The Effect of Penicillin on Hyaluronidase Production by Bacteria.

by

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INTRODUCTION

Penicillin, an antibacterial principle produced by certain strains of the mold Penicillium notatum or Penicillium chrysogenum, has been found to be highly effective in inhibiting the growth of most Gram positive as well as some Gram negative organisms. As will be indicated in the historical review, the exact mechanism of the antimicrobial activity is still unknown, although its mode of action has been the subject of much investigation.

Hyaluronidase, an extracellular enzyme produced by certain pathogenic bacteria, is considered to be a component of the invasive armament of these organisms by virtue of its spreading action in normal tissue.

The purpose of the investigational studies presented is the experimental examination of the effect of Penicillin on this enzyme, and of the response in hyaluronidase production by Streptococcus pyogenes to subinhibitory levels of the antibiotic during its metabolism. As a result of this investigation, some insight may be gained into the reaction of one facet of bacterial metabolism when submitted to sublethal concentrations of Penicillin, and thereby obtain the resultant "in vitro" effect produced by one of the possible constituents of bacterial invasiveness.

The studies presented here are part of a long range problem, which has been designed to differentiate the circumstances under which an antibiotic will inhibit bacterial cell reproduction, and circumstances under which less than this,

will inhibit one enzyme system, which in this case is hyaluronidase. This problem has a bearing on the whole understanding of the effect of antibiotic levels in the tissues of the body.

HISTORICAL REVIEW

PENICILLIN

I. MODE OF ACTION

(1) Introduction to Mechanism of Chemotherapeutic Activity

The antibiotic Penicillin, produced by the mold Penicillium notatum, was first described in 1929 by Fleming, who observed that this substance inhibited the growth of many Gram positive organisms. Recently this substance has been extracted, and has been found to be an unstable acid with the probable formula of $C_{14}H_{19}NO_6$ or $C_{14}H_{17}NO_5 \cdot H_2O$.(88).

By definition, "According to current usage, the term antibiotic designates a product of the metabolism of one microorganism that is antagonistic to the continuation of the normal life activities of another microorganism when present even in very low concentrations."(176).

The penicillins may be practically considered as being non-toxic to animal tissues, when used in therapeutic concentrations which exert a strong selective action against certain types of bacteria. At present, there is little evidence of differences in the mode of action of the various penicillins, F, G, K, X, etc..(92).

Penicillin appears to cripple the proliferative activities of susceptible microorganisms or otherwise interfere with important metabolic functions, followed by their final destruction. This activity of penicillin when conferred onto an organism produces a state of bacteriostasis. This state depends upon factors which affect the rate at which irreversible alterations go on within the inhibited cells, being conditioned by the

firmness of the combination between the compound and the susceptible cell as well as by the ease with which the combination can be dissociated. Thus, to be effective, it is not necessary for penicillin to kill an organism, but merely to attack some component nutrient metabolite, or essential enzyme system, thereby producing a metabolic block.

Penicillin is selective in its action, and "in vitro" studies have shown that it causes an actual decrease in the number of organisms, the bactericidal action taking place only if multiplication occurs.(88).

According to Waksman, (217) this decrease in number of organisms and their final destruction by an antibiotic, consists of the antibiotic's interference with certain essential metabolic processes of bacteria. This is accomplished:

(a) by oxidizing a metabolic substance which has to be reduced in the process of bacterial nutrition;

(b) by combining with the substrate or with one of its constituents, rendering it inactive for bacterial utilization;

(c) by competing for an enzyme required by the bacteria for an essential metabolic process;

(d) by affecting the surface tension of the bacterium;

(e) by influencing its respiratory mechanism;

(f) by interfering with bacterial cell division.

The antibiotic, penicillin, accomplishes its mode of action probably by a combination of two or more of these interfering actions. This conception of bacterial destruction by essential metabolite displacement or inactivation is in accord-

ance with Filde's thoughts pertaining to antibiotic activity. (51).

The penicillins are characterized by the fact that they inhibit susceptible bacteria more effectively both "in vivo" and "in vitro" when the environment is most favorable for growth and by the fact that within wide limits their activity is not affected by the number of bacteria present (12), whereas many other chemotherapeutic agents are less effective if bacterial contamination is heavy.

Pratt & Dufrenoy (176) have stated that impurities in amorphous (impure) penicillins make it difficult to properly evaluate the interpretations of earlier recorded results. Among the impurities may be traces of heavy metals, members of vitamin complexes, and various unidentified growth regulating factors. These impurities may shorten the lag period, and although themselves inactive, may increase markedly the effectiveness of the penicillin. These impurities may affect the systems regulating the respiratory processes of the bacterial cells, so that the cells become more susceptible to the action of penicillin.

Therefore, the activity of antibiotics is conditioned not only by their chemical constitution, but also by the nature of the living agents of disease and their environmental background as well. The exact mechanism of the antimicrobial activity of penicillin is still unknown although it appears to exert its effects by either inhibiting the growth or killing those organisms susceptible to it. Various factors, however, have an influence upon its antimicrobial activity, both "in

vitro" and "in vivo".

(2) Factors Influencing Antibacterial Activity "In Vitro"

From the standpoint of the chemical constitution of a chemotherapeutical compound, it may be mentioned that compounds endowed with surface activity are especially likely to disrupt cell membranes and denature the enzymes of the microorganisms, which results in their irreversible damage and death. As stated by Dubos (33), among the factors which appear to influence the specificity or selective activity of chemotherapeutic agents for microorganisms may be mentioned their acidic and basic properties, the nature and properties of their membranes, permeability, the relative importance of the metabolic and viability of their specific biochemical systems affected by compounds, the activity of autolytic enzymes, etc. Each of these properties can undergo variation either independently or simultaneously, and thereby produce mutants of reduced susceptibility or resistance to chemical agents of biologic or nonbiologic origin. (33). Even one condition, such as low temperature, which decreases the rate of division, tends to decrease the susceptibility of bacteria to the killing power of penicillin. (24), (93), (110).

One of the most important factors influencing penicillin's antibacterial activity, is the fact that among bacteria susceptible to penicillin, various strains of them may show marked differences in natural susceptibility to the compound, both "in vitro" and "in vivo". Strains of gonococci and beta hemolytic streptococci of group A have shown the greatest uniformity in natural susceptibility to penicillin "in vitro",

while strains of staphylococci, meningococci and Hemophilus hemolyticus have shown the widest variations. (141).

Bacteria are most susceptible to the action of penicillin when they are in the logarithmic phase of growth, and when the cells are dividing most rapidly, and are exhibiting the greatest need for oxygen. (176). Conversely, conditions which decrease the rate of division, tend to decrease the susceptibility of bacteria to the killing power of penicillin. (24), (93), (110).

Hobby, Meyer & Chaffee (87) have found that the effect of penicillin may be bacteriostatic or bactericidal, depending upon experimental conditions, and that the rate of killing depends on the organism. This was later confirmed by Lee et al. (109). Hobby et al (87) also found that penicillin was effective only at the stage of multiplication, and that the number of bacteria decreased at a constant rate until 99% were destroyed. No detectable amount of penicillin was absorbed or destroyed by the organism from the solution. (87).

Eagle & Musselman (44) have found that the concentration of penicillin "G" which killed a total of 41 strains of alpha and beta hemolytic streptococci, Staphylococcus aureus and Staphylococcus albus, Diplococcus pneumoniae and the Reiter treponema, at a maximum rate, was 2 - 20 times the minimum effective concentration level ("sensitivity" as ordinarily defined). With some organisms, even a 32,000-fold increase beyond this maximum effective level did not further increase the rate of its bactericidal effect. However, with about half the strains studied, when the concentration of penicillin

"G" was increased beyond that optimal level, the rate at which the organisms died was paradoxically reduced rather than increased, so that the maximum effect was obtained only within a relatively narrow optimum zone. They found marked differences between bacterial species and occasionally between different strains of the same species, with respect to the maximum rate at which they could be killed by the antibiotic in any concentration. Although there was a rough correlation between these two factors, there were many exceptions. Individual strains affected only by high concentrations of penicillin "G" might be killed rapidly, whereas strains sensitive to minute concentrations might be killed only slowly. Within the same bacterial suspension individual organisms varied only to a minor degree with respect to the effective concentrations of penicillin "G". (44).

Frieden & Frazier (54) have reported on the effects of a number of substances upon the sensitivity of Staphylococcus aureus to penicillin. Reduction in the concentrations of magnesium or phosphate reduces the sensitivity to penicillin, but absence of glycine exerts an opposite effect. Nicotinamide increases sensitivity to penicillin. Pyridoxine but not pyridoxal or pyridoxamine acts as a penicillin antagonist, but the effect appears to result from an "in vitro" inactivation of penicillin.

Pratt & Dufrenoy (174) found that trace amounts of cobalt ions lower the effective penicillin threshold on test plates, and "in vivo" enhancement of penicillin action was also observed. (177). Thus cobalt may act as a synergist toward a

penicillin reaction with sulfhydryl groups according to these investigators. (177).

Yanagita & Suzuki (222) found that with both Staphylococcus aureus and Escherichia coli addition of penicillin at various stages of growth caused a decrease in bacterial number only at the transition from lag to log phase and during the log phase of the growth curve.

(3) Induced Morphological Changes Occasioned by Growing in Penicillin

Fleming (49) originally observed that staphylococcus colonies around large colonies of Penicillium notatum became transparent and were obviously undergoing lysis. Garrod, (64) also observed this, and stated that penicillin is a slow-acting germicide (like the amino-acridines) and for this reason largely behaves like an antiseptic with the effects of its action influenced by changes in temperature, reaction of substrate, and by the age of the cultures. Hobby, Meyer et al, (88) however, state that lysis of susceptible organisms does not occur, nor is it absorbed from solution or destroyed by the organisms.

The majority of workers, however, report lysis of susceptible organisms during their destruction, but the mechanism of lysis is still obscure. Todd, (212),(213) reported that various penicillin-sensitive bacteria undergo lysis to a greater or less extent in broth cultures containing penicillin, especially pneumococci and strains of staphylococci. He found that a culture usually becomes more turbid before lysis begins, and concluded that multiplication is probably an essential preliminary to the lysis of bacteria through the production of

an autolysin composed of autolytic enzymes, with most rapid lysis occurring with organisms at their maximal rate of multiplication. According to Todd (213) the most probable explanation of lysis of bacteria in cultures containing penicillin is that the organisms are first killed by the penicillin and then autolyzed, while bacteria producing little autolysin are killed without undergoing lysis.

Gardner (62) was the first to point out that penicillin in subinhibitory concentrations causes morphological changes in certain bacteria. He observed that cultures of Clostridium welchii growing in a fluid medium containing penicillin of less than full inhibiting power manifested a flocculent growth with a heavy deposit instead of the more uniform turbidity. The majority of the cells of this culture took the form of unsegmented filaments ten or more times longer than the average normal cell. According to him, penicillin acts by preventing the division of bacterial cells with the production of abnormally large forms of cocci and long forms of bacilli which may undergo lysis by simple disruption or through the effects of impurities.

Since the time of Gardner's observation (62), several workers have noticed that during bacterial growth in sub-inhibitory levels of penicillin, there are produced pleo-morphic, diphtheroid, filamentous or "swollen" forms. (53), (175),(199), (105). Klimek et al, (105) have found that repeated selection of penicillin-resistant staphylococci eventually gave a cocco-bacillary or diphtheroid, Gram negative organism. Bellamy & Klimek (7) report that this resistant

organism is a slow-growing obligate aerobe which can rather easily return to sensitivity, again becoming a Gram positive coccus.

Other investigators (62), (87) have shown that the streptococci were among the organisms which displayed this pleomorphism to subinhibitory levels to penicillin. No such changes could be induced in the meningococcus. In the case of the staphylococci and the streptococci, the changes were somewhat different, insofar as they consisted of spherical enlargement of the cells and imperfect fission. Cells of Staphylococcus aureus under the influence of penicillin tend to form streptococcus-like chains, concomitantly losing their Gram positiveness, (36) while streptococci tend to form longer chains than normal. (62). At sub-bactericidal concentrations, penicillin stimulates metabolism and may act as a "growth factor" in promoting cell size, although at the same time it disharmonizes the processes of cellular enlargement and cellular division. (176).

Hobby & Dawson, (85), Miller & Foster (156) and other investigators have also stressed the fact that penicillin is particularly effective against susceptible bacteria which are about to divide. Knox, (106) has also observed that the effect of penicillin on sensitive bacteria depends upon the phase of growth during which penicillin is added to cultures. Young bacterial cells were found particularly susceptible and may undergo lysis while mature cells were found more resistant and were neither readily killed nor lysed.

Hobby et al (87) report that the action of penicillin on

hemolytic streptococci is not accompanied by lysis of the organism. According to Todd (212), pneumococcus types I, II, and III, Streptococcus viridans, hemolytic streptococcus groups A, C and G, staphylococci and Clostridium welchii are all lysed, while Escherichia coli and Pseudomonas pyocyaneus are not lysed. Knox (106) agrees with Todd (212) as to the phase of growth when lysis takes place, that is, the phase of multiplication, but disagrees with him on the point that hemolytic streptococci are lysed by penicillin. Thus, Knox confirms Hobby & Meyer's results. (87).

Gardner (63) states that the action of penicillin on sensitive bacteria has no particular connection with multiplication or division, though in the semi-resistant Staphylococcus typhi a disorder of division seems to be the minimal effect of feebly antibacterial concentrations. In his opinion the action of penicillin is on feeding bacteria, and begins directly they begin to feed.

Strong concentrations of penicillin in nutrient media were found to kill spores of bacteria slowly, without inducing or permitting germination. (107). Minimal concentrations of penicillin were found to permit the earliest phase of germination of a spore, followed gradually by the lysis of swollen and distorted cells, but generally without complete sterilization.

(4) Physiological and Metabolic Alterations Occasioned by Growth in Penicillin

According to Hotchkiss, (92) it has been realized for some time, that susceptible bacteria, if attempting to grow in the presence of penicillin, were rapidly rendered non-

viable, whereas non-proliferating cultures remained viable and relatively unaffected.

Staphylococci exposed to penicillin for a time, then washed, and transferred into normal media, continued to decrease in viability for a time depending upon the extent and duration of exposure. Parker & Marsh, (166) by making successive plate counts of staphylococci, showed that viability begins to decrease after a lag period, which is longer for lower concentrations. According to Yanagita & Suzuki (222), the gradual lysis of staphylococci by penicillin can be observed in dilute peptone and, without growth, in cysteine. Eagle (43) has found that the rate of killing of staphylococci, streptococci, pneumococci and spirochetes is not increased beyond a certain maximal rate, no matter how much penicillin is present, and with much higher levels of penicillin, Escherichia coli shows essentially the same response. (222). Hirsch & Dosdogru (83) measured the oxygen uptake of proliferating cultures of staphylococci to obtain a continuous record of the effect of penicillin and streptomycin. They found evidence of a lag dependent upon concentration of the antibiotic, and also reported that the respiration eventually diminished to a steady level, dependent upon the antibiotic's concentration. They found with penicillin at low concentrations, there was a pronounced immediate effect, followed by lysis, while lysis did not occur in higher concentrations, with these strains of staphylococci.

Sub-bacteriostatic concentrations of penicillin have been shown to enhance metabolic activity and the growth of

Staphylococcus aureus. (176), (28). This, in part, explains the narrow ring of enhanced growth that immediately surrounds each zone of inhibition on assay plates. Pratt & Dufrenoy (176), (37), (174) have suggested that they represent regions in which the test organisms, having been subjected to sub-bacteriostatic concentrations of penicillin without subsequent exposure to "static" or "cidal" concentrations, have been stimulated to a state of intense metabolism and growth, characterized by an abnormally high rate of respiration. Although this increased growth may be due to the fact that the effect of penicillin on cells promotes bacteriolysis through autolytic enzymes. (213). Thus, products liberated by dying bacteria may serve as growth factors for survivors. (176). Pratt & Dufrenoy (176) feel that penicillin exerts a secondary action by increasing the permeability of the cell wall of most organisms in a colony so that some of their constituents are released into the medium where they serve to enhance the metabolic activity and to stimulate the growth of more resistant neighboring cells. Such cells then would then become more susceptible to penicillin.

Gale (56) has developed and applied a procedure for the determination of the free amino acid content of bacterial cells to the study of the action of penicillin on the assimilation of amino acids by staphylococci and streptococci. Gale & Taylor (60) report that these organisms, grown in low concentrations of penicillin, have almost completely lost the ability to accumulate free glutamic acid inside the cell; whereas, without penicillin in the medium, the cells accumulate glutamic acid in

steadily increasing concentrations in media containing the amino acid. This decrease in glutamic acid accumulation is manifested by cells in media containing penicillin, or by washed suspensions of bacteria that have been grown in penicillin, but washed normal cells appear to be unchanged in the presence of penicillin. They, (60) reasoned that a specific injury had been done to the cell during growth in the presence of penicillin, and supposed that the resultant inability to use glutamic acid may prevent growth. The process of accumulation in free form is affected (92); the further building up internally of glutamic acid compounds is not. (57). Lysine accumulation, which appears to be a process of physical diffusion, remains unaffected; other amino acids were not investigated. The respiration, glucose-oxidation and fermentation of penicillin-tested cells were found to be normal.

According to Bailey & Cavallito (5), this loss of assimatory power of glutamic acid may be correlated with loss of viability. The internal metabolism of glutamic acid is normal in such cells; but since the passage of glutamic acid across the cell wall is blocked by the penicillin treatment, the metabolism of this amino acid is limited by the internal concentration. Gale & Taylor (59) and Gale (58) have shown that one of the earliest manifestations of the action of bacteriostatic concentrations of penicillin on Staphylococcus aureus is a blocking of the absorption of the essential metabolite, glutamic acid. This blocking of glutamic acid by penicillin becomes evident after very short contact of penicillin with the cell, before morphological changes are

apparent. This corroborates the fact, that penicillin interferes with the assimilation of essential growth factors by the bacterium.

According to Hotchkiss (92), the effect mentioned above, may possibly be connected with the finding of Schwartzman (191) that dicarboxylic and basic amino acids, and cysteine, counteracted to some extent the effects of penicillin upon growing Escherichia coli. Hotchkiss (91) had been studying the incorporation of various mixtures of amino acids into protein by respiring staphylococci, and on this system, crystalline penicillin "G" had no effect. After the appearance of Gale & Taylor's preliminary report (59), Hotchkiss tried staphylococci grown in penicillin, but even then did not find a decrease in capacity to take up glutamic acid alone or in mixtures (92). Hotchkiss felt that the probable failure of his attempt to confirm Gale & Taylor's findings, was not due to penicillin differences, but due to differences in the organism or cultural conditions. (92).

Penicillin and various other antibiotics have been shown to be inactivated by -SH compounds (16), (19), (20), (21), (66), and the mode of action of these antibiotics has been conceived as possibly dependent on the inactivation of -SH groups in the bacterial cell. As indicated by Lourie (115), antagonism or interference with chemotherapeutic action of a particular drug does not inevitably imply that the mode of action of that drug is by inactivation of chemical groups, essential for the parasite, which are similar to chemical groups of the antagonizing substance. However, Lourie (115)

feels that whether or not every instance of antagonism by -SH groups points to inactivation of such groups as the essential mode of action of the particular drug under consideration, there can be little doubt that -SH groups of the parasite are among the most vulnerable components of the cell. They provide a broader target than any individual metabolite, in that they are widely distributed, being essential for the activity of many important enzymes, such as, for example, glyoxalase, succinic acid, dehydrogenase, triose-phosphate dehydrogenase, and certain pyruvate oxidases. (115).

Pandalai & George (165) suggest that penicillin interferes in phases where nucleic acid plays a part in cellular processes. Nucleic acid is claimed to antagonize the bacteriostatic action of penicillin, and penicillin bacteriostasis may also be reversed by nucleic acid. (5).

Krampitz & Werkman (108) found that only impure preparations of penicillin inhibited dismutation of pyruvic acid. Pure penicillin "G" was reported to interfere with the dissimilation of ribonucleic acid and consequently with its assimilation during growth. According to their findings, both intact cells and enzyme preparations of Staphylococcus aureus failed to show any effect of penicillin on enzymes of intermediate products of glucose metabolism. This confirms earlier observations of Schuler, as reported by Bailey & Cavallito (5).

(5) Effect of Penicillin on Enzyme Systems and Toxin Production

The great diversity of chemical structures encountered among antibiotics has been quoted to reduce the possibility that

their antibacterial action is due to a single mechanism. (9). According to Bailey & Cavallito (5), very little definite success has been achieved in attempting to associate the action of antibiotics with inhibition of specific enzymes or enzyme systems. As previously mentioned, differences in reactivity of antibiotics toward sulfhydryl type compounds, should lead one to expect differences in the manifestation of inhibitory mechanisms at the enzyme or enzyme system level, or the intact cell level of investigation. (5).

Pertaining to the level of mechanism of action involving reaction of functional groups, Bailey & Cavallito (5) feel that the sulfhydryl inhibition theory is the only common denominator for a large group of antibiotics of heterogeneous chemical structure. The apparent alternative to such a mechanism would be to postulate specific metabolite - antimetabolite relationships for the many compounds, which has not yet been done for even one antibiotic.

Levaditi & Vaisman (113) have reported that the action of penicillin destroys the silver-binding power of the enclosing membrane of the sensitive cell. They state that penicillin action blocks the enzyme systems concerned with cell division, hence the hypertrophy of the bacteria in the stage preceding lysis.

Duguid (38) upholds this view, in that penicillin in low concentrations appears to interfere with the formation of the outer supporting bacterial cell wall in its mode of action on susceptible growing bacteria. Gale & Taylor (59) also suggest that penicillin either combines with, or produces a

reorganization of the cell wall, such that the assimilatory mechanism is blocked. Thus, cells in the presence of penicillin, have their assimilation of glutamic acid prevented, and consequently its internal concentration decreases.

Krampitz & Werkman (108) have stated that penicillin, on prolonged contact with Staphylococcus aureus cells, does not irreversibly denature any of the proteins of the enzyme systems involved in this organism's activity.

Contrary to previous impressions, one phase of the mechanism in penicillin's mode of action may be due to the fact that penicillin abolishes oxygen uptake by young organisms. Chain & Duthie (24) were one of the first to report that during the early lag and logarithmic phases of multiplication of staphylococci, penicillin in very small concentrations (0.04 to 0.1 unit per cc.) exerts a strong inhibitory effect on, and eventually completely stops, oxygen uptake by the organisms, while during the "resting" phase of growth, penicillin is without this effect, even in larger concentrations. They observed bactericidal effects of the compound upon staphylococci before actual cell division had occurred. Thus, penicillin appears to interfere with this metabolic function at least in the early stages of bacterial development. As previously stated, penicillin interferes with the dismutation of pyruvate by susceptible staphylococci, (219) thus probably affecting the way in which susceptible bacteria use energy rather than the processes by which energy is liberated. (90).

Hirsch & Dosdogru (83) report that both streptomycin and penicillin halt respiration of staphylococci after a latency

period. Two types of action of penicillin were reported; at 0.05 to 0.1 units per cc. there is a long latency and then a rapid decrease in respiration which accompanies disintegration of the cocci ("degenerative effect"); at 1.0 to 1,000 units per cc. a cessation of respiration results after a shorter latency period which corresponds to the bacteriostatic effect.

According to Pratt & Dufrenoy (176), any agent that impairs oxygen uptake correspondingly impairs the ability of the aerobic cell to absorb and to retain ions or molecules. They feel, that inhibition of the energy-providing respiratory system may be so great as not only to prevent absorption and retention of materials from the external environment, but to prevent retention by the cells of their normal constituents. (36).

Using sodium ribonucleinate as a substrate, Krampitz & Werkman (108) observed that the oxygen uptake of washed staphylococci and other bacteria is completely inhibited in the presence of high concentrations of penicillin "G". Similarity of the oxygen uptake curves representing the sodium ribonucleinate and endogenous activity, suggested that penicillin inhibited dissimilation of cellular ribonucleic acid. This experimental evidence suggested that the overall effect of penicillin is to block the enzymatic system involved in the dissimilation of ribonucleates, whether synthesized by the organisms or merely absorbed from the external substrate.

Dufrenoy & Pratt (37) have reported on some possible cytochemical mechanisms of penicillin action based upon observa-

tions of properties of areas on agar plates which show bacterial growth as compared with zones containing penicillin-inhibited microorganisms. On the basis of color reactions, lower sulfhydryl concentrations were found in zones of growth inhibition. An impairment of dehydrogenase activity was inferred. Bailey & Cavallito (5) have criticized this observation of differences in sulfhydryl concentrations between zones of inhibition and of growth, on the grounds that the growth zone contains more organisms than the inhibited areas.

Gros & Macheboeuf (69) have found that the hydrolysis of glycerophosphate by suspensions of Clostridium sporogenes proceeds to the same extent in the presence or absence of penicillin. Penicillin, however, inhibits the dephosphorylation of adenosine triphosphate by these suspensions. Working with the same organisms, they later found (70), that penicillin inhibits strongly, the hydrolysis of adenosinepyrophosphoric acid in non-proliferating suspensions.

According to these investigators (71), the mode of action of penicillin on bacteria may most plausibly be explained on the basis of inhibition of a metabolic process common to all susceptible organisms. Their former work (69), (70) with the action of enzymes connected with carbohydrate metabolism was exhibited also by organisms killed with toluene. This apparently established the pattern of enzymatic inhibition by penicillin, and they extended this line of investigation. They found that penicillin was able to inhibit Stickland reactions (donation of hydrogen from one amino acid to another) and similar oxidation-reduction processes based on enzyme systems

in which phosphorylated derivatives are important co-enzymes. Their observations agreed with the fact that there is an inhibition of glutamic acid utilization in the presence of glucose and penicillin. These investigators feel that the inhibition might be directed against the mobilization of phosphoric acid, which is essential for the utilization of glutamic acid. This is also supported by the fact that another amino acid (lysine), which does not require glucose to be present in order to be utilized by staphylococci, can be utilized freely in the presence of penicillin. (71).

Dixon (31) and Neter & Will (163) have reported that penicillin inhibits fibrinolysis by penicillin sensitive fibrinolytic strains of Staphylococcus aureus and hemolytic strains of Streptococcus pyogenes. Neter & Will (164) also observed that penicillin was without effect upon tetanus toxin "in vitro" in mice. Boor & Miller (17), (18) have observed that penicillin repeatedly administered in relatively large doses to mice and rabbits, exerted a considerable degree of protection against the lethal effects of meningococcal endotoxin, although there was no evidence of a detoxifying action of the compound "in vitro". They observed similar results with gonococcus endotoxin. Blair and co-workers (13) have reported that penicillin has no effect on pre-formed alpha-toxin of the staphylococci. Kilbourne & Loge (101), by measuring the degree of suppression of antistreptolysin formation, found that penicillin therapy suppressed the formation of antistreptolysin in hemolytic streptococcal pharyngitis, and was proportional to the duration of the penicillin action.

Hotchkiss (92) reports that penicillin does not appreciably affect bacterial amino acid utilization, glycerophosphatase nor muscle adenosinetriphosphatase. Torda & Wolff (216) state that penicillin in moderately large amounts had an inhibitory effect on acetylcholine production in a brain mince.

It appears to Benedict & Langlykke (9) at the present time, that highly reactive antibiotics interact with a number of enzyme systems or metabolites essential to the bacterial cell. They agree with Wintersteiner (221) that the known complexity of bacterial metabolism and enzyme systems on the one hand, and the great diversity of chemical structure encountered among antibiotics on the other hand, greatly reduces the possibility that their antibacterial action is due to a single mechanism, as envisaged in the sulfhydryl hypothesis. (66), (65), (67), (68).

(6) Evidences of Penicillin Combination or Accumulation Within the Cell

According to Hotchkiss (92), the observation, that it is only growing cells which are notably affected by penicillin, indicates the possibility that a metabolic process involved in growth may cause the accumulation or incorporation of penicillin into toxic concentration or combination at some point in the cell.

Pratt & Dufrenoy (175) described the use of dyes in following changes in cells induced by penicillin. Penicillin-treated Staphylococcus aureus failed to apportion vacuolar material to daughter cells during cell division. Under the

influence of penicillin, lipids were displaced from cells undergoing lysis and the liberated lipids were hydrolyzed into fatty acids.

The lag before sterilization begins, dependent as it is on concentration, seemed to Parker & Marsh (166), as conceivably a time in which either penicillin was accumulated, or else a competing metabolite became exhausted.

Synergism between penicillin and various other agents has frequently been reported. For example, Kirby & Dubos (102) have shown that the growth of Mycobacterium tuberculosis is markedly inhibited when the wetting agent Tween 80 is present. The presence of this wetting agent rendered the otherwise virtually insensitive organism highly susceptible to penicillin. From this observation, they thought that this might be construed as evidence that the vulnerable sites of the organism were not accessible to the inhibitor until a "wetting" or "penetrating" agent was added. Lysis, although indicated, was minimal and not progressive.

II. ACQUIRED RESISTANCE OF BACTERIA TO PENICILLIN "IN VITRO"

(1) Introduction to Acquired "In Vitro" Resistance

For the purpose of this review, the term "resistance" is defined as a decrease in the susceptibility of a naturally susceptible organism to penicillin, and "induced resistance" as that brought about by repeated subculturing of the organism in increasing concentrations of the antibiotic.

Spicer & Blitz (202) have reported that the destructive action of penicillin on sensitive bacterial strains is not

complete. A residuum of viable organisms always remains, which is capable of withstanding the destructive action of the antibiotic but is inhibited from multiplying in its presence. A peculiarity of penicillin's antimicrobial activity is the fact that, although in culture media at 37°C. it may kill large numbers of sensitive bacteria, it does not always, even in high concentrations, kill all the bacteria present. (107). Bigger (11) regards the survivors, which he terms "persisters", as being insensitive to penicillin because they are dormant non-dividing forms. However, Miller and his colleagues (157) suggest that the mode of action of penicillin may not be very different from that of the sulfonamides, since both act on some bacteria, while being completely ineffective on others, obviously due to fundamental differences in the biological characteristics or nutritional requirements of the two types.

The mechanism by which resistance to penicillin arises is not completely understood. Davies, Hinshelwood & Price (28-A) have postulated that the adaptation of an organism to an antibiotic may result through one or more of the following changes: (1) natural selection from an initially heterogeneous population; (2) actual modification of the individual cells due either to establishment in the cells of a mechanism alternative to that already in use, or the quantitative modifications of the existing mechanism, particularly the enzyme systems; or (3) a change in some center of organization of the cell. These possibilities resolve into two theories: (1) bacterial adaptation to penicillin is a manifestation of induced variation of originally sensitive

cells, and (2) bacterial adaptation to penicillin represents a biological selection of more resistant individuals out of a primarily heterogeneous bacterial population.

(2) Induced Resistance to Penicillin "In Vitro"

The capacity of various bacteria to acquire resistance or fastness to penicillin "in vitro" has been amply proven. There is indication that the resistance of a strain to one type of penicillin is the same for all types of penicillins, as three different penicillins cannot be differentiated by a penicillin "G" resistant strain of Staphylococcus aureus. (9). Therefore, strains of organisms that become resistant to one type of penicillin become correspondingly resistant to the others, since penicillin-fastness depends upon the slowing down of metabolic rates. (46).

A number of investigators have been able to produce induced resistance or fastness, of various susceptible bacteria to penicillin "in vitro" by culturing them in media in which the concentration of penicillin was progressively increased. (138), (189), (178), (218), (203), (214).

