THE INFLUENCE OF SEVERAL FACTORS ON THE ACTION OF PENICILLIN AGAINST SOME MEMBERS OF THE ENTEROBACTERIACEAE

Thesis for the Degree of Ph. D.

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Dale Emil Bordt

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This is to certify that the

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The Influence of Several Factors
on the Action of Penicillin Against
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Major professor

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By

Dale Emil Bordt

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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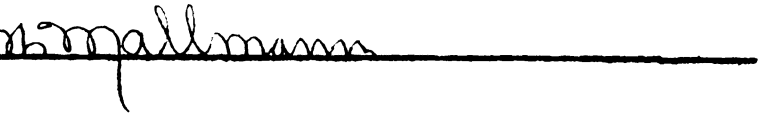
Approved 

AN ABSTRACT

Various factors were studied to determine their influence on penicillin action against selected members of the Enterobacteriaceae.

The medium used was the synthetic medium of Young, Begg and Pentz (1944) with lactose as the sole carbon source. In this medium 200 units of penicillin per ml were found to be bactericidal to Escherichia coli. Penicillin was most actively bactericidal when exposure took place after about six hours at 37 C, which corresponds to the length of lag of E. coli in this medium.

Dilution was not an effective means for reduction of penicillin where recovery of previously exposed organisms was desired. Penicillinase was found to destroy penicillin effectively and to allow recovery of previously exposed cells. A much higher concentration of the enzyme was required for recovery of cells exposed to penicillin than was necessary to inactivate the same amount of penicillin under identical conditions of time and temperature.

Salmonella pullorum was found to survive exposure to 200 units of penicillin per ml, provided a sufficient preincubation was employed before exposure. This was evidently due to the

fact that penicillin is active only against growing cells. The addition of approximately one percent sterile fecal material resulted in a bactericidal action of penicillin against S. pullorum. This was presumably due to the fact that the fecal material contributed growth substances to the medium, and thus established the penicillin effect.

Sodium arsenite in proper concentration was shown to be bacteriostatic against members of the genus Salmonella, but not against other Enterobacteriaceae. Sodium arsenite in a 0.002 percent concentration in this synthetic medium protected certain salmonellae from penicillin activity even in the presence of one percent fecal material.

The presence of two percent tryptose and 0.02 percent cysteine in the plating medium materially enhanced the recovery of S. pullorum from exposure to penicillin.

The addition of 0.5 percent lactose and 0.005 percent triphenyltetrazolium chloride to the plating medium resulted in colonies of diagnostic significance. Lactose non-fermenting organisms reduced the tetrazolium salt and formed dark red colonies, while those producing acid from lactose in general failed to reduce the salt and remained colorless or only faint pink.

A bisphenol, 2,2'methylenebis-4-chloro-6-isopropylphenol, added to a level of 0.001 percent in the plating medium

effectively inhibited certain gram positive cocci which survived penicillin exposure.

A series of human fecal specimens was examined to determine whether this penicillin enrichment technique could be used for the isolation of salmonellae from feces. The results of this limited series of examinations compare favorably with those obtained with several media presently used for routine diagnostic work.

Among the limitations of the method is the fact that certain organisms other than the salmonellae do survive exposure to penicillin and, when present in large numbers, produce overcrowded plates making isolation of typical colonies difficult. Organisms involved in this type of interference are (1) pseudomonads, (2) nutritionally deficient coliforms, (3) lactose non-fermenting or slow fermenting coliforms. Other limitations are the inability to recover quantitatively the salmonellae, and the relatively small inoculum necessary.

The method, with some modifications, could also be very convenient for isolation of naturally occurring biochemically deficient forms of bacteria.

Young, E.G., R.W. Begg and E.I. Pentz. Inorganic nutrient requirements of Escherichia coli. Arch. Biochem. 5:121-136. 1944.

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INTRODUCTION

Fleming (1929) made the historic observation that a filtrate of a Penicillium culture showed a remarkable selective action against the gram positive bacteria in general, and a considerably lesser action against most gram negative organisms. He was the first to take practical advantage of this fact by using penicillin in an agar medium to facilitate the isolation of Bacillus influenzae (a gram negative organism) from nasal washings which also contained an abundance of gram positive microorganisms.

MacLean (1937) also applied this same principle with marked success for the isolation of Hemophilus pertussis from whooping cough cases.

Pizzi (1945) reported the use of penicillin in a medium for the cultivation of Trypanosoma cruzi. The penicillin is nontoxic to the protozoan while inhibiting the growth of bacteria.

Burnet, Stone, and Anderson (1946) made use of a combination of sulfonamide and penicillin for the isolation of the virus of influenza B in chick embryos during an epidemic of influenza in Australia. The penicillin and sulfonamide were effective in inhibiting growth of bacteria

in the chick amnion, and sufficiently nontoxic to the embryo and the virus to allow invasion by and development of the virus to take place.

Hawking (1946) used ten units of penicillin per ml in a tissue culture of chicken macrophage reticulo-endothelial cells for the isolation and growth of Plasmodium relictum.

Lacy (1946) utilized the selective action of penicillin for the isolation of Brucella abortus from milk.

Morin and Turcotte (1946) used penicillin for the biological "purification" of vaccine emulsions. These authors point out that 56 units of penicillin per ml of vaccine prevented bacterial infection by vaccination without adversely affecting the immunological potency of the vaccinia virus.

Dienes (1947) reported the use of penicillin for the isolation of pleuropneumonia-like organisms from pathological specimens.

Howitt and Barnett (1948), working with poliomyelitis virus, report that the use of 1,000 units of penicillin and 20 mg of streptomycin per ml of fecal suspension made intraabdominal inoculation of monkeys possible without preventing paralysis.

Pramer, Houhannes and Royotzkie (1950) developed a method for the quantitative recovery of mycobacteria from sewage. They used Dubos' medium plus five units of peni-

cillin and 50 units of grisein per ml. They also pre-heated the sewage sample at 50 C for 60 minutes. This treatment was reported to suppress 87-94 percent of sewage organisms with no loss of Mycobacterium tuberculosis.

These papers are cited to give examples of the wide use of penicillin in the isolation or biological "purification" of selected organisms. They are by no means the only cases reported of the use of penicillin for this purpose. Penicillin and other antibiotics are used routinely in virus laboratories as a practical means of preventing bacterial infection of laboratory animals or chick embryos by bacterial contaminants in virus preparations.

In all of the mentioned procedures advantage is taken of the natural ability of certain organisms (such as viruses, protozoa and most gram negative bacteria) to resist the lethal action of a given concentration of penicillin. Under the same conditions, contaminants are killed or inhibited due to their natural penicillin sensitivity.

Hobby, Meyer, and Chaffee (1942), studing the mechanism of action of penicillin on pneumococci, hemolytic streptococci and staphylococci, concluded that penicillin is bactericidal under certain conditions. They found the rate of killing by penicillin to vary with different organisms, and also that penicillin appears to be effective only when

active multiplication of the exposed organisms takes place.

