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FACTORS INFLUENCING CHLORAMPHENICOL RESISTANCE OF *MICROCOCCUS PYOGENES* VAR. *AUREUS*

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CHAPTER I

INTRODUCTION

General Considerations of Antibiotic Resistance

The problem of microbial resistance to antibiotics is of practical and fundamental importance. The practical importance is obvious in the treatment of infectious diseases. Even more important, however, is the realization that studies on the mechanisms whereby drug resistance arises can contribute important knowledge toward a better understanding of comparative biochemistry and the general problem of drug tolerance.

The antimicrobial activity of antibiotics is one of the most widely studied and least understood subjects in the field of microbiology. The use of antibiotics is widespread and the basis for their choice is sound, even though there is little known about their mode of action. Antibiotics have been observed to inhibit or kill bacteria, activities that are respectively classified as bacteriostatic or bactercidal. The arbitrary division of these materials into these two groups is not entirely satisfactory, since some agents which are classified as bacteriostatic may, in sufficient concentration, initiate changes in the cells which

will lead to death unless the antibiotic is removed; hence, they are bactericidal, albeit slowly.

Complete understanding of the mechanism by which an antibiotic acts is likely to require knowledge at all of the following levels: a) that process by which the active agent attains a toxic concentration upon or within the afflicted cell. b) the interaction of this agent with some morphological element or some metabolic constituent of the cell. c) the nature of the interference with a normal cellular function and the immediate or general results of this interference. Eagle (1954) found that among bacteria which do not degrade penicillin, resistant strains regularly bind less penicillin than do sensitive strains; further all free extracts of these organisms also bind penicillin in relation to the sensitivity of the intact cell. Cooper and Rowley (1949) have demonstrated a lipid fraction of staphylococcal cells which will irreversable bind penicillin.

Only in those instances where it has been demonstrated that enzymes degrade antibiotics, i.e., penicillinase (Abraham and Chain, 1940) and chloramphenicol reductase (Merkel and Steers, 1953), is resistance a well understood phenomenon. Another, but less well understood, mechanism of resistance is a decreased affinity of the cellular components for the drug (Eagle, 1954) (Saz and Martinez, 1956).

In addition to the two mechanisms mentioned above, one might theorize that a natively nonsusceptible organism lacks the particular factor which renders other organisms susceptible or that this factor is efficiently protected. Since antibiotics might function as antimetabolites, their blocking action could result in an increase of some substrate

level which would serve to overcome the inhibition. This might also result in an increase of growth activity and could explain the stimulation that has been observed in the presence of low concentrations of an antibiotic (Smith, et al., 1949). It is also possible, however, that the organism in some ways succeeds in bypassing the point in its metabolic processes which is inhibited.

Yudkin (1953) has shown that any bacterial culture is composed of organisms which have a considerable range of resistance, with the majority of the cells having the same degree of resistance. As constant products of mutation minor variations of metabolic processes lead to the origin of new phenotypes. These new types possess systems of metabolism almost like the parent strain, since evolution characteristically proceeds in small steps. Many contributions to the literature give ample evidence that acquired resistance to antibiotics is genetically determined. This has been shown by several techniques one of which is the fluctuation test of Luria and Delbruck (1943) which has been employed by Demerec (1945) and Luria (1946) in studies of penicillin resistance in Staphylococcus aureus.

Drug resistant bacteria may result when the genetic change provides an alternative metabolic route or a more efficient method for synthesizing an antagonized essential metabolite (Broquist, et al., 1953). The occurrence of antibiotic resistant bacteria is a consequence of evolutionary pressures leading to the selection of types best fitted for survival. The absence of drugs in the natural environment puts no evident selective advantage on drug resistance as a hereditary trait in naturally resistant species. Obscure hidden advantages may exist or the

phenomenom of resistance may be selectively neutral. In sensitive species the resistant derivative arising as mutants are outnumbered and at a selective disadvantage in the absence of the drug; otherwise they would themselves comprise the wild type or possibly a so-called "naturally" resistant strain. When different kinds of drug resistant mutants arise some may be selectively favored by the environment. Thus in the laboratory mutants which resist an antibiotic without dependence upon extracellular inactivation are isolated with greatest frequency, although not exclusively (Eagle, 1954).

The demonstration that resistance commonly arises by mutation and selection does not exclude the possibility that under specific experimental or clinical situations another method of development may be operative. Numerous polemics have surrounded attempts to decide whether drug resistance is induced or selected in microorganisms. Occurrence of mutant cells in continuous culture devices, as shown by Novick and Szilard (1950) and Bryson and Szybalski (1952), is one of several ways of proving the spontaneous origin of bacterial clones resistant to harmful agents. Convincing evidence of a chromosomal or genic basis for the antibiotic resistance of these clones can be obtained from several types of experiments, notably transformation (Hotchkiss, 1951, 1955), transduction (Zinder and Lederberg, 1952) and recombination (Lederberg, 1951, Lederberg, et al., 1951, Cavalli, 1952).

Microorganisms that are resistant to an antibiotic but which do not require it for growth may usually be maintained through many generations in the absence of the drug without a substantial loss in resistance. In the absence of the selective action of the drug, additional genetic

changes or reverse mutations may occur with a resultant increased growth rate or viability and a corresponding reduction of resistance (Bryson and Demerec, 1955).

Physiological Aspects of Antibiotic Resistance

Investigations on the modes of action of antibiotics have been directed along three main lines. First, a direct approach has been employed by studying the effects of the drugs on metabolic processes and individual enzyme systems. These investigations have been carried out with both living cells and with cell free systems. The second approach, which is not quite so obvious and straight forward, involves a study of how microorganisms are able to avoid or overcome the action of drugs through a comparison of susceptible parental strains and resistant variants derived therefrom. These observable differences might be related to those functions with which the drug interfers. A third method of investigation, also indirect, involves the empirical search for specific chemical substances which are capable of antagonizing, either competitively or noncompetitively, the action of the drug. All of these methods have given some insight into how the antibiotics might function. Some of the results more pertinent to the general problem of chloramphenicol activity are presented below.

The effect of chloramphenicol on a large number of metabolic processes has been tested and, in almost every case, the results obtained have been of a negative nature. Smith, Worrel and Swanson (1949) demonstrated that the utilization of fats and esters by Escherichia coli was apparently inhibited by concentrations of chloramphenicol that were in-

sufficient to inhibit growth. Enzymatic studies on cell free systems showed that the response differed with the concentration of the drug; very low concentrations caused an inhibition and moderate concentrations caused stimulation of enzymatic activity. Concentrations of the antibiotic comparable to therapeutic levels caused almost complete inhibition of the esterase activity. The observed changes in esterase activity were similar to the change in growth response which were normally found over a wide concentration range of the antibiotic. Chloramphenicol had negligible activity against the esterase of animal tissue slices, indicating that the intact animal cell possessed some mechanism for protecting the enzyme of the cell from the drug action. Chloramphenicol has been found to inhibit the diamine oxidase of several genera of bacteria (Owen, et al., 1951). This diamine oxidase was also inhibited by monoamine and diamine derivatives of the antibiotic which have no antibacterial action.

Hahn, Wisseman and Hopps (1955) studied three different forms of bacterial energy utilization for possible interference by chloramphenicol. These included (1) the bioluminescence of Achromabacter fischeri as an example of electron transport, (2) phosphorylation involved in the dissimilation of glucose in E. coli as a key process of synthesis of high-energy phosphate bonds and their utilization in glucose metabolism, and (3) the motility of E. coli as a special example of the utilization of metabolic energy. None of these examples of energy metabolism were influenced significantly by chloramphenicol. The authors suggested that the drug does not interfere with the generation of a necessary supply of metabolic energy, nor does it act by a general inhibitory action on the

utilization of such energy.

Kushner (1955_a) found that oxidation of a variety of dicarboxylic acids by intact cells of Pseudomonas fluorescens was strongly inhibited by chloramphenicol. The oxidation of succinate, fumarate and malate by crushed bacterial cells was also inhibited by chloramphenicol. However, the dehydrogenases which first act on these compounds were not directly blocked. In a comparison of chloramphenicol sensitive and resistant strains Kushner (1955_b) found that the oxidation of these substrates by the intact cells of the resistant strain was not inhibited by the antibiotic. Chloramphenicol inhibited succinate oxidation to approximately the same extent in crushed, cell free preparations of resistant cells as in similar preparations of sensitive cells. His results indicated that, in P. fluorescens, structural integrity of the cell is essential for resistance to chloramphenicol.

A well defined physiological difference between aureomycin resistant and sensitive strains of E. coli has been demonstrated by Saz and Martinez (1956). They noted that with sensitive strains, the enzyme nitro reductase which can catalyze the reduction of chloramphenicol and other aromatic nitro compounds to the corresponding arylamines, could be inhibited by low concentrations of aureomycin; the enzyme from a resistant strain was not inhibited by aureomycin. The resistant enzyme contained a firmly bound conjugated flavin whereas the flavin of the sensitive reductase was easily dissociated by acid $(NH_4)_2\ SO_4$ precipitation and by precipitation of neutral 40 to 50 per cent $(NH_4)_2\ SO_4$ fractions with protamine sulfate. Apparently this treatment separated the reductase into its flavin component and apoenzyme. A flavin requirement of

the resistant reductase was not demonstrated by either of these procedures. Activity of the treated sensitive preparations could be restored by the addition of Mn++ and flavin mononucleotide. It was postulated that, in this instance, resistance reflects the synthesis of an altered enzyme with an increased affinity for coenzyme and a corresponding decreased affinity for the antagonist.

Comparisons of antibiotic sensitive microorganisms and resistant strains derived from them indicate that some alteration of physiological properties often accompanies the development of resistance. Carrere, Roux and Dautheribes (1951) induced resistance to aureomycin and chloramphenicol in Salmonella typhi, Proteus mirabilis and Fecalis alkaligenes and compared these to the original strains. They did not find any great differences in the biochemical properties of these bacteria except that the aureomycin resistant strain of Fecalis had acquired the ability to utilize citrate. On the other hand, pronounced differences were observed in the immunological characteristics. Antiserum prepared against each strain (both sensitive and resistant) would agglutinate the homogolous organism to the highest titer. Thus, aureomycin sensitive and chloramphenicol sensitive strains could be differentiated from their resistant variants and also the aureomycin resistant strain could be differentiated from the chloramphenicol resistant strain. Greater physiological differences between antibiotic resistant and sensitive strains have been observed (Ramsey and Padron, 1954; Padron, Smith and Ramsey, 1954). These workers started with a strain of Micrococcus pyogenes var. aureus which was inhibited by 4 ug of chloramphenicol per ml. and obtained strains which ranged in resistance from 8 to 1040 ug chloramphenicol per

ml. Resistance was found to be a stable characteristic and was not attributable to chloramphenicol reductase activity. Resistance was accompanied by a decreased requirement, both anaerobic and aerobic, for niacin, thiamin and arginine. The relative degree to which each strain was able to dispense with niacin and thiamin bore no relationship to the quantitative resistance of the strain. Padron, Smith and Ramsey (1954) found that the development of resistance to chloramphenicol by M. pyogenes was accompanied by a higher endogenous respiration, a decreased ability to oxidize pyruvate, a loss in ability to oxidize acetate and increased formation of lactic acid from glucose. A chloramphenicol sensitive strain of M. pyogenes was transferred in synthetic medium containing decreasing concentrations of niacin and/or thiamin until strains were developed which had minimal requirements for either or both vitamins (Ramsey, 1954). A comparison of the chloramphenicol resistance of these strains with that of the parent strain showed no difference in drug resistance. This indicated that the decreased vitamin requirements of chloramphenicol resistant strains observed by Ramsey and Padron (1954) were not necessarily the direct result of the increased resistance.

A strain of M. pyogenes was isolated by Wood and Steers (1954) that was resistant to the highest soluble concentration of chloramphenicol. Growth experiments using the postulated precursors of purines (glycine, formate, bicarbonate, ammonium ion, 4-amino-5-imidazole-carboxamide and the B vitamins) and isotopic studies using adenine-8-C^{14} and guanine-8-C^{14} demonstrated that the resistant strain could not utilize these simple precursors for growth and could not interconvert adenine and guanine. The parent sensitive strain, however, could utilize these simple

precursors for purine synthesis (it had no purine requirement) and could interconvert adenine and guanine. McVeigh and Hobdy (1952) compared a parent strain of M. pyogenes with strains that had been subcultured in increasing concentrations of chloramphenicol, streptomycin, aureomycin, subtilin and penicillin. Generally, the resistant strains grew more slowly than the parent strain. In contrast to the other resistant strains, those which had become resistant to penicillin had lost the ability to reduce nitrates, to liquify gelatin, to produce acids from sucrose, maltose, mannitol, lactose and glycerol and to grow anaerobically. They also failed to produce coagulase and to grow in the presence of 7.5 per cent sodium chloride, thereby indicating a loss of pathogenicity. The strains resistant to the other antibiotics had not lost completely any of these capacities. The penicillin resistant strains showed an increase in the ability to synthesize the vitamins and amino acids necessary for growth whereas the other resistant strains showed very little difference from the parent strain.

One of the more fruitful fields of investigation has been that dealing with protein synthesis and inducible enzyme formation. Hahn and Wisseman (1951) reported that chloramphenicol inhibited formation of the inducible enzymes required for the oxidation of lactose, maltose, arabinose and acetate by a strain of E. coli. Chloramphenicol inhibition of inducible enzyme (gluco-kinase) formation by E. coli was noted by Marmur and Saz (1953). Their results confirmed those of Hahn and Wisseman in demonstrating that oxidation of a substrate by adapted cells or cell free enzymes was not inhibited, but that cells of E. coli were not able to adapt in the presence of the drug. They developed a strain of E. coli

that was resistant to 200 ug chloramphenicol per ml and then compared the resistant and sensitive parent strain. The metabolism of gluconate was an adaptive process with both strains. In the presence of 42 ug chloramphenicol per ml the oxidation of gluconate by the parent strain was completely inhibited while the resistant organism utilized the gluconate in a manner which was not distinguishable from the control without the drug. It was concluded that in the sensitive strain chloramphenicol inhibited the formation of inducible enzymes and that the resistant strain was inhibited to a lesser degree. These results would be subject to more accurate interpretation if chloramphenicol concentrations proportional to growth inhibitory amounts for both strains had been used. Kushner (1955_b) in a comparable experiment found that chloramphenicol would inhibit the ability of a sensitive strain of P. fluorescens to form inducible enzymes required to oxidize benzoate, malonate and L-tryosine. Chloramphenicol had no effect on these adaptive phenomena in resistant strains. Bernheim (1954) demonstrated chloramphenicol inhibition of the formation and activity of the adaptive enzyme required for the oxidation of benzoate by P. aeruginosa. In a study of beta-galactosidase in Staphylococcus aureus Creaser (1955) observed that the concentration of amino acids in the medium determined the final concentration of the enzyme formed, but that the rate of formation was free of influence of this concentration. Concentration of the inducer played an important role in the amount of enzyme formed. He further found that omission of purines and pyrimidines from the medium resulted in a lower production of enzyme. Creaser tested fifteen antibiotics including chloramphenicol for effect on enzyme formation and found that all of them were capable of inhibiting the formation of

this inducible enzyme. These reports of inhibition of inducible enzyme formation by chloramphenicol have all indicated that some mechanism involved in protein synthesis is the site of chloramphenicol activity.