McKee & Rake (139), (138), increased the resistance of Staphylococcus aureus, Streptococcus pyogenes and pneumococci (types I, II & III) anywhere from 2 to 6,000 times that of the parent cultures by 8 to 60 serial passages through barely sub-inhibitory concentrations of penicillin. Resistance of the streptococci and pneumococci was not induced as readily or to as high a degree as in the case of Staphylococcus aureus. The acquired penicillin resistance, once acquired, appeared to be a permanent change. (138), (139). However, it has been stated

that acquired "fastness" to penicillin is only temporary in nature. The organisms return, after several generations, to their normal rates of growth and become susceptible again to the action of penicillin. (214).

Spink & Ferris (204), and Klimek et al (104) also are in disagreement with this observation relating to lack of loss of resistance over succeeding generations after withdrawal from penicillin. Spink & Ferris (204) believe that the resistance developed "in vivo" differs from induced resistance, the former being more permanent and due to the production of penicillinase by the resistant organisms. Klimek et al (104) showed that resistance could be lost, unless "fixed" in the organism by maintaining it for a time in the presence of the antibiotic.

According to Meads (141), hemolytic streptococci belonging to group A do not possess any natural resistance to penicillin, although they may acquire resistance "in vitro" and "in vivo". (196). Data obtained by Klein (103) demonstrates that streptomycin-resistant organisms are more frequently encountered among stock-culture collections than are those resistant to penicillin.

Several investigators (153), (154), (155), (197) have found that resistance appeared more rapidly to streptomycin than to penicillin, and believe that streptomycin acts to "select" naturally occurring resistant organisms present in the original cultures (meningococci).

Contrary to the theory that penicillin confers an effect on the susceptible organism, such, that it results in a meta-

bolism so altered, that the organism can grow in a normally inhibitory concentration of the antibiotic, is the theory proposed by Demerec (30) and Luria (117), which may be termed the "genetic theory".

This theory proposes, that within any bacterial population, individuals will be found in varying numbers that possess varying degrees of susceptibility to a given antibiotic. The susceptibility of a given strain of an organism to an antibiotic is, then, the antibiotic effect on the majority of the organisms in the test. There are, however, a few organisms present that are less susceptible than the majority. In the presence of the antibiotic the majority of the population is inhibited and only those few resistant forms or mutants can develop. Demerec (30) believes that this resistance is not induced by the action of penicillin on bacteria but originates through mutation. Thus, those individuals in possessing a natural resistance to penicillin, may have their degree of resistance increased by exposures to higher concentrations of the compound, and Demerec has interpreted this as being due to the summation of the effects of several independent genetic factors for resistance, which undergo consecutive mutation.

(3) Morphological Alterations Coincident With Acquired Resistance

Abraham and co-workers (1) originally assumed that organisms with acquired penicillin resistance do not show other changes in their biologic and metabolic characters. In general, more recent observations record that organisms developing only slight degrees of resistance show little or no morphologic

differences from the susceptible organism; when resistance is increased or marked, striking morphological changes have been reported.

Gardner (62) reports that resistant Clostridium perfringens cells attain lengths ten times greater than normal in the presence of penicillin; staphylococci show spherical enlargement and spindle formation. Bahn et al (4) rendered five strains of gonococci resistant to penicillin, and found all strains to consist of pleomorphic cells of increased size.

Hall & Spink (80), noted that cells comprising a colony from a highly resistant Brucella abortus strain contained tiny amorphous coccoid forms which stained faintly red, and also large coccoid forms which stained dark red. Klimek et al (104) found that Staphylococcus aureus growing in 1 mg. per cc. of penicillin was rod-like in shape and Gram-negative. The morphology and Gram-staining reaction reverted to that typical for Staphylococcus aureus when the resistant strain was subcultured repeatedly in penicillin-free broth. The altered morphology and staining reaction apparently became fixed with the resistant strain, if it was cultivated in penicillin broth for a long period, and no reversion occurred on subsequent withdrawal of penicillin.

The findings of Klimek et al (104) have been substantiated by Gale & Rodwell (61) who found no change in morphology or staining reaction in Staphylococcus aureus growing in 2,000 units per cc., but when its resistance had been increased to 6,000 units per cc., they found it to be highly pleomorphic and Gram-negative.

(4) Metabolic Alterations Occasioned by Induced Resistance

The majority of investigators are in agreement that there are no great biological differences between resistant and sensitive strains of the same organism (5), and this appears, in particular, to be more acceptable, when the degree of induced resistance is low.

There is general agreement that resistant organisms grow more slowly than do the parent strain, as noted by Hall & Spink (80), Seligmann & Wassermann (197), Klimek et al (104), Murray et al (162) and Schoenbach & Chandler(190).

Penicillin-resistant gonococci have been reported by Bahn et al (4), to ferment glucose at a slower rate than the sensitive parent strains. Murray et al (162) found two highly resistant Klebsiella pneumoniae strains to have lost the power of fermenting saccharose, of using citrate, and of producing acetylmethylcarbinol. In the experience of Lyons (119) with resistant staphylococci, the metabolism of these organisms has not undergone alteration during the process of developing fastness as evidenced by coagulase activity and mannite fermentation. Clinically, he observed no loss of virulence.

As previously mentioned in this review, it has been definitely indicated, (204), (118), (195), that induced penicillin-resistance does not necessarily involve the production of penicillinase.

Bellamy & Klimek (7) found that an extremely resistant Staphylococcus aureus had lost its ability to grow anaerobically, and they suggested that penicillin interfered with one or more essential components of the anaerobic energy mechanism of the

susceptible organisms. They further showed (8) that the resistant organism had acquired the ability to synthesize nicotinic acid, one of the essential vitamins for the growth of Staphylococcus aureus, and to produce penicillinase, but had lost the power to grow in 6.5% sodium chloride, to reduce nitrates, and to produce acid in saccharose, lactose, or mannitol. It retained its ability to ferment glucose. Except for the ability to synthesize nicotinic acid, these results suggest the relative loss of enzymatic abilities rather than a gain.

Gale (58) has shown that staphylococci, selected as penicillin resistance progressively increases, had a decreasing capacity to concentrate and store glutamic acid. Gale & Rodwell (61) noticed with highly resistant staphylococcal strains, derived by penicillin inducement, that they were increasingly independent of an external supply of the twelve or more amino acids needed by the parent strain. Thus, there is an inferred link between penicillin-resistance and amino acid utilization. The most highly penicillin-resistant of the strains they obtained, were Gram-negative, grow without added amino acids by being able to synthesize their own, and do not accumulate any free glutamic acid internally. Thus, they were able to grow and synthesize amino acids in a medium composed of salts, ammonium sulfate, glucose, cysteine and thiamine. Delayed and submaximal growth occurred when cysteine was omitted from the medium.

From this, it appears to Hotchkiss (92), as if the normal staphylococcus has compensated for a loss of synthetic abilities

by learning to store a supply of free amino acids, incidentally becoming Gram-positive, and for a reason as yet not clear, penicillin-sensitive. Resistance development to penicillin appears to be a reversion toward a more primitive saprophytic type of metabolism. (5).

HYALURONIC ACID

(1) Occurrence and Distribution

Hyaluronic acid is a mucopolysaccharide which was first isolated from bovine vitreous humor by Meyer & Palmer (149). It occurs in the mesenchyme, from which derive connective tissue, blood lymphatics, bone, cartilage, etc. (206). Since it is the chief ground substance of this type of tissue, it was subsequently soon isolated from human umbilical cord (150), bovine aqueous humor (151), bovine and human synovial fluid (152), Rous tumors of chicken (172), and pig skin (146). Hyaluronic acid is absent from blood, cerebrospinal fluid, and nasal and gastric mucin (42), but does occur in lung tissue (95).

Kendall and co-workers (100) were the first to identify hyaluronic acid as a constituent of the capsular material in Group A streptococci. Then Seastone (193) demonstrated its presence in the capsules of Group C streptococci of animal origin.

This substance is present in the mucoid or matt strains of the streptococci (194), (29). It is present only in the

encapsulated strains, but strains of encapsulated streptococci found in groups A, B, C, G, and M have been found that lack the substance in their capsules. (194), (131), (132).

Seastone (194) found that seventy-five of eighty-one Group A strains isolated from human sources, produced enough hyaluronic acid in the culture medium to be detected in dilutions of 1:10 or greater. All of the strains producing large amounts were encapsulated. None of the four strains belonging to B and C groups, produced detectable amounts of hyaluronic acid. No relationship between the hyaluronic acid content of the capsule and the type of Group A streptococcus has been described (179).

In young broth cultures of hyaluronic acid-producing strains of streptococci, the hyaluronic acid is closely associated with the cells, about which it is demonstrable as capsules by the India Ink technique or by special strains (35). In older cultures it passes into the broth, from which it may be recovered by chemical methods.

When Group C streptococci are treated with hyaluronidase, they become susceptible to phagocytosis; but, because subcultures form capsules, they obviously have not lost their hyaluronic acid-producing capacity (35).

Upon the addition of testicular (131) and leech (84) extracts, as well as hyaluronidase prepared from Group C streptococci (131), the capsules of Groups A and C streptococci disappeared almost immediately. A similar effect was observed "in vivo" in the peritoneal cavity of the mouse using leech extract (84). The capsule of Group B streptococci was not

affected (144).

(2) Chemical Structure

The exact chemical structure of hyaluronic acid is unknown (95). On hydrolysis, it yields equimolar concentrations of N-acetylglucosamine and glucuronic acid (150). Humphrey (95) states that existing evidence suggests that specimens of hyaluronic acid prepared from different sources are chemically identical. Blix & Snellman (14) have shown that hyaluronic acid prepared from umbilical cord has the longest particle length and to possess the greatest degree of polymerization, when compared to that obtained from synovial fluid or vitreous humor.

According to Meyer (206), the repeating unit in this substance is glucuronido-N-acetylglucosamine, polymerized by a glucosidic linkage of the latter. Besides these stable linkages, a labile acid anhydride linkage has recently been found, which appears to bind the high polymer to the giant molecules of the native gels.

(3) Chemical, Physical & Immunological Properties

In its native form, hyaluronate gives a typical mucin clot, forms gels, and is not apparently bound to protein (206). It has a high molecular weight of several million (206), and does not dialyze through collodion membranes of great porosity (42). It is soluble in water, viscid, and precipitable by acetic acid (42).

It occurs free or in a salt linkage, and is not chemically bound to protein; but it will combine stoichiometrically with the free amino groups of proteins at pH levels lower than the

iso-electric point of the proteins to form salts (42). Examples of these salts are the mucins or mucoids prepared by acidifying the diluted solutions of such fluids as vitreous humor or synovial fluid.

Fluids containing hyaluronic acid, have the acid migrate in an electric field at pH 7.6 - 7.8 with the same speed as the isolated pure acid (42). Isolated hyaluronate has all the characteristics of a highly asymmetrical molecule. Aqueous solutions possess bi-refringens of flow, and can be spun into threads of considerable tensile strength (144).

Hyaluronic acid is not antigenic (144), (145), (95). Humphrey (95), chemically coupled hyaluronic acid from umbilical cords to protein by an azobenzyl ether linkage, and found that this compound does not act as a specific hapten when injected into rabbits. Hyaluronic acid, prepared from mucoid streptococci, was isolated by Kendall & co-workers (100), and found to be serologically inactive. Seastone (193) was unable to produce detectable circulating antibodies against the capsules, when whole capsulated streptococci or purified capsular material was injected into rabbits.

(4) Purification and Preparation of Hyaluronic Acid as a Substrate

McClean & Hale (136) found that hyaluronic acid extracted from vitreous humor and purified for use as a substrate, suffered a spontaneous loss of viscosity on storage.

In the preparation of the substrate, emphasis has been laid on the removal of detectable protein constituents, and on subsequent isolation of an essentially homogeneous polysaccharide fraction. This removal of protein is necessary, insofar as

possible, as mucopolysaccharides bound to protein are less easily depolymerized than the corresponding protein-free compound (144). During purification steps, some degradation of carbohydrate structure may occur (127), and the macromolecular state may not be the optimum (205), (127), or the same, for each lot prepared from time to time. For these various reasons, the necessity of having one lot of purified substrate for a series of experiments is well illustrated.

The isolation of hyaluronic acid from human umbilical cords by various investigators, involves many modifications of the same general principles. Several methods have been reported in the literature (150), (183), (97), (127), (136), (137). However, the work of Morgan & King (160), Blix & Snellman (14) and Rogers (183) has revealed the necessity for using gentle methods of handling and treatment if the physical properties are to be preserved.

Considerable degradation of the polysaccharide occurs (183), when methods that incorporate boiling water for extractions are used, such as Madinaveitia & Stacey described (127). The use of glacial acetic acid in extraction and purification procedures (181) destroys in part, the viscosity of the mucopolysaccharide (136).

The usual method of precipitation of hyaluronic acid from an aqueous extract depends on the addition of three volumes of ice-cold ethanol containing a trace of sodium acetate, as a first step in precipitation (136). The final product contains about 7% total N. McClean (134) found, however, that if the alcohol is saturated with potassium acetate and brought to pH 9

to 10 with potassium hydroxide, the hyaluronic acid may be precipitated by the addition of only 1.25 volumes. This product contains considerably less protein than that derived by the original method (136) and contains 4.8% total N. by comparison (134).

Humphrey (97) subjected the crude acetone precipitate from acetone dehydrated human umbilical cords, as prepared by McClean & Hale (136) to various procedures designed to remove impurities, while avoiding any loss in viscosity. He found that the use of 90% (w/v) aqueous phenol did not remove fine protein particles (resembling haematin) which imparted an opalescence, but that a chloroform-amyl alcohol mixture, as originally described by Sevag (198), removed the remaining protein as a chloroform gel more advantageously. The use of this chloroform-amyl alcohol mixture, repeated three or four times, was wasteful, but yielded a clear, colorless and highly viscous solution from which hyaluronic acid or its potassium salt could be precipitated by ethanol. This product, on analysis for nitrogen and glucosamine, gave values in agreement with those of Meyer & Palmer (150).

(5) Relationship to Bacterial Virulence

Early work of Seastone (193) suggested the possible relation of hyaluronic acid to virulence of streptococci. He observed that highly invasive strains of Group C streptococci isolated from guinea pigs produced more mucopolysaccharide than non-pathogenic strains.

If hyaluronic acid is a factor in the virulence of streptococci, then it might be expected that hyaluronidase would exert

a protective effect in experimental infections. Hirst (84) found that mice infected with ten thousand minimum lethal doses of Group C encapsulated streptococci could be completely protected by treatment with leech extract. However, only slight evidence of protection was demonstrated with similar treatment of mice, experimentally infected with a Group A, type 3 streptococcus. He concluded that the virulence of Group C infections in mice is related to the hyaluronic acid content of the capsule.

McClean & Hale (136) failed to protect mice against either Group A or Group C streptococcal infections by the use of testicular extract. They thought that the activity of the enzyme was reduced owing to the inhibitory effect of serum.

Seastone (193) and Kass & Seastone (99) have reported positive results with crude testicular extracts containing hyaluronidase, in that experimental infections with Group A streptococci could be favorably altered. Heat-inactivated hyaluronidase failed to exert a beneficial effect.

Rothbard (188) has demonstrated that hyaluronic acid capsules of Group A streptococci interfere somewhat with the phagocytosis of these organisms. Therefore, to a certain degree, these capsules contribute to the virulence of these organisms, but Rothbard found that the M-substance is the more fundamental factor.

According to Dubos (35), in Group C streptococcal infections of animals, a close relationship between hyaluronic acid production and the virulence of these bacteria seems definite. In the case of Group A streptococci, however, even though

virulent strains are often heavily encapsulated, a comparable relationship between hyaluronic acid encapsulation and virulence has not been definitely established. Recently, Pike (170) demonstrated that mouse passage of capsulated Group A streptococci resulted in a substantial increase in virulence, but no consistent increase in the amount of hyaluronic acid produced. Pike (170) concluded that no evidence of increase in mouse virulence is correlated with hyaluronic acid production.

HYALURONIDASE

(1) Occurrence and Distribution

Duran-Reynals in 1928 and 1929 first described (39), (40) a substance in extracts of bull testicle that increased the area of the lesions following the injection of the infective virus material into mammalian skin. These fundamental experiments were based upon the primary observation that vaccinia-virus infection in the rabbit develops most rapidly and most certainly when the inoculation of the infective agent is made into the testis (207). McClean (128), (129) confirmed these early experiments in 1930, and he demonstrated, as did Duran-Reynals independently (89), that intradermal injection of a dilute suspension of India-Ink formed a dark spot in mammalian skin, which in the presence of testicular extract, spread rapidly over an unusually large area. As McClean (128) and Hoffmann & Duran-Reynals (89) demonstrated, the substance

increased the permeability of the tissues, facilitating the speed of dispersion of the fluids injected, whether the solution incorporated dyes, toxins, suspensions of India Ink, viruses or bacteria. These experiments established the presence in testis, of a substance now known as testicular hyaluronidase. Thus, it became clear that this enzyme was facilitating the passage of foreign particles, living or dead, between and around the cells of the cutaneous connective tissue.

The spreading factor has been demonstrated in the venoms of many snakes, such as the Copperhead and Black Tiger (23), many of the rattlesnakes (48), especially those of the Crotalus group (41), (130), in spider and bee venoms (23), in leech extracts (25), and various other insects. The fact that these spreading factors were in reality hyaluronidase was established by Chain & Duthie (23), Favilli (48) and McClean & Hale (136), (135).

Duran-Reynals (41) pointed out that this spreading factor is present in the filtrates of virulent strains of pneumococci and streptococci. Meyer, Dubos & Smyth (147) subsequently confirmed the presence of this spreading factor in the autolysates of a rough type II pneumococcus. Its occurrence was shown further in other types of pneumococci, both smooth and rough, virulent and avirulent (143), and by many strains of streptococci, staphylococci and by many anaerobes of the Clostridia group, such as Clostridium welchii and Clostridium septicum, but not by the causative organisms of tetanus, plague, gonorrhoea, typhus, etc., nor by viruses (215).

Chain & Duthie (23), (22) established the fact that spreading factor from various sources, that hydrolyzed the mucopolysaccharide, hyaluronic acid, to N-acetylglucosamine and glucuronic acid with a fall in viscosity, was hyaluronidase. The correlation of the mucolytic activity of mucinase (hyaluronidase) with the spreading property was also confirmed at about the same time by Favilli (48). A great deal of confirmatory experimental data was reported very shortly afterwards by McClean & Hale (135), (136).

(2) Mode of Action on Substrate

Hyaluronidase catalyzes the hydrolysis of hyaluronic acid to acetylated glucosamine and glucuronic acid (208).

The degradation of the substrate by the enzyme "in vitro" occurs in at least three phases. In the first phase, there is a destruction of the ability of a solution of hyaluronate to clot or gel when acetic acid is added in the presence of serum (134). This destruction of the clotting power of the substrate is an early stage in its degradation, which can be detected before any appreciable fall in viscosity occurs (134). In the second phase, there is a drop in viscosity without any free amino-sugar or reducing substances being detected; in the last phase, there is a progressive liberation of the latter substances, which reach a maximum in 24 to 48 hours (42).

Hyaluronidase acts upon hyaluronic acid, and closely related polysaccharides from placenta and tumor mucin, and lowers the viscosity of chondroitin sulfate from hasal septa (127). According to Meyer (206), the chemical basis of the spreading reaction "in vivo" is apparently the depolymerization

of the hyaluronate gel, by hydrolysis either of the glucosaminidic linkage or of the anhydride linkages. Hyaluronidases of different origin undoubtedly contain different enzymes, but their number and mode of action are not fully known (144).

Since there are two glucosidic linkages in hyaluronic acid, one belonging to the N-acetylglucosamine, the other to the glucuronic acid moiety, this suggests that the depolymerization and the hydrolysis into monosaccharides requires two enzymes (144). Meyer et al (143) compared hyaluronidases from various strains of pneumococci and streptococci, and from testis by viscosimetric and reductometric methods. The results indicated that hyaluronidases are mixtures of at least two enzymes, and that these component mixtures are in different ratios. This conclusion was based on the observation that pneumococcal hyaluronidase hydrolyzed the substrate almost to 100% of the theoretical amount, whereas testicular hyaluronidase, which showed a much faster rate in the viscosimetric tests than the bacterial enzyme, hydrolyzed the substrate to only approximately 50%. The testicular enzyme on prolonged hydrolysis exceeded the 50% reduction, but the total reduction was considerably short of 100%. The addition of pneumococcal hyaluronidase to the non-hydrolyzed residue brought about complete hydrolysis, while addition of fresh testicular enzyme had a negligible effect.

(3) Chemical and Physical Properties

Hyaluronidase is very soluble in water, soluble in glacial acetic acid (120) and phenol (120), but insoluble in 90% (w/v)

phenol (97) and most organic and many non-aqueous solvents. Heating a solution of this enzyme at 60°C. considerably or totally suppresses its activity, the inactivation being complete at 100°C. for all hyaluronidases (42), (26). They are inactivated by gentle shaking (23), and inhibited by chloroform, but not by toluene, merthiolate, phenyl mercuric nitrate or acriflavine (23). Contradictory results have been reported on their inactivation or further reactivation by iodine, sulfites, arsenites, and cyanides (181), (147), (23). Hale (79) demonstrated that the hyaluronidase from Clostridium welchii is not readily inactivated by oxygen, and does not require oxygen for its activity. He also showed that it does not promote the oxidation of reduced rosinduline or the reduction of indophenol while it is acting on hyaluronate at pH 7.0. Its enzymatic activity is not influenced by the absence or presence of copper. Hale (79) found that Clostridium welchii or testicular hyaluronidase is partially inactivated by $KMnO_4$ or I_2, and cannot be reversed by Na_2SO_4. Meyer et al (147) obtained a reactivation of hyaluronidase after being inactivated by I_2.

In solution, hyaluronidase shows a fair degree of stability between pH 4.6 and 9.0, but some streptococcal enzymes are unstable. (143), (186). Some discrepancies have appeared in the literature concerning their destruction in highly acid or alkaline pH ranges (120), (26), (23). Claude & Duran-Reynals (26) state that hyaluronidase withstands pH 2.0 and is not precipitated by HCl while being brought to this reaction.

Claude & Duran-Reynals (26) demonstrated that testicular

hyaluronidase is inactivated by pepsin and trypsin, but not by carboxypeptidase. This was confirmed by Chain & Duthie (23). Hyaluronidase gives the color test for proteins, and passes through Berkefeld filters, but not through semipermeable membranes which retain proteins (26), indicating that the enzyme has a high molecular weight, a conclusion not fully corroborated by ultracentrifugal studies (120).
In an electric field it migrates to the anode, in solutions between pH 6.2 to 6.9 (3). Hyaluronidase is not effected by X-Rays or ultraviolet light (3). This enzyme does not lower surface tension. (129), (121). It is inactivated by blood serum of several species (133), (82). McClean (133) found that mouse, rabbit and sheep sera inhibit both homologous and heterologous testicular enzymes. Leonard & Kurzrok (111) state that normal rat serum inhibits bull testis hyaluronidase, but, has no effect if diluted 100-fold, while immune rat serum still inhibits after such dilution.

A solution of hyaluronidase is partly precipitated by many reagents normally employed for protein removal, but is not, or only slightly precipitated by half-saturated ammonium sulphate (121) and by neutral lead acetate (158). Sodium flavianate (142), basic lead acetate (120), (124), or saturated ammonium sulphate (124) precipitates it almost totally. Hyaluronidase is strongly adsorbed to ortho aluminium hydroxide "C" (alumina "C" gamma) and to a lesser extent by other adsorbents (122), (136). Concentration in alundum thimbles results in preparations possessing 55 times the power measured in area of spread over the control, per 0.5 ml. of concentrate

(26). On the basis of some of the properties aforementioned, several methods of purification have been devised (158), (26), (121), (122), (125), (181), (23), (142), (124).

Madinaveitia (124) has used an ammonium sulphate fractionation of aqueous testicular extracts, followed by lead acetate precipitation under controlled pH, and he found that this method afforded a convenient purification. He demonstrated that kaolin and Fullers earth were better for adsorption than Kieselguhr after ammonium sulphate fractionation. This investigator also demonstrated (122) that the hyaluronidase of testicular extracts and of Clostridium welchii are strongly adsorbed by Alumina "C" gamma, from which they may be conveniently eluted with sodium carbonate or disodium hydrogen phosphate.

Robertson et al (181) added acetone to the solution of enzyme and then added $CaCl_2$ to adsorb the enzyme into the Ca_3PO_4 formed. A disappointing yield was afforded, although a 900-fold increase in concentration was obtained in the final product. Rogers (184) has used dialyzed iron at pH 5.6 for adsorbing out streptococcal hyaluronidase, with which he received a yield varying from 20% to 50%.

East & Madinaveitia (45) demonstrated that the beta-glucosaminase of testicular extracts is distinct from hyaluronidase, and its adsorption behaviour on kaolin is different. Hyaluronidase was found to be more readily adsorbed on kaolin than is beta-glucosaminase. The more dilute hyaluronidase is in solution, the more strongly it is adsorbed (45).

Rogers (186) has found that aqueous solutions of purified

streptococcal hyaluronidase are not stable at 0°C. to 4°C. unless protected by the presence of glycerol. When dialyzed for 6 hours at room temperature against undiluted glycerol and stored at -10°C., they suffer only a 10% to 20% loss of enzyme potency in nine months. During estimations of enzymic activity, the dilutions were made in gum arabic solution (0.5 gm./100 ml.), as partially purified enzymes were found to be rapidly inactivated by dilution in water (186). Comparatively crude preparations of enzyme exhibit the same effect (186).

(4) Influence of "In Vitro" Factors on Hyaluronidase Activity

1. Effect of pH The effect of pH on the rate of hyaluronidase activity varies with the source of the enzyme, the salt concentration and the assay method.

Meyer and co-workers (143) noticed in the reductometric procedure, that testicular hyaluronidase had a double optimum; one was at pH 4.5, the other at pH 5.7, which they interpreted as an indication for the presence of two distinct enzymes. Meyer et al (142) also showed that the pH optima in the reductometric procedure for pneumococcus and Clostridium welchii hyaluronidase was at 5.8. With the turbidimetric method of assay, the pH optimum of testicular hyaluronidase in M/10 acetate buffer in the presence of 0.15M sodium chloride was found to be 6.0 (144).

By the viscosimetric method, the assay has a marked dependency on the salt concentration necessary at different pH levels (134). It has been shown by McClean & Hale (136) and Madinaveitia & Quibell (125) that the concentrations of salt affects the viscosity-reducing activity of these enzymes, and

that this effect varies with enzymes from different sources and with the nature of the ions present.

Madinaveitia and associates (123) reported their failure to obtain correlation between skin-diffusing and viscosity-reducing activities of several enzymes from different sources. According to McClean (134), this could be explained on the basis that their viscosity estimations were carried out at pH 4.6 in a final concentration of M/6 buffer solution, whereas the skin diffusion test took place at about pH 7.0 in an isotonic salt concentration.

McClean (134) demonstrated by viscosimetric procedures, that there is a marked decrease in activity at pH 7.0 if the concentration of McIlvaine's buffer (140) is increased from M/60 to M/6 with testicular or Clostridium welchii hyaluronidase. However, at pH 4.6, there is a substantial increase in activity as the concentrations of buffer are increased, with other conditions remaining constant. Thus, McClean (134) has demonstrated that the apparent pH optimum of viscosity-reducing activity, and the titre of the enzyme activity are markedly affected by the concentration and nature of the salts in the reacting system.

The anomalous behaviour of hyaluronidase from streptococci, when assayed viscosimetrically, had been noticed (143), (131) to vary from that of the testicular enzyme, which decreased the substrate viscosity at a rate directly proportionally to the enzyme concentration (125). The enzymes from Clostridium welchii, Clostridium septicum, and Staphylococcus aureus behave the same as testicular hyaluronidase (136). Using the con-

ditions with which McClean & Hale (136) obtained a decrease in viscosity of substrate in a direct proportion to bacterial enzyme concentration, Hale (79) was unable to obtain similar results with streptococcal hyaluronidase. The streptococcal enzyme appeared to be destroyed or inhibited during its action (79) at the pH of 4.6. Hale then applied McClean's (134) aforementioned observations, and found that the Group A or Group C streptococcal enzymes differed from testis, Clostridium welchii and staphylococcal hyaluronidase, in that it is rapidly destroyed at pH 4.6, but is relatively stable at pH 7.0.

Rogers (186), since, has shown that streptococcal hyaluronidase shows optimal activity at a pH value of about 5.5, which is less than 1.0 unit above that at which inactivation occurs too rapidly for determinations to be made. At pH 4.6, in the presence of substrate, the inactivation which occurs is almost completely reversible on adjustment of the pH to 7.0. The final concentration of McIlvaine's phosphate-citrate buffer (140) was M/60, and that of sodium chloride at M/10. When the pH optimum of the enzyme was to be determined, the pH of the final buffer-hyaluronate mixture was always measured, as the hyaluronate itself has a considerable buffering action (186). The glass electrode was used for all pH measurements (186).

2. Effect of Salts Robertson and co-workers (181) reported that the dialyzed hyaluronidase of Clostridium welchii had no effect on a substrate of dialyzed synovial hyaluronic acid. Addition of phosphate or other salts in increasing concentrations resulted in increasing activity up to 0.08M. concentration.

McClean & Hale (136) and Madinaveitia & Quibell (126) reported a marked influence of salts, especially sodium chloride, on the activity of testicular hyaluronidase, as determined viscosimetrically. They found that the activity of the enzyme increases up to an optimum concentration of salt, which varies, for the type of enzyme used, from 0.1 to 0.3M. A further increase of sodium chloride causes a rapid fall in activity. If the concentration of NaCl is reduced below 0.1M, the reaction time is no longer inversely proportional to the concentration of enzyme.

McClean (134) during his development of the Mucin Clot Prevention Assay, confirmed these results on the effect of sodium chloride to enzyme activity, and found that the activity of hyaluronidase from testes increases with salt concentration up to at least 0.1M. concentration. He also demonstrated that the apparent pH optimum of viscosity-reducing activity and the titer of the enzyme are greatly affected by the concentration and nature of the salt in the reacting system (134).

Bergamini (10) has confirmed these observations, by demonstrating that the activity of hyaluronidase of testis, leeches, and of Clostridium welchii varies with the electrolytic content. He found that they were totally inactive in the absence of electrolytes. He demonstrated that NaCl, KCl, $CaCl_2$, NaI, KI, KBr, $NaNO_3$, Na_2SO_4, and $MgSO_4$ all activate the enzymes. With testicular hyaluronidase, the effects of different salts vary, being strongest with KI and NaI, and weakest with Na_2SO_4 and $MgSO_4$. All the other salts have a similarity with their effects. He (10) found that phosphate buffer

solution at pH 6.8 is less active than NaCl of the same concentration. These findings were supported by the viscosity changes demonstrated by the enzymic effects on the hyaluronate of umbilical cord.

Rogers (186) has found that with the use of McIlvaine's (140) phosphate-citrate buffer at M/60 and that of NaCl concentration at M/10, the optimal activity of streptococcal hyaluronidase was at pH 5.5 and of staphylococcal hyaluronidase at pH 6.6. He mentioned that if the use of other types of buffers and other ionic concentrations were employed, very considerable alterations, both in pH optima and in the shape of the pH activity curves, would probably be observed.