Foster and Wilker (1943), in a study of penicillin effects on Micrococcus pyogenes var. aureus, found that survival from penicillin exposure was not due primarily to highly resistant "persisters". On the contrary, cultures from surviving cells were found to have survival curves identical with the parent culture. They conclude that penicillin depends for its activity on the logarithmic prolongation of generation time. This suggests also that the survival was due to some special metabolic state of the cell at the time of exposure.

Bigger (1944) lent additional support to the hypothesis that only growing, or at least actively metabolizing cells are susceptible to the lethal action of penicillin. He found that by using a dilute solution of boric acid as a bacteriostat for M. aureus, the action of penicillin could be inhibited.

Hobby and Dawson (1944a,b) worked along similar lines. They showed that an increase in growth rate of hemolytic streptococci caused an increased rate of kill by penicillin. Conditions which decreased growth rate also decreased the rate at which penicillin acted. In addition, these workers observed that sulfadiazine, which inhibits growth, also inhibits penicillin action. It was also found that at 4 C,

penicillin action, as well as growth, was stopped or greatly retarded.

Lee, Foley, and Epstein (1944), working with Staphylococcus aureus FDA, came to the following conclusions:

1. The rate of kill by penicillin is greater in a medium where normally the growth rate is greater.

2. Physiologically young organisms are killed quicker than old ones. This was believed to be due to the shorter lag involved.

These workers also showed that greatly increasing the concentration of penicillin did not appreciably shorten the time required to kill all the organisms in a suspension. They showed a linear relationship during logarithmic growth between the logarithm of the viable population found in the control tubes and the logarithm of the number of viable organisms in the presence of 1.5 units per ml of penicillin. Their final conclusion also is that actual growth of the organism seems to be necessary for penicillin to kill.

Rantz and Kirby (1944), in their in vitro studies on the action of penicillin on staphylococci, observed that, in order to establish the penicillin effect, the organisms apparently must divide. They also confirm the observation by Foster and Wilker (1943) that the bacteria remaining viable after exposure to penicillin are in general as sen-

sitive to penicillin action as the parent strain. These workers interpret this to mean that survival is not due to an artificially induced penicillin resistance, but it is due merely to the fact that the surviving organisms were not in a metabolic state suitable for division to take place.

Schwartzman (1944), in studying the affect of penicillin on Escherichia coli, showed that methionine enhances the action of penicillin; and he also states that substances inhibitory to growth interfere with penicillin action.

Chain and Duthie (1945) came to the conclusion that penicillin acts as a bacteriostatic or bactericidal agent depending on concentration. These effects occur only under conditions that will allow for bacterial growth in the absence of penicillin. In a medium where growth is nutritionally impossible, penicillin is ineffective. They also observed that bacteriostatic concentrations of helvolic acid interfere with the action of penicillin on M. aureus.

On the other hand, Treffers (1946) found that inhibitory agents like iodoacetic acid, sodium azide, gentian violet and merthiolate potentiate the inhibitory action of penicillin. He postulates this potentiating effect to be due to these agents affecting a part or all of a common enzyme system.

Dufrenouy, Strait, and Pratt (1947) studied the effect

of penicillin on E. coli, Proteus vulgaris, M. aureus and Bacillus subtilis. As a result of these studies they came to the conclusion that penicillin affects both gram negative and gram positive organisms in essentially the same manner. The threshold concentration necessary for lethal action appears to be the only major difference between the two groups.

Krampitz and Workman (1947) showed that penicillin had no deleterious effect on the metabolism of glucose by M. aureus, and also that it had no effect on the enzymes concerned with the intermediate products of carbohydrate breakdown. In a synthetic medium, in the absence of penicillin, a gradual acceleration of oxygen uptake would occur after about five hours. In the presence of penicillin this acceleration of oxygen uptake did not occur. Control experiments showed the same accelerated uptake to occur with cells in a buffer solution without carbohydrate substrate. The substances being oxidized were evidently constitutents of the cells. During the course of rapid endogenous activity, pentose disappeared. The presence of penicillin inhibits this disappearance. These workers are of the opinion that the source of pentose is the ribose of ribose nucleic acid. They conclude that in some manner, penicillin interferes with the dissimilation of ribose nucleic acid and consequently with its assimilation

during growth.

Eagle and Musselman (1949) made the observation that beta hemolytic streptococci, when exposed to penicillin in a medium favorable for growth, did not resume growth immediately after addition of the penicillin destroying enzyme penase, but the organisms showed a slow recovery time. This time of recovery was directly related to the time of exposure. They also noted that large doses, up to 10,000 times necessary for inhibition, did not further accelerate bacterial death. On the contrary, in some cases such large doses even retarded lethal action. These workers also observed that when cells were exposed to penicillin in a medium unfavorable for growth and were then placed in a favorable medium, they did not require the recovery period necessary for cells which had been exposed in a favorable medium. Their conclusions, drawn about the action of penicillin, are as follows: "The Bactericidal action of penicillin may thus depend on the continuation, in a favorable medium, of certain metabolic activities in the cells in which the processes which normally lead to growth and cell division have been interrupted by penicillin." This is in agreement with the conclusions of Krampitz and Werkman (1947) previously cited. It was also noted that cells thus altered by penicillin exposure in a medium favorable for growth are able

to recover from this exposure only in a medium favorable for growth.

Davis (1948), and Lederberg and Zinder (1948) simultaneously reported the possibility of using penicillin for the isolation of biochemically deficient mutants of bacteria. These were the first two deliberate and practical applications of the previously discussed phenomenon that penicillin kills bacteria only when in a medium favorable for growth.

The technique of Davis (1948), using E. coli, was in general the following: A suspension of E. coli was irradiated with ultra-violet light to induce mutation. The cells were then incubated in a complete medium to allow the surviving ones to multiply. The cells were then washed with saline and placed in a minimal medium in the absence of tryptophane. After that they were exposed to 300 units of penicillin per ml for 24 hours. The cells were removed and plated on a complete medium. A high proportion of the colonies which then developed were found to be made up of cells requiring tryptophane for growth. These mutants, when exposed to penicillin in a complete medium, show no increased natural resistance to the antibiotic. It is apparent, then, that the surviving cells resisted the penicillin exposure because they were exposed in a meta-

bolically inactive state. This inactive state of the cells was due to the inability of the deficient medium to support their growth.

Since these original observations, many other workers have also used this approach for the isolation of bacterial mutants.

PURPOSE OF THE STUDY

The apparent success of the "penicillin enrichment technique" prompted the present investigation. It was considered worth-while to investigate the possibility of using this unique action of penicillin for the selection of bacteria from mixed populations containing closely related types.

A classical example of this type of situation involves the isolation of members of the genus Salmonella from fecal material containing large numbers of other members of the Enterobacteriaceae. With this object in mind, the influence of several factors on the penicillin susceptibility of coliform bacteria and various members of the genus Salmonella was chosen as the primary object of this study.

The main portion of this work will be concerned with the investigation of the effects of various conditions in a medium which modify the action of penicillin on representative members of the coliform group and the genus Salmonella.