More confirmatory and direct evidence of this has been obtained by other workers. Wisseman, et al., (1954) showed that assimilation of ammonia by E. coli was decreased by the presence of chloramphenicol. Furthermore, an abrupt cessation of protein synthesis occurred when the organism was placed in contact with concentrations of the drug that approximated that required for growth inhibition. Amino acid incorporation studies with labeled glycine showed that the amino acid was not taken into the protein but was incorporated into the nucleic acids at the same rate as it had been in the absence of chloramphenicol. The rates of ribnucleic and deoxyribonucleic acid formations were unaltered by the presence of chloramphenicol. The failure of chloramphenicol to inhibit a wide variety of metabolic processes lends support to the possibility that its action on protein synthesis may be specific. Further support to this concept was obtained by Hahn, et al., (1954), who demonstrated the inhibitory action of the L (+) erythro isomer of chloramphenicol on D (-) glutamyl polypeptide formation by Bacillus subtilis. The antipodal relation between the steric configuration of the component amino acids of the cellular proteins and the polypetide on the one hand and the configuration of the specific chloramphenicol isomer which inhibits the formation of each of these classes of substances on the other hand suggests that the drug affects reactions involving amino acids in which their steric configuration plays a determining role.

Maxwell and Nickel (1954) found that L-erythro chloramphenicol

had between 1 and 2 per cent of the activity of the D-threo isomer in inhibiting the early growth of E. coli. The L-threo and D-erythro isomers had less than 0.4 per cent of the D-threo activity. Bacteriostatic concentrations of D-threo and L-erythro chloramphenicol caused significant inhibition of protein synthesis but not of nucleic acid synthesis in non-proliferating suspension of E. coli.

Using C^{14} labeled glutamate, Gale and Folkes (1952) studied amino acid exchange and protein synthesis in S. aureus. They found that a mixture of purines and pyrimidines stimulated both nucleic acid synthesis and protein synthesis but had a negligible effect on amino acid exchange. Chloramphenicol inhibited the incorporation of glutamate into the protein fraction of the cells. This inhibition was far greater when the incubation was in a complete mixture of amino acids than when glutamate was used alone, thus indicating that the mechanism of protein synthesis was much more sensitive than that of amino acid exchange. Measurements of total protein also confirmed the fact that protein synthesis dropped to zero when chloramphenicol was added. The formation of nucleic acids was uninhibited by chloramphenicol and in some systems there was an actual stimulation of the synthesis of ribonucleic acid. Similar results have been obtained by Pardee and Prestidge (1956). This is of particular interest since under certain conditions (e.g. induction) the synthesis of nucleic acids parallels the synthesis of protein. This action of chloramphenicol was found to be in contrast to the action of bacitracin which inhibits the synthesis of both nucleic acids and protein to the same degree.

Hopps, Wisseman and Hahn (1954) investigated the influence of

chloramphenicol upon another major category of synthetic processes, polysaccharide formation. Neisseria perflava produces an amylopectin-like polysaccharide from sucrose. The failure of the drug to influence the manufacture of the polysaccharide is another factor which adds favor to the pinpointing of the site of action on protein synthesis.

The degree of sensitivity or resistance of any bacterial culture is relative. Since antibiotics function by interfering with the metabolism of the microorganism, almost any of the many other factors which influence metabolism could possibly alter the effects of the drug. Variation of the supply of some metabolic precursor or an alteration of the metabolic rate or protein synthesis could change the effectiveness of the drug rather markedly. Because of this, a reported inhibitory concentration of any antibiotic is significant only for those conditions under which that particular test was performed. The antibacterial activity of streptomycin is dependent upon the test medium (Rhymer and Wallace, 1947); for example, brain heart infusion and phytone inhibited streptomycin activity. Chatterway, et al., (1949) observed that penicillin inhibition was antagonized by yeast extract. Eagle and Musselman (1949) found that recovery of Streptococcus pyogenes following exposure to toxic concentrations of penicillin was conditioned by the surrounding menstruum; that medium which supported good growth of the organism in the absence of penicillin also prompted more rapid and complete recovery from the antibiotic. Wainwright and Mullaney (1954) demonstrated that susceptibility to penicillin may vary according to the source of carbon in the medium. The composition of culture media has been shown to influence growth inhibition of various microorganisms by antibiotic discs (Neter, Murdock and

Kurz, 1952). In brain veal agar or proteose No. 3 agar chloramphenicol was highly inhibitory for P. aeruginosa whereas on Endo agar the inhibitory effect was negligible. When E. coli was used as the test organism good inhibition was obtained on all three media. Chloramphenicol was found to be less effective in urine agar than in brain veal agar. Swendseid, Wright and Bethell (1952) noted that suspensions of human bone marrow produce a compound termed "hemofolin" when incubated with folic acid. "Hemofolin" was observed to reverse chloramphenicol inhibition of growth while folic and folinic acids would not. These results were considered to constitute evidence relating the activity of chloramphenicol to folic acid metabolism in bacteria.

These reports cited above were, for the most part, casual observations and not primarily investigations into the problem of antibiotic antagonism by complex materials. Foster and Pittillo (1953_a) initiated the first investigation of any magnitude and thoroughness. These workers were able to demonstrate an antagonism of dihydrostreptomycin, chloramphenicol, terramycin, penicillin and bacillin by basamine, peptone, liver extract and brain heart infusion. Basamine, a yeast extract, was the most effective of these materials. Welsch (1955) observed that the nature of the culture media influenced the phenotypic expression of a given mutation of E. coli to streptomycin resistance. Gunnison, et al., (1955) described the effect of environment on the bactericidal activity of antibiotics on nonmultiplying bacteria. They found that neomycin, polymyxin and streptomycin were more effective in the absence of nutrients than in nutrient broth; oxytetracycline and bacitracin were equally effective in either environment; penicillin had little effect in the

absence of nutrients.

Davis and Sevag (1955), working with several species of gram negative bacteria, noted that the composition of the media had a marked effect on the apparent resistance of the organisms to antibiotics. Surprisingly, it was observed that all of the organisms tested were more resistant to the effects of bacitracin, streptomycin and neomycin in a glucose salts medium than in nutrient broth, whereas the opposite effect was observed with penicillin, tetracyclines, chloramphenicol and carbomycin. Reactions toward polymyxin B varied from one medium to another and seemed to be a strain characteristic. Cohen and Pratt (1956) observed that tryptose and beef extract would reverse bacitracin activity against M. pyogenes, while proteose peptone exhibited the opposite effect, i.e., the organism was more sensitive to bacitracin in the presence of proteose peptone. Ramsey and Padron (1954) noted that M. pyogenes did not readily develop resistance to chloramphenicol in a synthetic medium while resistant strains were easily obtained through serial transfer in a complex broth (Difco AC) in the presence of the antibiotic. Also the synthetic broth would not support the growth of these resistant strains in the presence of chloramphenicol at a level of 50 per cent of that which previously allowed their growth in the more complex medium.

Further search for metabolites that might be capable of reversing the action of chloramphenicol has involved studies of the amino acids. The possibility of the antibiotic competing with amino acids in the various metabolic processes of the cell is an attractive one. Rebstock, et al., (1949) showed that the structure of chloramphenicol resembled an amino acid and identified several of the degradation products of the drug

as substituted amino acids. Woolley (1950) has shown that inhibition of E. coli by chloramphenicol was overcome by additions of phenylalanine to a glucose salts medium. The antagonism was noncompetitive and was demonstrable only in the presence of minimally effective concentrations of the drug. Of the other amino acids tested, only tyrosine and tryptophan demonstrated antagonistic activity. With Lactobacillus casei only phenylalanine was able to influence the toxicity of chloramphenicol. Bergmann and Sicher (1952) investigated the mode of action of chloramphenicol on wild type E. coli and a number of mutants. Inhibition of the wild type was fully reversed by anthranilic acid. It was suggested that chloramphenicol interfered in the conversion of anthranilic acid to indole. Glycine, which exhibits a slight retarding action on the growth of E. coli has been found to antagonize the action of chloramphenicol, whereas aspartic acid, which is highly inhibitory, enhances the action of the drug (Mentzer, et al., 1950).

Toxicity of dihydrostreptomycin and of L-tryosine for Aerobacter aerogenes and a mycobacterium was found by Pittillo and Foster (1954) to be reversed to a significant degree by phenylalanine. The toxicities of antibiotic 106-7 (cycloserine, Merck & Co.) and of chlortetracycline for A. aerogenes were reversed by glycine. It was of interest that the antibiotics which were reversed by the same metabolite were synergistically inhibitory when combined. Pittillo and Foster (1953) differentiated between streptomycin and dihydrostreptomycin inhibition by means of reversing metabolites. These closely related antibiotics had been regarded as identical in antimicrobiol growth effects and in enzymatic effects, as well as in dependency experiments. However, it was found that the

inhibitory effect of streptomycin was effectively reversed by guanylic acid, guanosine, cytosine, xanthosine, thymine and yeast autolysate but not by phenylalanine. When dihydrostreptomycin was tested against these same materials its activity was reversed effectively only by phenylalanine.

In a study of the role of amino acids on the sensitivity of A. aerogenes and P. aeruginosa to polymyxin B, Haas and Sevag (1953) observed that maximum resistance occurred in a glucose salts medium. The addition of the individual amino acids leucine, serine and methionine had no effect on the degree of susceptibility. However, when any combination of these amino acids was added to the medium the toxic action of the antibiotic was greatly enhanced. The use of casein hydrolysate also increased the effect of the antibiotic. Yoneda, et al., (1952) were able to show a competitive antagonism between pyridoxal and the antimicrobial agent isonicotinic acid hydrazide, and Lichstein (1955) found that preincubation of the cells with pyridoxal decreased significantly the degree of inhibition by isonicotinic acid hydrazide of the tryptophanase activity of E. coli. The amount of protection afforded by preincubation varied with the concentrations of pyridoxal and of isonicotinic acid hydrazide and to some extent with the time of preincubation. Foster and Pittillo (1953_b) tested various vitamins and a vitamin mixture for ability to reverse the inhibitory effects of chlortetracycline, chloramphenicol, penicillin, dihydrostreptomycin and oxytetracycline on a strain of E. coli. Chlortetracycline was the only antibiotic of the five tested in which antagonism by vitamins occurred to any extent; the antagonism by riboflavin was particularly noteworthy. Further examination, using twelve different

organisms that were sensitive to chlortetracycline, showed that this characteristic was not limited to the strain first tested. By using a riboflavinless strain of Bacillus subtilis it was possible to demonstrate a competitive relationship between aureomycin and riboflavin with an inhibition index of 8.

The investigation described herein was undertaken for the purpose of obtaining a better understanding of the effects of the physiological state of a microorganism upon the activity of chloramphenicol. Elucidation of the role of nutrient materials would lead to a better interpretation of laboratory tests designed to determine the antibiotic sensitivity of bacterial cultures. A comparison of the activity of these nutrients on chloramphenicol sensitive and resistant strains of M. pyogenes could lead to a better understanding of the physiological differences associated with increased resistance to chloramphenicol and the mode of action of the antibiotic.

CHAPTER II

EXPERIMENTAL

Bacterial Cultures

The test organisms used throughout this study were a chloramphenicol sensitive strain of Micrococcus pyogenes var. aureus (Strain S) and a resistant variant (Strain R) developed from it by serial transfer in increasing concentrations of chloramphenicol (Ramsey and Padron, 1954). Lactobacillus casei ATCC 7469 was used in certain phases of this work for the microbiological assay of riboflavin.

Stock cultures of the two strains of M. pyogenes were stored at 4 C on slopes of Difco AC agar. The agar slopes used for maintaining the resistant strain were supplemented with 500 ug of chloramphenicol per ml. Transfers were made at monthly intervals by inoculating AC broth, incubating at 37 C for 20 hours and transferring to the appropriate stock slope. These slopes were then incubated at 37 C until visible growth appeared. This time interval was usually 12 to 18 hours for Strain S and 3 to 4 days for Strain R.

The organisms to be used as an inoculum were prepared from 20-24 hour cultures grown at 37 C in Difco-A.C. broth or synthetic broth as desired for a particular experiment. The broth cultures were centrifuged, washed once in saline, and resuspended to their original volume

in either saline or a single strength basal salt solution. Dilutions of the washed suspensions were made in sterile distilled water except for the final dilution which was made in chilled synthetic broth without glucose or a single strength basal salt solution. This final dilution of inoculum was maintained in an ice bath during the period of inoculation; 0.05 ml of the desired dilution was used as the inoculum.

Chloramphenicol

Stock solutions of chloramphenicol were made in distilled water and stored at 4 C in foil or paper wrapped bottles to protect the antibiotic from light. The desired concentration of chloramphenicol was added to the medium prior to sterilization.

Synthetic Medium

The composition of the synthetic medium used is given in Table I. This medium was prepared in two parts. A double strength basal portion was prepared (Part A) and then supplemented with the vitamins listed in Part B. Since a single strength medium was desired, this was then diluted by the addition of distilled water and/or other supplements to give the desired final concentration. The glucose was autoclaved separately as a 20 per cent solution and added aseptically to the otherwise complete medium. For plating experiments the synthetic medium described above was supplemented with 1.5 per cent agar. In certain experiments it was necessary to omit some of the ingredients of the synthetic medium or to add increased quantities of some materials. Any variations from the composition of the synthetic medium indicated in Table I are noted in the text.