According to Meyer (144), the influence of salts, especially NaCl, was found to be much more marked with the turbidimetric and viscosimetric methods than with the reductometric method of assay. He demonstrated that the influence of sodium chloride on the rate of depolymerization depended on the source of the substrate. The differences observed by the chloride effect between the hyaluronate preparations seem to be due to the presence of competitive inhibitors in the substrate. Therefore, the NaCl effect depended on the purity and origin of the substrates, which in turn influenced the results obtained in the activity titres of enzyme preparations (144).

3. Hyaluronidase Production Increase by Adaptation Increase in hyaluronidase production by the addition of hyaluronate to the medium was first reported by McClean & Hale for Clostridium welchii (136), and then by McClean (133). Subsequently, the

stimulating effect of added hyaluronate was studied systematically by Rogers (183), (182). McClean (132) observed that the capacity of a strain of streptococcus to produce hyaluronidase apparently cannot be enhanced by repeated subculture in a medium containing hyaluronate.

Rogers (182) added 0.1% of crude potassium hyaluronate to culture medium prior to sterilization by autoclaving (183), omitting glucose, and received an increase by 20 times in the titre of hyaluronidase by the streptococci. When 0.1% hyaluronate and 0.25% glucose was added, very little enzyme was produced, although the addition of glucose greatly increased the amount of growth. Rogers (182) thought the most likely explanation for the apparent inhibition of hyaluronidase formation by glucose appeared to be the additional acid formed by glycolisis of the carbohydrate. This acid formation lowered the pH below the range in which hyaluronidase was produced. This fact was confirmed by himself later (183). By maintaining the pH of this hyaluronate-glucose medium at 7.4 by the addition of NaOH during the growth of the organisms, the titre of enzyme activity rose to 1:20,000 by the M.C.P. test. In order to avoid continuous neutralization of the cultures, 2.5% sodium glycerophosphate was added to the medium. Previous investigations by Rogers & Knight (187) had demonstrated that this substance had been found to stabilize the pH in cultures of Clostridium welchii. Rogers (182) found that the titre of enzyme synthesized in the presence of sodium glycerophosphate was the same as that in continuously neutralized cultures.

Rogers (182) thus demonstrated quite clearly, that the effect of the polysaccharide was to stimulate the synthesis of hyaluronidase by the organisms. The synthesis of hyaluronidase was almost directly proportional to the amount of crude hyaluronate added up to 0.5%, by Clostridium welchii and most strains of streptococci. However, one of two strains of Staphylococcus aureus produced a titre of 1:3,200 units which was not increased by hyaluronate, and the other had a low titre with or without added hyaluronate. The titre was not raised either, in a strain of Clostridium septicum (183), (182). Subsequently, Rogers (184), (183) discovered the hyaluronidase production of Staphylococcus aureus was raised by the addition of a papain digest of peptone to the medium, while with Clostridium septicum, he discovered that its production level could be increased by some specially prepared peptone. Streptococci and Clostridium welchii do not require these special factors (183).

Rogers (183) has conclusively demonstrated a common property is possessed by Clostridium welchii, streptococcus, staphylococcus and Clostridium septicum, in that their optimal formation of hyaluronidase occurs only in well-buffered media. He found that enzyme synthesis apparently ceases when the pH of the culture falls below 6.0. From experiments conducted with crude and purified potassium hyaluronate additions to media, he concluded that the enhancement of hyaluronidase production by streptococci and Clostridium welchii is a true adaptation to hyaluronate, and is not due to impurities in the polysaccharide preparations used. The evidence presented,

demonstrated that the ratio of increase in enzyme production to the amount of pure hyaluronate contained in the crude polysaccharide is constant (183). Purification of crude hyaluronate does not decrease its stimulating properties. Rogers (183) also showed that the addition of heparin and chondroitin sulphuric acid to the test medium had no influence upon hyaluronidase formation.

4. Inhibition of Hyaluronidase by Normal Sera "In Vitro"

Meyer and associates were the first to note the inhibitory action of normal human and rabbit serum on pneumococcal, and streptococcal hyaluronidase, as well as that produced by Clostridium welchii. The reductometrically determined activity of the hyaluronidase of pneumococci and Clostridium welchii was inhibited 25% to 50% by normal serum, while with streptococcus hyaluronidase, the inhibition was smaller. McClean (133) then briefly mentioned an inhibitor present in the normal serum of guinea pig, rabbit, sheep, horse, mouse and humans on the hyaluronidase prepared from bull, rabbit and mouse testes, and that from streptococcus and Clostridium welchii. This inhibitory factor of serum of different species varies greatly in its inhibitory activity (76). This inhibition has been thoroughly investigated by Haas (75).

Haas (75), on the basis of some by no means conclusive evidence (76), has claimed that this inhibitory action of serum is due to the presence in it of an enzyme which inactivates hyaluronidase. Haas (75) also states that serum obtained by clotting is less active in its inhibitory power than that obtained from blood defibrinated by stirring. He (75) has

demonstrated that the serum inhibitor (Antinvasin I) that inhibits hyaluronidase, has no effect in phosphate concentrations of 0.06M.

Hadidian & Pirie (76) obtained optimum activation of the serum inhibitor with 0.03 to 0.1M sodium chloride; when the concentration was raised to 0.2M., there was an 80% decrease in activity of the serum inhibitor pertaining to its inactivation towards hyaluronidase. When the addition of low phosphate concentrations (0.01 to 0.05M.) to serum and hyaluronidase is made, in the presence of no sodium chloride, the inhibition of hyaluronidase by the serum inhibitor continues, but when the concentration of phosphate is raised to 0.05 to 0.1M., the inhibition is reversed. The rate of change in either direction is very slow at 0.05M. concentration of phosphate. Phosphate concentrations of 0.075M. produces full inhibition. These experiments were conducted on mixtures of testicular hyaluronidase using serum obtained from defibrinated pig, rabbit, chichen and bovine blood with the same results. This work confirms the demonstration by Haas (75) that in the presence of such concentrations of phosphate (0.05 - 0.1M.), the activity of hyaluronidase in reducing the viscosity of hyaluronic acid is unaltered by incubation with serum. This inhibitor in serum is associated with a protein, and precipitates out in the globulin fraction of serum (133), (76).

According to Hechter & Scully (82), Hadidian & Pirie (76) have clearly demonstrated that the inhibition of hyaluronidase by serum is not an enzymic reaction, but is due to a reversible binding of the enzyme activity by the serum factor. Alterations

of various conditions (salt and phosphate concentrations) modify the rate of the inhibitory binding reaction as well as the rate of the release of hyaluronidase from its inactive form (82).

Leonard & Kurzrok (111) have found that when normal rat serum is diluted 1:10, the inhibition it possesses toward hyaluronidase activity is very slight, while at 1:100 it possesses no effect. Normal rat serum is the most potent inhibitor of testicular hyaluronidase, with chicken, rabbit, horse and human listed in the order of decreasing effectiveness (112).

Pike (168) has demonstrated the effect of serum in culture medium on the production of hyaluronidase by Group A streptococci. This work was conducted in plain infusion broth and Bacto Tryptose broth, avoiding any addition of phosphate and sodium chloride. The addition of 10% horse serum to the medium was shown to have an inhibitory effect on hyaluronidase production, but evidence was presented which indicates that the weak hyaluronidase activity of some Group A streptococci were not definitely affected by the serum addition, and some may actually be increased in their enzyme production by the addition of 10% normal horse serum.

5. Inhibitors of Hyaluronidase "In Vitro" Several compounds other than normal serum have been demonstrated to have an inhibiting or inactivating effect on the activity of hyaluronidase. McClean (133) first reported the inhibitory action of heparin and chondroitin sulfate on the "in vitro" decapsulation of streptococci by testicular hyaluronidase.

Three-tenths percent of the ammonium salt of heparin suppressed the decapsulation of an enzyme concentration equivalent to one viscosity-reducing unit. McClean also demonstrated (133) that hyaluronate partly depolymerized by precipitation with acetic acid likewise inhibited the decapsulation, while hyaluronate depolymerized enzymatically was without inhibitory effect. Gastric mucin also inhibited the decapsulation, but Shiga-Kruse polysaccharide and a blood group A hapten had no effect. As noted by Meyer (144), these inhibitors are, or contain, acid polysaccharides; the two neutral polysaccharides tested by McClean were without inhibitory action.

Meyer (144) confirmed the inhibitory activity of heparin on the hydrolysis of hyaluronate, and demonstrated that the percent of inactivation increased as the amount of heparin present was increased. However, in the presence of sodium chloride, no inactivation was created. This has been confirmed by Rogers (183). Meyer also demonstrated (144) that heparin desulfurated with oxalic acid-barium oxalate had no effect. The inhibition he obtained with chondroitin sulphate was less than with heparin, the ratio between the two being approximately 1:100.

Meyer and co-workers (142) found that the hydrolysis of free hyaluronate was twice as fast as that of an equimolar concentration of a protein salt, prepared from synovial fluid. Kass & Seastone (99) confirmed this observation later, showing that reduction of the turbidity of a preformed horse serum-polysaccharide precipitate required ten times the enzyme

concentration than that of the polysaccharide alone.

Beiler & Martin (6) investigated several substances possessing vitamin "P" activity for inhibitory effect on hyaluronidase "in vitro", and found only rutin possessed this ability, at a concentration of 1.0mgm./cc. Ascorbic acid and dicoumarol inhibited the enzymic action at concentrations of 0.1mgm./cc.

The "in vitro" activity of testicular hyaluronidase is not inhibited by sulfanilamide (6). McClean & Hale (136) have found that sulfanilamide and sulfapyridine do not inhibit the production of this enzyme by Clostridium welchii, nor do they inhibit the "in vitro" activity of the enzyme. Guerra (72) found that sulfadiazine did not decrease the spreading effect of hyaluronidase.

Discrepancies in the literature appear concerning the inhibitory activity of sodium salicylate (144), (211), (210), (6), (116), (73). Dorfman et al (32) confirm the observations (144), (72),(73) that this compound has been found to inhibit the spreading effect of hyaluronidase "in vivo". Hyaluronidase derived from Clostridium perfringens and bull testes was also inhibited by sodium salicylate "in vitro", but the concentrations necessary for "in vitro" inhibition are considerably higher than those obtained "in vivo" (32), (210), (144).

(5) Methods of Assay

For the determination of hyaluronidase, biological, chemical and physico-chemical methods have been used. Uncertainty as to the size of the hyaluronic acid molecule (206)

has hampered the establishment of standard assay methods and the adoption of a useful unit. This fact, and because it has been impossible as yet to obtain reproducible enzymatic activity on different hyaluronic acid preparations (184) makes it impossible to correlate different pieces of work conducted on the same subject by the same methods in terms of absolute values.

1. Biological Methods (a) Spreading Reaction in Skin This method has been reviewed by Duran-Reynals (42). India Ink is probably the best indicator, since the area of spreading is more circumscribed with it, than with diffusible dyes (144). The area of spread of the test solution is compared to the area of spread of a control solution lacking the enzyme.

An improved quantitative method for the estimation of the spreading reaction has been published by Humphrey (94), who injected the spreading agent intracutaneously into groups of six guinea pigs. The animals were killed 20 minutes after injection, the skin removed immediately, and the size of the bleb measured at the inner surface. He (94) calculated that the minimal diffusion dose is the least amount of enzyme which will produce a 20% increase of the mean area over the mean area of the controls.

According to Meyer (144), this method of assay cannot be considered as an accurate one, as some unspecified agents also give spreading reactions (144), because correlation with other methods is the poorest of all, and because of the pressure influence from the bleb (81).

(b) Streptococci Decapsulation

The decapsulation of mucoid hemolytic streptococci of Groups A and C has been compared with hyaluronidase activity estimated by other methods (133). According to Meyer (144), these organisms lose their capsules under various conditions without added enzyme. It has been suggested that the disappearance of capsules during the growth of cultures of these organisms may be due to an enzyme (142), (161). Although McClean (131) found a trace of hyaluronidase in 20-hour cultures of capsulated strains, he concluded that hyaluronidase and capsules composed of hyaluronic acid (100), (193) were mutually exclusive. To support this contention, Crowley (27) found no hyaluronidase production among 186 strains of encapsulated Group A streptococci.

Pike (169) has recently presented evidence that some strains of Group A streptococci which produce hyaluronic acid in capsular form, also produce hyaluronidase, which causes the disappearance of the hyaluronic acid from the culture fluid. The hyaluronidase was not released from the cells until after the capsules had begun to leave the cells and their hyaluronic acid dissolved in the medium. He concluded that certain mucoid strains produce hyaluronidase, but the activity of the enzyme was relatively weak and variable.

2. Physico-Chemical Methods (a) Mucin Clot Prevention Test

This test was first used by Robertson, Ropes & Bauer (181) and modified by McClean and others (137). This method is based on the observation that native hyaluronic acid in acidic solutions coprecipitates with protein in typical fibrous "mucin"

clot. After incubation with hyaluronidase, the quantity of the clot is reduced and the character of the precipitate changes from a fibrous to a flocculent precipitate, until finally, the solution remains clear. The chemical basis for the test appears to be the opening of the anhydride linkages of the hyaluronic acid by the enzyme (206). This test is suited for serial determinations, and is probably the most sensitive one for low concentrations of hyaluronidase (144), although its error is about ∔ or - 25%.

(b) Turbidimetric Method This method, first described by Kass & Seastone (99), is based on the observation that pure hyaluronate at pH 4.2 provides a fairly stable colloidal suspension with dilute serum, whereas depolymerized hyaluronate remains clear. This method is apparently based on the hydrolysis of the acetylglucosaminidic linkage (206). In a modification developed by Meyer & Hahnel (144) the time of incubation is kept constant; in the original procedure (99) the time is varied, the enzyme concentration being constant. It is considered accurate to about 10% ∔ or - reproducibility (144). Some trouble has been encountered with this mode of assay, through irregularities which may be traced to the protein employed in forming the turbidity with the substrate (192).

(c) Viscosimetric Method The first systematic use of the viscosity-reducing method in estimating hyaluronidase activity was reported by Madinaveitia & Quibell (125). Using a crude extract of acetone precipitated vitreous humor as substrate, he found that the time required to reach

half viscosity were inversely proportional to the concentration of enzyme. These investigators have also shown that the reaction time, that is, the time taken to reach half-viscosity level, is independent of the concentration of the substrate and of its original viscosity (125). McClean confirmed these facts in his work (136). For the purpose of hyaluronidase activity determinations, they provisionally defined a viscosity-reducing unit (V.R.U.) - "as that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed in 20 minutes." (136). McClean (134) then modified this assay procedure with corrected concentrations of sodium chloride and buffer molarities, and substituting McIlvaine's buffer (140) for the sodium citrate used formally (136). The values of sodium chloride, buffer concentrations and pH values have already been previously presented, together with the reasons for their use, in the section dealing with "Influence of In Vitro Factors on Hyaluronidase Activity". Rogers (183), (184), (186) employed this method of assay, as modified by McClean (134), for all his systematic investigations of streptococcal hyaluronidases.

The viscosity-reducing method is quantitative, accurate, but cumbersome. Its accuracy has been variously set at between ± or - 5% (125) to 10% (136), (134). Meyer (144) has reported that the viscosity of the substrate-buffer mixture remains constant for at least two weeks at 4°C. without preservative.

This method of assay depends on two reactions; the opening of anhydride linkages leading to the initial fall in viscosity, and the opening of acetylglucosaminidic bonds leading to the secondary fall in viscosity (206).

There are many modifications reported (144), (209), (220), (114), (200) which employ variations in substrate and sodium chloride concentrations, and employ different pH and temperature levels, with resulting variations in the potency of a defined unit of enzyme potency in each case.

3. Chemical Methods This is a reductometric method, in which the opening of both the glucosaminidic and the glucuronidic linkages is measured (206). The hyaluronidase activity is determined by measuring the increase in reducing sugar, or by the increase in liberated acetylglucosamine. In both methods, pure hyaluronate of known hexosamine and uronic acid content should be used. The main disadvantage of the reductometric method is its complicated kinetics. This results from the action of the different enzymes involved, since they simultaneously split the glucosidic linkages (144). The reductometric method has been used by Meyer (144) in the identification of hyaluronate from different sources.

Measurement in increase of acetylhexosamine (159) has been used by some investigators (23) using synthetic acetylglucosamine as a standard. Results were received that had a noted discrepancy of error, according to Meyer et al (143), that were in excess of six times the total weight of represented polysaccharide. This result was confirmed by Hahn (77) and by Humphrey (97).

Rogers (186) however, has recently devised an accurate method of measuring the activity of bacterial hyaluronidases by the amount of reducing sugars liberated under standard conditions from potassium hyaluronate, by using an equimolar solution of N-acetylglucosamine and glucurone as a control for Somogyi's cupric sulphate reagent (201) in the titrimetric procedure.

The reductometric method of assay requires larger enzyme concentrations and longer incubation time (206), and, excluding Rogers' method (186), cannot be used for the determination of hyaluronidase activity (144).

(6) Complexity of Hyaluronidases

A number of differences among hyaluronidases from different sources have been observed by various investigators. There has been previous mention in this review of a few of these dissimilarities. Hale (79) found streptococcal hyaluronidase more sensitive to pH levels than enzymes from staphylococci or Clostridium welchii. Rogers (183) observed that Group A and C streptococci and Clostridium welchii produced hyaluronidase adaptively, while staphylococci and Clostridium septicum did not respond to hyaluronate added to the medium. Pike (168) has demonstrated that horse serum enhanced the hyaluronidase activity of one strain of Group A streptococcus, but the same lot of serum under the same cultural conditions, inhibited the hyaluronidase production of another Group A strain.

Rogers (184), (183) obtained evidence of differences in the end products of hyaluronic acid hydrolysis by the enzymes

of testis, streptococci and Clostridium welchii. Rogers (183) showed that a digest of hyaluronate obtained with streptococcal hyaluronidase, possessed no stimulatory powers for production of the enzymes by streptococci. However, when a digest of hyaluronate obtained with testicular hyaluronidase was added, the stimulation of hyaluronidase production by streptococci was almost as great as with pure hyaluronate. Rogers (184) confirmed his results later, and demonstrated the same effect to be true for Clostridium welchii. He also found that potassium hyaluronate, hydrolyzed by streptococcal or Clostridium welchii enzymes does not stimulate hyaluronidase production by the streptococci. The streptococcal hydrolysate does, however, stimulate hyaluronidase production by Clostridium welchii, but the Clostridium welchii hydrolysate has but a small influence on this organism (184).

In the reductometric assay procedure, testicular hyaluronidase possesses a double pH optimum, one at pH 4.5, the other at pH 5.7, buffer molarities being constant, which has been interpreted by Meyer et al (143) as an indication of two distinct enzymes. Rogers (186) has demonstrated two pH optimum activity levels for streptococcal hyaluronidase as measured by the reducing sugar assay. One of these optima, at pH 5.7, agrees closely with the single optimum obtained from his viscosity-reduction assay experiments, which was pH 5.5. The other optimum level of enzymic activity as demonstrated by the reducing sugar assay was at pH 7.1.

According to Meyer (144), the dual nature of the two

glucosidic linkages in hyaluronic acid, one belonging to N-acetylglucosamine, the other to the glucuronic acid moiety, suggests that the depolymerization and the hydrolysis into monosaccharides require two enzymes. Meyer and co-workers (143) demonstrated this by a comparison of hyaluronidases of different origin, measured viscosimetrically and reductometrically. Their results indicated that hyaluronidases were mixtures of at least two enzymes, one attacking the long chain molecules, the other hydrolysing the aldobionic acid units formed (143). This conclusion was based on the observation that pneumococcus hyaluronidase hydrolysed the substrate almost to 100% of the theoretical amount, whereas testicular hyaluronidase, which showed a much faster activity rate in the viscosimetric tests than the bacterial enzyme, hydrolyzed the substrate to only approximately 50%. The testicular enzyme on prolonged hydrolysis exceeded the 50% reduction, but the total reduction was considerably short of 100%. The addition of pneumococcal hyaluronidase to the non-hydrolyzed residue brought about complete hydrolysis, while addition of fresh testicular enzyme had a negligible effect (144).

Hahn (77), (78), by the use of fractional salting-out procedures, utilizing ammonium sulphate and basic lead acetate, separated two enzymes from testicular extracts. One enzyme, which he termed a mucopolysaccharidase, hydrolyzes hyaluronate to the aldobionic acid stage, the activity of which he measured by the viscosity-reduction method. The other fraction, the activity of which was demonstrated and measured by reductometric

means, he called a muco-oligosaccharidase, which hydrolyzes the disaccharide to monosaccharides (77), (78). Since acetylglucosamine was liberated during hydrolysis by the mucopolysaccharidase, the free aldehyde group must belong to the hexosamine moiety (144), and muco-oligosaccharidase may be called a glucuronidase type of enzyme.

By immunological means, it may be shown that hyaluronidase from one source, produces specific antisera to that source (144). McClean (134) has found no crossing over with antisera against Clostridium welchii to Clostridium septicum. Streptococcal enzymes were found to be group, but not type specific. McClean (134) found the hyaluronidase from three different serological types of Group C streptococci appeared to be antigenically identical among themselves, although antigenically distinct from the Group A, type 4 streptococcal hyaluronidase studied. An antiserum prepared against hyaluronidase of bull testis inhibited this testicular enzyme, but did not inhibit a testicular enzyme from the mouse, or any of the bacterial enzymes (134). However, it is interesting to note that highly purified testicular hyaluronidase has never been obtained in antigenic form (136), (144).

(7) Relationship to Bacterial Virulence

The function of hyaluronidase in hemolytic infection has been postulated to be the action of this enzyme upon the ground substance, thereby favoring the spread of these agents in connective tissue by means of its depolymerizing activity (206). By the production of hyaluronidase, hyaluronic acid is broken down and made available for energy purposes for the metabolism

of the organism producing the enzyme (206). In addition, glucosamine, one of the degradation products, is readily utilized by a large number of organisms, and may help explain the numerous observations that other disease-producing agents are frequently found in association with hemolytic streptococci (206). Pathogenic bacteria, in which invasive qualities are closely allied with virulence, include staphylococcal, streptococcal, and gas gangrene infections (42). Many strains of these three produce hyaluronidase (42). The division of pathogens into those which do, and those which do not produce hyaluronidase, does not correspond to any other method of classification, and is only roughly correlated with invasive power (41), (130), (167), (173).

McClean (132) has demonstrated that nonencapsulated, hyaluronidase-producing strains of Groups A and C streptococci exhibited low virulence when injected intraperitoneally in mice, whereas encapsulated strains varied greatly in virulence. However, on intracutaneous inoculation of mice, some of the hyaluronidase-producing strains exhibited greater variance than the capsulated strains. McClean (131), however, considered that hyaluronidase production and capsule formation were mutually exclusive phenomenon. Other work (173), (169) which has been presented previously in this review, has shown that it is not mutually exclusive in all cases, and is variable as well.

Crowley (27) has been unable to find any correlation between hyaluronidase production and virulence for man. Of 127 strains isolated from cases of scarlet fever, only thirty-

five or 27.6% showed hyaluronidase activity; similarly only fifty strains or 36.8% of 136 strains from cases of pharyngitis showed production of the enzyme. The hyaluronidase-producing strains examined by Crowley (27) were not encapsulated. Hyaluronidase-producing organisms have been, likewise, isolated from healthy individuals (27). Hyaluronate was not added to the culture medium when testing for hyaluronidase activity (27).

According to the majority of investigators, no parallelism between hyaluronidase production and virulence has been demonstrated (35), (98), (47), (15), (27), (96), (132). According to Humphrey (96) type I pneumococci rarely produce the enzyme.

Duran-Reynals (42) and Topley-Wilson (215), in some cases, consider that a bacterial strain that is moderately virulent and produces hyaluronidases, may, when present only in small infective numbers at the site of entry, be rendered into a subinfective concentration. Because of the spreading action of the pathogenic material throughout the tissues, this enables the defensive mechanism of the body to cope with the organisms more easily. The phenomena of "Critical Concentration" thereby acts as a coincident factor also, in determining the virulence (42).

The combination of streptococcal hyaluronidase with other invasive enzymes, such as streptokinase and proteinase, is felt to enhance the ability of this organism to spread through body defences, as phagocytic cells, the production of a fibrin clot, as well as the natural presence of hyaluronic acid in the connective tissue (50). Dubos (34) finds favor

with this statement of combined effect. As an example, he states that destruction of muscle collagen by the collagenase of Clostridium welchii and the hydrolysis of cellular ground substance by hyaluronidase, enables the hemolytic and dermonecrotic lecithinase to diffuse more readily through the tissues and cause more extensive necrosis (34). Thus, the organisms then spread into the damaged area and the process is repeated.

Kass and co-workers (98) isolated 94 strains of Clostridium welchii from soil and feces, and tested them for mouse virulence. It appeared to these investigators, that regardless of the role of hyaluronidase in a gangrenous lesion, its production "in vitro" by a given strain of Clostridium welchii, bears no necessary relationship to the virulence of that strain for mice.

MATERIALS AND METHODS

Test Organism

A beta-hemolytic strain of Streptococcus pyogenes that produced hyaluronidase was the strain of bacteria whose reactions to the antibacterial action of penicillin were studied in the experimental work here reported. A subculture of this strain was received from Dr. Nuala Crowley, Cambridge, England. This strain had been typed and tested by herself, and found to be a Lancefield Group A, Griffiths type 4, and she numbered the strain P315. It had been isolated from a case of Scarlet Fever at Cambridge in 1942. This strain had been repeatedly tested for hyaluronidase-producing powers for a period of three months, by Dr. Crowley, and was termed by her to be a typical streptococcal hyaluronidase-producer, affording a measurable titre of enzyme activity in a medium without added hyaluronate. Hereafter, in this thesis, this strain of Streptococcus pyogenes will be termed "P 315 A-4".

This strain, on being received, was tested for possible contamination by being grown on blood-agar plates. A mass inoculum was then picked off the plates and inoculated into each of four "Pneumo Broth" tubes of culture medium. After 18 hours incubation at 37°C., the organisms were tested for purity of culture by the Gram Stain, and then centrifuged down. The concentrated organisms were then aseptically transferred to sterile Kahn tubes and lyophilized for storage purposes.

This strain was typically Gram-positive, in chains of 5-6 cocci on the average, and was verified to be a Lancefield Group

A streptococcus, according to the method (J. H. Brown) used in the Clinical Bacteriology Laboratory, McGill University. On the surface of a 24 hour old blood-agar plate, the colonies were of the smooth phase, and surrounded by a typical zone of hemolysis for a beta-hemolytic Streptococcus pyogenes. Cultures of this strain in nutrient broth and Hiss serum carbohydrate culture media produced typical biochemical and growth characteristics described for this species in "Bergey's Manual of Determinative Bacteriology". This strain was nonencapsulated, as determined by Baker's Method of capsule staining, employing the use of Ziehl-Neelsen's carbolfuchsin and Nigrosin.

Stock cultures of this strain were subcultured once a week onto a fresh blood-agar plate, and after 24 hours of incubation, stored in the refrigerator at 5°C.

Culture Media

With the exception of "Assay Broth" and "Beef Extract Broth", all media employed in the experimental studies here reported, were the standard media used in the Department of Bacteriology and Immunology at McGill University.

Assay Broth

This broth was prepared according to the formula below, and had the addition of 0.25% crude potassium hyaluronate added to it as a source of carbohydrate and for the adaptive production of hyaluronidase. This medium has been found satisfactory for its buffering capacity and enzyme production. The inclusion of 2.5% sodium glycerophosphate provides a concentration of 0.0793M. phosphate, which, together with other tissue phosphates in the medium and sodium chloride concentration are

felt to inactivate any inhibition produced by the addition of but 2% serum derived from clotted normal rabbit blood. The beef extract, neopeptone, and sodium glycerophosphate were obtained from one manufactured lot in each case. The crude potassium hyaluronate was prepared according to the same method in each production lot.

	per 100 ml. distilled water.
Lemco Beef Extract	0.3 gm.
Neopeptone (Difco)	1.0 gm.
# Salt solution	1.0 ml.
Potassium hyaluronate (crude)	0.25 gm.
Sodium glycerophosphate	2.50 gm.
Rabbit serum(from clotted normal rabbit blood)	2.0 ml.

- Salt Solution - To provide a final approximate concentration in the medium of

NaCl - - - 0.25%

KCl - - - 0.02%

$CaCl_2$ - - 0.01%

Method of Preparation

The crude potassium hyaluronate is dissolved in the water. The beef extract, neopeptone and salt solution are then added and dissolved. The pH of the solution is then brought to pH 7.4 to 7.5 with NaOH, as determined by the glass electrode. The sodium glycerophosphate is then added. The medium is now sterilized at 120°C. for 20 minutes and then allowed to stand overnight at room temperature. The rabbit serum is now added, and the medium seitzed through a Hormann #9 asbestos pad, with

a coarse sintered glass filter attached to the outlet of the Seitz filter. The medium is now asceptically dispensed in 9.5 ml. or 10 ml. quantities, to sterile, cotton-plugged test tubes of the same size, according to the specifications of the experiment involved. The tubed medium is now incubated for 72 hours at 37°C. as a means of verifying its sterility. The tubed medium is then checked, and transferred to a refrigerator at 5°C. for storage. Only sufficient medium is prepared in a quantity, such that it will be used inside of three weeks work. Concentration of medium through evaporation is thereby kept at a minimum. In no case, was any experiment conducted using medium from two lots of different preparation dates.

Beef Extract Broth

This culture medium was prepared exactly the same as "Assay broth", omitting the crude potassium hyaluronate, and substituting 0.25 gms. of Dextrose (Analar Reagent grade) per 100 ml. of culture medium.

Crude Potassium Hyaluronate

This preparation was conducted according to the method reported by McClean (134). The crude salt of hyaluronic acid may be used for the adaptive production of hyaluronidase, as a highly purified compound is not necessary (183).

Human umbilical cords were collected from the Montreal Maternity Hospital through facilities provided by the kind permission and cooperation of Dr. N. W. Philpott. These umbilical cords were squeezed free of blood, washed, and placed in covered jars containing acetone, from whence they were collected

every three days from the Case Room.

Method of Extraction and Partial Purification

I. After four or more weeks dehydration under acetone in the refrigerator at 5°C., sufficient umbilical cords were ground in a hand meat grinder, such, that when dried by evaporation and then by evacuation over $CaCl_2$, the material on regrinding would yield about 100 grams of very fine dried material.

2. To this material in a two litre flask was added 1 litre of chilled distilled water. The mixture was stoppered and placed in the refrigerator for 24 hours with some added chloroform for purposes of preservation from bacterial action. During the aqueous extraction, the contents of the flask were occasionally shaken.

3. After 24 hours extraction, the contents of the flask were squeezed through eight layers of coarse cheesecloth. The filtrate was then placed in stoppered flask with a few ml. of chloroform and stored in the refrigerator. The umbilical cord mince was then extracted with an additional litre of distilled water as described in Step #2, and replaced in the refrigerator for 24 hours.

4. The second squeezing of the material was then conducted. The two filtrates were combined, and the solution centrifuged at 1,500 R.P.M. for 20 minutes in a refrigerated centrifuge. The supernatants were aspirated off by means of a suction trap connected to a water-pump. The precipitate was discarded.

5. The combined supernatants were now adjusted to pH 9.0 to

9.5 by addition of KOH, and then chilled to 5°C. in the refrigerator.

