Cx5	1.26	1.5	1.1
Cx50	1.49	1.9	1.2
Cx1250	1.75	2.0	1.4
Cx7500	1.33	1.6	0.9
RF-S	1.25	1.4	1.1
RFx5	1.28	1.6	1.2
RFx50	1.62	1.8	1.1
RFx175	1.64	2.0	1.2
RFx1000	1.53	1.8	0.9

time the average diameter of the cells of the resistant cultures was about 0.44 microns larger than that of the sensitive cells. On further increase in resistance the average diameter of the cells became smaller. Table 9 also shows that resistant cells are more variable in size than are the parent sensitive cells. While the average difference between the largest and smallest cells measured was 0.3 micron for the four sensitive strains, the value was 0.7 micron for the four resistant cultures which had attained a 1000 fold increase in resistance.

DISCUSSION

The present investigation indicates that cultures of M. pyogenes var. aureus, when subcultured in the presence of penicillin, became increasingly tolerant to it. All the strains appeared capable of acquiring a higher degree of resistance than they attained. One can only speculate on how resistant an organism could become if subcultured in the presence of penicillin for an extended period of time. As a culture acquires resistance, growth becomes less luxuriant. Eventually, this would be a limiting factor in its ability to acquire further tolerance to penicillin. Nevertheless, it is a tribute to the versatility of a bacterium that the descendants of a culture are capable of living and growing in the presence of a comparatively large amount of a toxic substance which, a few weeks previously, would have been lethal in a very high dilution.

There are two theories concerning the mechanism whereby bacteria acquire resistance to penicillin in vitro. The first is that acquired resistance is only an adaptive change on the part of the organism, that is, a result of interaction between the antibiotic and the bacterium. The second, as first presented by Demerec (1948), postulates that resistant bacteria arise by a process of spontaneous mutation, the penicillin acting only as a selective agent by the destruction of sensitive cells. According to Demerec's hypothesis, resistance to penicillin acquired in vitro could be expected to proceed in a stepwise fashion. Figure 1, which shows

the pattern of development of resistance of the four strains used in this study demonstrates no such stepwise increase in resistance. However, a strain would often grow in the presence of a certain concentration of penicillin through several subcultures, then suddenly become tolerant to much larger concentrations.

When comparing the virulence of sensitive and resistant bacteria it was necessary to consider all the factors which affect virulence. Factors such as volume of inoculum, suspending medium, route of inoculation, size, age, weight and species of animal can be easily controlled. To a less extent the log phase of the organism can be regulated. However, in vitro produced resistant micrococci exhibit a slower rate of growth than do sensitive cultures. This slower rate of growth naturally affects virulence. An organism that divides slowly will have less chance to produce a fatal infection because the host's defensive mechanism will have more time to destroy the parasite. Certainly some, and possibly a considerable part, of the decreased virulence of resistant micrococci is due to this decreased rate of growth. Then, loss of virulence may not be due entirely to dissociation phenomena brought on by the organisms having acquired a resistance to penicillin.

On the other hand, the argument might be presented that this slowed rate of growth should not be considered in evaluating the results of virulence tests on resistant micrococci. It might be said that this is one of the ways in which the organism was changed

by subculture in penicillin and that this was one of the characteristics of the resistant organism which was altered.

Micrococcal infections are usually localized. In a localized infection the slow rate of division of a resistant micrococcus might not be important from the point of view of virulence because the organism is usually comparatively well isolated from the defensive mechanisms of the host. However, when the virulence of a resistant micrococcus is tested by intravenous inoculation of rabbits whose defensive mechanisms have ready access to the injected bacteria the results can be far different. It is difficult to control or to evaluate the effects of this slow rate of growth when comparing the virulence of sensitive and resistant organisms but it must be taken into consideration.

SUMMARY

In vitro induced resistance of *M. pyogenes var. aureus* to penicillin is accompanied by changes in virulence, hemolytic activity, some biochemical reactions, gram stain reaction and morphology. The most striking change was in virulence. All four strains lost their virulence for rabbits as penicillin resistance was acquired. Two of the resistant cultures became non-hemolytic, while the other two still retained some hemolytic activity. All highly resistant strains failed to ferment glycerol and to grow in the presence of 6.5 per cent sodium chloride while only one strain lost the ability to liquefy gelatin. Many of the cells in the two most resistant cultures became negative to Gram's stain. On continued exposure to penicillin the cells of the cultures enlarged. After becoming highly resistant the size of the cells returned to near normal.

Resistant cultures showed no change in coagulase production, chromogen production, nitrate reduction or in fermentation of dextrose, lactose, maltose, mannite or sucrose.

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