TABLE I

SYNTHETIC MEDIUM

Part A: Double Strength Basal Medium
(Weight per Liter)

K_2HPO_4	4.0 gms.
$(NH_4)_2SO_4$	2.0 gms.
$MgSO_4$	0.4 gms.
$MnSO_4$	20 mg.
$FeSO_4$	20 mg.
NaCl	10 mg.
Tryptophan	50 mg.
Cystine	50 mg.
Casamino acids	2.0 gms.
Glucose*	40 gms.

Part B: Vitamin Supplement#
(Final concentration, ug per ml)

Niacin	2.0	Inositol	1.0
Thiamin	2.0	Para-amino benzoic acid	1.0
Riboflavin	1.0	Folic acid	0.01
Pyridoxamine	1.0	Biotin	0.01
Calcium pantothenate	1.0	Cyanocobalamine	0.00015

pH 7.0

*Glucose was autoclaved separately and added aseptically to the sterile medium to give a final concentration of 2 per cent.

#20 ml of each vitamin stock solution (100 fold final concentration) was added to each liter of double strength basal medium.

Since the primary object of the study was to determine the role of physiological conditions in resistance, a number of techniques were used in order to avoid, as much as possible, erroneous conclusions inherent in any such study. First, in order to circumvent the possibility that what appeared to be stimulation was actually selection of random mutants, inocula of small size were used except in those experiments in which the effect of varying inocula sizes were examined. Further, it was necessary to establish parameters which could be used for assaying stimulation. The synthetic medium was used as a "base line" menstruum with a minimal activity in counteracting the antibiotic; a complex medium (Difco AC agar) was used as the "stimulatory medium." All materials to be tested were compared with these two extremes. The material to be examined for activity was pipetted into sterile plates and 15 ml of the synthetic agar medium containing the desired concentration of chloramphenicol was then added to the supplement. Thorough mixing of the supplement with the medium was found to be essential in order to obtain uniform results.

Plating Techniques

The plating technique, essentially the same as that employed by Foster and Pittillo (1953_a), consisted of pipetting 0.05 ml of the diluted cell suspension into the solidified agar in two or more plates and spreading it over the entire surface with a sterile bent glass rod. Colony counts were made after 1, 2, 3, 5, 7 and 10 days incubation at 37 C. A Quebec colony counter, which gave an magnification of 2, was used to count all plates.

Broth Cultures

Several different techniques were used to follow the growth of the bacterial cultures in liquid media under experimental conditions. Examination of broth cultures for visible turbidity was used as a screening technique to detect chloramphenicol reversing activity. Although such a method cannot be considered to be quantitative, it will be shown that the results obtained by the method were qualitatively similar to the more delicate and quantitative methods described below. Because of this, and also because of the relative simplicity of the method, its use in preliminary experiments was considered to be justified.

Another technique was the determination of viable cell count at various time intervals. Dilutions of aliquots of the bacterial culture were made in distilled water and one ml portions of several dilutions were inoculated into duplicate petri plates. Twenty ml quantities of agar medium were then poured into each plate and the sample and agar mixed thoroughly. The plating medium consisted of 0.5 per cent glucose, 0.3 per cent yeast extract, 1.0 per cent peptone and 1.5 per cent agar. The plates were allowed to harden and then were incubated at 37 C. Colony counts were performed at 24 and 48 hours. Graphic representation of the growth of a bacterial culture is usually best achieved by using a logarithmic scale for whatever measurement of the density of the culture is made. Moreover, as Monod (1949) has pointed out, both diagrams and associated calculations are more readily interpreted if logarithms to base 2 are used instead of common logarithms (i.e., base 10); an increase of 1 unit on the logarithmic scale then corresponds to the equivalent of

one division of each of the cells present at the beginning of the interval. Therefore, the tables prepared by Finney, et al., (1955) were used to calculate the logarithm to the base of 2 of the number of viable organisms. This value was then used in the graphic presentation of these results.

For turbidimetric growth curves one drop of an appropriate dilution of a washed cell suspension was inoculated into photometric tubes (15 mm diameter) which contained 5 ml of a broth medium. Turbidimetric measurements were made at intervals using a Bausch and Lomb Spectronic 20 Colorimeter set at a wavelength of 525 mu. Color error was minimized by using an uninoculated tube of medium as a blank. When the effect of materials with varying degrees of color were being examined, appropriate blanks were prepared.

Preincubation Experiments

Sterile molecular membrane filters were inoculated by spreading 0.05 ml of the inoculum over the surface of the membrane. The inoculated molecular membranes were placed on drug free media. After the desired preincubation interval, the membranes on drug-free agar plates were transferred to plates of the same type media containing graded concentrations of chloramphenicol. The plates were incubated at 37 C and counts were made at daily intervals for 7 days. Multiplication of the organism during the preincubation period was controlled by the fact that they become fixed on the membranes and any multiplication occurring will be clonal in nature and therefore will not affect the total colony count.

Cell Generation Time on Agar Media

The generation time on agar plates was determined by the method of Spiegelman, Sussman and Pinska (1950). The inoculum was spread over the surface of a number of agar plates. At varying periods of time replicate plates were respread with a sterile bent glass rod and then incubated. Respreading of the plates should not result in an increased colony count until there has been some multiplication of cells. As demonstrated by this procedure, the average time required for the first cell division to occur was 4 hours.

Riboflavin Assay and Degradation

Degradation of riboflavin was accomplished by adjusting aliquots containing 10 ug per ml to pH values of 5.0 and 9.0 and irradiating them for a period of one hour with a Mineralite U. V. Lamp (Model R51) placed at a distance of 8 cm from the surface of the solution. The depth of the solution was 1.1 cm and the solutions were continuously agitated by a magnetic stirrer during the period of irradiation. Residual riboflavin was determined by the growth response of Lactobacillus casei (ATCC 7469) to varying concentrations of the irradiated solutions added to Riboflavin Assay Medium (Difco). An untreated aliquot was used as the standard riboflavin solution. A standard microbiological response curve was prepared by plotting the turbidimetric readings for each level of the riboflavin standard solution used against the micrograms of riboflavin contained in the respective tubes. From this standard curve, the riboflavin count of the tubes containing irradiated solutions of riboflavin could be determined by extrapolation.

CHAPTER III

RESULTS

Padron (1953), using the method of Smith and Worrell (1949) for the detection of arylamine, found that chloramphenicol resistant strains of M. pyogenes did not possess an increased capacity to degrade chloramphenicol (enzymatically) to the corresponding arylamine. The role of enzymatic degradation of the antibiotic as a mechanism of resistance was reinvestigated by use of a microbiological assay for chloramphenicol. The resistant strain of M. pyogenes (Strain R) was grown in a subinhibitory concentration of chloramphenicol (500 ug per ml) in A C broth; an uninoculated flask was incubated as a control. Both broths containing chloramphenicol were then sterilized by Seitz filtration and aliquots were added to sterile A C broth to give a series of tubes containing concentrations of chloramphenicol ranging from 0 to 10 ug per ml. The tubes were then inoculated with the sensitive parent strain of M. pyogenes (Strain S) and incubated for a total of 10 days. No decrease in chloramphenicol could be detected in the medium in which Strain R had grown. These results were in agreement with those of Padron and show that enzymatic degradation of chloramphenicol is not a major factor in the resistance of Strain R to the antibiotic.

Chabbert and Debruge (1956) found that chloramphenicol resistant strains of staphylococcus produced in ordinary media a substance which

was diffusible in agar and able to inhibit the action of doses of chloramphenicol normally effective against susceptible strains. Therefore, the filtrate of a broth culture of Strain R grown in the absence of chloramphenicol was examined for the presence of a chloramphenicol antagonist which might have been excreted into the medium by the resistant organism. The first method used consisted of placing sterile filter paper discs impregnated with filtrate of Strain R on the surface of synthetic agar plates which contained chloramphenicol and which were seeded with Strain S. In order to examine larger quantities of filtrate, agar plates similar to those described above were prepared and discs of agar 15 mm in diameter were removed by use of a cork borer. Up to 0.5 ml of the test material could be placed in the resulting wells. Controls of uninoculated A C broth were used. In no instance, utilizing these two test systems, could any antagonism of drug activity be demonstrated. Therefore, the factor which endows Strain R with its resistance to chloramphenicol is not reflected by the presence in the filtrate of any material which would enhance the growth of Strain S in the presence of chloramphenicol.

Padron (1953) found that synthetic broth would not support growth of M. pyogenes in 50 per cent of the concentration of chloramphenicol that was growth limiting when tested in A C broth. The effect of increasing concentrations of chloramphenicol upon the time required for growth initiation and the rate of growth of Strain S in a synthetic medium is shown in Figure 1. Each increasing concentration of chloramphenicol causes a prolongation of lag and a lower rate of cellular multiplication. An investigation of the role of A C broth in permitting

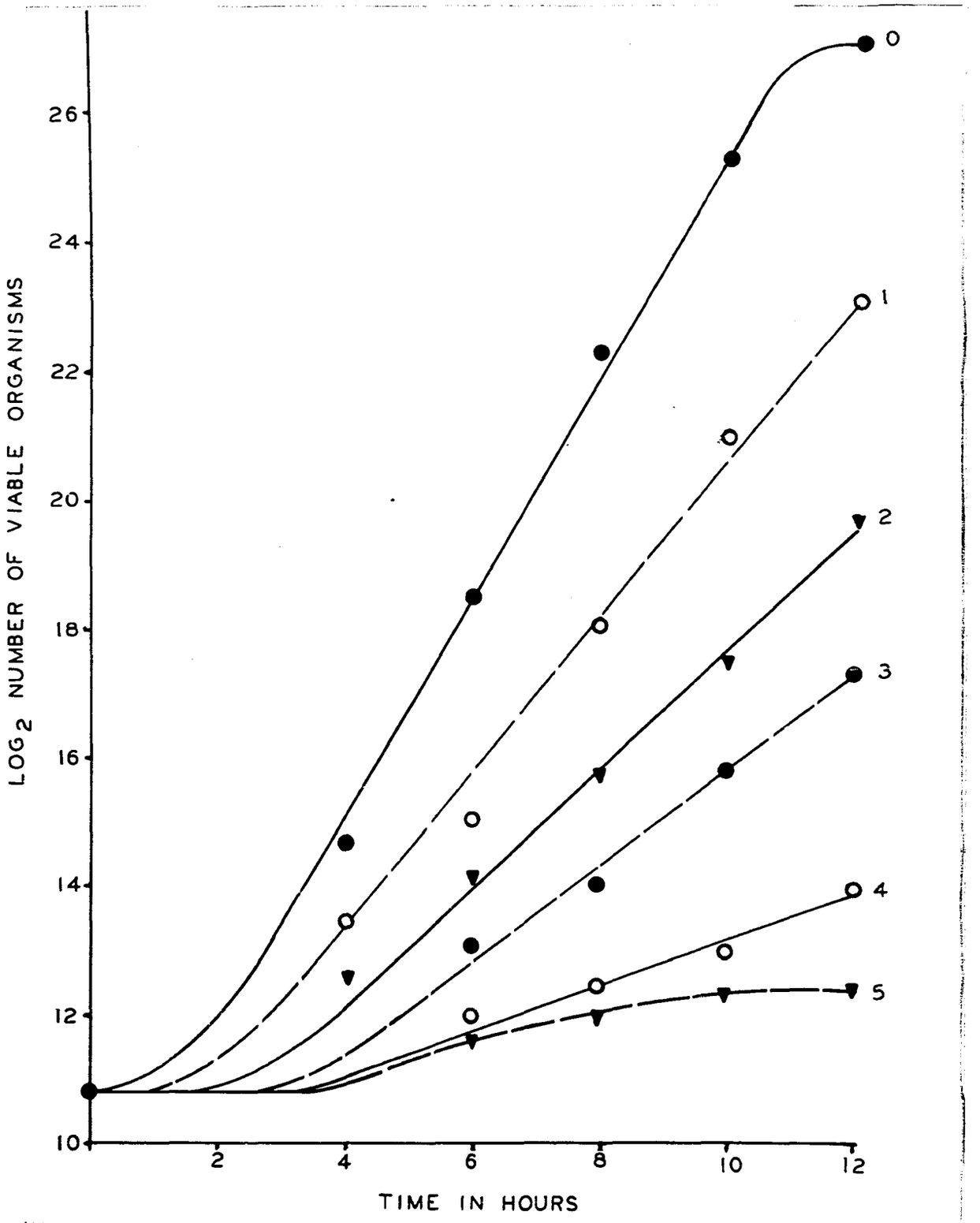

Figure 1. The effect of increasing concentrations of chloramphenicol on the growth of Micrococcus pyogenes (Strain S). The concentration of chloramphenicol in micrograms per ml of synthetic medium is shown by the numbers at the end of each line in the figure.

growth in relatively high chloramphenicol concentrations was made. In a preliminary screening it was found that the addition of as little as 0.34 gms of dehydrated A C broth per 100 ml of synthetic broth would have a demonstrable effect on the ability of Strain S to grow in the presence of chloramphenicol. Various ingredients of A C broth were examined for their ability to antagonize the activity of chloramphenicol when added to synthetic broth (Table II). Whole A C broth permitted growth of Strain R in 400 ug chloramphenicol per ml, whereas unsupplemented synthetic broth permitted growth in only 100 ug chloramphenicol per ml. In like manner, synthetic broth supplemented with yeast extract or proteose peptone No. 3 antagonized the action of chloramphenicol; yeast extract appeared to be superior to the complete A C complex. The effect of yeast extract was reflected both in the resistance of the culture and in the more rapid rate of growth in the presence of any given concentration of antibiotic. A quantitative plating assay of the stimulatory activity of yeast extract is seen in Figure 2. Here, viable cell counts of synthetic broth cultures with and without a yeast extract supplement are compared. It may be seen that yeast extract exerts a dramatic effect on both the time required for growth initiation and the rate of growth once it commences. Lag time was, in this instance, reduced by 1 hour and the generation time has been reduced from 76 minutes to 24 minutes. By contrast, malt extract, while apparently not inhibitory in the absence of chloramphenicol, caused a suppression of growth in the lowest concentration of antibiotic tested (Tables II and VI).