6. The hyaluronic acid was now precipitated out as the potassium salt by adding 1¼ volumes of chilled potassium acetate-saturated 95% ethyl alcohol. The mixture was slowly stirred with a glass stirring rod, and the potassium hyaluronate precipitated out quickly in a characteristic clot.
7. This clot was broken up, washed three times with chilled 95% ethyl alcohol and once with ether.
8. The potassium hyaluronate was then dried "in vacuo" over P_2O_5, ground in a mortar to a fine powder, and stored over $CaCl_2$ in the refrigerator.

Purified Potassium Hyaluronate

The method employed for producing potassium hyaluronate in a purified form for use as a substrate in the viscosimetric assay, was that of McClean & Hale (136) as modified by McClean (134), and incorporated the utilization of a method of protein denaturation described by Sevag (198) and applied by Humphrey (97) and Meyer & Palmer (150).

Method of Purification

1. The aqueous extract was obtained in the same fashion as described in steps #1 to #4 of the procedure for aqueous extraction in the "Method of Extraction and Partial Purification" of crude potassium hyaluronate as previously described.
2. To the chilled filtrate was added 5% w/v potassium acetate

and allowed to dissolve with the help of stirring. The hyaluronic acid - protein complex was precipitated by five volumes of 95% ethyl alcohol. This mixture was allowed to stand in the refrigerator overnight.

3. The precipitate was removed on a coarse grade filter paper under suction. It was removed from the filter paper and dissolved in approximately 800 to 1,000 ml. of distilled water, depending on the amount of precipitate. The solution was then brought to pH 7.0 to 7.2 by KOH.
4. To each 100 ml. of hyaluronate solution was now added 25 ml. of chloroform and 2½ ml. of n-Butyl alcohol. This solution was now mechanically shaken for 20 hours at room temperature in a separatory flask.
5. The solution was then centrifuged in a refrigerated centrifuge at 1,500 R.P.M. for 20 minutes. The supernatant was aspirated off by suction and precipitated by five volumes of 95% ethyl alcohol, after addition of 2½% potassium acetate. This solution was left in the refrigerator overnight.
6. The precipitate was removed as in step #3 and step #4 was repeated.
7. The mixture was centrifuged again as in step #5, and to the supernatant, that was removed by aspiration, was added 15 ml. of saturated alcoholic potassium acetate. The potassium hyaluronate was precipitated by 5 volumes of cold acetone.
8. The potassium acetate was washed with acetone, dried "in vacuo" over P_2O_5, ground to a powder in a mortar and stored

over $CaCl_2$ in the refrigerator.

9. Four lots of potassium hyaluronate, prepared in the above manner, were dissolved in a minimum of distilled water (2,000 ml.) with the aid of a Waring Blendor, and the pH was adjusted to 9.0 with KOH. Then 500 ml. of chloroform and 50 ml. of n-Butyl alcohol were added to the solution. This mixture was then mechanically shaken for 21 hours at room temperature.

10. The mixture was then centrifuged in a refrigerated centrifuge at 1,800 R.P.M. for 30 minutes, and the supernatant aspirated off by suction. The hyaluronate was precipitated by 1¼ volumes of chilled potassium acetate-saturated 95% ethyl alcohol. The mixture was then slowly stirred with a glass stirring rod, and the potassium hyaluronate precipitated out in a clot.

11. This clot was broken up, washed three times with chilled 95% ethyl alcohol, and then once with ether. The potassium hyaluronate was then dried "in vacuo" over P_2O_5; ground in in a mortar to a fine powder, and stored over $CaCl_2$, in screw-capped vials in the refrigerator.

Triplicate samples were assayed for their total Nitrogen content by the Micro-Kjeldahl method. The averaged results afforded a total Nitrogen value of 3.81%. This compared very favorably with results obtained by other investigators (150). The biuret test was negative for a 0.2% solution.

This one lot of purified potassium hyaluronate was used as the source of substrate for all assays conducted by the viscosimetric method.

Preparation of Substrate for Viscosimetric Assay

For reasons presented in the Historical Review, the viscosimetric assay was conducted according to the method of McClean & Hale (136) as modified by McClean (134) and Rogers (186). Using these methods, entail the use of a solution of 0.2% purified potassium hyaluronate at pH 5.7, to which is added a solution of sodium chloride dissolved in McIlvaine's (140) citric acid - phosphate buffer, such, that the buffer is at a final M/60 concentration and the sodium chloride at a final M/10 concentration in the reacting solution of enzyme-substrate.

1. McIlvaine's Standard Buffer and Sodium Chloride Solution

Stock solutions of 0.1M. citric acid and 0.2M. disodium hydrogen phosphate were made from reagent grade chemicals, and were stored in the refrigerator at 5°C. The proper amounts of each were combined to obtain pH 5.7 and then diluted to obtain a final combined molarity of M/10.

Sufficient sodium chloride was added to an aliquot of this buffer, such, that when 1 ml. of buffer-saline solution was diluted 1:6, the final molarity of added NaCl was M/10.

2. Potassium Hyaluronate A solution of 0.2% purified potassium hyaluronate was made in distilled water. The pH of this solution was brought to 5.7 by the use of HCl, as measured by the glass electrode.

3. Substrate Mixture

Four volumes of the 0.2% hyaluronate solution are mixed with one volume of the buffer-saline. The pH of the combined substrate-buffer-saline mixture is checked to insure that it is correct, and then the solution is filtered through

a chemically clean sintered glass filter of coarse porosity, with the aid of suction, stoppered, and placed in the refrigerator at 5°C., and used within 48 hours.

During an assay, one volume of enzyme dilution is added to these combined five volumes of substrate-buffer. The final molarity of the buffer is then at the required M/60 concentration. The final concentration of NaCl is M/10 through the predetermined weighing of the salt, as mentioned above.

Ostwald Viscosimeters

The viscosimeters used had a bulb capacity of 2 ml. and an efflux time varying from 65 to 90 seconds, when loaded with a constant charge of 3 ml.

Cleaning of Viscosimeters

The viscosimeters were always cleaned and prepared for use by an approved method (2), (52). After use, the viscosimeters were rinsed with tap water, and then filled with chromic acid cleaning solution, and left in the cleaning solution for 24 hours. The cleaning solution was then removed, and the viscosimeter filled and emptied with ten rinses of tap water, followed by three rinses of distilled water. A rinse of 95% ethyl alcohol was then made, followed by ether, and then dried by a current of air. They were stored in clean, stoppered tubes until used.

Calibration of Viscosimeters

An automatic water-bath was brought to a constant temperature of 34°C. A sensitive control thermostat was afforded by the use of resultant changes in temperature upon a mixture of nine parts of ethyl ether and one part of carbon disulfide.

By the use of special clamps, a viscosimeter placed in the water-bath is maintained in a vertical position, with the upper meniscus over one inch below the surface of the water in the water-bath.

The efflux times of ten chargings of distilled water for each viscosimeter were made by the use of a stopwatch, after the contents of the viscosimeters had reached the same temperature as the water-bath. The readings were averaged, and the viscosimeter with the slowest efflux rate was selected as the control viscosimeter. A calibration was then computed for each viscosimeter, in order that its efflux rate may be expressed in terms of the control viscosimeter's value. This factor of difference, determined through the use of distilled water, was the same when a mixture of 50% glycerine and water was used.

The nature of the viscosity-reducing assay necessitated the use of several viscosimeters in one day's experimental work. The validity of this means of calibration is based upon the following reasons:

(1) Physical and chemical constants remaining the same, it is an accepted fact that the relative viscosity obtained for a given solution and its solvent is the same value obtained by the use of any viscosimeter.

(2) Madinaveitia & Quibell(125), and confirmed by McClean (136), have found that "the time required to reach half-viscosity was inversely proportional to the concentration of enzyme" (125). These investigators have also shown that "the reaction time, that is, the time taken to reach half-viscosity level, is independent of the concentration of

the substrate and of its original viscosity" (125). Thus, regardless of viscosimeter, the decrease in relative viscosity proceeds at the same rate for a given substrate if the enzyme potency at the outset was the same in each case. Therefore, with these aforementioned conditions, regardless of efflux rate of a viscosimeter, the relative viscosity is the same in any viscosimeter at any given time during the substrate degradation. Therefore, during degradation, the same curve of decrease in relative viscosity will be obtained from each viscosimeter, and, at any given stated time, when computed against accumulative time, the same decrease in relative viscosity will be found.

(3) As the same curve, designating decrease in relative viscosity when computed against accrued time, is produced by any two viscosimeters, the difference betweenthe two viscosimeters resolves into the fact that more readings may be obtained in a given time with the faster viscosimeter. Therefore, with the degree of degradation equal, the same difference in relationship between efflux rate between two viscosimeters will be found as at the start, when no degradation had started. In the viscosity-reducing assay used, after the efflux time had been corrected by the viscosimeter's calibration factor, half this efflux time was added to the accumulative time at the beginning of the run, against which the relative viscosity change was being computed. Thus, a correction was made for the fall in viscosity that occurred during the reading of a flow-time.

Protocol Graph #1 is an example for illustrative purposes of the application of this conversion factor to one viscosimeter as measured against the control or "constant" viscosimeter, when the same enzyme concentration was used.

Viscosimetric Assay

The loss of viscosity in a substrate has been used to follow the course of hyaluronidase action quantitatively. According to Seligmann & Wassermann (197), this method has the primary advantage that loss of viscosity appears to be the first change that can be recognized during the enzymic decomposition of hyaluronic acid. Factors influencing this "in vitro" change are more likely therefore, to affect the action of hyaluronidase "in vivo" (197), than those influencing the later stages of decomposition of the molecule to reducing sugars,which are brought about by other enzymes (198), (199). For this reason, and others already stated in the Historical Review, the viscosity-reducing assay method was used, according to the method developed by McClean & Hale (136), and modified by McClean (134). The developmental observations and claims for accuracy have been confirmed by Hale (79) and Rogers (186).

As stated by Madinaveitia & Quibell (125), and confirmed by McClean & Hale (136), a Viscosity Reducing Unit (henceforth to be termed V.R.U.) may be provisionally defined as "that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed in 20 minutes".

During the estimation of enzymic activity, dilutions were

made, for reasons previously presented in the Historical Review (186), in 0.5% gum arabic solution, that had been filtered through a coarse grade chemically clean sintered glass filter.

For the individual assay, in each case, one prepared lot of gum arabic solution, buffer-saline (solvent), and substrate-saline-buffer solution were used throughout. The assay was conducted according to the following steps:

1. Solvent Control

The control flow-time of solvents was obtained in the following manner. Three ml. of the previously stated buffer-saline were added to 12 ml. of distilled water and mixed. One ml. of gum arabic solution was then added to 5 ml. of this diluted buffer-saline mixture in a test-tube and thoroughly mixed. This solution was then placed in the water-bath at 34°C. At the same time, a viscosimeter was placed in the water-bath. The materials were allowed to come to the temperature of the water-bath for a timed interval of ten minutes. After this interval, 3 ml. of the solvents were transferred to the viscometer. The fluid was blown (not sucked) into the bulb in order to avoid the formation of bubbles. The first reading was taken one minute after addition to the viscosimeter, by means of a stop-watch, and recordings were made for ten flow-times.

2. Substrate Control

The control flow-time of the substrate was obtained in the aforementioned manner, with the following materials. These materials consisted of five ml. of thoroughly mixed substrate-buffer-saline mixture added to 1 ml. of the gum

arabic solution.

3. Enzyme Reaction Measurement

The assay for the activity of an enzyme sample was obtained by the following procedure. A viscosimeter was placed in the water-bath. Four ml. of substrate-buffer-saline mixture were accurately dispensed to a test-tube. Four ml. of the appropriate dilution of enzyme, prepared by serial dilution in gum arabic solution, were dispensed to another test-tube. All stages of serial dilution of the enzyme were prepared by the use of separate chemically clean pipettes. Both solutions were then placed in the constant temperature water-bath. At the end of a timed ten minute interval, 0.8 ml. of the enzyme dilution were accurately dispensed to the 4 ml. of substrate mixture. A stop-watch, which is started at this moment, will record the accumulative reaction time (R). The enzyme-substrate mixture was quickly, but thoroughly mixed, and three ml. were run into the warmed viscosimeter.

The fluid was then blown into the bulb of the viscosimeter, and the efflux rate recorded by a second stop-watch, the time at which the reading was started being recorded from the stop-watch measuring accumulative reaction time (R). The readings were thereafter taken at frequent intervals until the flow-time of the reacting system was less than half the sum of the substrate control and solvent control efflux times.

The flow-time readings for the efflux rate of the substrate control and solvent control were then averaged, and corrected by the calibration factor of the viscosimeter used. The individual flow-time readings of the enzyme-reacting system under

test were corrected for the viscosimeter in use. The values received on either side of the half viscosity level are plotted against time (R), and the point at which the line crosses the half viscosity level is taken as the reaction time of the enzyme sample under test.

A correction is made for the fall in viscosity that occurs during a reading of the flow-time; half the corrected flow-time is added to the time at the beginning of the run of the fluid through the viscosimeter.

In order to facilitate calculation of relative viscosities, half-viscosity levels and enzyme strength, all the flow-times are considered to be relative to a flow-time of 100 for the M/60 citric acid-phosphate buffer, M/10 saline, and gum arabic mixture, comprising the solvent control.

Thus: $\frac{\text{Flow-time of "substrate control"}}{\text{Flow-time of "solvent control"}} \times 100 =$ Relative viscosity of the substrate

The relative viscosity of the reacting mixture is obtained by:

$$\frac{\text{Flow-time of substrate-saline-buffer plus enzyme dilution}}{\text{Flow-time of "solvent control"}} \times 100 =$$

Relative viscosity of the reacting mixture at the reaction time recorded.

A typical calculation of the activity of a sample of enzyme:

Reaction time to half-viscosity level (R) = 30 minutes

Amount of dilution of enzyme sample (D) = 60(dilution 1/60)

Then viscosity-reducing units(already defined) =

$$\frac{D \times 20}{R} = \frac{60 \times 20}{30} = 40 \text{ V.R.U.}$$

Before a final flow-time assay is made, the dilution of the enzyme must be brought to such a concentration, as to provide

a half-viscosity level in a reaction time falling between 15 to 45 minutes. Thus, an accuracy of replication is afforded between 5% (125) to 10% (134), (136).

Mucin Clot Prevention (M.C.P.) Test

This assay method was used in the preparation of the partially purified hyaluronidase, for enzyme detection purposes, and as a semi-quantitative method for assay of titre activity. This test was conducted according to the method of McClean et al (137), and modified as to the type of buffer according to Friou & Wenner (54-A).

Preliminary observations indicated that the use of the crude potassium hyaluronate as substrate at 0.2% concentration was satisfactory for this test. The preparation of this crude potassium hyaluronate has been recorded previously.

Preparation of Substrate Mixture

For each test, substrate was freshly prepared in the following proportions:

	Ratio
0.2% crude potassium hyaluronate dissolved in distilled water	1
Normal rabbit serum diluted 1:10 in 0.85% saline	1
McIlvaine's buffer (140) at pH 6.8 M/20	2

Conduction of Test

1. A preliminary test was conducted at a tenfold dilution to ascertain the approximate end-point of activity of the enzyme. Then the test was conducted at twofold serial differences.
2. The appropriate dilutions of enzyme were made in chilled distilled water in bulk. Then 0.5 ml. amounts of each dilution were added to Wassermann tubes standing in ice-water. A control consisted of 0.5 ml. distilled water.

3. One ml. of the above substrate mixture was added to each tube, including the control. Addition of the substrate was made starting with the tube containing the highest dilution of enzyme. With a piece of wax paper over the finger each tube is inverted once to mix the solution.
4. The test mixtures were incubated at 37°C. for exactly 20 minutes.
5. The rack of tubes were then placed in ice-water to cool rapidly for a period of 5 minutes.
6. There was now added 0.2 ml. of 2N. acetic acid to each tube, while they remained in the ice-water bath.
7. The tubes were then held up to the light, gently shaken, and read. The results were expressed as follows:

No clot or threads of precipitate - - enzyme present.

Threads of precipitate - - - - - - - - plus or minus presence.

Clot - - - - - - - - - - - - - - - - - - - no enzyme present.

The dilution showing no clots or threads was taken as the highest effective dilution of the enzyme (137). McClean et al have expressed their results in terms of the original dilution of enzyme in 0.5 ml. and disregarded the subsequent dilution with the substrate mixture. The same practice was followed when this method of assay was used.

Partially Purified Hyaluronidase Preparation

This partial purification of hyaluronidase was obtained by the use of the observations of Madinaveitia and others, that the use of the neutral salt ammonium sulphate almost totally or completely precipitates out the enzyme at 70% concentration, or full saturation. At half-saturation, only a small amount of

the enzyme precipitates out. The partial purification and concentration of the enzyme was conducted according to the following steps:

1. Five drops of an 18 hour old culture of Streptococcus pyogenes P315 A-4 grown in glucose beef-infusion broth were inoculated into 100 ml. of "assay broth"(previously described). This culture was then incubated for 18 hours at 37°C.
2. This culture was then tested for purity of the strain of streptococci it contained, by microscopic examination, and by streaking a loopful of the culture on the surface of a blood-agar plate. This inoculated plate was examined after subsequent incubation at 37°C.
3. The culture was centrifuged for one hour at 2,000 R.P.M. in a refrigerated centrifuge. The supernatant, removed by aspiration with suction provided by a water-pump, was then passed through a Hormann #9 asbestos pad inserted in a small Seitz filter. The filtrate was collected in a receiving flask, which was placed in an ice-water bath., by the aid of suction. The filtrate possessed a pH of 6.75 as determined by the glass electrode, and had a volume of 82 ml.
4. Two mls. of the filtrate were removed for assay of the enzyme activity by the Mucin Clot Prevention Test. The remainder of the filtrate was placed in a cellophane dialyzing bag, and dialyzed against chilled 0.85% saline at pH 7.0 to 7.2 in the refrigerator. Two drops of toluol were added to the surface of the enzyme solution for preservative purposes. A negative pressure was maintained on the surface of the saline, such as to maintain constant volume of the enzyme

solution. The activity of the enzyme filtrate was found to be present at a titre of 1:6,000.

5. Dialyzing was conducted for four days, with the saline being changed twice daily, and then stopped. The enzyme solution was removed from the dialyzing bag, and the volume was found to be 90 ml. The enzyme solution was then placed in a flask and maintained at ice-water bath temperature. To the enzyme solution was added 90 ml. of a saturated chilled solution of ammonium sulphate with a corrected pH value of 6.6. The resultant mixture was intermittently slowly stirred, and was left at ice-water bath temperature for one hour.
6. The cloudy solution was then centrifuged at 2,000 R.P.M. for one hour in the refrigerated centrifuge. The supernatant was removed by the aforementioned method and replaced in a flask in an ice-water bath. Ammonium sulphate crystals were now added at intervals, with intermittent, slow stirring, until a few undissolved crystals remained at the bottom of the flask. The solution was left for one hour, as before.
7. The solution was then centrifuged by the aforementioned method, and the supernatant discarded. The precipitate was dissolved in chilled neutralized 0.85% saline, so that the final volume was 10 ml. This concentrated solution of enzyme was dialyzed in the same manner as formerly described.
8. The barium chloride test (U.S.P. XII) was used to detect the end of dialyzing as determined by absence of sulphate.

The presence or absence of sulphate crystals was determined under the microscope near the end of the dialyzing period.

9. The enzyme solution, after dialyzing, was then passed through a coarse grade, chemically clean sintered glass filter. The volume was measured prior to filtration and found to be 9.1 ml. This partially purified enzyme concentrate was then dispensed in 0.25 ml. quantities to Kahn tubes, and lyophilized. The tubes were then sealed with "Pyseal" and stored in the cold at 5°C.

A Mucin Clot Prevention Assay conducted on this partially purified enzyme, using the same lot of crude potassium hyaluronate as substrate material, was found to be active at a titre of 1:19,000 for 0.25 ml. of lyophilized material. This represents, with an assay accuracy of + or - 25%, a yield of 70.9% of the original material, with an increase in enzyme concentration by 12.3 times per ml. of partially purified enzyme. This partially purified hyaluronidase, after 10 months in the dried lyophilized state, upon reassaying demonstrated no detectable loss in its titre of activity.

Kolmer Serial Dilution Assay for Penicillin

This method (107) was found convenient and accurate for assaying the sensitivity of the Streptococcus pyogenes P315 A-4 to penicillin, as well as determining the potency of penicillin samples at high increments of dilution. The reference strain used for comparison of sensitivities was the Oxford strain of Staphylococcus pyogenes. A subculture of this strain was made from a culture of the stock strain, which was kindly furnished

by Dr. G. G. Kalz. This strain was kept as a stock reference culture, being maintained on a nutrient agar slope by weekly transfer, and stored in the refrigerator at 5°C. during the intervals between transfers.

The reference standard penicillin used, was crystalline sodium penicillin "G", obtained through the courtesy of Dr. C.W. J. Armstrong, Laboratory of Hygiene, Ottawa. This reference standard possessed a potency of 1,650 I.U./mgm. Each vial of standard was stored over activated alumina, and the double container placed in a ground-glass stoppered bottle containing $CaCl_2$, and stored in the refrigerator.

Conduction of Assay

1. Each assay was conducted in duplicate for each sample or strain being tested. A separate, cotton-plugged, sterile, chemically clean pipette was used for each dilution in each assay, unless otherwise indicated.
2. For each specimen, there was arranged eleven sterile cotton-plugged Wassermann tubes. One and one-half ml. of beef-infusion broth was placed in the first tube, and 1 ml. in each of the remaining tubes. Glucose broth was not used, because the presence of this or other sugars in the medium may produce irregular breaks in the curves of inhibition. Apparently this is due to an alteration or partial inactivation of the penicillin, which occurs when sterile penicillin and glucose broth are allowed to stand before inoculation. It may also occur during the period of incubation.
3. Approximately four or five mgms. of reference standard

penicillin were weighed out in a weighing bottle by the use of a chainomatic balance. A solution of this penicillin was prepared in cold, sterile 0.85% saline solution at pH 7.0, and carefully diluted to a final concentration of 4 units per ml.

4. Addition of 0.5 ml. of the aforementioned dilution of penicillin was then made to the tube containing 1½ ml. of broth, mixed, and 1 ml. transferred to the next tube, mixed, and subsequently serially diluted to the tenth tube, where 1 ml. was discarded after mixing.

   The tubes now contain 1.0, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.016, 0.008, 0.004 and 0.002 units of penicillin respectively.

5. The Oxford strain of Staphylococcus aureus, which had been cultivated in glucose beef-infusion broth for 22 hours at 37°C. was diluted 1:1,000 with sterile beef-infusion broth. The strain of Streptococcus pyogenes under test, was cultivated and diluted in the same fashion, after shaking for one minute with sterile glass beads, to break up clumps. For purposes of shaking, the cotton plug was aseptically removed, and a sterilized rubber stopper inserted. Purity of the strains of organisms used, was confirmed by streaking the organisms on the surface of blood-agar plates prior to dilution, and then incubated with the assay tubes. With a separate sterile 1 ml. pipette for each strain of organism, addition of 1 drop of the diluted culture was made to all tubes of the duplicate series in each case. Care was taken to place the drop of diluted culture directly to the surface

of the medium, and not on the sides of the test-tubes.

6. A culture control was prepared for each series. This consisted of 1 ml. of beef-infusion broth, to which was added one drop of the diluted culture from the same pipette used at the same time as addition to the test series.
7. The inoculated series of tubes were mixed and incubated at 37°C. for 18 hours.
8. If the culture control showed good growth, the series of tubes carrying the varying amounts of penicillin were examined. The smallest amount of penicillin in units (highest dilution) showing inhibition of growth was recorded for each series. In this fashion, the sensitivity of a strain of Streptococcus pyogenes was obtained. The sensitivity of the Oxford strain of Staphylococcus aureus served as a control, on the results of the individual assay, as well as an automatic check on the weighing of the penicillin reference standard.
9. When this assay was used to test the potency of the penicillin being used in the experimental work, the test organism used was the Oxford strain of Staphylococcus aureus. The penicillin reference standard was weighed out, as well as the specimen under test, in the method aforementioned. The reference standard was diluted to contain 4 units per ml., and the specimen being tested was diluted to an expected 4 units per ml. as well.

The test was conducted the same as has been already outlined, the tubes in all duplicate series being inoculated with

the diluted Staphylococcus aureus culture only. The potency of penicillin was found by multiplying the highest inhibiting dilution of the specimen by the smallest inhibiting amount of the reference standard penicillin in units. The result expresses the amount of penicillin in units per 1 ml. of specimen being tested as per the following example:

Highest inhibiting dilution of specimen = 1:32

Smallest inhibiting amount of reference standard penicillin = 0.031 units/ml.

32 X 0.031 = 0.992 units of penicillin per 1 ml. of specimen, as compared to the reference standard containing 1 I.U./ml.

This method of assaying strain sensitivity and penicillin specimen potency provided a comparatively quick method of testing, and yielded potency titres or sensitivity titres as desired, that checked in the duplicate series every time. On testing, with several preliminary assays, it was found that the error of difference between drops of cultures diluted 1:1,000 as delivered by Exax recalibrated pipettes, could not be detected by the assay's dilution increment difference.

Crystalline sodium Penicillin "G":

All experiments were conducted with the use of crystalline sodium penicillin "G" bearing a labelled potency of 1:660 I.U./mgm., and which demonstrated a potency of 1,650 I.U./mgm. as obtained by the aforementioned method of assay during the period of its use. As previously described, (Historical Review) the use of any penicillin other than a crystalline type, may have adverse experimental effects, through incorporated impurities.

Bacterial Population Measurement

In order to avoid the larger degree of error incurred by

the use of bacterial plate-count methods or direct counts with the use of a bacterial counting chamber, measurement of turbidity due to bacteria alone was made by the use of an Evelyn colorimeter, as a means of estimating bacterial populations. The employment of turbidimetric measurements for determination of bacterial populations has had a wide application in the field of bacteriology (80-A). During the experimental investigations, actual bacterial numbers in a population were not required, but rather relative differences in the percentage of growth in various populations of the same bacterial species were desired.

A set of colorimeter tubes that had been selected to agree within less than two units on the galvanometer scale were used (74). Precautions were observed in the conduction of the assay that agreed with accepted methods (200-A), and the directions of the manufacturer of the instrument. Ten ml. of broth culture were used in each instance of measurement. A sample of the lot of broth used in the individual assay was used as a standard blank, thus no correction factor was necessary in the readings, other than the galvanometer scale calibration corrections, thereby eliminating a possible source of error.

A #660 filter produced maximum transmission for the control assay broth, and this filter was used throughout the investigations. Before use, the instrument was turned on for 10 minutes with filter inserted. The tube containing the uninoculated broth as a blank was inserted, and the galvanometer adjusted until it read 100. The test solution, after twirling insured proper dispersion of bacteria, was

then inserted, and the reading recorded. After each reading, the blank was reinserted to see that the galvanometer reading had not changed from 100. This procedure was repeated until three readings insured dispersion was as complete as the instrument could detect.

Readings were recorded to the nearest $\frac{1}{4}$th between galvanometer scale units, and by means of a table expressing density from the galvanometer reading (L = 2-log"g" against galvanometer reading "g"), the percent deflection as measured by the galvanometer reading was transposed into terms of absolute density. As broth was used as a blank, the absolute density change in each case was due to bacteria alone.

Sulphate Identity Test (United States Pharmacopeia XII.)

Solutions of sulphates, yield with a 1 Normal barium chloride solution, a white precipitate, which is insoluble in HCl. Hydrochloric acid produces no precipitate when added to solutions of sulphates.

Cleaning of Glassware and Sintered Glass Filters

Colorimeter tubes, viscosimeters, all pipettes and sintered glass filters were cleaned by the use of acid cleaning solutions. Viscosimeters and pipettes were cleaned by the use of chromic acid, prepared (89-A) by the addition of one litre of concentrated sulphuric acid to 35 ml. of saturated sodium dichromate. The pipettes and colorimeter tubes were then rinsed at least ten complete times with tap water, followed by three complete rinses of distilled water.

Owing to the difficulty of removing dichromate from sintered glass filters, the acid cleaning solution described

by Morton (161-A) was used. These filters were rendered chemically clean by the following method:

1) If the filter was pathogenically contaminated, it was autoclaved. If not, it was mechanically cleaned by rinsing the filter from the reverse side by means of a rubber tube attached to a water tap.
2) The filter was rendered chemically clean by placing in an acid cleaning solution of concentrated sulphuric acid with the addition of a little $NaNO_3$ and $NaClO_4$. This mixture was heated to 90°C., and let stand at room temperature overnight.
3) Ten rinses of tap water were then made, followed by a rinse of distilled water. A 50 ml. size requires 500 ml. of distilled water to be run through it, in order that the last portion of distilled water passed through it to be of the same pH as the distilled water used.

Beckman pH Meter

This instrument was employed for the determination of the pH of all aqueous solutions. The instrument was standardized each time used, by the use of 0.1M. potassium hydrogen phthalate at pH 4.0.

Pipettes:

For the preparation of molar solutions and buffer solutions, retested Exax Ostwald - Folin pipettes were used wherever possible. These pipettes were recalibrated according to accepted procedures (27-A).

K-Exax retested serological pipettes or other types of similar grade, were used for the quantitative transfer of

solutions.

"Special Pasteur Pipettes", prepared (180) to deliver the same number of drops per ml., were used where growth rate measurements were being made, in order to obtain inoculations of the same amount.

EXPERIMENTAL

I. "The Effect of Crystalline Sodium Penicillin "G" on the Depolymerization of Hyaluronic Acid by Partially Purified Hyaluronidase."

This test was designed to demonstrate any "in vitro" inhibition of hyaluronidase activity in its depolymerizing action upon hyaluronic acid, as measured by the viscosity-reducing assay. Partially purified hyaluronidase was used, since it has been demonstrated to be more susceptible to environmental factors (186), and also, it was felt that less penicillin-inhibitory metabolic by-products would be present to exert their effect upon the antibiotic.

Method of Conduction

The crystalline sodium penicillin "G" was previously tested for its potency per mgm., according to the method already described in "Materials and Methods". A sample of this penicillin was then carefully weighed out, and diluted in chilled sterile 0.85% saline to a potency of 12 I.U./ml. This solution was divided. One portion was autoclaved at 120 C. for 20°minutes, and the other portion was stored in an ice-water bath as the active penicillin sample. The inactivated sample would serve as a control.

A tube of lyophilized partially purified hyaluronidase was dissolved in 1 ml. of 0.5% gum arabic and diluted in gum arabic solution to 1:100, and then stored in an ice-water bath until used.

Equal quantities of the enzyme and active penicillin were then mixed and assayed for viscosity-reducing activity according to the methods already described (Materials &

Methods). The penicillin potency was now 6 I.U./ml. Then equal quantities of the inactivated penicillin sample were mixed and assayed in the same manner.

At another date, this test was repeated using penicillin at a final concentration of 25 I.U./ml. At this time, preliminary trial runs were conducted, so that the activity of the enzyme would be lessened, and bring the half-viscosity level reaction time close to 15 minutes for the computation of results to be made.

Results of Experiment I.