The two organisms chosen for the initial portion of the study were:

(1) A typical strain of E. coli freshly isolated from human feces.

(2) A strain of Salmonella pullorum freshly isolated

from chicken feces.

S. pullorum was chosen to represent the genus Salmonella because it is one of the least resistant of the salmonellae to the action of penicillin. Thomas and Levine (1945) showed that as little as 10 units per ml inhibited growth in beef extract broth. Also, S. pullorum is considered not to be highly pathogenic for man, which minimizes the danger of accidental infection while counting colonies on plates, etc. in the laboratory.

THEORETICAL CONSIDERATIONS

According to the studies on the mode of action of penicillin previously cited, the following conditions must be met in order to obtain the desired selective kill of the coliforms:

1. Conditions must be formulated which are favorable for the growth of the coliforms. The presence of penicillin in sufficient concentration should then be bactericidal to this group.

2. These conditions must at the same time be such that members of the genus Salmonella, which may be present, will not be able to initiate growth; but will, nevertheless, remain viable in a metabolically inactive state.

In order to attain such conditions, physiological differences between the two groups of organisms were considered. The outstanding difference appears to be the ability of the coliforms to utilize lactose as a source of carbon in an otherwise inorganic medium, and the inability of members of the genus Salmonella to do so.

On this basis, then, a synthetic medium with lactose as the sole carbon source was considered adequate to fulfill

the necessary requirements. A medium of this type is that described by Young, Begg, and Pentz (1944).

In an intensive study of the nutrient requirements of E. coli, these authors found the optimum inorganic requirements to be the following:

NaCl	0.5%
$(NH_4)_2SO_4$	0.8%
KH_2PO_4	0.2%
Na_2HPO_4	0.2%
Mg	0.5 γ per ml
Fe	0.56 γ per ml

In the present study the magnesium was supplied in the form of $MgSO_4 \cdot 7H_2O$ (0.005 grams per liter) and the iron in the form of $FeSO_4 \cdot 7H_2O$ (0.00278 grams per liter). The pH of this inorganic base medium after autoclaving is pH 6.5. The authors found that this base medium plus 3.0 percent glycerol supported the growth of E. coli as well as did nutrient broth, except that a longer lag period was observed.

This inorganic base medium was dispensed in 50 ml amounts in 125 ml flasks and sterilized by autoclaving for 10 minutes at 121 C. Lactose, as the sole carbon source, was added aseptically from a similarly autoclaved solution prior to inoculation to give the desired final concentration.

DETERMINATION OF THE EFFECTS OF SEVERAL FACTORS ON PENICILLIN ACTION

The first consideration in this study was to determine the conditions necessary for the optimum lethal effect of penicillin on *E. coli* in the synthetic medium of Young, Begg, and Pents (1944). For convenience this medium will hereafter be referred to as YBP medium.

From the evidence obtained by the work of the several authors quoted previously, conditions optimum for most rapid initial growth of *E. coli* should also be optimum for penicillin action against this organism. Hobby, Meyer and Chaffee (1942); Bigger (1944); Hobby and Dawson (1944); Lee, Foley, and Epstein (1944); Rantz and Kirby (1944); Schwartzman (1944); Chain and Duthie (1945); Dufrenoy, Strait and Pratt (1947); Krampitz and Werkman (1947); Eagle and Musselman (1949).

EXPERIMENT I.

Determination of the Optimum Concentration of Lactose in YBP Medium for the Minimum Lag of *E. coli*.

Procedure: Fifty ml quantities of YBP medium containing varying concentrations of lactose were prepared and seeded with 0.5 ml of a 1-200,000 dilution of a 24-hour brain heart

infusion broth culture of *E. coli*. Duplicate one-ml quantities were plated in brain heart infusion agar at hourly intervals. After a 24-hour incubation period, the colonies were counted and recorded as in Table 1. The incubation temperature was 37 C.

Discussion: As can be seen in Table 1, the optimum concentration of lactose for shortest lag of *E. coli* is from 0.25 to 0.5 percent.

These results also show that the length of lag at the optimum lactose concentration is approximately five to six hours.

Before an attempt was made to demonstrate the bactericidal action of penicillin against *E. coli* in this medium, a consideration of the concentration of penicillin which must be used was necessary.

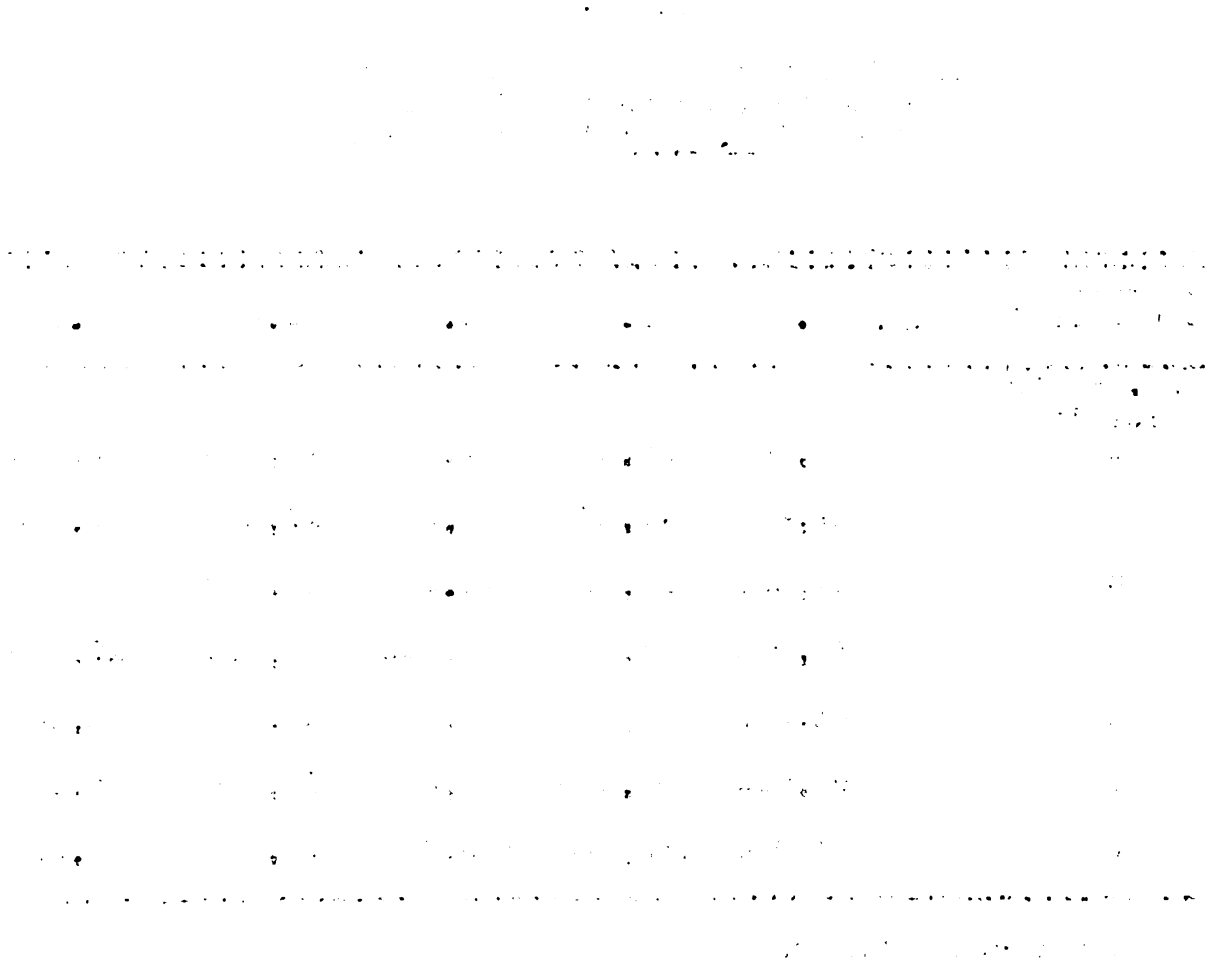
Thomas and Levine (1945) studied the *in vitro* effects of penicillin on various intestinal bacteria and found that the order of sensitivity was as follows: *Salmonella*, *Eberthella*, *Proteus*, *Shigella*, *Escherichia*, and *Aerobacter*. Most *Escherichia* strains required from 50 to 100 units per ml for complete inhibition in beef extract broth, while *E. communior* and *A. aerogenes* usually required over 100 units per ml for inhibition. On this basis then, a concentration of 200 units per ml was chosen to insure kill of even the