The interrelationships of yeast extract, proteose peptone No. 3

TABLE II

ANTAGONISM OF CHLORAMPHENICOL BY COMPONENTS OF A C BROTH[1]

Supplement added to synthetic broth, per cent	Chloramphenicol concentration - ug per ml								
	0	10	25	50	100	200	300	400	500
None	24*	48	60	108	120	—**	—	—	—
A C Broth#	24	24	24	48	72	120	156	240	—
Yeast Extract 0.3	24	24	48	72	72	96	120	144	180
Proteose peptone No. 3 1.0	24	48	48	72	96	144	180	216	—
Malt Extract 0.3	24	—	—	—	—	—	—	—	—

[1]Test organism: M. pyogenes, Strain R.
*Hours required for visible growth to appear.
**Complete inhibition
#Not incorporated as a supplement to synthetic broth.

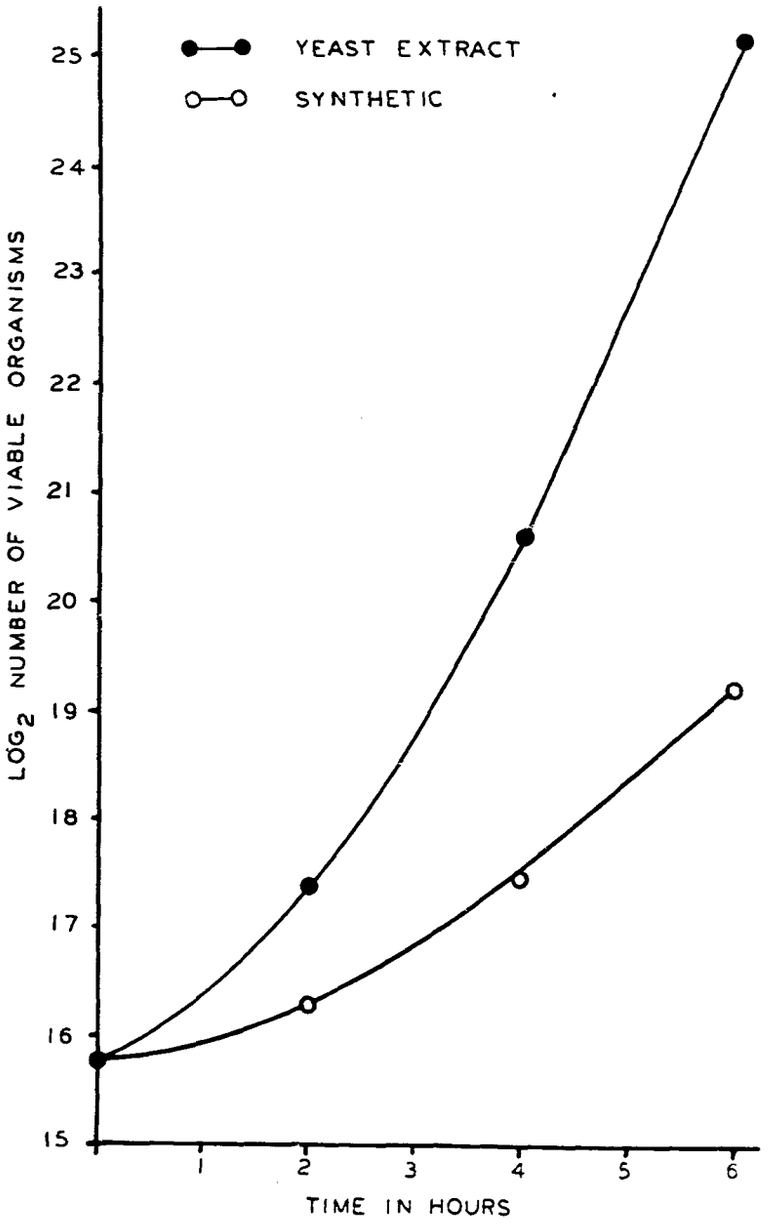

Figure 2. The stimulatory effect of yeast extract on the growth of Micrococcus pyogenes (Strain R). The yeast extract was added as a supplement to the synthetic medium at a concentration of 0.3 per cent.

and malt extract are shown in Table III. It is obvious that the greatest antagonistic activity of A C broth is derived from two of its components, yeast extract and proteose peptone No. 3. The malt extract when combined with either or both of these materials reduces their activity. This, as well as the results shown in Table II, indicates that malt extract contains some factor which has a marked inhibitory effect on the test organism in the presence of chloramphenicol.

In view of the demonstrated fact that the physiological condition of the test medium can play an important role in drug resistance, it might be expected that inoculum size would also be a contributing factor to resistance, since inoculum size will often determine whether or not a culture will grow even in the absence of an inhibitor. Table IV shows the results of an examination of the influence of inoculum size on chloramphenicol resistance in synthetic broth and in a yeast extract supplemented broth. The reduced resistance in synthetic broth that accompanies dilution of the inoculum is quite marked. In the presence of yeast extract this effect is also quite evident. However, in this case there is a plateau effect where subsequent dilution, within the ranges tested, had little further effect on the level of chloramphenicol required to inhibit growth. This is an example of the normal ranges of resistance that occur within a given bacterial culture. As described by Yudkin (1953), the resistance of a given population follows a gaussion distribution and it might be expected that a more concentrated inoculum would contain more cells representing the extreme ranges of sensitivity and resistance while the more dilute inoculum would contain only those cells which were representative of the majority of the population of the

TABLE III

THE EFFECT OF COMBINATIONS OF SOME COMPONENTS OF A C BROTH ON CHLORAMPHENICOL ANTAGONISM#

Supplement added to synthetic broth per cent	Chloramphenicol concentration - ug per ml							
	0	25	50	100	200	300	400	500
None	24*	84	108	**	___	___	___	___
Proteose peptone No. 3 1.0 Yeast extract 0.3 Malt extract 0.3	24	48	60	72	108	144	___	___
Proteose peptone No. 3 Yeast extract	24	48	48	72	96	108	168	___
Proteose peptone No. 3 Malt extract	24	72	144	168	___	___	___	___
Yeast extract Malt extract	24	48	60	72	108	144	___	___
A C Broth[1]	24	48	54	72	108	144	156	___

#Test organism: M. pyogenes, Strain R.

*Hours required for visible growth to appear.

**Complete inhibition.

[1]Not incorporated as a supplement to synthetic broth.

TABLE IV

THE EFFECT OF THE CONCENTRATION OF INOCULUM ON CHLORAMPHENICOL RESISTANCE OF MICROCOCCUS PYOGENES (STRAIN R)

Chloramphenicol concentration ug per ml	Reciprocal of Dilution of Inoculum									
	0	1	2	3	4	0	1	2	3	4
	Synthetic medium plus 0.3 per cent yeast extract					Synthetic medium				
0	18*	18	18	18	18	18*	42	48	42	48
10	18	24	42	42	42	24	96	144	—	—**
25	24	42	72	48	60	72	144	—	—	—
50	42	72	90	72	90	90	—	—	—	—
100	60	90	108	96	102	96	—	—	—	—
200	72	114	—	—	—	—	—	—	—	—
300	102	144	—	—	—	—	—	—	—	—
400	144	—	—	—	—	—	—	—	—	—
500	216	—	—	—	—	—	—	—	—	—

*Hours required for visible growth to appear.
**Complete inhibition.

culture.

Several dilutions of inocula were used in an experiment designed to test a number of complex materials for their ability to antagonize chloramphenicol. The experiment was not designed to obtain a quantitative measure of activities but was a screening test to determine the presence or absence of such activity. Complex materials were added to synthetic broth containing graded concentrations of chloramphenicol. The concentration of the complex materials (Table V) was based on the physical characteristics of the material and the concentrations that are used in ordinary bacteriological media. The synthetic broth supplemented with yeast extract had already been shown to be a rather potent antagonist of chloramphenicol activity and was used here as an example of a material with such activity. It will be noted that the yeast extract, liver extract and tryptose phosphate broth (Difco) allowed growth in tubes containing up to and including 500 ug per ml of chloramphenicol when tested with undiluted inoculum. A decreased effect, proportional to cell numbers of the other two inocula sizes, was found with yeast extract and tryptose phosphate broth. The liver extract, however, was not as effective when tested with the more dilute inocula. The tryptose phosphate broth was not used as a supplement to the synthetic broth, but was included in this experiment as an example of a complex broth that is commonly used for the cultivation of some of the more fastidious microorganisms. Although not included in this experiment, proteose peptone No. 3 has been found to exhibit results similar to those found with yeast extract.

Hemoglobin, human plasma and egg yolk had little effect with

TABLE V

ANTAGONISM BETWEEN COMPLEX SUBSTANCES AND CHLORAMPHENICOL IN LIQUID MEDIA

Supplement added, per cent	Dilution of inoculum[1]	Chloramphenicol concentration ug per ml								
		0	10	25	50	100	200	300	400	500
None	10^{0}	6*	18	30	42	72	120	168	—**	—
	10^{-2}	12	42	72	96	—	—	—	—	—
	10^{-4}	18	72	96	—	—	—	—	—	—
Yeast extract, 0.3	10^{0}	6	12	18	24	42	60	96	120	168
	10^{-2}	6	24	36	54	96	120	—	—	—
	10^{-4}	12	42	54	96	144	—	—	—	—
Bacto-liver, 1.0	10^{0}	6	18	24	36	48	84	96	144	216
	10^{-2}	6	30	54	72	—	—	—	—	—
	10^{-4}	12	42	72	96	—	—	—	—	—
Bacto-hemoglobin 0.3	10^{0}	6	24	36	42	60	120	168	216	—
	10^{-2}	12	30	42	72	96	120	192	—	—
	10^{-4}	18	42	72	90	102	192	—	—	—

TABLE V, Continued

ANTAGONISM BETWEEN COMPLEX SUBSTANCES AND CHLORAMPHENICOL IN LIQUID MEDIA

Supplement added, per cent	Dilution inoculum[1]	Chloramphenicol concentration ug per ml								
		0	10	25	50	100	200	300	400	500
Serum Plasma 1.0	10^{0}	6*	18	24	36	42	96	144	—**	—
	10^{-2}	12	36	42	72	96	144	—	—	—
	10^{-4}	24	42	72	96	120	—	—	—	—
Egg Yolk 1.0	10^{0}	6	18	36	48	84	144	160	—	—
	10^{-2}	12	36	72	96	144	168	—	—	—
	10^{-4}	18	54	96	144	240	—	—	—	—
Bacto-Peptone 0.5	10^{0}	6	12	18	30	48	96	120	192	—
	10^{-2}	12	36	60	96	—	—	—	—	—
	10^{-4}	18	42	72	120	—	—	—	—	—
Neopeptone 0.5	10^{0}	6	12	18	42	60	120	216	240	—
	10^{-2}	12	42	72	96	144	216	—	—	—
	10^{-4}	18	72	120	—	—	—	—	—	—

TABLE V, Continued

ANTAGONISM BETWEEN COMPLEX SUBSTANCES AND CHLORAMPHENICOL IN LIQUID MEDIA

Supplement added, per cent	Dilution of inoculum[1]	Chloramphenicol concentration ug per ml								
		0	10	25	50	100	200	300	400	500
Egg Albumen 1.0	10^{0}	6*	18	24	42	96	—**	—	—	—
	10^{-2}	18	42	84	144	—	—	—	—	—
	10^{-4}	24	72	120	—	—	—	—	—	—
Skim Milk Powder 0.5	10^{0}	6	18	36	42	72	120	192	—	—
	10^{-2}	12	72	144	—	—	—	—	—	—
	10^{-4}	18	96	—	—	—	—	—	—	—
Asparagus Juice (Canned) 1.0	10^{0}	6	12	24	48	72	120	—	—	—
	10^{-2}	12	120	—	—	—	—	—	—	—
	10^{-4}	18	—	—	—	—	—	—	—	—
Tryptose Phosphate Broth#	10^{0}	6	12	24	42	54	96	120	144	216
	10^{-2}	12	24	42	72	96	144	—	—	—
	10^{-4}	12	42	72	120	—	—	—	—	—

[1]Test organism: M. pyogenes, Strain R.
*Time, in hours, required for visible growth to appear.
**Complete inhibition.
#Not tested as a supplement to synthetic broth; resuspended as recommended by Difco and tested as such.

the heavier inoculum but all exhibited a definite antagonistic action to chloramphenicol when tested with smaller inocula. Peptone exhibited activities that were quite similar to those obtained with liver extract although it was less effective when tested with the undiluted inoculum. Antagonism was demonstrated with neopeptone only with the intermediate inoculum size. Egg albumin was inhibitory to more concentrated inoculum and to a lesser degree to the smaller inocula; dry milk yielded similar results. Asparagus juice was quite inhibitory, particularly with smaller inocula.

The ability of complex materials to antagonize chloramphenicol activity is reflected by both the total concentration of chloramphenicol required to prevent growth and by the time required for growth to appear in subinhibitory concentrations. For example, with an inoculum dilution of 10^{-2}, yeast extract increases the inhibitory concentration of chloramphenicol from 50 to 200 ug per ml and at the same time permits growth in 50 ug chloramphenicol per ul in 54 hours as compared to 96 hours for the unsupplemented control tube.