The results of the experiment measuring the effect of penicillin in a final concentration with the enzyme of 6 I.U./ml. were not able to be computed, owing to the large concentration of enzyme present. The results are presented in Graph #1. A sufficient number of flow-times were taken, to obtain a correct impression of the effect of penicillin upon the enzyme's activity as it was exhausted in each case. The actual flow-time readings and relative viscosity computations are presented under the heading of Protocol #1.

Flow-time readings and relative-viscosity computations of the effect of penicillin in a final concentration with the enzyme of 25 I.U./ml. are presented under the heading of Protocol #2, together with their representative graph.

The result obtained indicates that the sample of enzyme containing 25 I.U./ml. of penicillin possesses 3.53% more activity than that with the inactivated penicillin.

Discussion of Experiment #I.

Although two different viscosimeters were used with the

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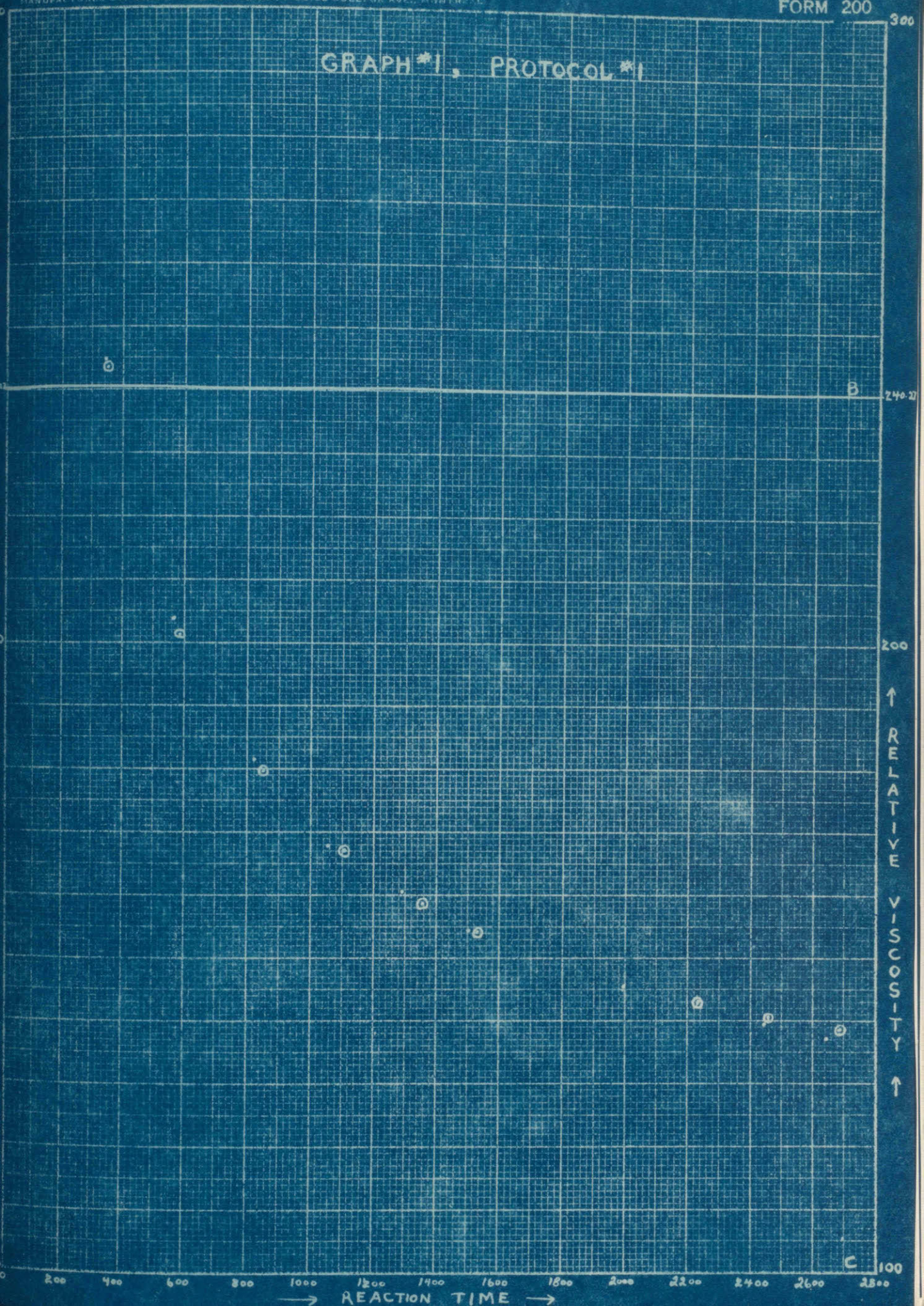
GRAPH #1, PROTOCOL #1



assay incorporating 6 I.U./ml. of penicillin, the similarity of the shape of the curves, together with the similarity of the results obtained, demonstrates the accuracy in the application of the calibration factor to correct flow-times to one constant or control viscosimeter. The similarity of the results also indicates that penicillin does not inhibit the depolymerizing activity of hyaluronidase on its substrate hyaluronic acid under "in vitro" conditions.

The same viscosimeter was used to assay the effect of 25 I.U./ml. of penicillin on the enzyme. As 3.53% more activity was obtained with the active penicillin, this indicates that penicillin at this concentration produces no inhibitory action upon the enzyme's "in vitro" action upon its substrate. It is readily seen that a 3.53% error was incurred in the assay, which has a reputed error of 5% (125) to 10% plus or minus replicative error (134), (136) as indicated in the Historical Review.

II. "A Test for Enzyme Potency Loss Over a Period of Nine Hours While at a Temperature of 5°C."

This test was conducted for the purpose of estimating whether there was any loss incurred during storage of the buffered culture filtrate containing the enzyme while at a temperature of 5°C. for a period of nine hours. This test was considered necessary, since the completion of some expected experiments was felt to be of this duration of time.

Method of Conduction

One drop of an 18 hour culture of Streptococcus pyogenes P315 A-4, grown in glucose beef-infusion broth, was inoculated into each of two tubes containing 10 ml. of "assay broth".

These were incubated for 18 hours at 37°C.

At the end of this incubation period, a loopful from each of the inoculated cultures was streaked onto the surface of separate blood-agar plates, and Gram-stains were prepared from each culture. The stained slides were examined for purity of streptococcal culture before work was continued. The inoculated blood-agar plates were incubated for confirmation of the direct examination.

The two tubes were then mixed, and ten ml. extracted, and passaged through a micro-Seitz filter. The filtrate was then transferred to a chilled water-bath at 5°C. in the refrigerator. The remainder of the culture was than passaged through the Seitz filter, and the filtrate tested for its pH by the glass electrode. This was found to be at a pH of 6.55, well above the level of pH 6.0 at which enzyme synthesis is reported to be halted (183).

The viscosity-reducing assay was then conducted in the usual manner as previously described, after preliminary trial runs were made to estimate a proper dilution in gum arabic solution that would produce a half-viscosity level reaction time within 15 to 45 minutes.

After the expiration of nine hours, one ml. of the enzyme that was stored at 5°C. was carefully diluted to the same extent as before, and the viscosity-reduction assay was conducted in the same manner.

Results of Experiment II.

The activity of the initial sample of enzyme was found to consist of 150.81 V.R.U. After nine hours storage at

5°C. in its buffered filtrate, the activity was found to be 151.26 V.R.U. The flow-time readings and relative viscosity computations are presented under the heading of Protocol #3 for both enzyme samples.

Discussion of Experiment II.

The results appear to indicate that no loss occurs during storage of the enzyme in its buffered filtrate at 5°C. for this period of time. The error in this replicative assay is therefore 0.29%.

The buffering capacity of this medium for this strain of streptococcus appears to be capable of keeping the reaction of the medium above the level at which inactivation of enzyme synthesis takes place, as discussed in the Historical Review, and confirms Rogers' investigations (182), (183), (187).

III. "A Test for Hyaluronidase Loss Through Adsorption to Seitz Pads".

This test was conducted for the purpose of estimating whether there was any loss of enzyme activity through adsorption of a higher level taking place in the first fraction of filtrate obtained, than in latter fractions. This was deemed advisable, since, if there was loss of enzyme, the first fraction of filtrate would subsequently cause a dilution of the last volume of filtrate passaged through the asbestos pads. This would create an error in assaying samples of cultures possessing varying levels of enzyme activity, since retention of enzyme through adsorption may not prove to be a direct relationship in its power. If this retentive power is present, this property may possibly necessitate a larger volume of low activity enzyme culture medium to be passaged,

before its retentive capacity for the enzyme is neutralized by a sufficient volume of the culture medium containing the enzyme. Therefore, if the adsorptive effect of the asbestos pads promote enzyme retention, this effect would prove stronger upon the first fraction passaged through the pad. If this effect was demonstrated, then a solution of low activity enzyme in culture medium would proportionally lose a higher percentage of enzyme than an equal quantity of high activity enzyme in culture medium, since asbestos pads are both quantitative and qualitative in their adsorptive capacity and properties respectively.

Method of Conduction

One drop of an 18 hour culture of Streptococcus pyogenes P315 A-4 grown in glucose beef-infusion broth, was inoculated into each of six tubes containing 10 ml. of "assay broth". These were incubated at 37°C. for 20 hours. Two tubes of uninoculated "assay broth" were incubated along with the inoculated culture medium.

At the end of this incubation period, the cultures were tested for purity of bacterial type by the method described in the preceding experiment.

To each tube was then added three or four glass beads, and the cotton plugs replaced by clean rubber stoppers. The tubes were shaken then, for exactly one minute to help break up clumped bacteria. The contents of the six tubes were then mixed.

Ten ml. of the uninoculated control assay broth were dispensed to a chemically clean colorimeter tube, and in-

serted into the instrument and the galvanometer brought to 100% transmission. Ten ml. of the bacterial culture were dispensed then into each of three colorimeter tubes, and the bacterial populations measured by the method already described in "Materials & Methods".

The contents of two of these tubes were then passaged through a separate micro-Seitz for each of the samples, with the aid of suction, in the following manner. The first fraction of filtrate in each case was removed by pipette withdrawal, until they were approximately the same. The amount of filtrate in each case was approximately 1,70 ml. and 1.8 ml. respectively. These samples were labelled, stoppered and transferred to a chilled water-bath in the refrigerator. Clean receiving tubes were then attached to the micro-Seitz in each case and the filtration continued until approximately five ml. were contained in the receiving tubes. Exactly four ml. of filtrate were then extracted from each tube and discarded. Filtration was then continued, until no more filtrate was appearing from the pad. These samples of filtrate were appropriately labelled, stoppered and transferred to the chilled water-bath in the refrigerator.

The viscosity-reducing assay was then conducted in the usual manner on each of the four samples of filtrate, using the same dilution of enzyme in gum arabic for each of the four filtrates.

Results of Experiment III.

Two of the results obtained from measuring the bacterial population of this mixture were the same, and the other was

within ¼ of the galvanometer's unit.

The flow-time readings and relative-viscosity computations were calculated in the same manner as that used for Experiment II. The results, expressed in viscosity-reducing units are shown in Table I.

TABLE I.

	First Fraction of Filtrate	Second Fraction of Filtrate
Specimen A - - - - -	196.01 V.R.U.	185.50 V.R.U.
Specimen B - - - - -	181.56 V.R.U.	179.78 V.R.U.

When the first fractions are totalled, as well as the last, and the result obtained in values of percent difference, the last fraction is demonstrated to contain 3.29% less enzyme than the first fraction of filtrate.

The greatest variance, that is, between the largest value obtained and the smallest of the four samples of the same enzyme, expressed in values of percent difference is 8.3%.

Discussion of Results of Experiment III.

The error encountered between three samples of the same bacterial population, as measured turbidimetrically, has been found to be ¼ of one galvanometer unit.

According to the results obtained, there is no loss of enzyme in the first filtrate fraction, through retention on adsorptive asbestos Seitz filter pads.

The greatest error of variance obtained between four fractions of the same culture filtrate, that is, between the values of 196.01 and 179.78 viscosity-reducing units, was

8.3%. This was the greatest variance error between the four flow-time measurements, together with the simultaneous application of the calibration error between the different viscosimeters used. Error of variance between any one of the obtained results and the averaged values of the four results, would be of necessity much less. This value of 8.3%, designating the largest variance error, is well within this assay's replicative error. (136), (134).

IV. "The Effect of Sub-Bacteriostatic Concentrations of Penicillin "G" on the Production of Hyaluronidase "In Vitro".

An experiment has been previously described, illustrating that penicillin "G" has no inhibiting effect upon the enzyme's degradation of its purified specific substrate.

This experiment was designed to illustrate the effect of graded inhibitive, sub-bacteriostatic concentrations of penicillin upon the metabolism of the hyaluronidase-producing bacteria, Streptococcus pyogenes P315 A-4, insofar as its ability to produce this enzyme was concerned, when in contact with its adaptive producing environment.

Method of Conduction

The sensitivity of this strain to penicillin was conducted according to the assay procedure previously described in the section entitled "Materials & Methods". The range of penicillin concentration that provided no growth, inhibited growth and no apparent inhibition of growth was thereby obtained.

Separate assays were then conducted to measure the amount of hyaluronidase produced by a culture metabolising in an inhibiting, sub-bacteriostatic concentration of penicillin "G".

The following procedure was used in each assay of hyaluronidase production under these conditions.

From the stock culture, maintained on blood-agar plates, a tube of glucose beef-infusion broth was incubated at 37°C. for 18 hours.

A sample of crystalline sodium penicillin "G" of known potency, was accurately weighed out, and serially diluted in sterile, chilled 0.85% saline to predetermined potency concentrations. To each tube of "assay broth" containing 9½ ml. of culture medium, was added 0.5 ml. of diluted penicillin at such a concentration, that the final dilution in "assay broth" of 1:20, would afford the desired potency in each case. As previously mentioned in the section entitled "Materials & Methods", attention is brought to the fact, that, all "assay broth" used in each separate assay, was composed of one lot of prepared medium, and no mixing of prepared lots was made in the individual experimental assays.

Two tubes of "assay broth" in each case of desired penicillin concentration were conducted in each assay. Four tubes of "assay broth" were used as controls, and each had an addition of 0.5 ml. of 0.85% sterile saline added to it. Two of these broths were uninoculated, and served as control blanks for the turbidimetric measurement of bacterial populations. The other two broths served as duplicate controls for hyaluronidase production by the strain of the streptococcus when not in the inhibiting presence of penicillin "G".

The tubes of media with and without added penicillin were twirled to afford mixing, and then one drop of glucose beef-

infusion broth culture was carefully dispensed to the top of the "assay broths" with penicillin and the control "assay broths" with no penicillin from the same pipette, held in a vertical position. Care was taken to insure that no inoculum was allowed to come in contact with the sides of the tubes of medium. All tubes were then carefully twirled to insure proper mixing of the inoculum in the inoculated tubes, and then all test media and controls were incubated at 37°C. for 18 hours.

At the conclusion of incubation, the cultures were tested for purity of bacterial content according to the method as previously described in Experiment II. The cultures were then prepared for turbidimetric measurement and the bacterial populations measured according to the previously described method in Experiment III, and in the section entitled "Materials & Methods". Prior to actual measurement, the contents of the duplicate broths containing the same concentration of penicillin were mixed. The duplicate tubes of uninhibited culture were also mixed. This afforded an actual measured ten ml. volume for withdrawal and addition to the colorimeter tubes.

After measurement, the galvanometer readings were corrected for galvanometer error, according to the calibration chart furnished with the instrument, and then the readings were transposed to terms of absolute density due to bacteria alone. From these terms, percent inhibition due to penicillin were easily calculated.

The contents of the colorimeter tubes were separately passaged through a dry, sterile micro-Seitz, and then the

receiving tubes were stoppered and placed in a chilled water-bath in the refrigerator at 5°C. and immediately assayed for enzyme potency.

The viscosity-reducing assay was conducted in the usual manner, as previously described, and the results obtained in V.R.U./ml. of filtrate. An example of one of these assays is furnished under the heading of Protocol #4.

The absolute density due to bacteria alone of the uninhibited population in each case was brought to the density of the uninhibited control culture by the factor of difference. This factor of difference was then applied to the amount of hyaluronidase produced by the penicillin-inhibited culture, so that the enzyme production levels were expressed on the basis of the same bacterial populations. The number of units produced by the inhibited culture were then expressed in terms of relative percent difference of production of enzyme from that of the control culture, which was represented as 100%.

Since these enzyme production values have been expressed in terms of percent difference with equal bacterial populations, the percent difference in relative adaptive response in production of hyaluronidase is representative of that difference per average streptococcal cell.

Results of Experiment IV.

The results of each assay of this experiment are provided in terms of percent in Table II, and pictorially presented on Graph #2.

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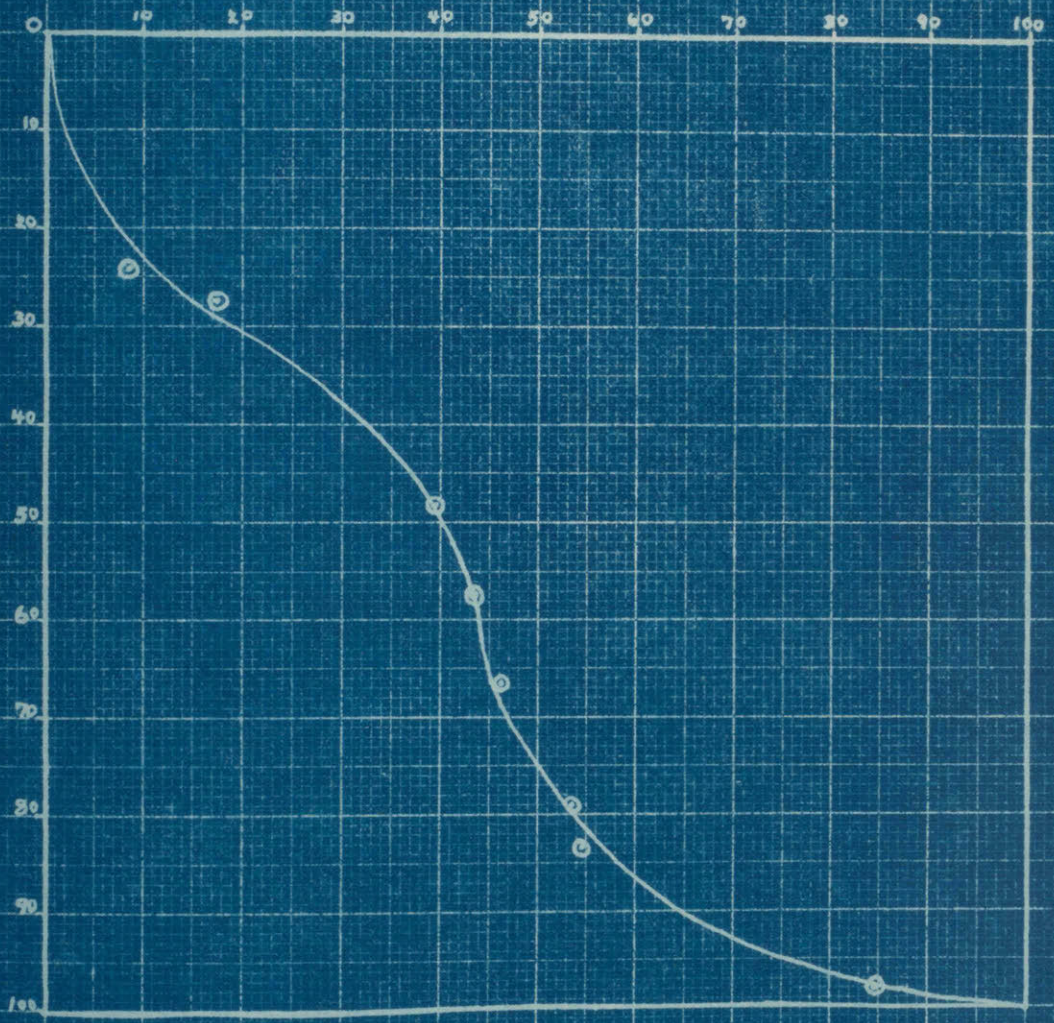
GRAPH #2



ABSCISSA:- PERCENT INHIBITION OF GROWTH BY PENICILLIN.

ORDINATE:- PERCENT REDUCTION IN LIBERATION OF HYALURONIDASE PER CELL.

TABLE II.

# Inhibition of Growth by Penicillin "G"	% Reduction in the Production of Hyaluronidase per Streptococcal Cell
8.67	24.13
17.69	27.13
39.56	48.31
43.35	57.38
46.30	66.77
53.55	79.00
54.42	83.47
84.57	97.82

Discussion of Results of Experiment IV.

According to the results obtained, it may be seen that very small inhibitive concentrations of penicillin "G" produce an immediate altered response in the production of this extracellular enzyme into its environment. As the degree of inhibition is heightened by larger concentrations of penicillin in the medium, there is a progressive reduction in the production of hyaluronidase into the medium.

From these results, it cannot be determined whether this reduction in enzyme production is due to an alteration in cell membrane permeability, or due to a direct effect on the bacterial cell's metabolism, or a combination of both effects.

It is an accepted fact that inhibiting sub-bacteriostatic concentrations of penicillin lower the over-all general viability of a susceptible bacterium in "in vitro" conditions. It has been demonstrated that viability commences to decrease after a lag period, which is longer for lower concentrations (166).

It has been demonstrated that penicillin either combines with, or produces a reorganization of the bacterial cell wall (59) such that assimilatory mechanism is blocked. Hyaluronic acid thereby may be non-assimilated for the production of the enzyme, as well as for purposes of energy. Hyaluronidase appears to be the metabolic by-product of the assimilation of hyaluronic acid by the bacterium. When in an "in vitro" environment the enzyme does not appear to be of benefit to the bacterium's metabolism when the carbohydrate is supplied in the form of hyaluronic acid.

Bacteria are most susceptible to the action of penicillin when they are in the logarithmic phase of growth and are exhibiting the greatest need for oxygen (176). Penicillin in very small concentrations, exerts a strong inhibitory effect on the oxygen uptake of organism's during the early lag and logarithmic phases of multiplication (24). Hyaluronidase is produced in its largest amount during the logarithmic phase of growth. Through the inhibition of oxygen uptake, the assimilation of hyaluronic acid possibly, and other essential nutient metabolites for energy purposes, and the adaptive production of the enzyme, is possibly inhibited because of lowered metabolic functioning.

Inhibition of streptococcal fibrinolysin and its production by penicillin has been reported by Dixon (31) and Neter & Will (163). The metabolic function producing this enzyme may possibly be of a closely allied mechanism to that producing hyaluronidase, as both are part of the invasive armament of this organism.

From the results here reported, it has been demonstrated that penicillin progressively inhibits the "in vitro" production of this spreading factor when the bacterium is in association with its adaptive environment. From these results, it may be suggested, that when a hyaluronidase-producing organism is in "in vivo" conditions, in association with connective tissue and the ground substance of cellular substance, that sub-bacteriostatic levels of penicillin would possibly reduce its hyaluronidase - production. This would thereby reduce the spreading effect of this enzyme through the tissues, and thereby possibly minimize part of the invasive mechanism of the bacterium producing this enzyme, and slow down the spread of this type of organism through the tissues.

V. "A Method of Inducing Penicillin Resistance to a Susceptible Bacterium Possessing an Adaptive Enzyme System."

It has been demonstrated that increasing, inhibitive, sub-bacteriostatic concentrations of penicillin progressively reduces or inhibits the penicillin susceptible bacterial cell's ability to produce the adaptive extracellular enzyme hyaluronidase, in its medium. Experiments were then devised to investigate the ability of a penicillin resistant strain of this bacterial species to produce the enzyme.

During the development of induced resistance, it was decided to alternately passage the streptococcal strain through "assay broth" containing hyaluronic acid, and then onto blood-agar plates, as it was thought that the adaptive response of the enzyme production would be kept at as high a level as possible. Differences would thereby be more pronounced if

they occurred, and thereby more accurately detected. This same technique would also afford a means of demonstrating whether the capacity of this strain to produce hyaluronidase would be enhanced by repeated subculture. As mentioned in the Historical Review, McClean (132) could not enhance the productive capacity of a strain with which he was working.

Method of Conduction

The method of inducing acquired resistance by subculturing strains of bacteria in small, progressively increasing sub-bacteriostatic concentrations of penicillin has been used by several investigators. (Historical Review).

The culture of Streptococcus pyogenes P315 A-4 was inoculated into three tubes of "assay broth" containing varying and highly inhibiting concentrations of penicillin "G". A control was established by inoculating a tube of "assay broth" from the same stock culture of Streptococcus pyogenes P315 A-4, and to which also was added the same amount of sterile 0.85% saline as that added to the "assay broth" containing the dilution of penicillin. These tubes were incubated for 24 hours at 37°C., and then examined.

The tube showing growth in the highest concentration of penicillin was then selected for transfer. A sterile, stainless steel, penicillin assay cup, as used in the penicillin cup-assay plate method, was then slightly warmed at its bevelled edge, and placed on the surface of each of two blood-agar plates. These cups were then filled to the brim with a dilution of penicillin "G" containing 5 I.U./ml. Two loopfuls

of the culture growing in the highest concentration of penicillin in the "assay broth" were then streaked over these blood-agar plates up to the assay-cup containing penicillin. The control culture was also streaked onto the surface of a blood-agar plate containing no assay-cups with penicillin. These plates were incubated for 24 hours at 37°C.

From the edge of the area of inhibition about the cup containing penicillin was picked a large inoculum of the organisms and inoculated into three tubes of "assay broth" containing varying dilutions of penicillin. A large inoculum was similarly picked from the control blood-agar plate and transferred to a tube of "assay broth" containing no penicillin.

This alternative transferring from broth to plate was conducted until forty-four transfers had been made into "assay broth" and forty-three onto plates. The penicillin concentration was able to be slowly increased at various times by increments of 0.0005 I.U./ml.

This method allowed an automatic check to be made on the purity of the strains by examination of the blood-agar plates. An examination was also made on each plating of the developing resistant strain for the development of any very resistant colonies appearing in the area of inhibition about the assay cup. Towards the end of the transferring, it was seen that the resistant strain grew very well in the presence of about four times as much penicillin as did the control passaged strain, and that for the last five transfers, the top level of inhibition was not increasing to the next small increment

of higher concentration of penicillin.

At this time, a penicillin assay was conducted for the sensitivity of the penicillin-resistant strain, (hereafter to be termed strain "R"), the control "assay broth" passaged strain, (hereafter to be termed strain "N"), and the non-passaged stock culture that was the parent of the other two strains, (hereafter to be termed strain "C").

This assay was conducted in duplicate for each strain according to the method outlined in the section "Materials & Methods". A complete set of assays for strain sensitivity were conducted with the use of a separate pipette for each dilution in a series, and a complete set using but one pipette throughout a single series of serial dilutions.

Each of the "N" and "R" strains were then lyophilized in "pneumo" broth.

Results of Experiment V.

After the forty-first transfer, and thereafter until the eighty-seventh, it was noticed that the resistant strain became more floccular, and settled out from the broth early in its growth cycle, and possessed what appeared to be a prolonged lag phase. The "N" strain became progressively more disperse in its growth, with but little tendency to precipitate out in 24 hours of incubation. The small amount of precipitated growth that did fall out, was very fine, and not floccular in its appearance.

Very resistant colonies were not observed at any time in the area of inhibition, and the edge of the perimeter of inhibition appeared clean cut and well-defined.

The results of the assay for penicillin sensitivity for the three strains appear in Table III. With the use of a separate pipette for each dilution, it is seen that an induced resistance has been acquired by the "R" strain over the "N" strain by 4.03 times. No change in penicillin susceptibility has been incurred by the "N" strain.

Discussion of Experiment V.

Exactly one increment's difference in dilution in the respective sensitivity to penicillin was recorded for each strain by using one pipette in the conduction of a serial dilution, indicating a considerable carry-over of penicillin with a strain that is not too sensitive to the antibiotic, and a correspondingly smaller carry-over with a more sensitive strain, that produced the same effect.

The observation, that no highly resistant strains developed to penicillin, is not unusual and is to be expected. As related in the "Historical Review", hemolytic Group A streptococci do not possess any natural resistance to penicillin (141). Investigators have found (138), (139) that acquired resistance "in vitro" was not induced as readily with streptococci as with other organisms, or as to high a degree. Induced resistance of four times that of the parent strain is about the average that is obtained.

VI. "Morphological and Biological Characteristics of the Three Strains of Streptococcus pyogenes P315 A-4."

During the development of induced resistance to penicillin "G" by the "R" strain, changes in the type of growth in "assay broth" were observed for the "N" and "R" strains. It was

TABLE III.

Tube No.		1	2	3	4	5	6	7	8	9	10	11
Dilution		1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	None
Unit		1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004	0.002	----
1. Strain "R" Series	A	-	-	-	-	+	++	+++	++++	++++	++++	++++
	B	-	-	-	-	+	++	+++	++++	++++	++++	++++
2. Strain "N" Series	A	-	-	-	-	-	-	+	++	++++	++++	++++
	B	-	-	-	-	-	-	+	++	++++	++++	++++
3. Strain "C" Series	A	-	-	-	-	-	-	+	++	++++	++++	++++
	B	-	-	-	-	-	-	+	++	++++	++++	++++
4. Strain "R" Series	A	-	-	-	-	-	+	++	++++	++++	++++	++++
	B	-	-	-	-	-	+	++	++++	++++	++++	++++
5. Strain "N" Series	A	-	-	-	-	-	-	-	+	++++	++++	++++
	B	-	-	-	-	-	-	-	+	++++	++++	++++
6. Strain "C" Series	A	-	-	-	-	-	-	-	+	++++	++++	++++
	B	-	-	-	-	-	-	-	+	++++	++++	++++
7. Oxford Strain of S. aureus Series	A	-	-	-	-	-	-	-	+	++++	++++	++++
	B	-	-	-	-	-	-	-	+	++++	++++	++++

Legend:- Series 1, 2, and 3 were conducted with a separate pipette for each dilution.

Series 4, 5, 6, and 7 were conducted with an individual pipette for each separate series, in the preparation of serial dilutions.

Observations were recorded at the termination of 21 hours of incubation at 37°C.

It was deemed advisable to examine these strains for morphological changes in the presence and absence of penicillin, and to observe their similarity or dissimilarity in various differential media and biochemical tests.

Method of Conduction

An 18 hour glucose beef-infusion broth of each strain was used as the source of inoculum. One drop of each strain was inoculated into a tube of "assay broth" with and without added penicillin. The dilution of penicillin was calculated in each case to cause 30% to 40% inhibition of growth for each strain. This information was derived from the results obtained in the assay of sensitivity to penicillin for each strain.

One loopful of each strain was inoculated into each of duplicate tubes containing various differential Hiss Serum broths and bacteriological media tabulated in Table IV. Blood-agar plates were also streaked with a loopful of each strain. All media were then incubated for a period of 24 hours at 37°C. Observations were then recorded, and all the media except the blood agar plates and "assay broths" were returned to be incubated an additional 24 hours.

Gram-stains were made of the "assay broth" cultures. The blood-agar plates were examined at 24 hours incubation.

Results of Experiment VI.

All colonies of the three strains were of the "smooth" phase. On the average, isolated colonies of the "R" strain appeared to be slightly smaller than the "C" strain, and the colonies of the "N" strain were very slightly larger and more

convex than those of the "C" strain. There was no apparent difference in the size of the zone of hemolysis between the three strains.