TABLE 1

The Effect of Varying the Lactose Concentration on the Initial Growth of *E. coli* in YBP Medium

Lactose Concentration (%)	0.1	0.25	0.5	1.0	2.5
Hrs. after seeding					
0	17,000*	18,000	17,500	18,100	16,000
1	17,400	17,800	18,000	17,300	17,400
2	17,200	18,300	19,200	18,900	18,000
3	18,000	23,100	20,300	20,500	17,800
4	31,000	46,000	44,000	32,000	21,400
5	63,000	84,000	78,000	48,000	24,300
6	74,000	123,000	119,000	77,000	38,000

* average count per ml



more resistant coliform strains. The use of such a relatively high concentration of penicillin is further justified by the work of Eagle and Musselman (1949), who showed that large doses of penicillin, even up to 10,000 times that necessary for inhibition, did not accelerate bacterial death.

The next logical step in this investigation was to determine the bactericidal activity of penicillin against E. coli in YBP medium.

Before this could be determined, however, some means had to be devised to reduce the residual penicillin concentration below the threshold value for the organism prior to plating.

Two theoretically possible methods to accomplish this presented themselves:

(1) The penicillin could be diluted beyond the threshold concentration before plating.

(2) The penicillin could be destroyed by incubation in the presence of a sufficient amount of a penicillin-destroying enzyme.

Spicer and Blitz (1948), working with Streptococcus viridans, showed that when penicillin was removed by dilution, no colonies developed upon plating the cells in an agar medium. When the penicillin was removed by sufficient penicillinase, however, the exposed organisms regained their viability and developed colonies in agar poured plates. The

reason for the cells failure to regain viability after penicillin removal by dilution is indicated by the work of Mass and Johnson (1949). These authors, using radioactive penicillin, showed a specific uptake of penicillin by susceptible cells independent of the extracellular concentration. This penicillin was not removable by dilution, and could be responsible for the residual effect after dilution of the antibiotic in the suspending medium beyond the threshold level for the organism in question.

It was desired to determine whether this residual effect is also operative in the case of S. pullorum. To determine this the following was done:

EXPERIMENT II.

The Effect of Removal of Penicillin by Dilution on the Recovery of S. pullorum.

Procedure: A 1-100,000 dilution of a 24-hour culture of S. pullorum was prepared in 0.85 percent saline. Penicillin was added to a level of 200 units per ml. After 10 minutes this suspension was diluted 1-100 in saline and plated in duplicate in 10 ml of brain heart infusion agar. This gave a final concentration of 0.2 units per ml in the agar plate, which is far below the threshold concentration of about 10 units per ml for S. pullorum (Levine 1945). After 24-hours incubation, no colonies developed, as can be

seen from Table 2. The control showed no loss of viability in the saline during this short exposure, nor did 0.2 units of penicillin per ml in the agar medium prove inhibitory to cells not previously exposed to the higher concentration of the antibiotic. On the basis of this evidence it is apparent that dilution could not be the method to use for the removal of penicillin for recovery of S. pullorum. These findings are also in agreement with those of Spicer and Blitz (1948).

The other alternative, as indicated by these same authors, involves the enzymatic inactivation of penicillin by the enzyme penicillinase.

Abraham and Chain (1940) were the first to report the existence of a bacterial enzyme capable of destroying penicillin. They showed that various bacterial extracts and culture filtrates destroyed penicillin with varying degrees of effectiveness.

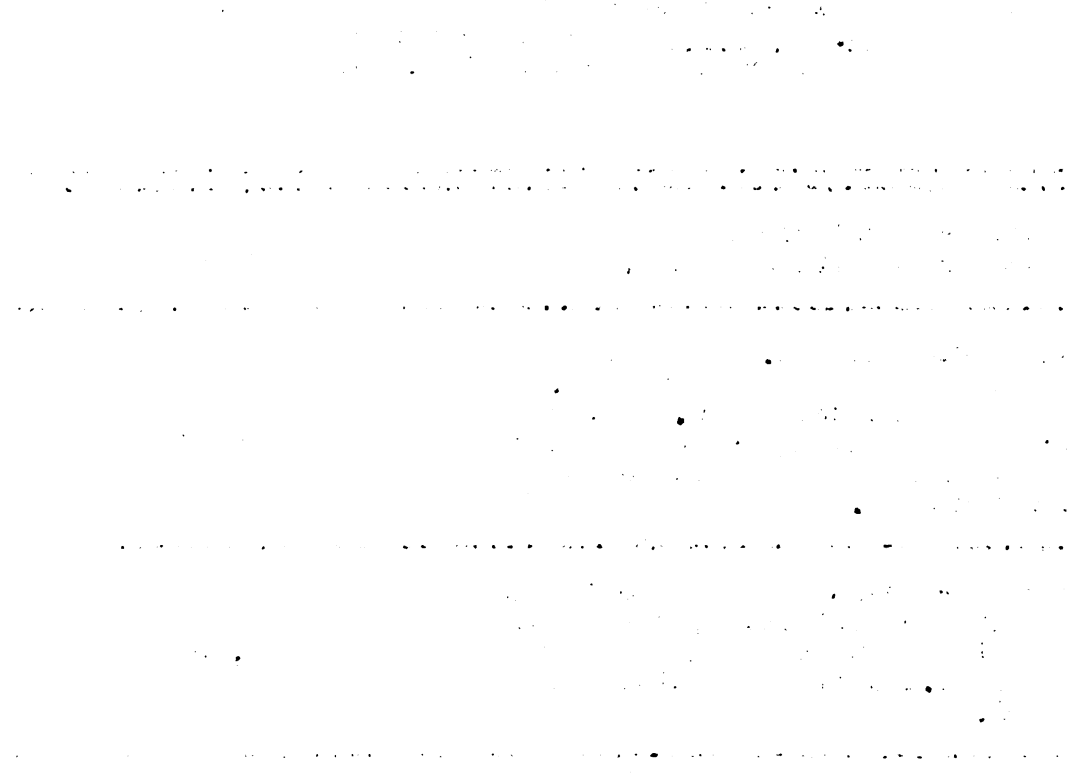
Hobby, Meyer and Chaffee (1942) also confirmed the production of a penicillinase by a strain of E. coli.