A more quantitative measure of the activity of a number of complex materials was obtained through the use of plating experiments. The results were qualitatively similar to those obtained with liquid media and are presented in Table VI. A significant factor in the interpretation of plating experiments was the length of time required for the development of the total plate count in the presence of even rather low concentrations of chloramphenicol. This necessitated prolonged incubation of plates containing chloramphenicol in order to obtain the total count. The greatest difference observed between the response of Strain

TABLE VI

ANTAGONISM BETWEEN COMPLEX SUBSTANCES AND CHLORAMPHENICOL IN SOLID MEDIA

Chloramphenicol ug per ml	Time, Days	Colony count* on synthetic agar plus supplement, 0.3 per cent								
		None	Yeast Extract	Proteose Peptone No.3	Liver	Malt Extract	Beef Extract	Peptone	Beef Heart infusion	A C control
0	1	0	570	422	419	0	540	340	380	630
	2	190	595	447	444	250	577	375	407	656
	3	214	601	437	455	255	603	372	417	656
10	2	0	0	0	0	0	0	0	0	0
	3	0	369	251	253	0	317	86	171	317
	5	101	364	253	282	1	317	93	157	321
	7	140	423	263	345	3	317	101	189	321
25	5	0	171	64	22	0	158	27	143	171
	7	30	203	67	39	0	171	31	152	178
	10	31	203	67	41	0	171	32	152	178
50	5	0	38	0	0	0	15	0	0	5
	7	2	82	14	4	0	62	2	33	27
	10	2	86	22	20	0	65	4	43	33

TABLE VI, Continued

ANTAGONISM BETWEEN COMPLEX SUBSTANCES AND CHLORAMPHENICOL IN SOLID MEDIA

Chloramphenicol ug per ml	Time, Days	Colony count** on synthetic agar plus supplement, 0.3 per cent							
		None	Yeast Extract	Proteose Peptone No.3	Liver	Malt Extract	Beef Extract	Beef Heart infusion	A C control#
0	1	0	348	317	336	289	244	314	264
	2	231	348	317	336	292	250	314	264
3	2	40	383	335	313	141	246	371	147
	3	130	307	345	392	162	259	401	160
5	2	34	278	286	282	0	122	301	127
	3	39	293	294	309	0	128	322	140
	5	42	293	309	319	0	128	322	140
	7	58	293	309	340	72	128	322	140
10	3	0	0	0	0	0	0	0	0
	5	0	226	226	134	0	40	73	100
	7	0	241	226	165	0	40	157	102

*Test organism: M. pyogenes, Strain R.
**Test organism: M. pyogenes, Strain S.
#Not tested as supplement to synthetic agar; resuspended as recommended by Difco and tested as such.

S and Strain R to complex supplements lay in the larger numbers of colonies of Strain R which developed on drug free plates that were supplemented with the complex material (compare columns 3 and 4, Table VI). This increase in colony count was also observed with Strain S, but the relative increase was not as great (columns 3 and 4, Table VI).

Here again, yeast extract and proteose peptone No. 3 and, in addition, beef extract and Bacto-liver, were the most effective antagonists to chloramphenicol action. The most significant point of the results of both tube and plate tests appears to be the parallel activity of the supplements in both their stimulatory and antagonist effects. For example, (even in the absence of chloramphenicol) the yeast extract supplement to the synthetic agar base stimulated three times as many cells of Strain R to grow as in the unsupplemented base (Table VI). Moreover, the supplement promoted more rapid growth of the inoculum cells since approximately 95 per cent of the total colony count was visible in 24 hours as compared with no visible colonies on the unsupplemented medium in the same time interval. Thus, one cannot ascribe an antagonistic effect *per se* to the supplement since the apparent antagonism observed may be accounted for by the stimulatory effect. This is in contrast to the results reported by Foster and Pittillo (1953) who found that *E. coli* colony counts were no higher in supplemented than in unsupplemented medium in the absence of antibiotics.

Quantitative studies were made on two of the more potent antagonists, yeast extract and proteose peptone No. 3. For this experiment replicate plates of synthetic agar were supplemented with graded amounts

of the materials plus 0, 10 or 25 ug chloramphenicol per ml when tested with Strain R and with 0 and 5 ug chloramphenicol per ml when tested with Strain S. The response obtained with the two supplements was found to be quite different. (Figures 3 and 4). Even the lower concentrations of proteose peptone No. 3 exhibited a striking ability to antagonize the action of chloramphenicol. The response to the lower concentrations of yeast extract was not as great but the activity of higher concentrations equaled that of the same concentration of proteose peptone No. 3. The response of both Strain S and Strain R to the material was the same in the absence as well as in the presence of chloramphenicol. The antagonism caused by both materials does not appear to be competitive since these substances do not counteract corresponding quantities of chloramphenicol at all levels tested. These results again suggest the possibility that negation of chloramphenicol inhibition is a non-specific effect resulting from growth stimulation.

Attempts to identify the components of the complex materials that were responsible for the antagonistic activity were without success. Yeast extract and proteose peptone No. 3 were absorbed with norite at pH values of 3, 7 and 10 and then filtered. The filtrates were adjusted to neutrality and compared with untreated materials for their ability to antagonize chloramphenicol activity. Those materials which had been absorbed with norite at pH 7 and 10 had not lost any of their activity, while that absorbed at pH 3 possessed less activity. Repeated attempts to absorb these factors on norite and subsequently elute them for testing failed. Both the norite absorbed material (pH 3) and the material eluted from the norite at a pH of 10 were toxic for Strain R. Controls contain-

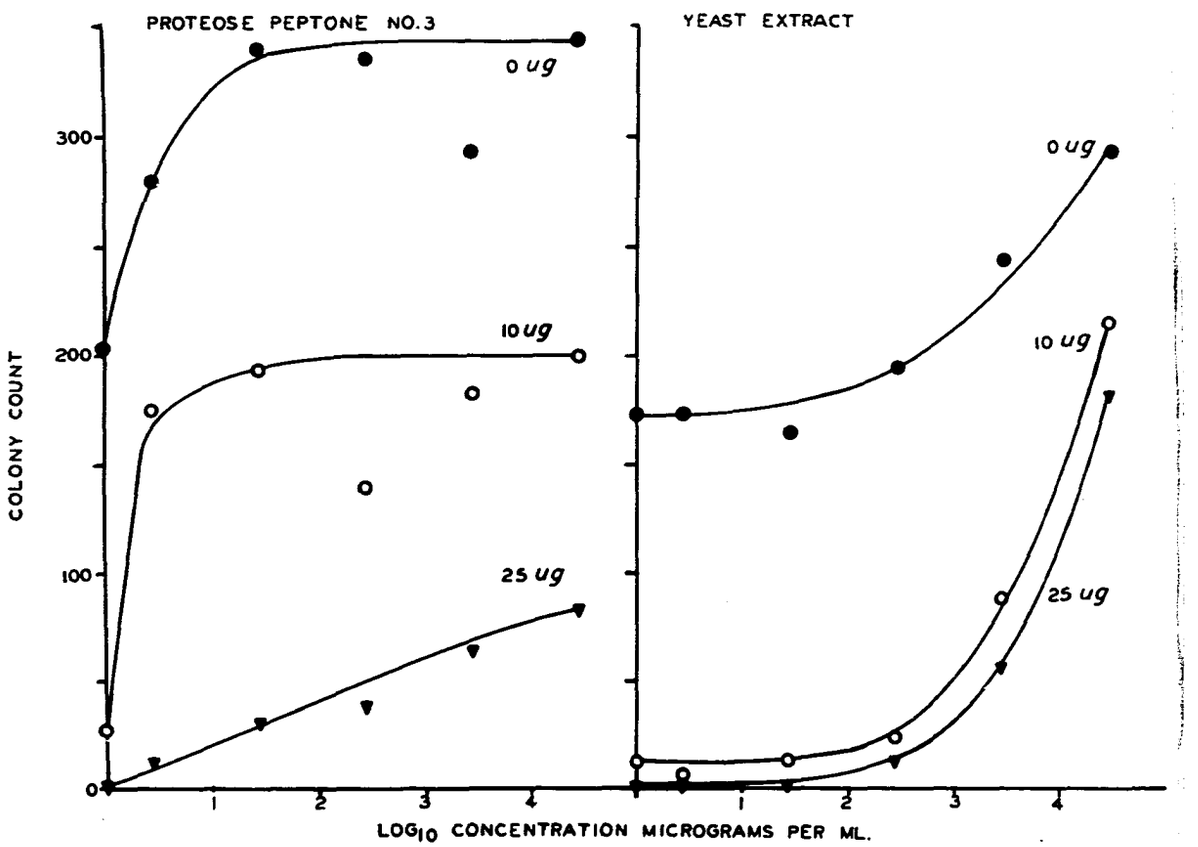

Figure 3. Assay of growth enhancement of Micrococcus pyogenes (Strain R) and chloramphenicol antagonism by proteose peptone No. 3 and yeast extract. Chloramphenicol concentration per ml is given by the numbers at the end of each line in the figure. 2 days incubation.

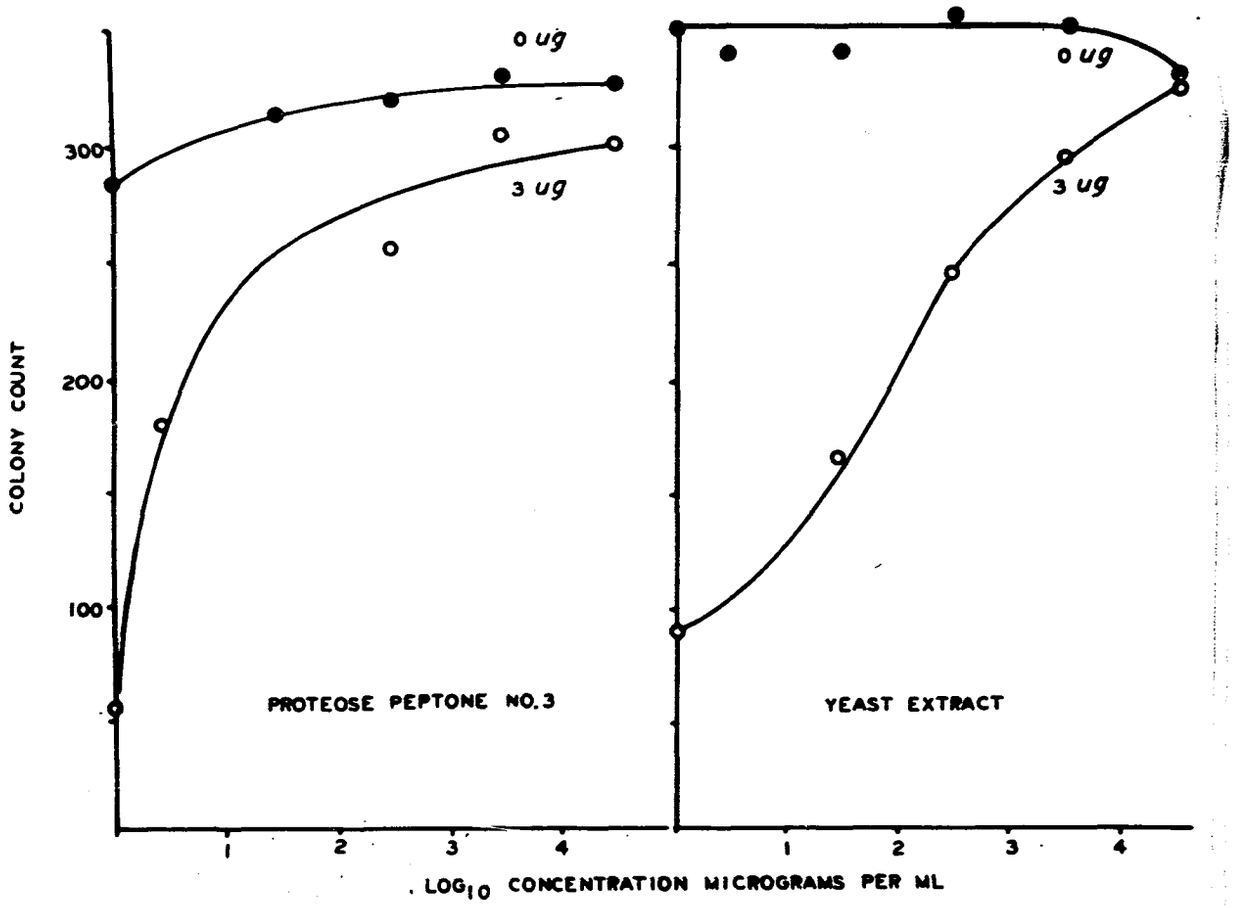

Figure 4. Assay of growth enhancement of Micrococcus pyogenes (Strain S) and chloramphenicol antagonism by proteose peptone No. 3 and yeast extract. Chloramphenicol concentration per ml is given by the numbers at the end of each line in the figure. 2 days incubation.

ing an amount of NaCl equal to that resulting from adjusting the pH did not demonstrate any inhibitory action. Similar treatment did not reduce the inhibitory action of the malt extract.

In order to examine more critically the respective roles of stimulation and direct antagonism, experiments were performed in which the chloramphenicol resistance of cells preincubated both in the presence and absence of yeast extract was compared with the resistance of cells placed directly in contact with the antibiotic. The results from an experiment with Strain S (Figure 5) indicate that two separate and distinct processes may operate to endow cells with a higher degree of resistance. First, the incorporation of yeast extract protects the cells from the deleterious effect of the antibiotic. The "control" curve of Figure 5A refers to membranes inoculated and placed directly on yeast extract agar supplemented with graded amounts of chloramphenicol (0 to 10 ug per ml). Comparison of this curve with the "control" curve of Figure 5B in which synthetic agar was used indicates that yeast extract in some way directly antagonizes the inhibitory effect of the antibiotic. These results are in accord with those of Foster and Pittillo (1953_a).