Gram-stained preparations of the "R" strain in the absence of penicillin showed the resistant strain to be Gram-positive, with chains of colli comprised of as many as 20 individual cells. In comparison to the other two strains, the average cell size was smaller. In the presence of penicillin, the "R" strain was Gram-positive, with very long chains. In an individual chain, there was striking dissimilarity between the size and shape of the cells. Some of the pleomorphic cells were bloated, oval, and bacillary in shape, while others were exceedingly small in size. Some chains appeared to be chains of only bacillary types. Some cells in a chain had a tendency to not retain the gentian violet as well as others.

Gram-stained preparations of the "N" strain in the absence of penicillin showed this strain to be uniformly Gram-positive, with an average chain length of two to three cells. This arrangement was strikingly dissimilar to the "C" and "R" strains. The cells were uniform in size, and appeared slightly larger than the "C" strain. In the presence of penicillin, the "N" strain was seen to be Gram-positive with much pleomorphism, cell distortion, bloated cells and the presence of frequent oval types. Chain length was increased, but no bacillary cells in chains were seen.

Gram-stained preparations of "C" strain in the absence of penicillin proved to be uniformly Gram-positive, with an

average chain length of 6 - 8 cells. The cells were uniform in their size, being slightly smaller than the "N" strain. In the presence of penicillin, the strain appeared the same as that of the "N" strain.

The results of the biochemical and differential media tests are demonstrated in Table IV. There were no striking dissimilarities observed in the metabolic functions between the three strains. The difference between acid production in starch and galactose Hiss Serum broths is felt to be a slight quantitative difference rather than a qualitative metabolic difference.

Discussion of Experiment VI.

Metabolic differences amongst the three strains were found to be the same, insofar as the tests employed were able to detect. This is in agreement with the findings of most investigators, especially when the degree of induced resistance is low (5),(Historical Review).

Morphologically, the differences were pronounced. The chain length arrangement was the chief difference between the "N" and "C" strain, although the "N" strain appeared to have an increased size, both in respect to cell as well as colony size. The "R" strain was felt to be smaller on the average with respect to colony size, and definitely with respect to cell size. The longer chain length and pleomorphism exhibited by the "R" strain in the absence of penicillin, was typical of an induced resistant strain of streptococci.

VII. "Growth Rates of the Three Strains of Streptococcus pyogenes P315 A-4."

During the acquisition of induced resistance to penicillin,

TABLE IV.

	Strain "R" 24hrs.	Strain "R" 48hrs.	Strain "N" 24hrs.	Strain "N" 48hrs.	Strain "C" 24hrs.	Strain "C" 48hrs.
Gelatin: growth	≠	≠	≠	≠	≠	≠
	≠	≠	≠	≠	≠	≠
liquefaction	-	-	-	-	-	-
	-	-	-	-	-	-
Litmus Milk:	-	-	-	-	-	-
	-	-	-	-	-	-
Sodium Hippurate:- (Coffey & Foley's)	-	-	-	-	-	-
	-	-	-	-	-	-
Peptone Water: growth	≠	≠	≠	≠	≠	≠
	≠	≠	≠	≠	≠	≠
NH_4 formation	-	-	-	-	-	-
(after 5 days incubation)	-	-	-	-	-	-
Hiss Serums:-						
Trehalose	AC	AC	AC	AC	AC	AC
	AC	AC	AC	AC	AC	AC
Sorbite	-	-	-	-	-	-
	-	-	-	-	-	-
Starch	A	A	A	A	A	AC
	A	A	A	A	A	AC
Raffinose	≠	≠	≠	≠	≠	≠
	≠	≠	≠	≠	≠	≠
Inulin	-	-	-	-	-	-
	-	-	-	-	-	-
Maltose	AC	AC	AC	AC	AC	AC
	AC	AC	A	AC	A	AC
Levulose	AC	AC	AC	AC	AC	AC
	AC	AC	AC	AC	AC	AC
Galactose	A	AC	A	AC	"A"	A
	A	AC	A	AC	"A"	A
Glycerine	≠	≠	≠	≠	≠	≠
	≠	≠	≠	≠	≠	≠
Lactose	"A"	"A"	"A"	"A"	"A"	"A"
	"A"	"A"	"A"	"A"	"A"	"A"
Glucose	AC	AC	AC	AC	AC	AC
	A	AC	AC	AC	AC	AC
Mannite	-	-	-	-	-	-
	-	-	-	-	-	-
Salicin	A	AC	A	AC	A	AC
	A	AC	A	AC	A	AC
Sucrose	AC	AC	AC	AC	AC	AC
	AC	AC	AC	AC	AC	AC

Legend:-

- = No change
≠ = Growth

A = Acid production and no clot

AC = Acid production and clot

"A" = Acid production weak and no clot.

it was noticed that the "R" strain had also acquired an apparent prolongation of the lag phase during the early stages of its life cycle. As this fact had an important bearing on future experiments, it was decided to investigate the amount and duration of this change in its growth rate.

Method of Conduction

Subcultures from the stock cultures maintained in the refrigerator on blood-agar plates of strains "R", "N" and "C" were subcultured to blood-agar plates every day for five consecutive days, employing transfer of large inocula. Each strain was then inoculated into a tube of glucose beef-infusion broth, and incubated for 24 hours at 37°C. These strains were then Gram-stained for determination of bacterial content.

Sterile beef-infusion broth from one preparation lot had 1% (w/v) of glucose added to it. This broth was then clarified by being passaged through a Seitz "K" clarifying pad, and the filtrate passaged through a chemically clean sintered glass filter. Except for the clarification, this medium was the same as the glucose beef-infusion broth made in the Department of Bacteriology & Immunology, McGill University. This medium was then accurately dispensed in volumes of 9½ ml. per tube. All tubes were of the same size. This medium was autoclaved at 120° for 20 minutes, cooled and inoculated.

The separate strains for inocula were aseptically dispersed by shaking with three glass beads for one minute by a previously described method. Inoculation was then made by the use of the special Pasteur pipettes described in the section entitled "Materials & Methods". One drop of the 24

hour old culture for each strain, was added carefully to the top of duplicate tubes of this broth in sufficient numbers for the observations desired. After inoculation, the culture strains were examined for purity by previously described methods utilizing blood-agar plates. The series of cultures were incubated at the same time at 37°C. in a water bath.

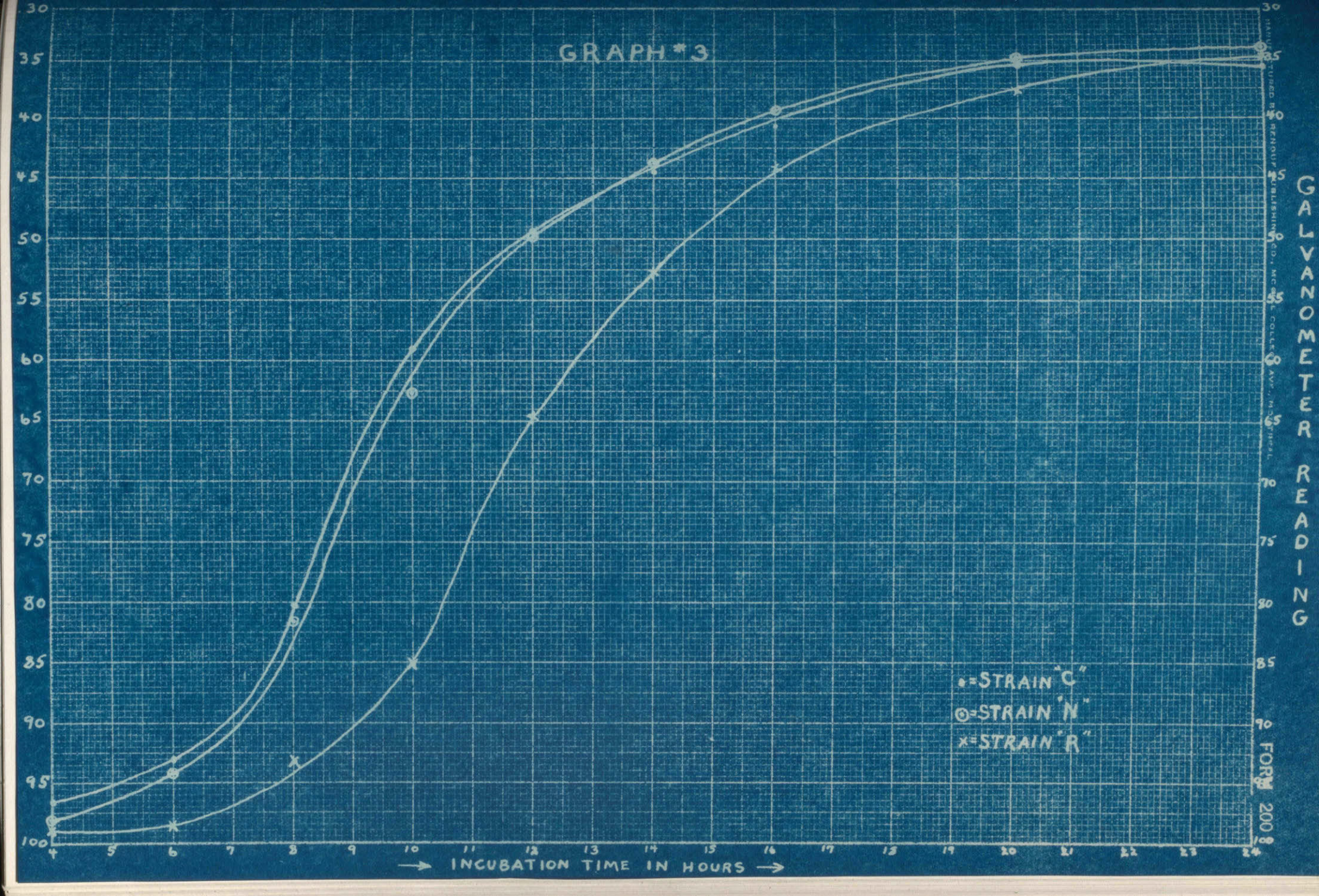
After a predetermined elapsed interval of time, duplicate tubes of broth culture for each strain were taken from the water-bath. The cultures were shaken with glass beads, for purposes of dispersal by previously described methods, and the duplicate cultures mixed in each case. Ten ml. were extracted and added to a chemically clean colorimeter tube, and the amount of growth measured turbidimetrically and recorded. The colorimeter tubes were then emptied and placed in a solution of fresh chromic acid, washed and dried in a hot air oven in preparation for the next measurement.

Results of Experiment VII.

The growth rates of the three strains are provided in Table V, and pictorially presented on Graph #3.

Discussion of Experiment VII.

A slightly greater lag phase is apparent with the "N" strain than that with the "C" strain. However, during the logarithmic phase of growth, the "N" strain exceeds the rate of growth by the "C" strain. The "R" strain possesses a decided and prolonged lag phase, which is most apparent at ten hours of incubation. Its growth rate then speeds up between twelve to fourteen hours of incubation, and after twenty hours


GRAPH #3
GALVANOMETER READING
INCUBATION TIME IN HOURS
•=STRAIN "C"
⊙=STRAIN "N"
×=STRAIN "R"
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
4
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24

TABLE V.

Elapsed Time in Hours	Strain "C"	Strain "N"	Strain "R"
4	96^{2}	98^{0}	99^{0}
6	93^{0}	94^{0}	98^{2}
8	80^{1}	81^{2}	93^{1}
10	59^{1}	62^{3}	85^{1}
12	49^{2}	49^{3}	64^{3}
14	44^{2}	43^{3}	52^{3}
16	40^{3}	39^{1}	44^{1}
20	35^{1}	34^{3}	37^{3}
24	35^{2}	33^{3}	34^{3}

Legend:-

The values for each strain are expressed in units recorded from the galvanometer of the Evelyn Colorimeter.

of incubation, the three strains are fairly even in their populations.

With the use of this "R" strain in future measurements for relative hyaluronidase production, in consideration of the population levels during incubation periods, it was decided to extend the period of incubation time from 18 to 21 hours. Through population levels of the three strains being closer, transposition of values obtained would entail less accumulative error when the percent of relative adaptive activity was expressed in terms of the same population level.

VIII. "Hyaluronidase Production Levels of the Three Strains in the Absence of Penicillin."

This experiment was designed with the knowledge that the

differences obtained with the differential and test media were very slight, if at all. It was desired to know if the capacity for hyaluronidase production of the "N" strain was enhanced through the passage of this strain in the specific adaptive substrate for its enzyme, and also what effect the acquisition of induced penicillin resistance had on the enzyme production of the "R" strain with respect to the parent strain "C".

Method of Conduction

This experiment was conducted on two different dates in exactly the same manner.

Duplicate tubes of "assay broth" and "beef-extract broth" were inoculated for each of the strains "R", "N" and "C", in exactly the same manner, as previously described in Experiment IV, from 18 hour incubation cultures of the respective strains in glucose beef-infusion broth. As no penicillin was added, 0.5 ml. of 0.85% sterile saline was added to each tube. Therefore, nutrient conditions were the same in each case as the control culture of Experiment IV. Incubation was conducted for a period of 21 hours.

Bacterial population measurement, enzyme activity measurement, and the values obtained were expressed in terms of relative percent difference in the amount of enzyme found in the medium, according to the methods described in Experiment IV. Bacterial populations have been brought to the same value, through selection of the strain showing the largest amount of growth, and this factor of difference applied to the enzyme values obtained. With this common base line, relative percent

differences in enzyme activity value were readily obtained. The relative percent differences obtained from the two experiments were then averaged, and the results are provided below. The term "amount of enzyme produced" is used with the understanding that it represents the amount of enzyme detectable in the filtrate of the media, and that hyaluronidase is described as an extracellular enzyme.

Results of Experiment VIII.

TABLE VI.

Percent Enzyme Produced in Beef-Extract Broth Relative to Strain "N" as 100%.

Strain "C" produces 12.85% more enzyme than strain "N".

Strain "R" " 17.20% less " " " " .

The fundamental ability to produce hyaluronidase by the strain "C", or parent strain, apparently is greater than either the "N" or the "R" strain.

TABLE VII.

Percent Enzyme Produced in Hyaluronic Acid Broth Relative to Strain "N" as 100%.

Strain "C" produces 133.45% more hyaluronidase than strain "N".

Strain "R" " 56.20% " " " " " .

The production of hyaluronidase in the broth containing hyaluronic acid demonstrates that a larger amount of adaptive enzyme is produced per cell by strain "C", than by the "N" or "R" strain. Therefore, apparently adaptive production of hyaluronidase by a strain is not enhanced by passage in the enzyme's specific substrate. From the results obtained, the production of hyaluroni-

dase appears to be decreased by the passaging, when the strain is cultured again under normal conditions in the enzyme's adaptive environment.

TABLE VIII.

Percent Adaptive Increase of Hyaluronidase Production by Hyaluronic Acid Addition.

Strain "C" demonstrates an adaptive increase of enzyme production in "assay broth" by 2,859.7% than it produces in beef-extract broth.

Strain "R" demonstrates an increase of 2,323.3%

Strain "N" " " " " 1,331.2%

These relative percent values illustrate the fact, that although, the fundamental ability to produce hyaluronidase without its adaptive substrate by the resistant strain has been lowered, the difference in adaptive increase when hyaluronic acid is added is almost as large as that of the parent strain. The actual amount of enzyme formed per cell, though, because of its lowered fundamental ability to produce it, is much less than that of the parent strain, "C".

Discussion of Experiment VIII.

These changes in adaptive production levels, as well as the change in the fundamental production levels of the individual strains, may possibly be explained on the basis of either altered metabolic activity of the individual strain, or on the basis of altered membrane permeability, or as a coincident result of both of these effects.

The "C" strain, or control parent strain, which has not

been passaged in the hyaluronic acid broth ("assay broth") shows a response by adaptive production of 133.45% more enzyme in relation to that of the "N" strain (Table VII), and a fundamental ability to produce 12.85% more enzyme than the "N" strain without the addition of hyaluronic acid. This reduction in adaptive response and fundamental activity to produce the enzyme by the "N" strain may possibly be interpreted as a change in its adaptive metabolic activity. The "N" strain may have become accustomed to this continued adaptive stimulation, and because of this impression on its adaptive mechanism, the response in enzyme production has been decreased.

The "R" strain, which has been passaged in hyaluronic acid broth the same number of times as the "N" strain, while acquiring adaptation to penicillin, shows by adaptive response, the production of 56.2% more enzyme in relation to that of the "N" strain, but 77.25% less than that of the "C" strain in the same type of medium (Table VII). However, because of its fundamental lessened ability to produce the enzyme, the actual percent increase by adaptive response is 74.5% as much as that of the "C" strain (Table VIII). During the adaptation of "R" strain to penicillin, the passaging of this strain through hyaluronic acid broth has possibly not created as great an impression on its adaptive mechanism as on that of the "N" strain. This may be attributed possibly to the disturbed metabolism created by penicillin adaptation simultaneously, and the adjustment to lessened production was not as smooth as in the case of the "N" strain. Also, due to

the fact that fewer generations were grown in the case of the "R" strain, through penicillin inhibition, adjustment was not possible to as high a degree.

Gale (55) has stated, that "If the rate of breakdown of a substrate is limited by the rate of diffusion of that substrate through the cell membrane, then it follows that apparent variations in enzyme activity may be due to alterations in the permeability of the membrane.". The "N" strain, and to a lesser extent, the "R" strain, through several substrate passagings, may have had the permeability of their bacterial membranes altered in such a fashion, that hyaluronic acid or its hydrolyzed end-products has been decreased and its utilization thereby lessened. This possible partial "substrate block" would account for the decreased adaptive production of hyaluronidase by "N" strain. Through reasons of simultaneous adaptation to penicillin already described, for the "R" strain, relative percent adaptive production of this strain is greater (Table VII) than the "N" strain, but the actual adaptive increase over its lowered fundamental production (Table VII) is about 25% less than that of "C" strain relative to "N" strain (Table VIII).

IX. <u>"The Effect of Sub-Bacteriostatic Concentrations of Penicillin "G" on the Production of Hyaluronidase "In Vitro" by Strains "N" and "R".</u>

An experiment has been previously described, illustrating the inhibiting effect of penicillin "G" on the adaptive production of hyaluronidase by strain "C", or the parent strain. Results have been obtained, that demonstrate passage of this organism through hyaluronic acid has altered its adaptive re-

sponse in the production of hyaluronidase. This result was revealed by strain "N". The penicillin resistant strain "R" has also been altered in this respect to a lesser extent, but its fundamental ability to produce the enzyme has been decreased to greater extent. Therefore, this experiment was designed to see if these two strains reacted in the same manner as the parent strain to progressively inhibiting sub-bacteriostatic levels of penicillin concentration "in vitro", with respect to their adaptive production of hyaluronidase.

Method of Conduction

Several assays were conducted with progressively inhibiting concentrations of penicillin "G" added to the "assay broth" medium, with each strain. Each assay was conducted on one strain only, with its uninhibited control culture, the culture medium of which contained 0.5 ml. of sterile 0.85% saline, instead of the graded dilution of penicillin in saline.

The methods used in each assay were exactly the same as described in Experiment IV, except that the incubation period was for 21 hours duration, for reasons already demonstrated and described in Experiment VII.

The methods of measurement, computation and expression of results in percent relative difference on the same bacterial population concentration, were the same as described and used in Experiment IV.

Results of Experiment IX.

The results are recorded in Table IX and X, and pictorially

presented on Graph #4. It was noted that the percent of inhibition obtained with each strain was a smooth gradation as the concentration of penicillin was increased, indicating that the "R" strain was uniformly susceptible to the relatively higher levels of penicillin concentration. Representative examples of flow-times and computations are provided for each strain under Protocol #5 and #6.

TABLE IX.

% Inhibition of Growth of strain "N" by penicillin "G"	% Increase in Production of Hyaluronidase per Streptococcal cell of "N" strain	% Decrease in Production of Hyaluronidase per streptococcal cell of strain "N"
0.85	30.53	
2.42	38.05	
4.79	75.79	
9.48	79.76	
15.65	137.71	
17.14	160.40	
20.83	195.14	
27.33	200.53	
43.82	116.29	
58.38	75.43	
70.63	40.25	
73.79		8.50
74.33		38.29
87.85		78.14

TABLE X.

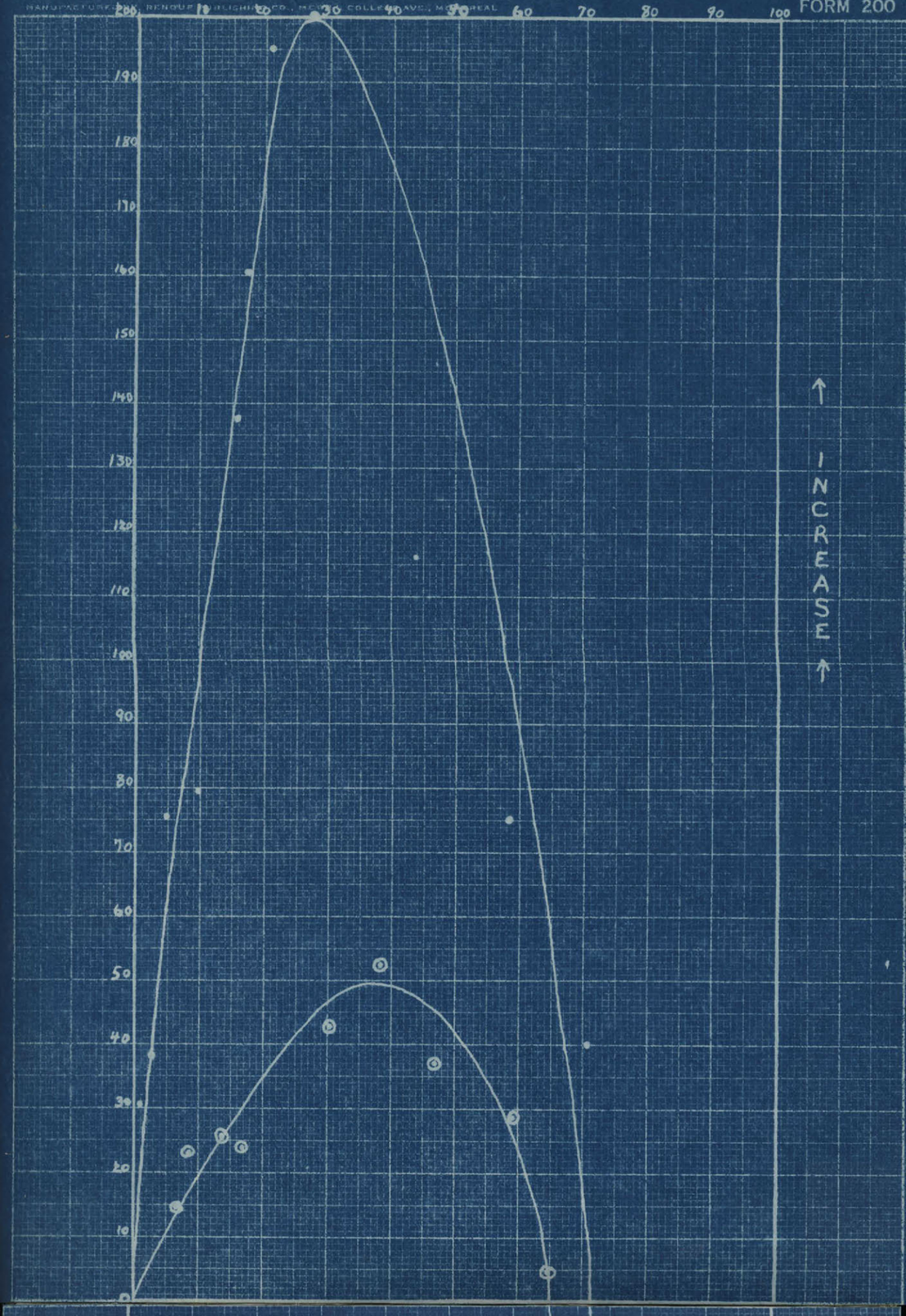
% Inhibition of Growth of strain "R" by penicillin "G"	% Increase in Production of Hyaluronidase per Streptococcal cell of "R" strain	% Decrease in Production of Hyaluronidase per Streptococcal cell of strain "R"
6.72	14.69	
8.52	23.15	
13.49	25.78	
16.73	23.95	
30.31	42.82	
38.32	52.52	
46.84	37.23	
59.27	28.86	
64.62	4.82	
70.45		26.31
81.81		60.59
89.95		83.43

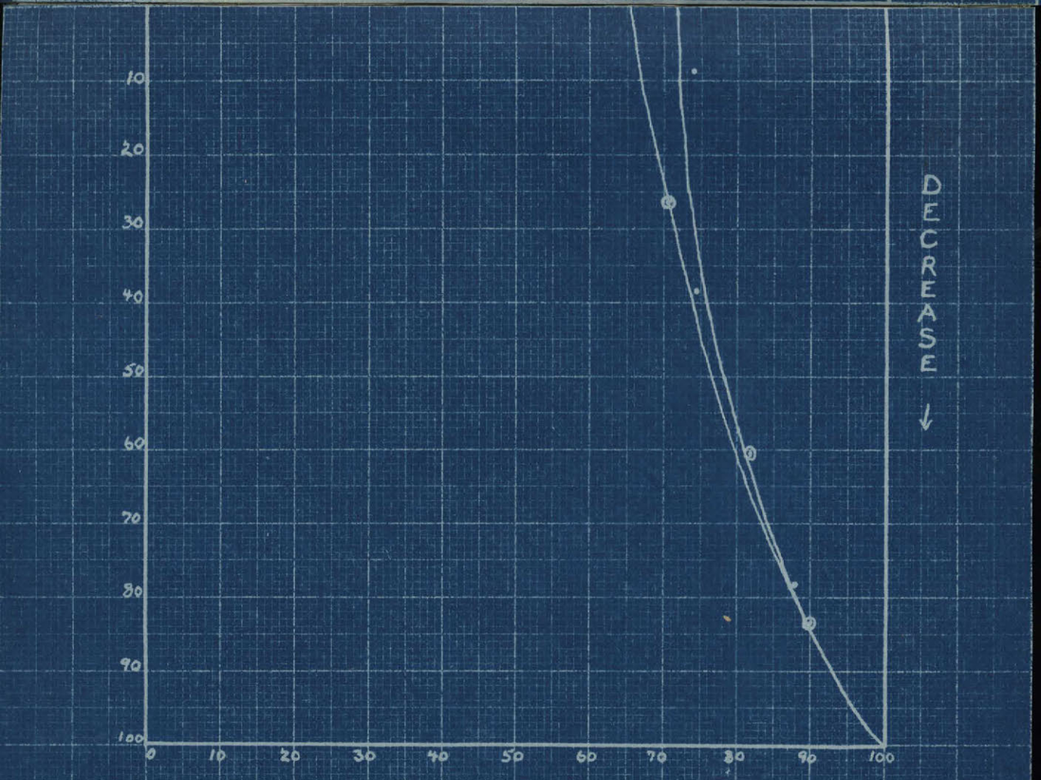
From the tables or the graph, it may be readily observed, that even a very slight degree of inhibition produces an immediate increase in the production of enzyme. At the stage of about 65% to 75% inhibition, the amount of enzyme produced per cell, irrespective of the strain, is about the same until complete inhibition is reached.

Discussion of Experiment IX.

Unlike the parent strain, which demonstrates an immediate decrease in enzyme production, both the "R" and "N" strain demonstrates a relative increase in their production, compared to their uninhibited controls, until 65% to 75% inhibition was reached, when they both demonstrate a decrease in production.

GRAPH #4





● = STRAIN "N"

◎ = STRAIN "R"

ABSCISSA :- PERCENT INHIBITION OF GROWTH BY PENICILLIN.

ORDINATE :- PERCENT LIBERATION OF HYALURONIDASE PER CELL.

This decrease is almost the same for each strain, until 100% inhibition is reached.

The results of this experiment apparently indicate the possibility that passaging these strains in the hyaluronic acid broth promoted a change in their metabolism of this compound, and in the membrane permeability of the "N" strain to this substance.

As stated by Gale (55) and Deere (29-A), (29-B, certain antiseptics and drying solutions will render a cell membrane that is impermeable to a substrate to a permeable state through the injury, so that the cell displays normal activity to the substrate. As related in the "Historical Review", numerous investigators have definitely demonstrated various alterations of the bacterial cell membrane through the action of penicillin upon it. If a partial alteration has occurred in the cell membrane of strain "N", such that its permeability to hyaluronic acid or its hydrolyzed end-products have been partially inhibited, this alteration apparently is progressively and quickly removed by the presence of small concentrations of penicillin so that its adaptive enzyme production is rapidly restored and increased.

As observed in the previous experiment, the relative adaptive response of the "N" strain is 135% less than the adaptive response of the "C" strain. From Graph #2, it may be seen that the adaptive response is approximately 95% higher at 30% inhibition by penicillin, when the 135% difference in basic adaptive response is removed. These percent values are relative to the uninhibited controls of the two

strains in question. It has been demonstrated and suggested (28), (176) that sub-bacteriostatic concentrations of penicillin enhance metabolic activity with an attendant high rate of respiration. Although no increase in adaptive production was demonstrated with the parent strain, because it was apparently at its highest degree of adaptive response, the presence of penicillin may have stimulated the adaptive mechanism of the "N" strain. This adaptive mechanism was already in an enhanced state, and was made apparent once the altered permeability was removed. Therefore, hyaluronic acid passaging of the "N" strain may have enhanced the capacity of the adaptive mechanism and was not evident until the alteration in permeability of the membrane was removed.

In the disruption of the bacterial cell membrane that has been proposed by the action of penicillin, the effect may have produced a disorganization of the already substrate altered membrane. All or part of the previously mentioned 95% relative increase in enzyme production may have been intracellular hyaluronidase.

The "R" strain apparently reacts to a lesser degree than the "N" strain. The impression created by substrate passaging is evident, but not as strongly impressed. Fewer generations of the "R" strain were adapted to the hyaluronic acid, and the metabolic alteration was less in the adaptive response. This result was apparent from the results of the previous experiment. Although it possessed a fundamental lower capacity to produce the enzyme, its relative adaptive response per cell was 56.2% higher than the "N" strain, on

the basis of equal fundamental enzyme producing capacities. The adaptive response over its beef-extract broth production is 74.5% higher than the "N" strain in the absence of penicillin (Table VIII).

The similarity at the point of degree of inhibition that the decrease in enzyme production begins on the graph, is suggestive that possibly the same metabolic effect was conferred on the "R" strain as has been described for the "N" strain because of hyaluronic acid passage. However, the degree of alteration was to a lesser extent owing to lowered proliferative and metabolic functional powers through simultaneous acquisition of penicillin resistance of a low order.

Although the "R" strain possesses approximately 80% of the relative adaptive capacity of "C" strain, in adaptive response over its fundamental production (Table VIII), its actual relative potency in enzyme activity per cell in relation to "N" strain, is 77.25% less than that of "C" strain (Table VII). From Graph #2, it may be seen that the largest spread in activity occurs at approximately 40% inhibition of growth, where there is a difference of 100% in enzyme activity. From this, it appears that the adaptive response of "R" strain is approximately 23% higher at 40% inhibition by penicillin than the "C" strain when the 77.25% in basic adaptive response is removed. Again, these percent values are relative to the uninhibited controls of the two strains in question. Compared to the "N" strain at 30% inhibition, previously shown to be 95% higher than the "C" strain, this illustrates that the "R"

strain's highest adaptive response in the presence of penicillin is about one quarter of that of the "N" strain's highest adaptive response under similar conditions. This illustrates the possible fact that the "R" strain metabolically reacted similarly to hyaluronic acid passage as the "N" strain did, but owing to lowered metabolic functioning and viability through acquisition of resistance to penicillin, the response to enzyme production in the presence of penicillin is approximately one quarter that of the "N" strain at their respective peaks of relative adaptive response, when computed against their corresponding respective inhibition value obtained from the parent strain "C".