Lawrence (1943), in an attempt to devise a sterility test for penicillin preparations, showed that takadiastase and clarase were effective in destroying penicillin. Later, however, Lawrence (1944) reported that the active penicillin destroying substances in clarase were certain filterable substances of bacterial origin. These bacteria were identified

TABLE 2

The Residual Bactericidal Effect of Penicillin on *S. pullorum* after Reduction of Penicillin Concentration by Dilution

Control - count per ml before penicillin exposure.	9,600
Count after 10 min. exposure to 800 units of penicillin per ml. Penicillin reduced to 0.2 units per ml by diluting 1-100 and plating one ml in 10 ml of brain heart infusion agar.	0
Control - count per ml. No initial exposure to penicillin, but plated in brain heart infusion agar containing 0.2 units of penicillin per ml.	10,500



by him as belonging to the Bacillus cereus type which were present as contaminants in some clarase preparations.

Woodruff and Foster (1945) made a rather intensive study of a bacterial penicillinase preparation. These workers found that penicillinase is destroyed rapidly by temperatures above 50 C, but that it is quite stable at 4 C. In addition, they found that the preparation was stable over a remarkably wide pH range of from 3.0 to 11.0, with an optimum activity of from 6.5 to 8.0. It was also found that penicillinase activity was maximum at 37 C, and that SH-containing compounds such as sodium thioglycollate and cysteine enhanced its activity. These authors also found that penicillinase activity is not limited to bacteria, but that certain yeasts, actinomycetes and other fungi show it to varying degrees.

Bacto-penase concentrate, a product of the Difco Company of Detroit, Michigan was used here. According to the manufacturers, one ml of penase concentrate is capable of inactivating 500,000 units of penicillin in two hours when incubated together in 15 ml of fluid thioglycollate medium or brain heart infusion broth at room temperature.

Different lots of penicillin and penase are apt to vary slightly in potency. Therefore, it was considered necessary to assay the potency of this particular lot of penase with the lot of penicillin used in order to express the penase

potency in terms of the penicillin used.

EXPERIMENT III.

The Assay of Bacto-penase Concentrate Carried Out According to the Procedure Outlined in the Difco Manual (Ninth edition), 1953.

Procedure: Dilutions of Bacto-penase Concentrate of 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, and 1-10 were prepared in sterile distilled water. One ml of each dilution was added to a series of test tubes containing 15 ml of Bacto-Fluid Thioglycollate Medium. One ml of a solution of penicillin containing 50,000 units per ml was added to each tube and allowed to remain at room temperature for two hours. Each tube was then inoculated with one ml of a 1-1,000 dilution of a 24-hour broth culture of M. pyogenes var. aureus P209, and incubated at 37 C for 24 hours. The highest dilution permitting growth in this period was determined, and this dilution of Bacto-penase Concentrate was multiplied by 50,000 to determine its potency in terms of the number of units of penicillin inactivated by one ml of the enzyme preparation. The results of this assay are given in Table 3.

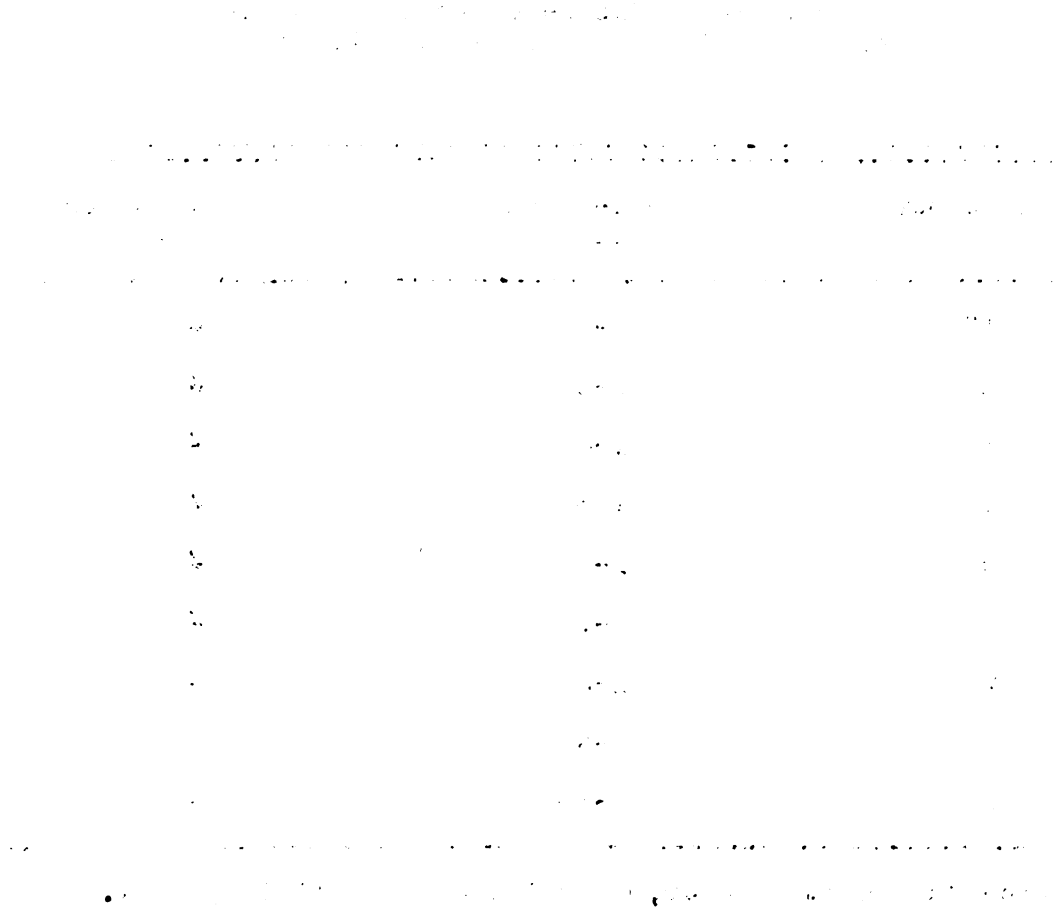
Discussion: From these results it is evident that 350,000 units of penicillin can be inactivated by one ml of penase in two hours at room temperature. The potency then is 350,000 "units" per ml.