The second effect to be noted from Figure 5 is that of preincubation. Allowing the cells to metabolize for four hours prior to exposure to chloramphenicol also endows the organisms with a higher degree of resistance. Yeast extract serves to magnify the effect of preincubation and the two effects appear to be additive. Lichstein (1955) has recently described an interesting phenomenon wherein preincubation of E. coli in the presence of pyridoxal or its coenzyme protects the tryptophanase system of the organism against the inhibitory effect of isoni-

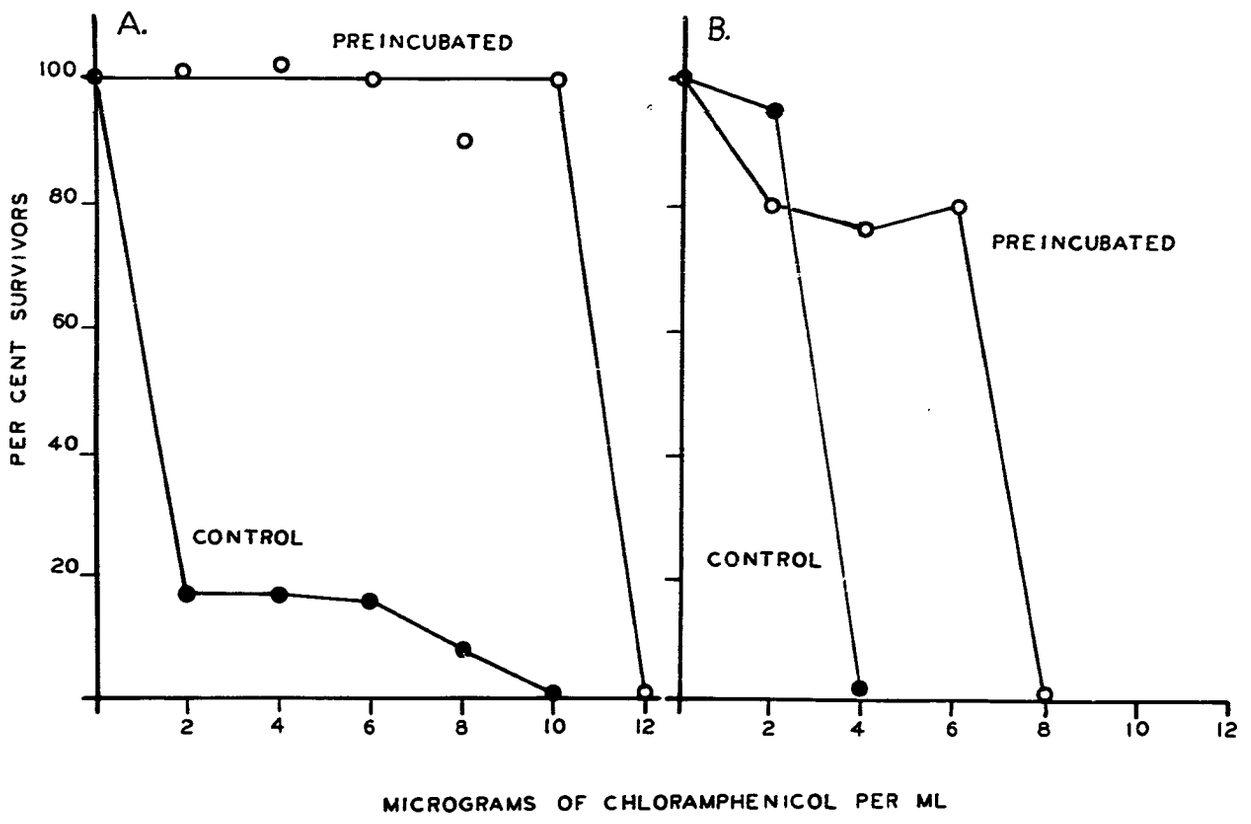

Figure 5. The effect of preincubation of Micrococcus pyogenes (Strain S) on chloramphenicol resistance. A- Synthetic agar supplemented with 0.3 per cent yeast extract. B- Unsupplemented synthetic agar. Preincubation time, 4 hours.

cotinic acid hydrazide. It may well be that a similar mechanism is operating in the present case. For example, preincubation may allow the cells to synthesize a necessary metabolite with which chloramphenicol interferes. At the same time, yeast extract may serve as an exogenous source of the same metabolite. Either effect would endow the organism with a higher degree of resistance, and it is not unlikely that the two effects would be additive.

It should be noted that the preincubation time employed with Strain S correlates closely with the time required for initiation of growth. Therefore, at most, the cells had undergone only one cell division prior to exposure to chloramphenicol. That the effect is not due to increased cell numbers is shown in Table VII. Preincubation for as short a period as 30 minutes results in a striking increase in survivors and allows growth in the presence of 10 ug chloramphenicol per ml, a concentration which completely inhibits growth under normal conditions. This increase continues progressively with the time of preincubation until a maximum effect is obtained at approximately 60 minutes preincubation.

The role of preincubation in increasing the number of survivors in the presence of chloramphenicol and in increasing the resistance of the culture to the antibiotic is applicable to both strains. In Figure 6 are presented results which show that preincubation of Strain R will give results that are comparable to those obtained with Strain S (Figure 5).

Preincubation of Strain S on synthetic agar from which one of the organic ingredients had been omitted (vitamins, amino acids or

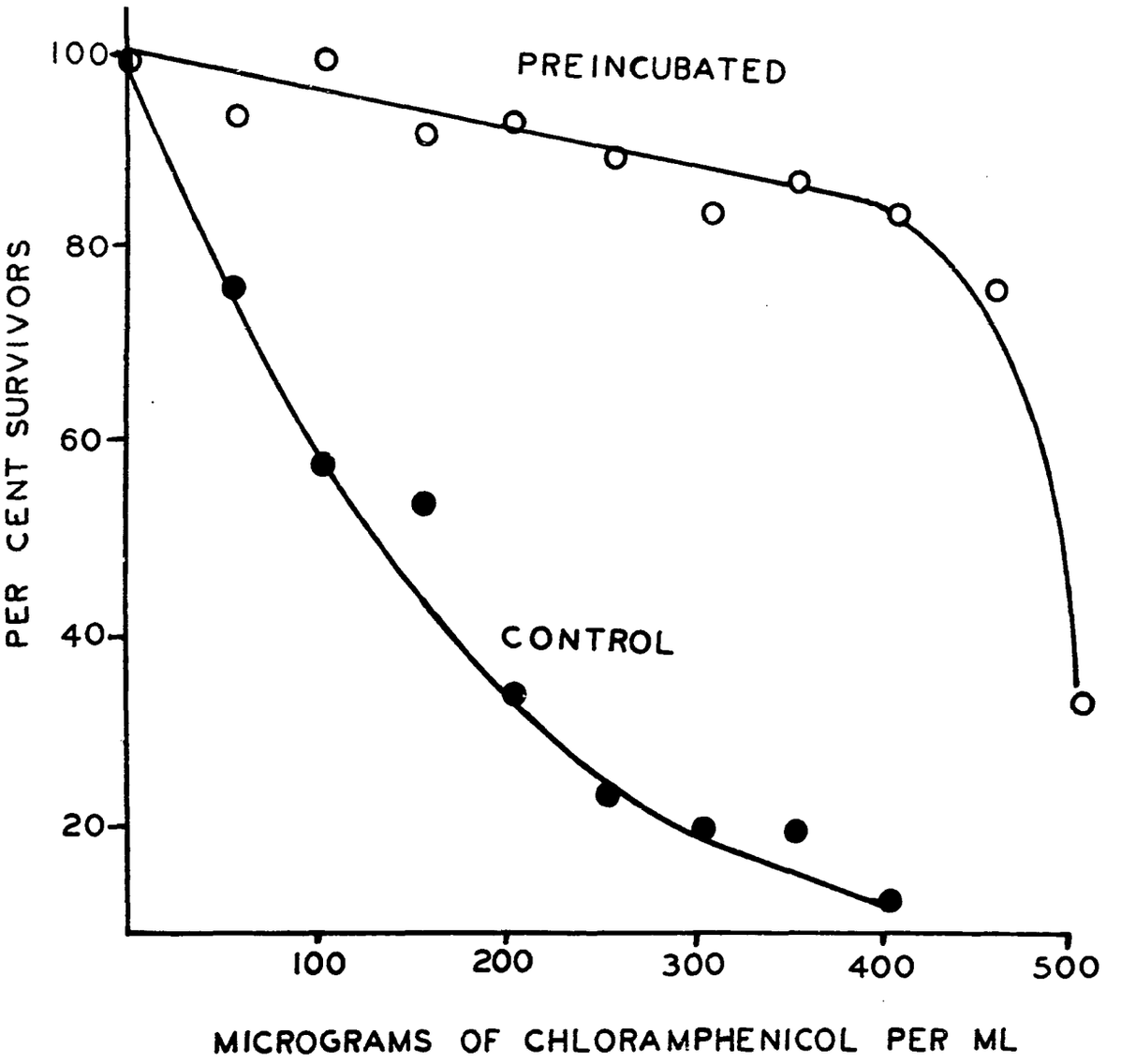

Figure 6. The effect of preincubation of Micrococcus pyogenes (Strain R) on chloramphenicol resistance. Preincubation time, 100 minutes. Synthetic medium supplemented with 0.3 per cent yeast extract.

TABLE VII

THE EFFECT OF PREINCUBATION TIME ON THE SURVIVAL OF *MICROCOCCUS PYOGENES* (STRAIN S) IN THE PRESENCE OF CHLORAMPHENICOL

Time of pre-incubation, min.#	Colonies appearing in 160 hours on yeast extract agar containing chloramphenicol, ug per ml.				
	2	4	6	8	10
0	52*	68	37	37	0
30	76	58	58	50	6
60	88	96	130	94	65

#Preincubation on yeast extract agar without chloramphenicol.
*Expressed as per cent of colonies developing on medium without chloramphenicol.

glucose) did not significantly alter the effect of preincubation observed on a complete synthetic medium. Preincubation on agar plates which contained individual components of the synthetic medium (glucose, amino acids or a mixture of B-vitamins) did not increase the chloramphenicol resistance of the culture. However, those cultures preincubated on agar containing only amino acids showed a slight increase in the number of colonies that developed in the presence of subinhibitory concentrations of the antibiotic. These results would imply that the preincubation effect is related to the over-all metabolisms of the organisms and not to one particular sequence of enzymatic reactions. The nature of the substance(s) present in yeast extract which are responsible for direct antagonism of chloramphenicol has not been determined. However, studies have been conducted on the effect of known materials (purines, pyrimidines and vitamins) on resistance.

The addition of adenine, uracil, guanine and xanthine to synthetic broth at a concentration of 25 ug per ml caused a slight increase in the sensitivity of Strain S to chloramphenicol (Table VIII). When all four of these materials were added to the medium at one time growth occurred in a concentration of chloramphenicol equal to the control which contained no added purines or pyrimidines. It would appear that purines and pyrimidines are not the constituents of complex materials which are responsible for an increased resistance to chloramphenicol.

Determination of vitamin requirements in the presence of chloramphenicol was accomplished by omitting single vitamins from the complete synthetic medium which contained graded concentrations of the antibiotic. Due to the variable lag time and growth rate of Strain R in the presence

TABLE VIII

THE EFFECT OF PURINES AND PYRIMIDINES ON THE SENSITIVITY OF *MICROCOCCUS PYOGENES* (STRAIN S) TO CHLORAMPHENICOL

Synthetic broth* supplemented with:	Highest concentration of chloramphenicol allowing growth at 120 hours, micrograms per ml
None	8
Adenine	4
Uracil	6
Guanine	6
Xanthine	6
Adenine, Uracil Guanine and Xanthine	8

*Riboflavin omitted from synthetic broth.

of different concentrations of chloramphenicol the results were recorded as the presence or absence of visible growth after 128 hours incubation at 37 C (Table IX). The omission of all vitamins from the medium did not allow growth even in the absence of the antibiotic. The omission of niacin, or thiamine, for which partial requirements had been previously demonstrated in the absence of the antibiotic, (Ramsey and Padron, 1954), allowed scanty growth only in the lower concentrations of chloramphenicol. In the absence of calcium pantothenate, pyridoxamine and inositol growth occurred in slightly higher concentrations of antibiotic than in the control containing all vitamins. Omission of cyanocobalamine, folic acid, para-aminobenzoic acid and biotin had a negligible effect on the level of chloramphenicol in which growth occurred.

One striking feature of this experiment is the observation that omission of riboflavin from the medium allowed rapid growth in 500 ug chloramphenicol per ml as compared to growth in only 200 ug chloramphenicol per ml in the presence of riboflavin. It is unlikely that this inhibition resulting from the incorporation of riboflavin into the medium was due to antagonism of other vitamins or to the destruction of essential nutrients (Wagener-Jauregg, 1954). In a medium containing only niacin and thiamin growth occurred in the presence of 400 ug chloramphenicol per ml (Table IX). However, when riboflavin was added growth was inhibited by 300 ug chloramphenicol per ml. When riboflavin solutions were sterilized by filtration and added to the medium (after sterilization) the same effect was observed. Therefore, it was concluded that inhibition was not due to destruction or binding of nutrients by riboflavin, but rather that riboflavin exerted a direct inhibitory effect on the organism.

TABLE IX

EFFECT OF VITAMINS ON SENSITIVITY OF

MICROCOCCUS PYOGENES (STRAIN R)

TO CHLORAMPHENICOL

Vitamin omitted	Highest concentration of chloramphenicol allowing growth at 128 hrs. Micrograms per ml
None	200
All	0
Thiamin	100
Niacin	100
Riboflavin	500
Ca pantothenate	300
Pyridoxamine	250
Inositol	250
p-aminobenzoic acid	200
Biotin	200
Folic acid	200
Cyanocobalamin	150
All except thiamin, niacin and riboflavin	300
All except thiamin, niacin	400

The effect of increasing concentrations of riboflavin on growth in the presence of varying concentrations of chloramphenicol is shown in Figure 7. The principal effect of riboflavin appears to be an increase in the time required for the organism to initiate growth since there was no great difference in the slope of the exponential phase of growth or in the total amount of growth. In the absence of chloramphenicol, 1 ug riboflavin per ml increased lag time by 2.5 hours; 2 ug riboflavin per ml increased lag by 4 hours. In the presence of 100 ug chloramphenicol per ml the inhibitory effect is increased, but, if considered on the basis of the percentage increase of lag, the results are similar. In the presence of 200 ug chloramphenicol per ml a different effect is noted; the increase in lag due to 1 ug riboflavin is proportional to that occurring in the other series, while 2 ug riboflavin per ml prevented growth initiation within the time of observation (100 hours). It would seem that chloramphenicol and riboflavin are additive in preventing growth initiation.

It is of particular interest that an enhancement of chloramphenicol activity is obtained through the use of a material which seems to have a slight inhibitory effect upon the time required for a culture to initiate growth. This lends support to the observations that chloramphenicol activity can be antagonized by the stimulation of metabolic activity.