It appears at this point, from the observations obtained, that there was a strong possibility of a partial alteration in the permeability of the membrane of the "N" strain to the hyaluronic acid or its hydrolyzed end-products, so that its assimilation was reduced, and the adaptive response was of necessity lowered. Owing to the disruptive powers of penicillin (Historical Review) upon bacterial cell membranes, this alteration was then removed, or else disrupted, and assimilation of the substrate for total adaptive response was regained.

The alteration to the hyaluronic acid did not appear to occur in the membrane of the "R" strain, since its adaptive response (Table VIII) in the absence of penicillin is almost the same as the parent strain "C". It is possible that its adaptive response was lowered because of the lowered viability of its metabolism when acquisition of induced pen-

icillin resistance was obtained.

The actual increase in adaptive response by the "N" strain above the parent strain "C", may be through enhancement in adaptive capacity, because of the passagings in the hyaluronic acid. This enhancement in adaptive capacity was not evident until the alteration in permeability was removed so that proper assimilation and utilization of the adaptive substrate could be made. This enhanced capacity, though, may be a coincident effect with the presence of penicillin on the metabolism of the organism. The adaptive response in hyaluronidase production may have been increased through stimulation brought about by the effect of penicillin upon an adaptive mechanism that has been enhanced in its function through the "training" it has received.

The actual increase in adaptive response of the "R" strain above the parent strain "C", through the reaction to the presence of penicillin, does not reach as high a peak as the "N" strain, and is about one quarter of it. Less possible "training" of the adaptive mechanism was secured, since fewer generations were exposed to the substrate, and was coincident with the simultaneous reorganization of the cell to the new penicillin adaptation.

However, all or part of the relative increase observed over the uninhibited control culture may be due to the measurement of the intracellular content of enzyme, or is a coincident effect with the previously described stimulation of an enhanced adaptive mechanism. The majority of investigators agree that the effect of penicillin does not promote lysis of the normal

streptococcal cell (87), (106). The reorganization of the "R" strain in the presence of penicillin while acquiring induced resistance, may have altered the permeability of the membrane to the intracellular enzyme and other intracellular contents, thereby promoting the loss of the intracellular enzymic contents. The same effect may have taken place with the "N" strain, coincident with the disruption in removal of the altered permeability to hyaluronic acid or its hydrolyzed end-products. Altered fundamental production of the "R" strain would demonstrate the difference in enzymic adaptive production increase between the two levels of hyaluronidase liberation.

The organisms comprising a bacterial population demonstrate varied degrees of resistance to the action of penicillin (11), (107), (202). Because of this, as the concentration of penicillin is increased, a correspondingly larger number of the organisms in the population have had the intracellular enzyme released to the medium. This was not evident with the parent strain "C", since it demonstrated an immediate decrease in production of the enzyme. This strain had suffered no reorganization to penicillin by becoming resistant, and had not incurred a possible adaptation to the hyaluronic acid, providing a reorientation of the cell membrane that was removed in the presence of penicillin.

X. "Rapid Transfer of Variant Strains in the Absence of Penicillin and Hyaluronic Acid."

This experiment was designed in an effort to see whether the altered enzyme production by the hyaluronic acid passaged strains was a strongly impressed factor, or was easily lost.

It was also designed to see whether rapid transfer in the absence of penicillin would create a return of the penicillin resistant strain to the original sensitivity of the parent strain.

Method of Conduction

From the refrigerated stock cultures on blood-agar plates, separate tubes of glucose beef-infusion broth were inoculated with the "R", "N" and "C" strains of Streptococcus pyogenes P315 A-4, and incubated for 18 hours at 37°C. These cultures were used as inoculum for a penicillin assay of the sensitivity of each strain. This assay was conducted according to the methods already described in the section entitled "Materials & Methods", with the use of a separate, cotton-plugged, sterile, chemically clean pipette for each dilution.

The "R" and "N" strains were then inoculated between 8.00 and 9.00 a.m. into plain beef-infusion broth and incubated at 37°C. At 5.00 p.m. to 6.00 p.m. of the same day, a large loopful of each culture was inoculated onto the surface of a separate blood-agar plate, and properly streaked over the surface. These inoculated plates were then incubated overnight at 37°C. On the following morning, the procedure was repeated. This method of alternate rapid transfer afforded an easy automatic check on the purity of each strain. After twenty-five consecutive transfers in broth and the same number on blood-agar plates, an assay for penicillin sensitivity was conducted for each strain in duplicate in the manner already described, using the known sensitivity of the parent strain as a control.

Each strain was then inoculated into glucose beef-infusion broth and incubated for 18 hours at 37 C. Predetermined dilutions of crystalline sodium penicillin "G" were made in chilled, sterile 0.85% saline in such a concentration as to afford approximately 40% inhibition of growth in "assay broth" for each strain. Inoculations were made in the usual manner already described, and incubation was conducted at 37°C. for 21 hours. The usual control culture was made in duplicate for each strain, as well as two uninoculated broth controls for colorimeter blanks.

After incubation, prior to the viscosity-reducing assay, Gram-stained smears were made of each strain, both from the uninhibited control cultures and the penicillin inhibited cultures. Blood-agar plates were streaked with an inoculum from each strain, and examined after incubation at 37°C. for 24 hours.

The method of viscosity-reducing assay, measurement of bacterial populations, and details of expressing enzyme activity and relative percent values were according to the method described in Experiment IV.

Results of Experiment X.

The results of both penicillin assays for strain sensitivity were exactly the same, and had not changed. The results were the same as obtained in Experiment V, as shown in Table III, Series 1, 2 and 3.

The morphological characteristics of the colony and cell appearance of the three strains were unchanged from the re-

sults formerly obtained, as described in Experiment VI.

The results of the viscosity-reducing assay provided the following results:

Strain "R":- At 39.46% inhibition of growth by penicillin "G", there was a relative adaptive increase of 13.39% from the normal uninhibited control culture in the production of hyaluronidase per cell.

Strain "N":- At 40.34% inhibition of growth by penicillin "G", there was a relative adaptive increase of 218.36% from the normal uninhibited control culture in the production of hyaluronidase per cell.

Discussion of Experiment X.

After fifty rapid transfers in media containing no penicillin or added hyaluronic acid, it was seen that the resistand strain had made no attempt to return to its original sensitivity. The induced resistance had taken several transfers in broth and agar containing penicillin before it was secured at this resistance, and this penicillin adaptation may be "fixed" (104) because of its maintenance in the presence of the antibiotic for a long time. Other investigators have found penicillin resistance that was acquired "in vitro" to be of a permanent nature. (138, (139).

Owing to the lack of time, more subculturing and testing could not be conducted.

Although it is difficult to comment on the results of but one viscosity-reducing assay, the results indicate that the

"N" strain has not changed in its relative adaptive response in the presence of inhibiting sub-bacteriostatic concentrations of penicillin. This would indicate that the alteration conferred by passage in hyaluronic acid broth appears to be a strongly impressed alteration that is not removed in fifty consecutive passages in the absence of the added compound.

It appears that the alteration conferred by hyaluronic acid passage on the "R" strain has not been as strongly conferred as that on the "N" strain, as a reversion has commenced towards natural relative adaptive response as indicated by the parent strain. This is to be expected, as fewer generations were proliferated in the presence of the hyaluronic acid, and as a simultaneous adaptation took place, the adaptation to hyaluronic acid was not as orderly.

GENERAL DISCUSSION

Throughout the report here presented, there were brief discussions given of the conclusions that could be drawn from the respective experimental findings.

Some of the experimental results here reported confirmed findings previously reported by other investigators. The strain of Streptococcus pyogenes P315 A-4 that acquired induced penicillin resistance, presented morphological and biological characteristics typical of induced resistance, and confirmed the observations of other investigators (62), (197), (196). The acquired penicillin resistance, once acquired, appeared to be a permanent change (104), (138), (139). The penicillin resistant strain has been shown to grow more slowly than the parent strain, possessing a prolongation of the lag phase (8), (197), (162), (190).

The experimental results here reported, have demonstrated that partially purified hyaluronidase, in its more susceptible state (186), suffers no inhibition by the presence of large concentrations of penicillin. However, small inhibiting concentrations of penicillin that decrease the amount of bacterial growth, produce an immediate, altered response to the liberation of this enzyme into its medium. As the degree of inhibition of growth is heightened by larger concentrations of penicillin, there is a progressive reduction in the production of hyaluronidase from the normal per cell, as determined by percent relative adaptive response on the basis of equal bacterial populations. The literature to date has not

been found to contain these findings. As reported in the "Historical Review", sulfa compounds do not appear to affect the depolymerizing action of hyaluronidase or its bacterial production (6), (136), (72). However, the anti-bacterial mode of action of these two chemotherapeutic agents is not similar. As previously stated, inhibition of streptococcal fibrinolysin and its production by sub-bacteriostatic concentrations has been reported (3), (163). From the results obtained "in vitro", it may be possible, that a hyaluronidase-producing organism "in vivo" would be reduced in its ability to spread in tissues when subjected to sub-bacteriostatic levels of penicillin.

It has been demonstrated in this report, that the productive capacity of this strain of Streptococcus pyogenes to produce hyaluronidase could not be enhanced by repeated subculturing in a specific adaptive substrate for the enzyme's production while the strain was in its normal environment. This confirms McClean's work (132). However, with the conditions used, an actual reduction in adaptive response was recorded for this variant strain.

When hyaluronic acid was used as the major source of carbohydrate, it appeared that the assimilation of this polysaccharide was reduced by a possible alteration in cell membrane permeability to this substance, thereby reducing the degree in adaptive formation of the enzyme. The fundamental ability of this strain to form the enzyme in the absence of added polysaccharide was slightly lowered. The same organism, when passaged in this polysaccharide as the source

of carbohydrate along with the presence of penicillin, suffered a small reduction in its adaptive response, but a reduction of approximately twice as much as the other passaged strain in its fundamental ability to produce the enzyme. This lowered fundamental ability of the penicillin resistant strain to produce hyaluronidase is about 30% less than the parent strain (Table VI). Since lowered metabolic activity is expected in a resistant strain, and as its adaptive response is 80% of normal (Table VIII) in the absence of penicillin, as compared to the parent strain, it appears that penicillin resistance of the order obtained is not metabolically tied up with this adaptive enzyme system.

In the presence of inhibitive sub-bacteriostatic concentrations of penicillin, the proposed alteration in membrane permeability of the non-penicillin resistant variant strain was removed or disrupted, and the relative adaptive response in enzyme production returned, together with an increase in the amount of adaptive enzyme produced per cell. A smaller increase in adaptive response was recorded for the strain that had been induced to penicillin resistance, when in the presence of penicillin.

The actual increase in adaptive response by the non-penicillin resistant hyaluronic acid passaged strain may be through enhancement in its adaptive production capacity, but was not evident until optimum assimilation of the polysaccharide was afforded by the presence of penicillin. No increase in adaptive response was seen by the penicillin resistant strain, when in the absence of penicillin, or in its

adaptive capacity. However, in the presence of penicillin, an increase in adaptive response was recorded to the extent of one-fourth that of the non-penicillin resistant hyaluronic acid passaged strain.

It appears possible then, that penicillin exerts a stimulatory action upon an enhanced mechanism at certain concentrations. Sub-bacteriostatic concentrations of penicillin have been shown to enhance metabolic activity by some investigators (28), (176). This stimulatory action is not evident upon the parent strain, as its adaptive mechanism has not been enhanced in its response by passaging in the hyaluronic acid.

Attention has been drawn previously to the fact, that this proposed stimulatory action by penicillin action on the variant strains "N" and "R", may be directed at the permeability of the cell's membrane, rather than at the metabolic function of the adaptive mechanism. Increase in the liberation of hyaluronidase in the presence of penicillin by strain "R" may be through the loss of intracellular enzyme into its environment through the promotion of a destructive action on an already reorganized membrane obtained by the acquisition of penicillin resistance. Disruption of an altered permeability to hyaluronic acid or its hydrolyzed end-products by penicillin with the "N" strain, may have rendered the membrane in a state incapable of retaining its intracellular hyaluronidase content.

It has been demonstrated by both passaged variant strains, that when the concentration of penicillin passes a certain level, a rapid decrease in adaptive production of hyaluronidase

occurs. This is to be expected, as the viability of the bacterial cell is progressively decreased as the concentrations of the antibiotic are increased.

After several subcultures in media containing no added hyaluronic acid or penicillin, the penicillin resistant strain had lost none of its resistance to the antibiotic. This strain however, produced a lower relative adaptive response in the presence of pemicillin than it did formerly. A reversion to the parent strain was indicated. No reversion was apparent in the case of the non-penicillin resistant strain under similar circumstances, indicating that strong impressions had been created by the passaging in hyaluronic acid broth.

It is improbable that variants such as these two hyaluronic acid passaged strains would arise under "in vivo" conditions. If such variants did arise through conditions of prolonged harboring of the organism, or through acquired penicillin fastness, the "in vitro" results would indicate, that under normal conditions, their ability to invade tissues would be reduced. However, if small sub-bacteriostatic levels of penicillin were introduced, their ability to liberate hyaluronidase into their environment would be enhanced to a higher level than that of the parent strain. This would cause an increased ability for the individual organism to spread through the tissues.

SUMMARY AND CONCLUSIONS

The following conclusions were arrived at from the observations of the experimental work here reported, as obtained from the strain of beta-hemolytic streptococcus used:

1. The methods used in the extraction and purification of hyaluronic acid produced a relatively pure salt of hyaluronate.
2. The methods used for partial purification and concentration of streptococcal hyaluronidase were demonstrated to be convenient and comparatively non-destructive insofar as could be determined on a yield basis and by its typical viscosity-reducing activity. In the partially purified state, after lyophilization, it was found to be stable for at least ten months.
3. Streptococcal hyaluronidase has been demonstrated not to be adsorbed on a fractional basis by adsorptive asbestos pads.
4. It has been found comparatively difficult to induce acquired penicillin resistance to a penicillin susceptible strain of beta-hemolytic streptococcus, and when it is acquired, it is of a low order and appears to be permanent in nature.
5. An induced penicillin resistance to a strain of beta-hemolytic streptococcus produces cellular changes.
6. When a strain of beta-hemolytic streptococcus is subcultured many times in a nutrient broth containing added hyaluronic acid, cellular changes and chain length of the variant strain are altered.

7. Crystalline sodium penicillin "G" produces no inhibiting effect upon the depolymerization of hyaluronic acid by streptococcal hyaluronidase under "in vitro" conditions.
8. As the sub-bacteriostatic concentration of crystalline sodium penicillin "G" is increased, a progressively increasing inhibitory action is produced upon the amount of hyaluronidase liberated into the medium by the organism's adaptive response during its metabolism, as determined by percent relative adaptive response on the basis of equal bacterial populations.
9. From the observations recorded from experimental work, it appears that the acquisition of induced penicillin resistance is not actively associated with the metabolism of this adaptive enzyme system.
10. When a hyaluronidase-producing strain of beta-hemolytic streptococcus is subcultured many times in a broth containing added hyaluronic acid, the amount of hyaluronidase produced by adaptive response is greatly reduced as compared to the relative amount produced by the parent strain. When a similar hyaluronidase-producing strain derived from the same parent culture is subcultured in the same manner, but in the presence of sub-bacteriostatic concentrations of penicillin, the adaptive response in hyaluronidase production is only slightly reduced as compared to the relative amount produced by the parent strain.

11. According to the results obtained, when a hyaluronidase-producing beta-hemolytic streptococcus is subcultured in nutrient broth containing added hyaluronic acid, an alteration in permeability of the bacterial cell membrane is caused, thereby permitting a reduced assimilation of hyaluronic acid or its hydrolyzed end-products. This promotes a lowered adaptive response in hyaluronidase production. When small, inhibitive sub-bacteriostatic concentrations of penicillin up to a certain level are added to this culture, the relative adaptive response is regained to a level higher than that of the parent strain. This appeared probably due to the disrupting effect of penicillin on the membrane, permitting normal assimilation of hyaluronic acid or its hydrolyzed end-products to be regained. Since an increase in the relative adaptive response in enzyme liberation was obtained higher than that of the parent strain, it appeared that penicillin over a certain range of concentration, produced a stimulatory effect upon an adaptive enzyme system that had been enhanced in its adaptive capacity through subculturing in its specific substrate, or was a result of liberation of the intracellular content of the enzyme.
12. It appeared that no alteration in membrane permeability occurred with a hyaluronic acid passaged strain that was induced to simultaneous penicillin resistance, with respect to hyaluronic acid or its hydrolyzed end-products.

Lowered metabolic activity was produced through the acquisition of penicillin adaptation with this strain. A similar effect was produced on this strain's adaptive response by the presence of penicillin over a certain range of concentration, but it was at a much reduced level as compared to the non-penicillin resistant hyaluronic acid passaged strain.

13. The penicillin resistance of the resistant strain appeared to be of a permanent nature.

14. After several subcultures in media containing no added hyaluronic acid or penicillin, the non-penicillin resistant strain produced the same level of relative adaptive response in hyaluronidase liberation in the presence of penicillin as it did prior to its subculturing. The penicillin resistant strain produced a lower relative adaptive response in the presence of penicillin than it did formerly. A reversion to the parent strain was indicated.

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Protocol #1.

Solvent Control:-
#2 Viscosimeter(Factor 1.084)

Flow-Times:-

#1.	86.9 secs.
#2.	87.0
#3.	87.0
#4.	86.8
#5.	87.0
#6.	86.9
#7.	87.0
#8.	86.8
#9.	87.0
#10.	87.0

Average = 86.94 X 1.084
= 94.25296 secs.

Substrate Control:-
#3 Viscosimeter(Factor 1.348)

Flow-Times:-

#1.	4 min. 27.0 secs.
#2.	4 min. 26.1
#3.	4 min. 25.1
#4.	4 min. 26.1
#5.	4 min. 27.0
#6.	4 min. 25.1

Average = 266.07 X 1.348
= 358.66236 secs.

Enzyme / Heated Penicillin:-
#1 Viscosimeter(No Factor)

Reaction Time	Flow-Times
#1. 3'50"	3'50.8"
#2. 7'50"	3'12.2"
#3. 12'15"	2'51.1"
#4. 16'16"	2'38.4"
#5. 20'10"	2'32.2"
#6. 23'53"	2'25.6"
#7. 32' 6"	2'17.2"
#8. 39'38"	2'11.4"
#9. 42'58"	2' 9.3"
#10. 47'28"	2' 6.8"

Enzyme / Active Penicillin:-
#4 Viscosimeter(Factor 1.0157)

Reaction Time	Flow-Times
#1. 3'57"	3'46.0"
#2. 8'10"	3' 6.8"
#3. 12'45"	2'47.0"
#4. 17' 8"	2'35.3"
#5. 21'23"	2'27.3"
#6. 24'18"	2'23.2"
#7. 36' 2"	2'12.7"
#8. 39'50"	2'10.7"
#9. 43'42"	2' 8.8"
#10. 54'28"	2' 3.5"

Solvent Control:- 94.25296 secs.

Substrate Control:- 358.66236 secs.

Relative Viscosity:- $\frac{358.66236}{94.25} \times 100 = 380.54$

Enzyme / Heated Penicillin:- #1 Viscosimeter; No Factor.

	Reaction Time	Relative Viscosity
#1.	345.4 secs.	244.8
#2.	566.1	203.9
#3.	820.5	181.5
#4.	1055.2	168.06
#5.	1286.1	160.4
#6.	1505.8	154.4
#7.	1994.6	145.5
#8.	2443.7	139.4
#9.	2642.6	137.2
#10.	2911.4	134.5

Enzyme / Active Penicillin:- #4 Viscosimeter; Factor = 1.0157.

	Reaction Time	Relative Viscosity
#1.	351.7 secs.	243.5
#2.	584.86	201.3
#3.	849.8	179.97
#4.	1106.8	167.36
#5.	1357.8	158.74
#6.	1530.7	154.32
#7.	2229.39	143.00
#8.	2456.37	140.85
#9.	2687.41	138.8
#10.	3330.71	133.09

Legend: Graph #1, Protocol #1.

A - Substrate Control.

B - Half-Life Time.

C - Base-line of Solvents Control = 100.

. - Heat Inactivated Penicillin / Enzyme.

⊙ - Active Penicillin / Enzyme.

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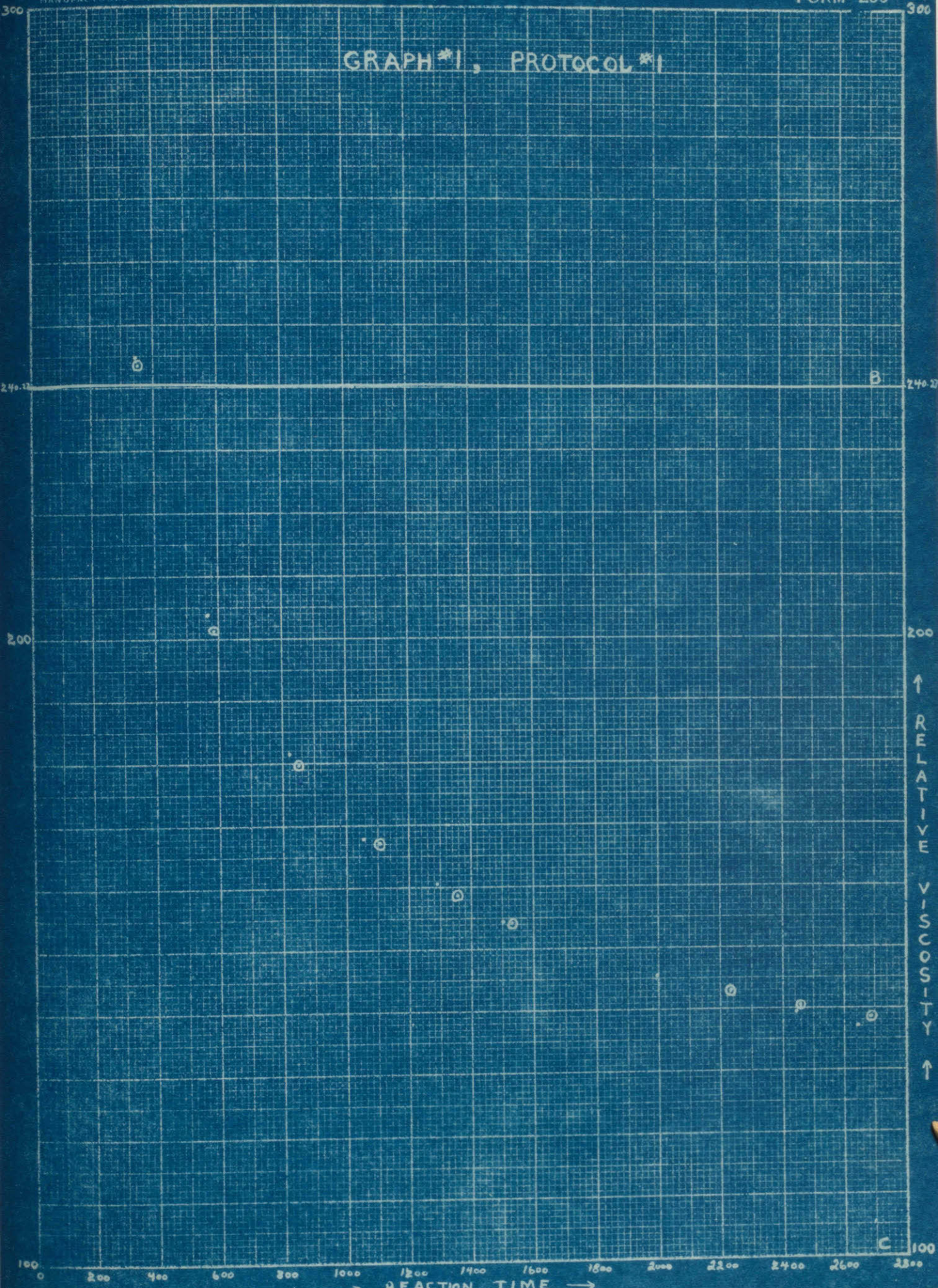
GRAPH #1, PROTOCOL #1



Protocol #2.

Solvent Control:-
#4 Viscosimeter(Factor 1.0157)

Flow-Times:-

#1.	92.8 secs.
#2.	92.6
#3.	92.8
#4.	92.8
#5.	92.7
#6.	92.8
#7.	92.6
#8.	92.8
#9.	92.8
#10.	92.7

Average = 92.74 X1.0157
= 94.196018 secs.

Substrate Control:-
#4 Viscosimeter(Factor 1.0157)

Flow-Times:-

#1.	6 min. 2.1 secs.
#2.	6 min. 2.2
#3.	6 min. 2.0
#4.	6 min. 2.0
#5.	6 min. 2.3
#6.	6 min. 2.1
#7.	6 min. 2.2
#8.	6 min. 1.9
#9.	6 min. 2.2
#10.	6 min. 2.1

Average = 362.11 X 1.0157
= 367.795127

Enzyme / Active Penicillin:-
#4 Viscosimeter(Factor 1.0157)
Dilution = 1/400.

Reaction Time	Flow-Times
#1. 2'28"	4'49.0"
#2. 8'27"	4' 5.7"
#3. 13'27"	3'42.9"
#4. 18'00"	3'25.0"
#5. 22' 9"	3'15.1"
#6. 26'13"	3' 6.4"
#7. 30' 6"	3' 0.3"

Enzyme / Heated Penicillin:-
#4 Viscosimeter(Factor 1.0157)
Dilution = 1/400.

Reaction Time	Flow-Times
#1. 2'29"	4'53.2"
$2. 8'41"	4' 7.7"
#3. 14' 5"	3'42.2"
#4. 18'38"	3'27.3"
#5. 23'12"	3'15.8"
#6. 27'28"	3' 7.9"
#7. 35'36"	2'55.4"

Solvent Control:- 94.196018 secs.

Substrate Control:- 367.795127 secs.

Relative Viscosity:- $\frac{367.795127}{94.19} \times 100 = 390.48$

Enzyme / Heated Penicillin:- #4 Viscosimeter; Factor 1.0157;

Dilution 1/400.

	Reaction Time	Relative Viscosity
#1.	297.90 secs.	316.17
#2.	646.79	267.10
#3.	957.84	239.60
#4.	1223.27	223.54
#5.	1491.43	211.14
#6.	1743.42	202.62
#7.	2225.07	189.14

Enzyme / Active Penicillin:- #4 Viscosimeter; Factor 1.0157;

Dilution 1/400.

	Reaction Time	Relative Viscosity
#1.	294.76	311.64
#2.	631.77	264.95
#3.	920.19	240.36
#4.	1184.1	221.06
#5.	1428.08	210.38
#6.	1667.66	201.00
#7.	1897.56	194.42

Legend of Graph for Protocol #2.

C = Substrate Control.
D = Half-Life Time.
E = Base-line of Solvents Control = 100.
. = Heat Inactivated Penicillin / Enzyme(A).
⊙ = Active Penicillin / Enzyme (B).

Computation:-

A = Heated Penicillin at dilution 1/400;
half-lifetime = 880 secs.

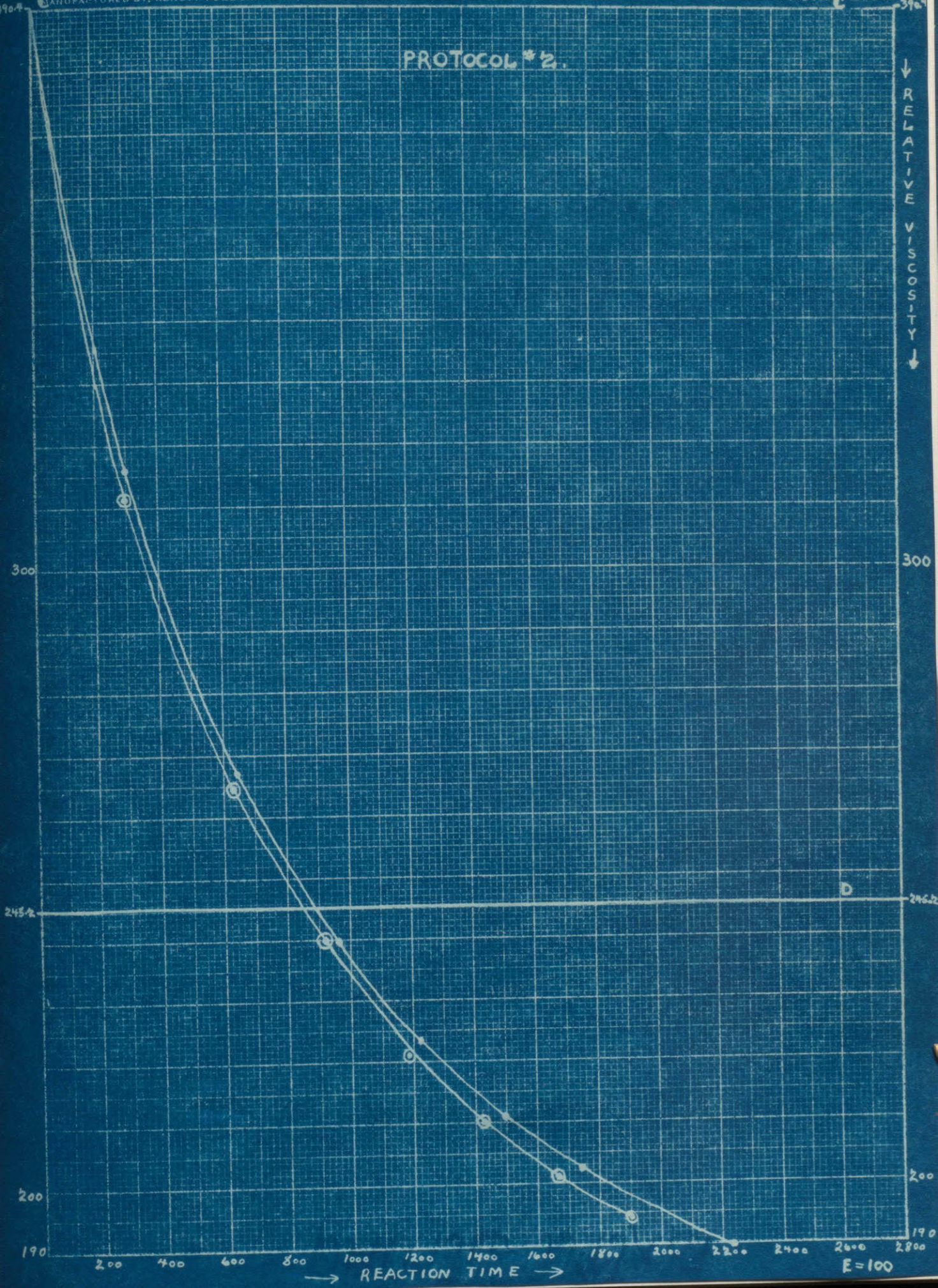
$$A = \frac{D \times 20}{R} = \frac{400 \times 20}{14.66} = 545.70 \text{ V.R.U.}$$

B = Active Penicillin at dilution 1/400;
half-lifetime = 850 secs.

$$B = \frac{D \times 20}{R} = \frac{400 \times 20}{14.16} = 564.97 \text{ V.R.U.}$$

$$B = \frac{564.97}{545.70} \times 100 = 103.53\% - 100.00\% = 3.53\%$$

Therefore B, or the enzyme with the active Penicillin with a concentration of 25 I. U./ml. possesses an activity of 3.53% more than the enzyme possessing the heat-inactivated penicillin.