TABLE 3

The Potency of Bacto-penase Concentrate Assayed Against Penicillin G Potassium

Tube Number	Bacto-penase dilution	Turbidity after 24 hours
1*	1-2	+
2	1-3	+
3	1-4	+
4	1-5	+
5	1-6	+
6	1-7	+
7	1-8	-
8	1-9	-
9	1-10	-

* Each tube contained 50,000 units of penicillin per ml.



It must be recognized that in the assay of penase, the susceptible bacteria were added to the assay tubes after penicillin inactivation. In actual sterility-testing procedures, or in procedures designed to recover bacteria after penicillin exposure, a very different situation exists. That is, the bacterial cells are in contact with penicillin before the addition of penase, and remain so during the entire inactivation procedure.

It was considered important, therefore, to determine whether the concentration of penase adequate for inactivation of a given amount of penicillin will also be adequate to allow for recovery of susceptible cells which had been previously exposed to the penicillin for a short time.

EXPERIMENT IV.

Determination of the Amount of Penase Necessary for Quantitative Recovery of M. aureus P209, Exposed to 200 Units of Penicillin per ml in 0.85 Percent Saline.

Procedure: A 24-hour culture of M. aureus P209 was diluted 1-10,000 in saline and 200 units of penicillin per ml were added. One-ml quantities were removed after 10 minutes and placed in nine ml of saline, brain heart infusion broth and fluid thioglycollate medium, containing various amounts of penase. After two hours at room temperature one-ml quantities were plated in brain heart infusion agar and

incubated for 24 hours at 37 C. The results are shown in Table 4.

Discussion: These results show that a much higher concentration of penase is necessary for the recovery of bacteria from penicillin exposure than is necessary for the mere inactivation of penicillin the same length of time. It is evident from these data that treatment with 200 inactivating units of penase for two hours is insufficient for recovery of M. aureus, exposed to 800 units of penicillin in a medium unfavorable for growth. A concentration as high as 35,000 inactivating units for four hours is necessary to cause quantitative recovery in the three media tested. Brain heart infusion broth appears to be the most efficient medium for penicillin inactivation. However, since the counts after five hours inactivation in fluid thioglycollate medium and brain heart infusion broth exceeded the count in the control tube, it is evident that some recovery and multiplication occurred.

The next experiment was designed to determine the concentration of penase necessary for quantitative recovery of S. pullorum from a short exposure to 200 units of penicillin per ml in a medium unfavorable for growth.

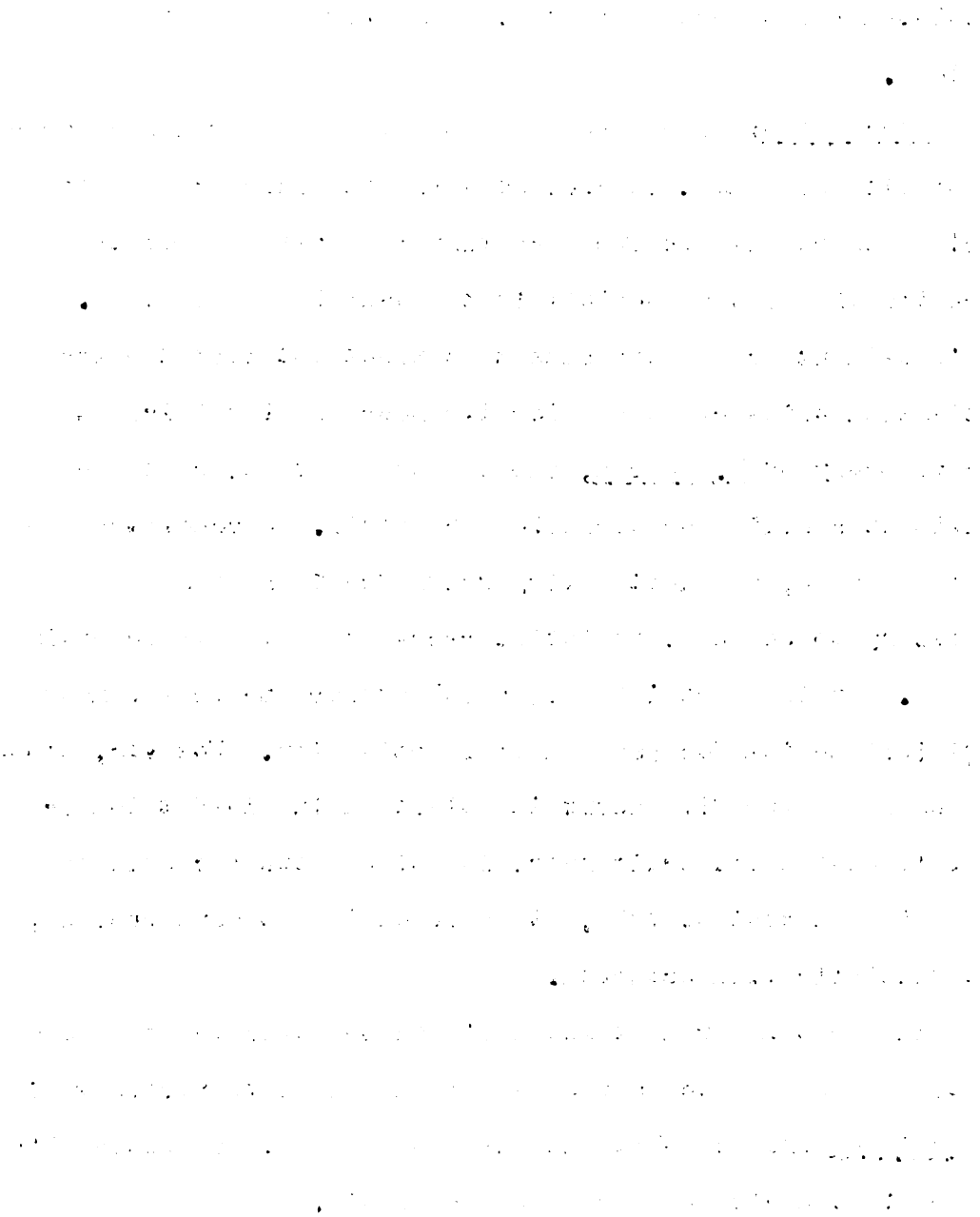


TABLE 4

Determination of the Amount of Penase Necessary for Quantitative Recovery of M. aureus P209 Which has been Exposed to 200 Units of Penicillin per ml in 0.85 Percent Saline

Medium used for inactivation	0.85% saline			Brain heart infusion broth			Fluid thioglycollate medium		
Units of Penase	200	3,500	35,000	200	3,500	35,000	200	3,500	35,000
Inactivation time in hours									
0	0*	0	1	0	0	5	0	0	5
2	0	0	2	0	0	196	0	0	54
4	0	0	370	0	0	540	0	0	550
Control-no Penicillin at 0 hours		370			380			380	

* average count per ml

EXPERIMENT V.

The Determination of the Amount of Penase Required for a Quantitative Recovery of S. pullorum from 10 Minutes Exposure to 200 Units of Penicillin per ml in 0.85 Percent Saline.