Partial degradation of riboflavin by ultraviolet light was accompanied by a reduction in inhibitory activity. For example, the sample irradiated at pH 9.0, which contained lumiflavin as the main degradation product (Wagner-Jauregg, 1954), had lost its inhibitory

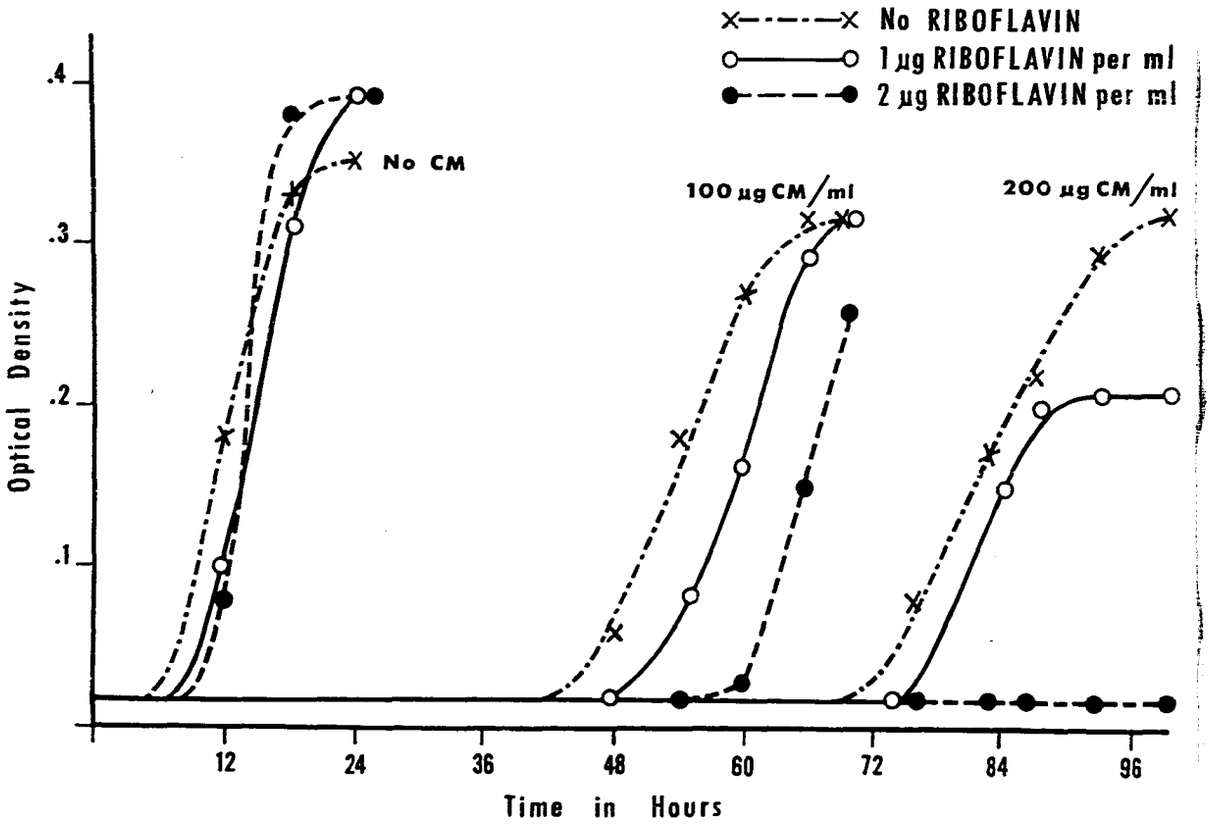

Figure 7. Prolongation of the initial stationary phase of growth by riboflavin and chloramphenicol (CM). Micrococcus pyogenes (Strain R) test organism.

effect; growth was identical to the control which contained no riboflavin. Furthermore, microbiological assay indicated that this treatment resulted in approximately 65 per cent loss of riboflavin activity (Figure 8). Partial destruction of riboflavin and a corresponding partial reduction of toxic activity resulted from irradiation at pH 5.0. It would seem that under these conditions riboflavin per se is the agent responsible for the prolongation of lag and alteration of the molecule results in decreased activity. However, alteration of the molecule does not always result in an inactivation since riboflavin-5-phosphate was found to be equal to riboflavin in inhibitory activity. Further evidence for the direct participation of riboflavin was provided by chromatographic assay of riboflavin obtained from four commercial sources (Sigma Chemical Co., Fisher Scientific Co., California Foundation for Biochemical Research, Nutritional Biochemicals Co.). Although each sample exerted some toxic effect, the greatest inhibition was observed when the most chromatographically pure substance (Sigma Chemical Co.) was tested for activity. Thus, it appears that riboflavin per se or a very closely related compound is the inhibitory agent.

The active chelating properties of riboflavin have been described by Albert (1953) who noted an unusual avidity of riboflavin for ferrous iron. The removal or reduction of the level of certain trace elements in the medium would be sufficient to explain the inhibitory action of the riboflavin. In order to examine this possibility, a two-fold increase in concentration of the various metals normally included in the medium were tested for their ability to reverse riboflavin in-

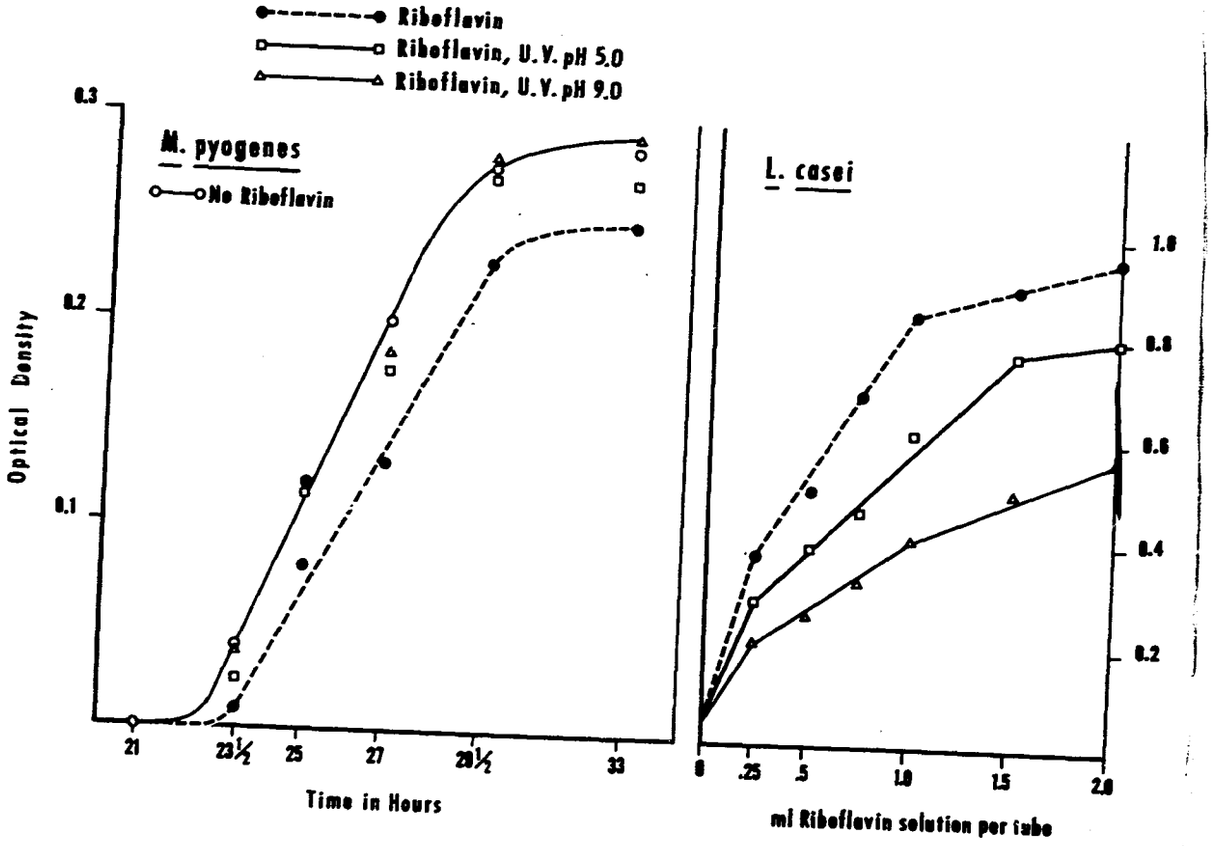

Figure 8. Reduction of the inhibitory action of riboflavin by ultraviolet light irradiation. Aliquots tested with Lactobacillus casei diluted to equivalent of 0.1 ug per ml. Aliquots tested with Micrococcus pyogenes (Strain R) at a concentration of 2 ug per ml.

hibition (Table X). In the control series without riboflavin growth was quite uniform in all tubes. In the presence of 2 ug riboflavin per ml growth was slower in all tubes except those containing a two-fold concentration of ferrous iron (4.0 ug per ml); in this instance growth was equal to that obtained in the control tubes without riboflavin. A two-fold concentration of manganese (6.5 ug per ml) caused a striking inhibition of growth in the presence of riboflavin. Furthermore, increasing concentrations of ferrous iron did not proportionally reverse the inhibition caused by higher concentrations of either manganese or riboflavin. Ferrous iron was more effective in antagonizing the inhibition resulting from increased manganese concentration than that which resulted from high riboflavin concentration. Cobalt and molybdenum were without effect.

Increasing concentrations of riboflavin were progressively effective in inhibiting growth initiation although there was little alteration in the amount of total growth attained (Figure 9). Manganese also exerted a toxic effect when present in sufficiently high concentrations. The inhibitory action of either manganese or riboflavin was greatly enhanced by an increased concentration of the other. A comparison of the results presented in part A and part B of Figure 9 shows that continued incubation permits the organisms to overcome this inhibition. This is in complete accord with the results presented in Figures 7 and 8 which indicate that the inhibition is manifested primarily as a prolongation of lag time. The inhibition caused by high concentrations of riboflavin in the absence of added manganese could be due to contaminating traces of manganese in the various ingredients of the

TABLE X

THE EFFECT OF INCREASED CONCENTRATION OF METALS ON GROWTH INHIBITION BY RIBOFLAVIN#

Riboflavin concentration	Micrograms cation per ml*			
	None	K^+ 1,1796.0	Mn^{++} 6.5	Mg^{++} 78.4	Fe^{++} 4.0
None	.30**	.28	.30	.30	.30
2 ug/ml	.18	.20	.04	.18	.30

#Test organism - M. pyogenes (Strain R).
*Two-fold normal concentration.
**Optical density at 30 hours.

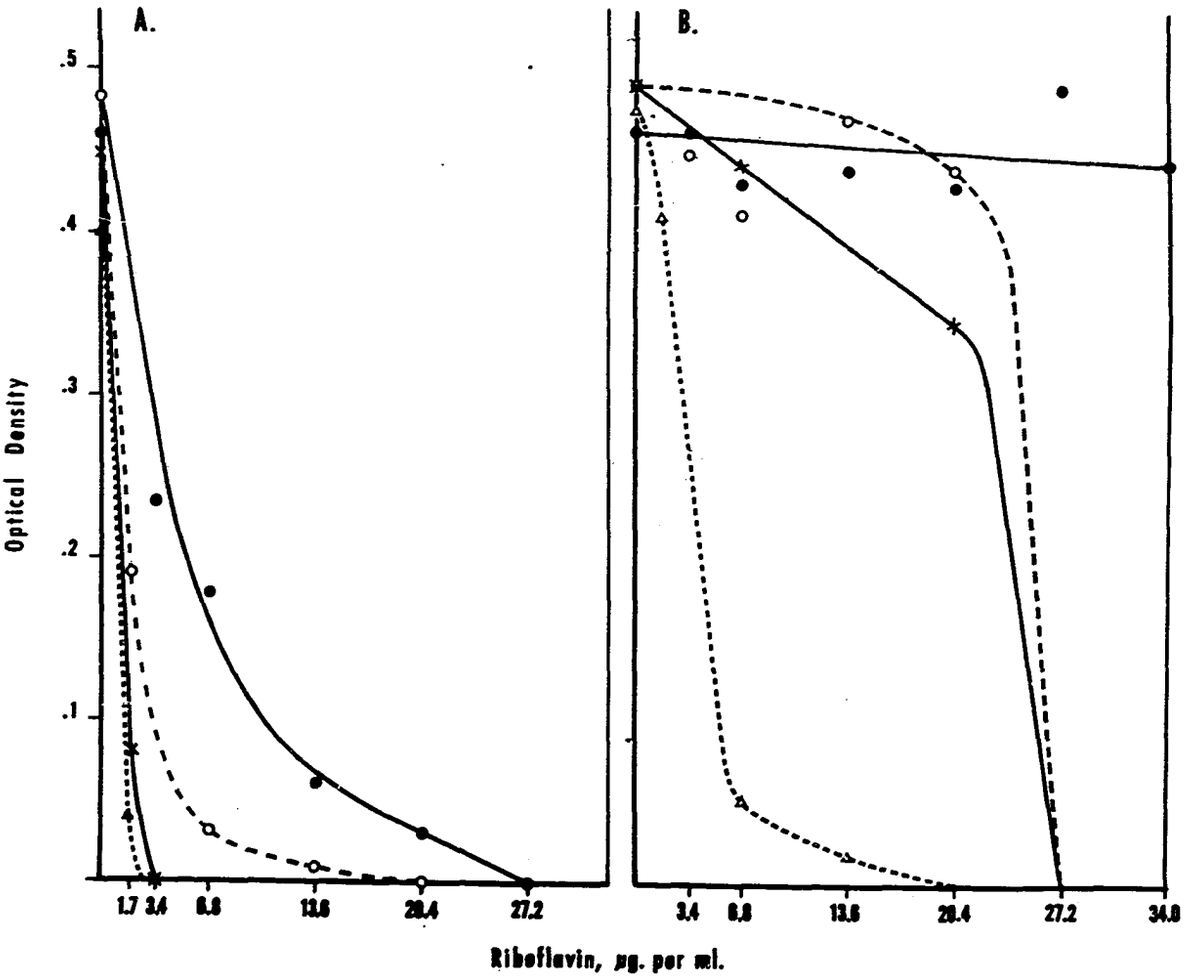

Figure 9. The effect of increasing concentrations of riboflavin and manganese upon growth inititation. Micrococcus pyogenes (Strain R). (Test organism) A. 29 hours incubation. B. 44 hours incubation. ●---● no manganese; 0---0 0.5 ug manganese per ml; X---X 1.0 ug manganese per ml; Δ---Δ 2.0 ug manganese per ml.

medium since no effort was made to purify the various constituents. In like manner, since the test organism was capable of synthesizing riboflavin it is possible that at least part of the observed toxicity of manganese could be due to the combination of manganese with endogenously produced riboflavin.