Enzyme at 9.45 a.m.:- #1 Viscosimeter; No Factor;
Dilution 1/230.

	Reaction Time	Relative Viscosity
#1.	315.2 secs.	352.18
#2.	695.0	311.74
#3.	1075.75	283.83
#4.	1442.25	263.82
#5.	1771.05	248.65
#6.	2066.25	238.54
#7.	2348.25	230.12
#8.	2618.15	223.59
#9.	2875.4	217.79
#10.	3127.95	212.63

Enzyme at 6.45 p.m.:- #1 Viscosimeter; No Factor;
Dilution 1/230.

	Reaction Time	Relative Viscosity
#1.	316.8 secs.	349.23
#2.	754.65	315.21
#3.	1104.15	280.46
#4.	1430.1	263.50
#5.	1744.6	249.81
#6.	2038.7	239.49
#7.	2352.85	231.38
#8.	2633.75	227.74

Legend of Graph for Protocol #3.

A = Substrate Control.
B = Half-Life Time
D = Base-line of Solvents Control = 100.
. = Enzyme at Start - 9.45 a.m.(C).
⊙ = Enzyme at 6.45 p.m.(9 hours old) (E).

Computation:-

"C" = Enzyme at 9.45 a.m.; at dilution 1/230;
half-lifetime = 1,830 secs.

$$\text{"C"} = \frac{D \times 20}{R} = \frac{230 \times 20}{30.5} = 150.81 \text{ V.R.U.}$$

"E" = Enzyme at 6.45 p.m.; at dilution 1/230;
half-lifetime = 1,825 secs.

$$\text{"E"} = \frac{D \times 20}{R} - \frac{230 \times 20}{30.41} = 151.26 \text{ V.R.U.}$$

$$E = \frac{151.26}{150.81} \times 100 = 100.29\% - 100.00\% = 0.29\%$$

Therefore E, or the enzyme held at 5°C. for nine hours demonstrates no loss in potency while held under these conditions in its buffered filtrate.

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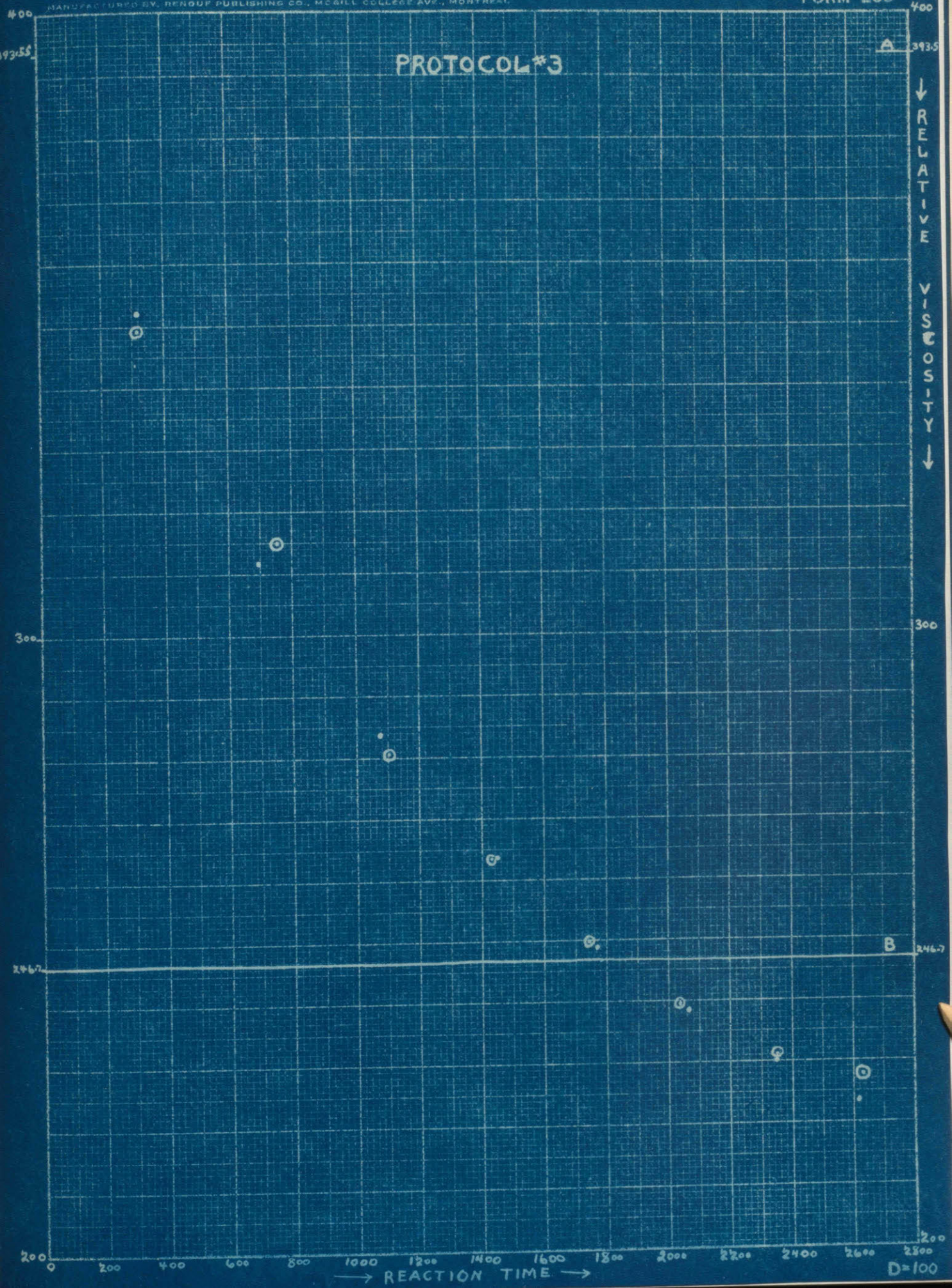

Protocol #4.

Solvent Control:-
#4 Viscosimeter(Factor 1.0157)

Flow Times:-

#1.	94.9 secs.
#2.	94.7
#3.	94.7
#4.	94.8
#5.	94.7
#6.	94.7
#7.	94.8

Average = 94.75 X 1.0157
= 96.23757 secs.

Substrate Control:-
#4 Viscosimeter(Factor 1.0157)

Flow Times:-

#1.	6 min. 7.4 secs.
#2.	6 min. 7.4
#3.	6 min. 7.5
#4.	6 min. 7.4
#5.	6 min. 7.3

Average = 367.4 X 1.0157
= 373.16818 secs.

Enzyme;No Penicillin;Dil'n.1/160.
#1 Viscosimeter(No Factor)

Reaction Time	Flow-Times
#1. 2'52"	5' 9.6"
#2. 9'30"	4'27.2"
#3. 15'00"	4' 3.0"
#4. 20' 8"	3'45.5"
#5. 24'50"	3'33.3"
#6. 29'28"	3'23.6"
#7. 33.55"	3'14.9"
#8. 38' 8"	3' 8.6"
#9. 42'20"	3' 3.3"
#10. 46'30"	2'58.6"

Enzyme;With Penicillin;Dil'n.1/20.
#1 Viscosimeter(No Factor)

Reaction Time	Flow-Times
#1. 2'56"	5'12"
#2. 9'25"	4'34.5"
#3. 15'23"	4'13.2"
#4. 20'50"	3'59.1"
#5. 26' 8"	3'46.4"
#6. 31' 7"	3'36.9"
#7. 35'56"	3'29.1"
#8. 40'37"	3'23.6"
#9. 45'12"	3'18.0"
#10. 49'40"	3'12.6"

Solvent Control:- 96.237575 secs.

Substrate Control:- 373.16818 secs.

Relative Viscosity:- $\frac{373.16818}{96.23} \times 100 = 387.78$

Enzyme; No Penicillin:- #1 Viscosimeter; No Factor;

Dilution 1/160.

Reaction Time	Relative Viscosity
#1. 326.8 secs.	321.72
#2. 703.6	277.66
#3. 1021.5	252.52
#4. 1320.75	234.33
#5. 1596.65	221.65
#6. 1869.8	211.57
#7. 2132.45	202.53
#8. 2382.3	195.98
#9. 2631.65	190.48
#10. 2879.3	185.59

Enzyme; With Penicillin:- #1 Viscosimeter; No Factor;

Dilution 1/20.

Reaction Time	Relative Viscosity
#1. 332.0 secs.	324.22
#2. 702.25	285.25
#3.1049.6	263.11
#4.1369.55	248.46
#5.1681.2	235.26
#6.1975.45	225.39
#7.2260.55	217.29
#8.2538.8	211.57
#9.2811.0	205.75
#10.3076.3	200.14

Legend of Graph for Protocol #4.

A = Substrate Control.

B = Half-Life Time.

E = Base-line of Solvents Control = 100.

. = Enzyme with Penicillin (C).

⊙ = Enzyme with No Penicillin (D).

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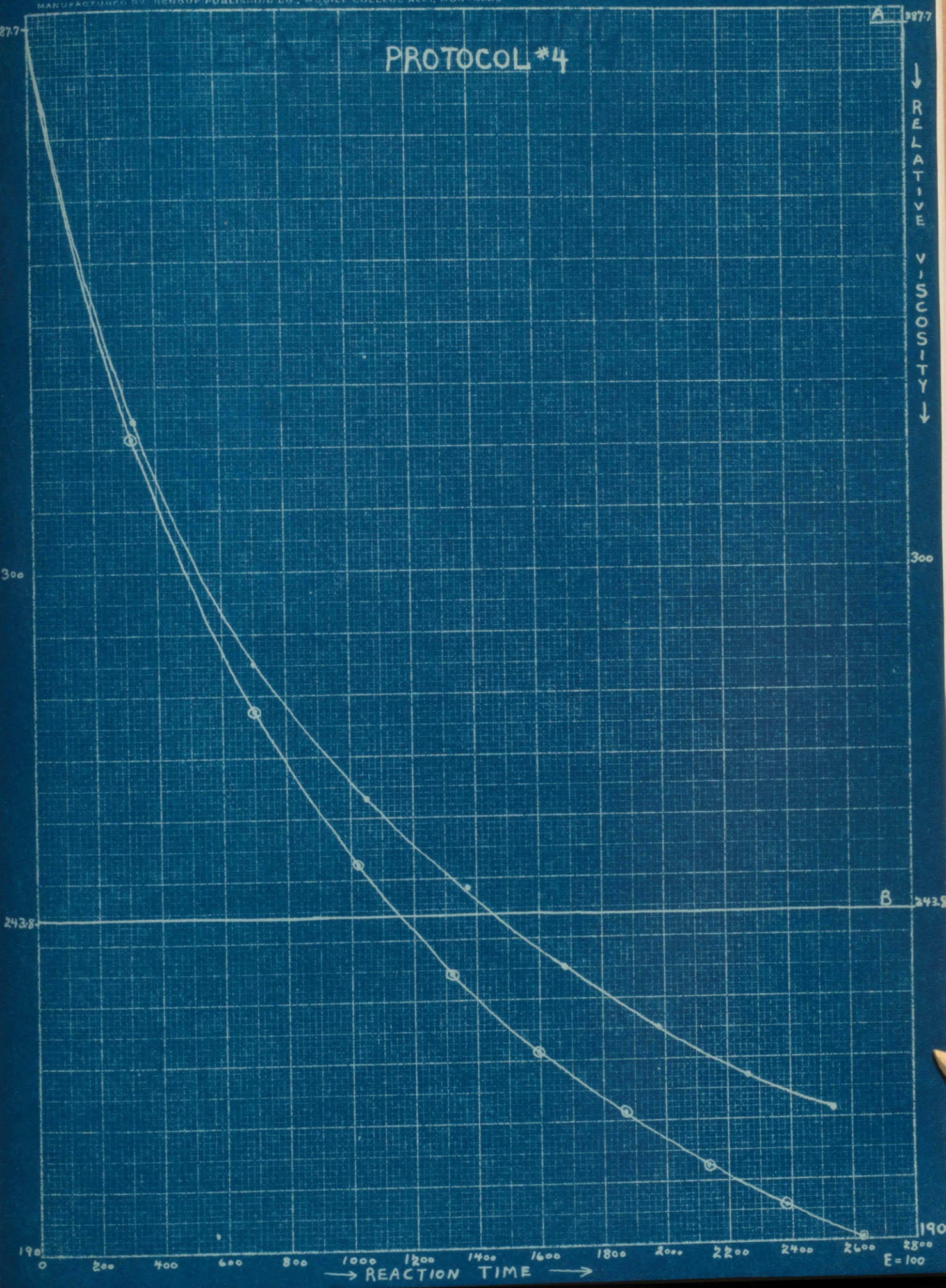
PROTOCOL *4



Computation:-

"C" = Enzyme with Penicillin at dil'n. 1/20;

half-lifetime = 1,465 secs.

$$"C" = \frac{D \times 20}{R} = \frac{20 \times 20}{24.46} = 16.353 \text{ V.R.U.}$$

"D" = Enzyme with No Penicillin at dil'n. 1/160;

half-lifetime = 1,145 secs.

$$"D" = \frac{D \times 20}{R} = \frac{160 \times 20}{19.083} = 167.68 \text{ V.R.U.}$$

Absolute Density Readings Due to Bacteria Alone:-

"C" = .0642 (Percent Transmission = 86^{1})

"D" = .1382 (Percent Transmission = 72^{3})

Therefore, "C" in terms of "D" = $\frac{.1382}{.0642} = 2.1526$

"C" = 2.1526 X 16.353 = 35.2014 V.R.U.

Therefore $\frac{35.2014}{167.68} \times 100 = 20.9932\%$ of normal of hyaluronidase is formed per Streptococcus cell, when such an inhibiting amount of Penicillin is contained in the culture medium, affording an inhibition of =

$$\frac{.0642}{.1382} \times 100 = 46.45\%$$

$$= 100.00\% - 46.45\% = 53.55\%.$$

Therefore, when sufficient penicillin is added to the culture medium to cause 53.55% inhibition of growth, there is a reduction of 79.0068% in the liberation of hyaluronidase per cell of Streptococcus pyogenes P315 A-4.

Protocol #5.

Solvent Control:-
#2 Viscosimeter(Factor 1.2214)

Flow Times:-

#1.	78.9 secs.
#2.	78.9
#3.	79.0
#4.	79.0
#5.	78.9
#6.	79.0
#7.	79.0
#8.	78.9
#9.	79.0
#10.	78.9

Average = 78.95 X 1.2214
= 96.429530 secs.

Substrate Control:-
#4 Viscosimeter(Factor 1.0157)

Flow Times:-

#1.	6 min. 16.3 secs.
#2.	6 min. 16.3
#3.	6 min. 16.2
#4.	6 min. 16.3
#5.	6 min. 16.2
#6.	6 min. 16.3
#7.	6 min. 16.3
#8.	6 min. 16.3
#9.	6 min. 16.2
#10.	6 min. 16.3

Average = 376.27 X 1.0157
= 382.177439 secs.

Enzyme; No Penicillin;Dil'n.1/300.
#1. Viscosimeter(No Factor)

	Reaction Time	Flow-Times
#1.	2'32"	5'10.2"
#2.	9' 8"	4'18.9"
#3.	14'33"	3'52.0"
#4.	19'15"	3'35.8"
#5.	23'52"	3'22.4"
#6.	28' 2"	3'13.2"
#7.	32'10"	3' 5.3"
#8.	36'12"	2'59.1"
#9.	40' 5"	2'53.8"
#10.	43'52"	2'49.5"

Enzyme;With Penicillin;Dil'n.1/350.
#2 Viscosimeter(Factor 1.2214)

	Reaction Time	Flow-Times
#1.	2'26"	4'17.2"
#2.	7'54"	3'42.4"
#3.	12'34"	3'21.6"
#4.	16'58"	3' 7.5"
#5.	21'26"	2'55.5"
#6.	25'18"	2'48.1"
#7.	28'56"	2'41.9"
#8.	32'47"	2'35.8"
#9.	36'18"	2'31.2"
#10.	39'39"	2'27.3"

Solvent Control:- 96.429530 secs.

Substrate Control:- 382.177439 secs.

Relative Viscosity:- $\frac{382.177439}{96.43} \times 100 = 396.32$

Enzyme; No Penicillin:- #1 Viscosimeter; No Factor;

Dilution 1/300.

	Reaction Time	Relative Viscosity
#1.	307.1 secs.	321.68
#2.	677.45	268.48
#3.	989.00	240.58
#4.	1262.9	223.78
#5.	1533.2	209.89
#6.	1778.6	200.35
#7.	2022.65	192.16
#8.	2261.55	185.73
#9.	2491.9	180.23
#10.	2716.75	175.77

Enzyme; With Penicillin:- #2 Viscosimeter; Factor 1.2214;

Dilution 1/350.

	Reaction Time	Relative Viscosity
#1.	303.07 secs.	325.77
#2.	609.81	281.69
#3.	877.11	255.35
#4.	1132.5	237.49
#5.	1393.17	222.29
#6.	1620.65	212.91
#7.	1834.87	205.06
#8.	2062.14	197.33
#9.	2270.33	191.51
#10.	2468.95	186.57

Legend of Graph for Protocol #5.

A = Substrate Control.

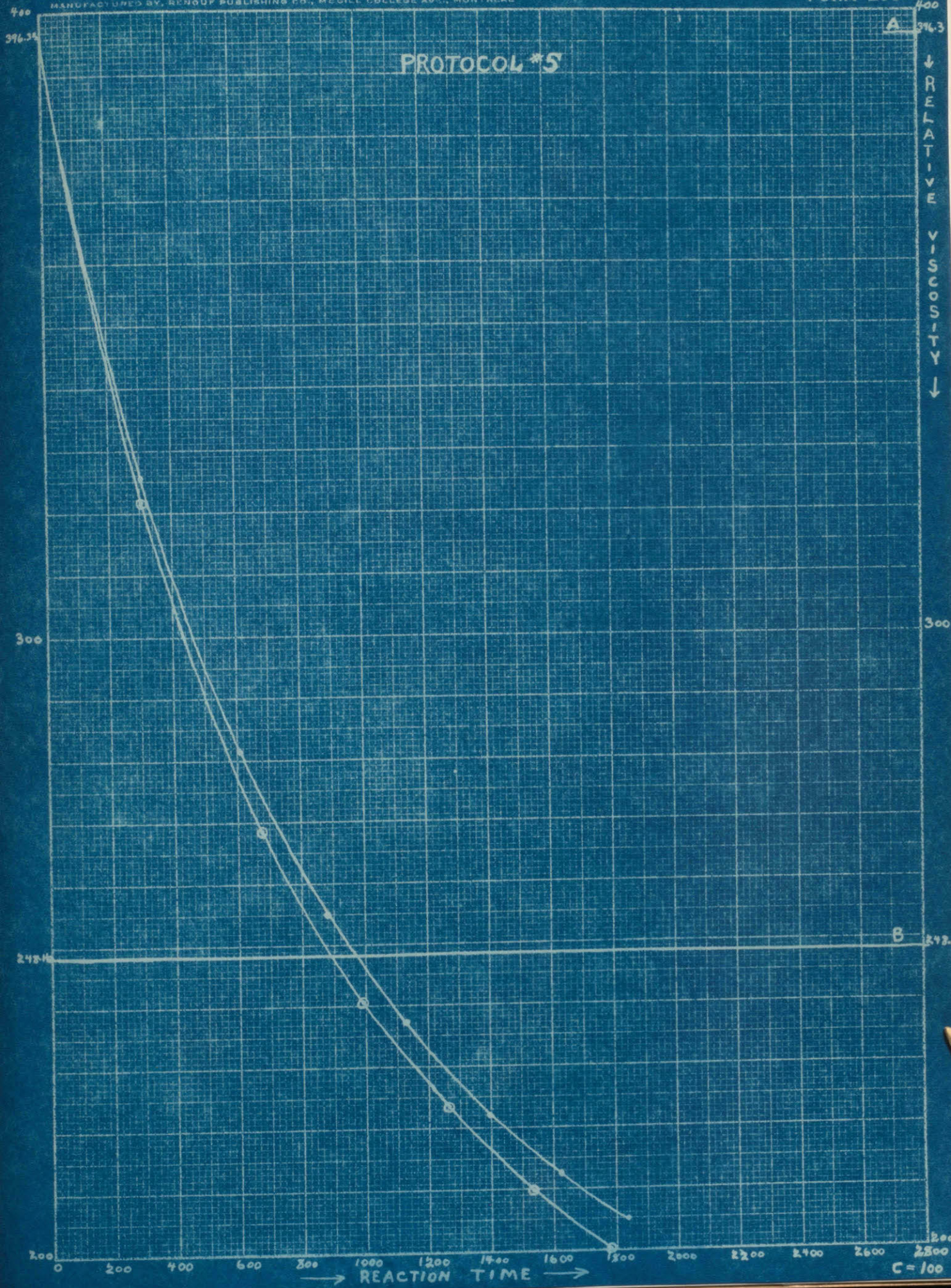
B = Half-Life Time.

C = Base-Line of Solvents Control = 100.

. = Enzyme with Penicillin (E).

⊙ = Enzyme with No Penicillin (D).

PROTOCOL #5



Computation:-

"E" = Enzyme with Penicillin at dil'n. 1/350;

half-lifetime = 965 secs.

$$\text{"E"} = \frac{D \times 20}{R} = \frac{350 \times 20}{16.08} = 435.32 \text{ V.R.U.}$$

"D" = Enzyme with No Penicillin at dil'n. 1/300;

half-lifetime = 900 secs.

$$\text{"D"} = \frac{D \times 20}{R} = \frac{300 \times 20}{15} = 400. \text{ V.R.U.}$$

Absolute Density Readings Due to Bacteria Alone:-

"E" = .1707 (Percent Transmission = 67^2)

"D" = .1973 (Percent Transmission = 63^2)

Therefore, "E" in terms of "D" = $\frac{.1973}{.1707} = 1.1558$

"E" = 1.1558 X 435.32 = 503.142856 V.R.U.

Therefore, $\frac{503.142856}{400} \times 100 = 125.78\%$ of normal of hyaluronidase is formed per Streptococcus cell, when such an inhibiting amount of Penicillin is contained in the culture medium, affording an inhibition of =

$$\frac{.1707}{.1973} \times 100 = 86.51\%$$

$$= 100.00\% - 86.51\% = 13.49\%.$$

Therefore, when sufficient penicillin is added to the culture medium to cause 13.49% inhibition of growth, there is an increase of 25.78% in the liberation of hyaluronidase per cell of Streptococcus pyogenes P315 A-4, Strain "R".

Protocol # 6.

Solvent Control:-
#4 Viscosimeter(Factor 1.0157)

Flow-Times:-

#1.	93.5 secs.
#2.	93.4
#3.	93.6
#4.	93.4
#5.	93.4
#6.	93.5
#7.	93.5
#8.	93.6
#9.	93.5
#10.	93.5

Average = 93.49 X1.0157
= 94.957793 secs.

Substrate Control:-
#4 Viscosimeter(Factor 1.0157)

Flow-Times:-

#1.	6 min. 7.8 secs.
#2.	6 min. 7.9
#3.	6 min. 7.9
#4.	6 min. 8.0
#5.	6 min. 7.9
#6.	6 min. 7.9
#7.	6 min. 7.8
#8.	6 min. 7.9
#9.	6 min. 7.9
#10.	6 min. 8.0

Average = 367.9 secs. XI.0157
= 373.67603 secs.

Enzyme;No Penicillin;Dil'n.1/230.
#1 Viscosimeter(No Factor)

	Reaction Time	Flow-Times
#1.	2'28"	5'34.4"
#2.	9' 7"	4'56.0"
#3.	15'41"	4'29.5"
#4.	21'57"	4'10.5"
#5.	27'33"	3'56.12
#6.	32'33"	3'46.5"
#7.	37'19"	3'38.5"
#8.	41'52"	3'32.3"
#9.	46'12"	3'26.8"
#10.	50'27"	3'21.9"

Enzyme;With Penicillin;Dil'n 1/350.
#2 Viscosimeter(Factor 1.2214)

	Reaction Time	Flow-Times
#1.	2'23"	4'19.3"
#2.	7'37"	3'51.1"
#3.	12'39"	3'29.8"
#4.	16'57"	3'17.3"
#5.	21'00"	3' 8.0"
#6.	24'58"	3' 0.1"
#7.	28'42"	2'53.9"
#8.	32'27"	2'48.0"
#9.	36' 3"	2'43.4"
#10.	39'32"	2'39.9"

Solvent Control:- 94.957793 secs.

Substrate Control:- 373.67603 secs.

Relative Viscosity:- $\frac{373.67603}{94.95} \times 100 = 393.55$

Enzyme; No Penicillin:- #1 Viscosimeter; No Factor;

Dilution 1/230.

	Reaction Time	Relative Viscosity
#1.	315.2 secs.	352.18
#2.	695.0	311.74
#3.	1075.75	283.83
#4.	1442.25	263.82
#5.	1771.05	248.65
#6.	2066.25	238.54
#7.	2348.25	230.12
#8.	2618.15	223.59
#9.	2875.4	217.79
#10.	3127.95	212.63

Enzyme; With Penicillin:- #2 Viscosimeter; Factor 1.2214;

Dilution 1/350.

	Reaction Time	Relative Viscosity
#1.	301.35 secs.	333.55
#2.	598.13	297.27
#3.	887.12	269.87
#4.	1137.49	253.79
#5.	1374.81	241.83
#6.	1607.98	231.67
#7.	1828.2	223.69
#8.	2049.59	216.10
#9.	2262.78	210.19
#10.	2469.65	205.68

Legend of Graph for Protocol #6.

A = Substrate Control.

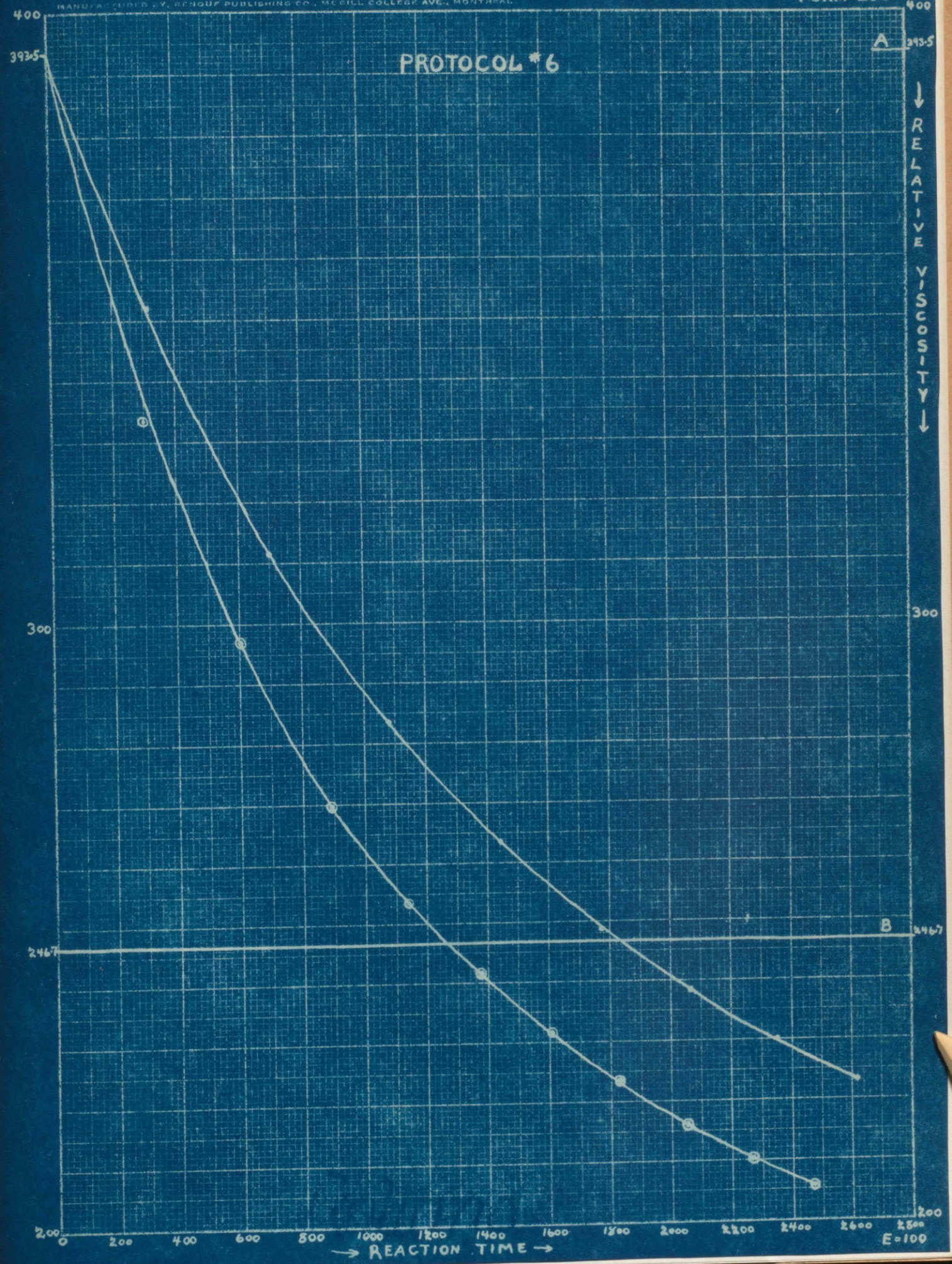
B = Half-Life Time.

E = Base-Line of Solvents Control = 100.

. = Enzyme with No Penicillin (C).

⊙ = Enzyme with Penicillin (D).

FORM 200



Computation:-

"C" = Enzyme with No Penicillin at dil'n. 1/230;

half-lifetime = 1,830 secs.

$$\text{"C"} = \frac{D \times 20}{R} = \frac{230 \times 20}{30.5} = 150.81 \text{ V.R.U.}$$

"D" = Enzyme with Penicillin at dil'n. 1/350;

half-lifetime = 1,275 secs.

$$\text{"D"} = \frac{D \times 20}{R} = \frac{350 \times 20}{21.25} = 329.41 \text{ V.R.U.}$$

Absolute Density Readings Due to Bacteria Alone:-

"C" = .2218 (Percent Transmission = 60°)

"D" = .1612 (Percent Transmission = 69°)

Therefore, "D" in terms of "C" = $\frac{.2218}{.1612} = 1.3759$

"D" = 1.3759 X 329.41 = 453.235219 V.R.U.

Therefore, $\frac{453.235219}{150.81} \times 100 = 300.53\%$ of normal of hyaluronidase is formed per Streptococcus cell, when such an inhibiting amount of Penicillin is contained in the culture medium, affording an inhibition of =

$$\frac{.1612}{.2218} \times 100 = 72.67\%$$

$$= 100.00\% - 72.67\% = 27.33\%$$

Therefore, when sufficient penicillin is added to the culture medium to cause 27.33% inhibition of growth, there is an increase of 200.53% in the liberation of hyaluronidase per cell of Streptococcus pyogenes P315 A-4, Strain "N".