Procedure: A 24-hour brain heart infusion broth culture of S. pullorum was diluted 1-10 million in 0.85 percent saline, and penicillin was added to a level of 200 units per ml. Five ml quantities were removed after 10 minutes and penase was added to give the final concentrations shown in Table 5. After a two-hour incubation at 37 C, one ml quantities were plated in brain heart infusion agar. Woodruff and Foster (1945) showed 37 C to be the optimum temperature for penase activity, and on that basis it was adopted here. The results are shown in Table 5.

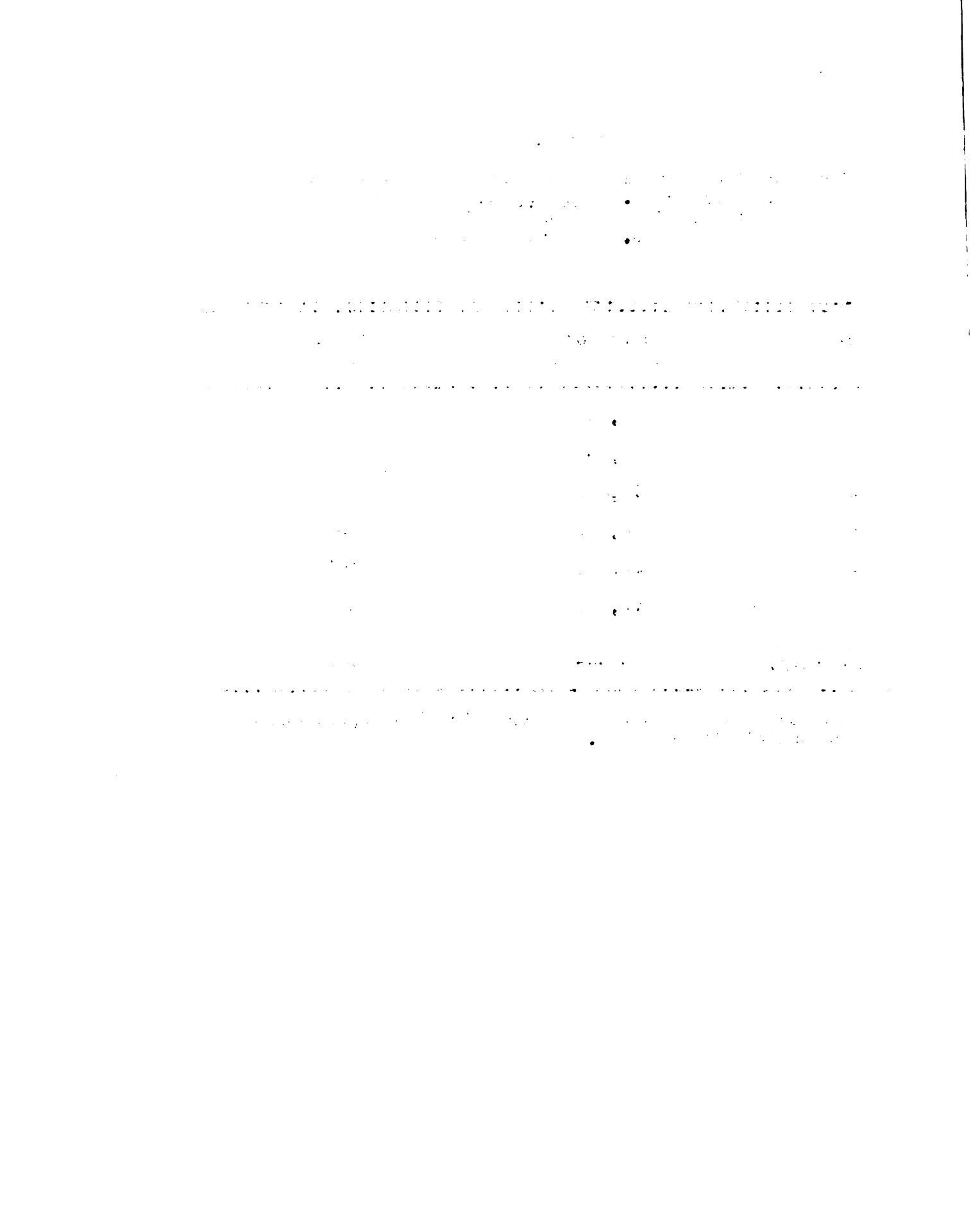
Discussion: An examination of these data reveal that in order to inactivate the 1,000 units of penicillin contained in the five ml of saline sufficiently in two hours at 37 C to allow for quantitative recovery of S. pullorum, at least 35,000 inactivating units of penase were necessary. This is a ratio of 35 units of penase to one unit of penicillin. This proportion of penase to penicillin was used in further experiments for determining conditions optimum for kill of E. coli.

TABLE 5

The Amount of Penase Required for a Quantitative Recovery of *S. pullorum* from Exposure to 200 Units of Penicillin per ml in 0.85 Percent Saline

Tube*	Units of penase added	Count per ml
1	3,500	0
2	8,750	0
3	17,500	0
4	26,250	82
5	35,000	144
6	70,000	139
Control (no penicillin)	------	149

* Each tube contained five ml for a total of 1,000 units of penicillin per tube.



EXPERIMENT VI.

Determination of the Optimum Time of Incubation of *E. coli* in YBP Medium Plus 0.25 Percent Lactose for the Maximum Kill by 200 Units of Penicillin per ml.

Procedure: Five-tenths ml of a 1-200,000 dilution of a 24-hour brain heart infusion broth culture of *E. coli* was seeded into 50 ml of YBP medium plus 0.25 percent lactose. Two hundred units of penicillin per ml were added and incubated at 37 C. At two hour intervals, five-ml quantities were removed and incubated for two hours at 37 C in the presence of 35,000 inactivating units of penase. At the end of two hours, duplicate one-ml quantities were plated in brain heart infusion agar. The colonies were counted after 24 hours at 37 C. The results are given in Table 6.

Discussion: These results show that an exposure of 13 hours to 200 units of penicillin per ml in YBP medium with 0.25 percent lactose will result in a 99.7 percent reduction in viability of *E. coli*.

Several workers, Hobby, Meyer and Chaffee (1942); Bigger (1944); Hobby and Dawson (1944); Lee, Foley and Epstein (1944); Rantz and Kirby (1944), have found that penicillin is most rapidly lethal for bacteria when the cells are in an actively growing state. On this basis, then, the most advantageous time for the addition of penicillin should be at the end of

TABLE 6

The Bactericidal Effect of 200 Units of Penicillin per ml on *E. coli* in YBP Medium with 0.25 Percent Lactose at 37 C

Hours exposed	Surviving cells per ml
0	10,800
1	4,700
3	1,990
5	700
7	380
9	300
11	180
13	109

the lag period of growth or in the early phase of logarithmic growth. An experiment was next performed to determine whether or not this is actually the case with *E. coli*.

EXPERIMENT VII.

Determination of the Influence of Pre-incubation Time on Penicillin Action Against *E. coli*.