The dependency of the riboflavin-manganese inhibitory effect upon aerobiosis was demonstrated in a plating experiment (Table XI). Under both anaerobic and aerobic conditions only slight variations were observed in the number of colonies developing on the plates which did not contain riboflavin. Under aerobic conditions riboflavin and manganese combinations were quite inhibitory; the presence of 5 ug riboflavin and 2 ug manganese per ml prevented the development of visible colonies during the period of observation. Riboflavin, without added manganese, caused some reduction of the number of colonies which developed anaerobically. On all plates which contained both riboflavin and manganese anaerobiosis caused a marked negation of the inhibitory effect. For example, 45.7 per cent of the number of colonies appearing on the anaerobic control plate with neither riboflavin or manganese, developed on the plates containing 4 ug manganese and 5 ug riboflavin per ml. This combination was completely inhibitory under aerobic conditions. There is a slightly greater ability of inoculum cells to overcome the riboflavin-manganese inhibition in broth cultures than on the surface of agar plates incubated aerobically (compare Figure 9 and Table XI). This might be due to a lower oxygen tension of the fluid medium.

TABLE XI

ANTAGONISM OF RIBOFLAVIN-MANGANESE INHIBITION BY ANAEROBIOSIS[#]

Condition of incubation	Riboflavin ug/ml	Micrograms of manganese per ml				
		0	1	2	3	4
Aerobic	0	56*	51	60	55	46
	5	50	19	0	0	0
Anaerobic	0	59	54	60	49	46
	5	40	26	15	23	27

[#]Test organism - M. pyogenes (Strain R).
*Average colony count at 72 hours.

CHAPTER IV

DISCUSSION

The investigations of the capacity of the resistant strain to inactivate chloramphenicol showed that there was no apparent ability to inactivate the antibiotic such as that noted in E. coli by Merkel and Steers (1953). This is in agreement with the results obtained by Padron (1953) using another technique. No extracellular material capable of antagonizing chloramphenicol was secreted by the resistant strain of M. pyogenes. This suggests that the resistance of this strain is probably a result of decreased permeability or of an altered metabolic route or enzyme system which does not have the same sensitivity to the drug, rather than by drug inactivation or production of an excess of some essential metabolite. As mentioned previously, Kushner (1955_b) noted an apparent decrease in permeability accompanying chloramphenicol resistance in P. fluorescens and Saz and Martinez (1956) noted that a tetracycline resistant strain of E. coli possessed an enzyme with a decreased affinity for the drug. Although the results reported here do not offer proof of such a mechanism in M. pyogenes they do eliminate other possibilities and make altered permeability and/or metabolism more probable explanations of resistance.

In any study of microbial growth processes the importance of

starting with a small number of relatively homogenous cells can hardly be overemphasized. In the present study this has been repeatedly observed where the effect of a substance on growth, with and without chloramphenicol, can vary markedly with the cell concentration. For example, some of the complex materials were able to stimulate some of the cells contained in a large inoculum to grow in the presence of chloramphenicol, while in the same experiment, the same test material would not support growth in increased concentrations of chloramphenicol when a more dilute inoculum was used. This type of effect was obtained with skim milk powder and neopeptone (Table V). The opposite effect was noted with human plasma and egg yolk. These two substances failed to permit growth in increased concentrations of chloramphenicol when a heavy inoculum was used; on the other hand, they did protect a more dilute inoculum from concentrations of the antibiotic higher than that which was inhibitory in unsupplemented synthetic medium. The increased resistance of all inocula sizes that results from the addition of yeast extract to the medium might be a combination of the two effects previously mentioned or might be due to an entirely different phenomenon. It is probable that several factors are involved in the antagonism of chloramphenicol activity by complex materials. One factor is the stimulation of some cells which have a relatively high resistance to the antibiotic but which require some nutrient not provided in the synthetic medium. Another factor, which is more applicable when dilute inocula are used, is a stimulation of the metabolic processes of a majority of the cells present and is reflected in an increased rate of growth and metabolism.

Thus, one cannot ascribe an antagonistic effect <u>per se</u> to a

substance since the antagonism observed may be accounted for *in toto* by the stimulatory effect. Working with *E. coli* Foster and Pittillo (1953_a) found that cell counts were no higher in supplemented medium than in unsupplemented medium in the absence of antibiotics. Thus, they believed that growth stimulation played no part in negation of the antibiotic effect. Discrepancies between their results and those presented here may be due to at least two factors. First, and most important, is the test organism used in the two instances; *E. coli* is a nonfastidious, rapidly growing organism and therefore probably is less affected by stimulatory compounds while *M. pyogenes* is slower growing and responds in a positive manner to many stimulatory factors, both known and unknown. The second factor to be considered is the period of observation employed in the two instances. In these experiments it has been necessary to commence observation at a relatively early hour following inoculation and to continue observations up to 10 days. Foster and Pittillo (1953_a) made only one observation at 36 hours after inoculation which could have two effects: 1) stimulation of growth of a rapidly growing organism would not be observed, and 2) lack of prolonged observation would not show growth in the absence of complex materials.

The response obtained with quantitative studies of yeast extract and proteose peptone No. 3 was found to be quite different. Proteose peptone No. 3 was the more active of the two with all concentrations of antibiotic tested (Figures 3 and 4). This difference in response might be explained in either of two ways. First, the antagonist(s) found in the two materials may be identical in nature but present in proteose peptone No. 3 in greater amounts than in yeast extract. Second,

the antagonists may be two different compounds and the one present in proteose peptone No. 3 is the more active of the two. Whichever is correct, the antagonism of chloramphenicol observed with both materials does not appear to be competitive since the substances do not counteract corresponding quantities of chloramphenicol at all levels tested. However, the most significant feature of the results is the parallel activity of the two substances in stimulating growth both in the presence and absence of chloramphenicol. These results again suggest that negation of chloramphenicol inhibition is probably a nonspecific effect resulting from growth stimulation.

Further evidence for the stimulatory nature of the observed antagonism has been demonstrated by preincubation experiments.. It may well be that a mechanism, similar to that observed by Lichstein (1955), is operable in this case. For example, preincubation may allow the cells to synthesize a necessary metabolite with which chloramphenicol interferes. At the same time, yeast extract may serve as an exogenous source of the same metabolite or its precursor. Either effect would endow the organisms with a higher degree of resistance, and it is not unlikely that the two effects would be additive. These results would imply that the preincubation effect is related to the over-all metabolism of the organism and not to one particular sequence of enzymatic reactions.

Chloramphenicol has been shown to exert a strong inhibitory action on microbial protein synthesis (Gale and Folkes, 1952; Maxwell and Nickel, 1954; Wisseman, et al., 1954). Hence, the hypothesis that

the antibiotic may act simply as an antagonism of one amino acid or another has been especially attractive (Mentzer, et al., 1950; Woolley, 1950; Bergmann and Sicher, 1952). Nevertheless, examination of the evidence in support of this proposed mechanism of action reveals that it is derived from experiments in which reversal of growth inhibition by the respective substances was generally minimal and was demonstrable only under special circumstances. Moreover, results of this kind have not been limited to selected amino acids; indeed, a wide variety of other substances has also been found to yield comparable degrees of reversal of growth inhibition (Swenseid, et al., 1952; Foster and Pittillo, 1953_a). It is conceivable that a complex relationship of antagonists exists here as in the case of the sulfonamides. However, a recent report by Hopps, et al., (1956) indicates that the minimal "reversal effects" mentioned above may be due to indirect factors which stimulate bacterial growth and whose relation to chloramphenicol inhibition is more nonspecific in nature than is usually included in the concept of antimetabolites.

The demonstration that substances which stimulate growth may also indirectly negate antibiotic activity is not surprising in view of other findings. For example, Eagle and Musselman (1949) found that recovery of Streptococcus pyogenes following exposure to toxic concentrations of penicillin was conditioned by the surrounding menstruum; that which supported good growth of the organism in absence of the antibiotic also prompted more rapid and complete recovery from penicillin. The work of Wainwright and Mullaney (1954) demonstrated that susceptibility to penicillin can vary according to the source of carbon found in the

medium. In view of this, it is logical to assume that complex materials could exert a similar effect, particularly with a fastidious organism such as M. pyogenes, by constituting a preformed supply of essential intermediates necessary for optimum growth and metabolism of the organism. This would be particularly true where resistance depended on adaptive mechanisms, e. g., in low concentrations of antibiotic (Eagle, et al., 1952). These results are in accord with those obtained by Wisseman, et al., (1954) who found that reversal of chloramphenicol activity against E. coli by amino acids and peptides was not apparent when growth stimulation by these materials was rigidly controlled. Also worthy of note is the observation that cells possessing a high state of metabolic activity are more resistant to chloramphenicol than cells with a low metabolic rate. This is in contrast to the well known fact that penicillin is bactericidal only against actively metabolizing cells (Hobby, et al., 1942). However, in the case of a bacteriostatic agent such as chloramphenicol, metabolic activity may produce just the opposite effect by providing the cell with a variety of intermediate compounds which are antagonistic to the antibiotic. In view of the fact that both preincubation and complex materials may antagonize chloramphenicol activity and that there is an additive effect of these two factors, it would seem that stimulation of metabolic activity is the main pathway by which chloramphenicol activity is antagonized by complex materials.

Demonstration of a relationship between the results presented herein and the well documented fact that chloramphenicol specifically inhibits protein synthesis (Gale and Folkes, 1952; Pardee and Prestidge, 1956) has not been possible on an experimental basis. However, certain

pertinent observations which indirectly bear on this problem might be cited. First, the fact that proteose peptone No. 3 was the most potent antagonist of chloramphenicol tested might indicate that a peptide or polypeptide is being supplied, the synthesis of which is inhibited by chloramphenicol. Secondly, the fact that accelerated metabolism and growth endows the cells with a higher resistance might be considered indirect evidence that more rapid protein synthesis is responsible for resistance.

The search for materials capable of antagonizing chloramphenicol activity revealed what appeared to be a potentiation of chloramphenicol activity by riboflavin (Table 10). Further investigation showed that a marked inhibition of growth also occurred in the absence of chloramphenicol when both riboflavin and manganese were in the medium. This would indicate that this inhibition is a result of the formation of an inhibitory riboflavin-manganese complex rather than an additive effect of the two agents. The observed enhancement of chloramphenicol activity can be considered a result of the additive effect of two inhibitory agents comparable to the greater effect of chloramphenicol noted by Ramsey (1957) when the temperature of incubation deviated from the optimum temperature for growth. The inhibition caused by higher concentration of riboflavin in the absence of added manganese could be due to contaminating traces of manganese in the various ingredients of the medium since no effort was made to purify the various constituents. In like manner, since the test organism was capable of synthesizing riboflavin it is possible that at least part of the observed toxicity of manganese could be due to the effect of the combination of manganese with endogen-

ously produced riboflavin. The active chelating properties of riboflavin would support the idea that a complex which interferes with formation or function of an essential metallo-enzyme was responsible for toxicity. It might be expected that an increased concentration of the normal metal constituent of the enzyme system would counteract the inhibitory effect of manganese, at least to some extent. This was found to be the case with ferrous iron, although the antagonism was not stoichiometric, and above certain levels of manganese no reversal by iron was apparent. The high avidity of the riboflavin molecule for ferrous iron as compared to manganese would indicate that inhibition was more complex than simple competition of metal ions for a site of attachment on the riboflavin molecule.

Examples of toxic chelate complexes have been demonstrated by Rubbo, et al., (1950) and Albert, et al., (1956). The over-all picture of the toxic action of 8-hydroxy-quinoline (oxine), and 2-mercaptopyridine N-oxide, as furnished by their results is that an iron or copper complex acts as a catalyst which promotes the destructive oxidation of a group (e.g., an -SH group) on a metabolite or enzyme which is vital to the cells. This toxic action can be prevented by the addition of cobalt to the medium which probably replaces the cobalt (removed by the chelating agent) that normally protects the group. This concept is supported by the fact that the toxic action can also be prevented by lowering the oxidation-reduction potential of the medium thus preventing the destructive oxidation, or bringing into play a new metabolic pathway which circumvents it. Such an alternate route has been demonstrated by Lenhoff, et al., (1956) in P. fluorescens; at high oxygen tensions, the

flavoprotein content, as measured by diaphorase activity, increased, while at lower oxygen tensions the cytochrome content of the cells was increased. The formation of an increased amount of cytochrome was also found to be dependent upon the presence of iron. It is possible that the ability of iron to antagonize the riboflavin-manganese inhibition of M. pyogenes is due to the increased formation of cytochromes which afford an alternate electron route rather than to a competition between the metal ions for a place in a flavin enzyme system.

CHAPTER V

SUMMARY

A chloramphenicol resistant strain of Micrococcus pyogenes var. aureus did not inactivate chloramphenicol or produce an extracellular substance capable of reducing the toxicity of chloramphenicol for the sensitive parent strain.

Complex biological materials such as yeast extract and proteose peptone No. 3 were capable of antagonizing the bacteriostatic action of chloramphenicol. These materials also supported more rapid growth of a larger number of cells of the inoculum. Quantitative assay of the activity of yeast extract and proteose peptone No. 3 revealed markedly different types of response curves for each of these materials. The activity was not due to competitive antagonism but seemed to be the indirect result of stimulation by these materials.

Preincubation of M. pyogenes in the absence of chloramphenicol prior to exposure to the antibiotic resulted in an increased resistance to the drug. This resistance was apparently due to a high rate of metabolic activity. The enhanced resistance to chloramphenicol which was obtained by adding a complex material such as yeast extract to the medium appeared to be additive with the effect of preincubation.

No purines, pyrimidines or vitamins were found which would

antagonize chloramphenicol activity.

Riboflavin, in the presence of manganese, inhibited the growth of M. pyogenes. The inhibitory action was shown to be dependent upon aerobiosis and was characterized predominantely as a prolongation of the lag phase. Increasing concentrations of either manganese or riboflavin, in the presence of a constant quantity of the other, enchanced the inhibitory effect. Ultraviolet irradiation reduced the toxicity of riboflavin in proportion to the amount of riboflavin degraded. Riboflavin-5-phosphate was equally as inhibitory as riboflavin. The inhibitory effect observed in the presence of low concentrations of riboflavin and/or manganese could be reversed in a non-stoichiometric manner by ferrous iron.

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