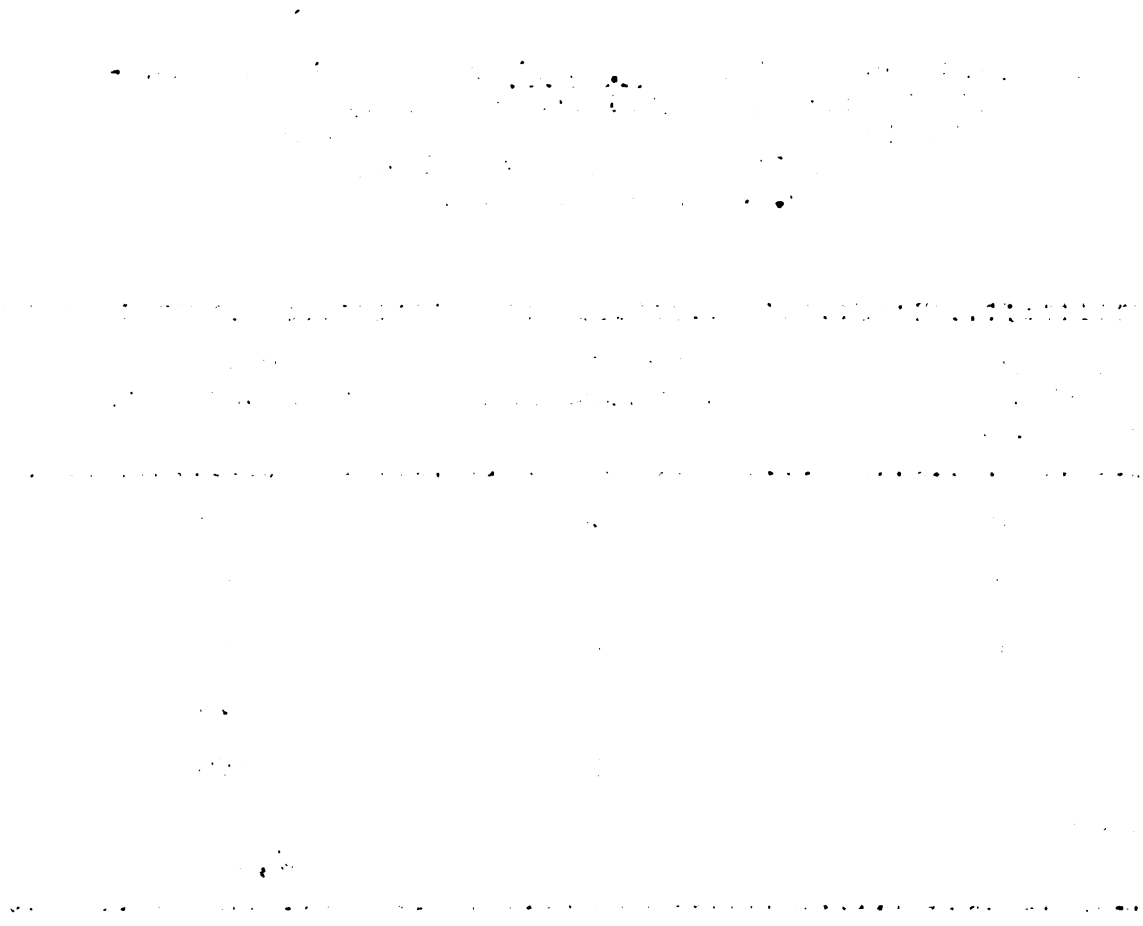
Procedure: Identical flasks containing 50 ml of YBP medium with 0.25 percent lactose were seeded with 0.5 ml of a 1-20,000 dilution of a 24-hour brain heart infusion broth culture of *E. coli*. These flasks were incubated at 37 C and 200 units of penicillin per ml were added to consecutive flasks at two-hour intervals. At nine hours from the time of seeding of the flasks, five-ml quantities were removed from each, and 35,000 inactivating units of penase were added. After two hours at 37 C, one ml quantities were plated in duplicate in brain heart infusion agar.

An examination of the results given in Table 7 shows that a six-hour pre-incubation and a three-hour penicillin exposure is more effective for the kill of *E. coli* than is a nine-hour penicillin exposure without a pre-incubation. This is in agreement with the findings of the authors cited above. Referral to Table 1 shows that after six hours incubation in YBP medium plus 0.25 percent lactose, *E. coli* is in an actively dividing state. This fact is apparently

TABLE 7

The Efficiency of Kill of *E. coli* by 200 Units of Penicillin per ml as Influenced by Length of Pre-incubation and Penicillin Exposure Time in YBP Medium Containing 0.25 Percent Lactose

Length of pre-incubation period, in hours	Penicillin exposure time in hours	Numbers of cells surviving
0	9	238
2	7	185
4	5	177
6	3	210
8	1	265
Control 0	0	24,000



responsible for the more rapid kill by penicillin added at this point, than when it is added before the apparent growth phase has begun. From the above findings it becomes evident that an optimum pre-incubation time for maximum kill of E. coli can be established.

EXPERIMENT VIII.

Determination of the Optimum Pre-incubation of E. coli in YBP Medium for Maximum Kill by Penicillin.

Procedure: The pre-incubation time here was varied and the penicillin exposure time was kept constant. Fifty-ml quantities of YBP medium in 125 ml flasks with 0.25 percent lactose were seeded with 0.5 ml of a 1-2,000 dilution of a 24-hour brain heart infusion broth culture of E. coli. These flasks were incubated at 37 C and at hourly intervals penicillin was added to give a final concentration of 200 units per ml. An arbitrary three hour penicillin exposure period was then employed. At the end of the penicillin exposure, five-ml quantities were removed from each flask and placed in test tubes in the presence of 35,000 inactivating units of penase. After two hours at 37 C, duplicate one-ml quantities were plated in brain heart infusion agar. The colonies were counted after 24 hours at 37 C.

Discussion: From the results of this experiment given in Table 8, it is evident that a pre-incubation of six hours

TABLE 8

The Influence of Pre-incubation Time on the Kill of *E. coli* by 200 Units of Penicillin per ml in YBP Medium Containing 0.25 Percent Lactose

Hours pre-incubation at 37 C	Count per ml after 3-hr penicillin exposure
3	TNTC*
4	TNTC
5	1360
6	910
7	1570
8	1980

Control - no pre-incubation and no penicillin exposure - 24,100 per ml

* TNTC - too numerous to count.

before the addition of penicillin is optimum for maximum kill of *E. coli* in YBP medium plus 0.25 percent lactose.

These results further confirm the hypothesis that penicillin action is greatest when the exposed cells are in an actively growing state. Table 1 shows that after six hours incubation in YBP medium plus 0.25 percent lactose without penicillin, *E. coli* is past its lag phase and is in an actively dividing state.

Having established the optimum pre-incubation time before penicillin exposure for maximum kill of *E. coli*, the next step was the determination of the optimum penicillin exposure time after pre-incubation for the optimum kill of *E. coli*.

EXPERIMENT IX.

Determination of the Effect of Exposure Time on the Kill of *E. coli* by 200 Units of Penicillin per ml after a Six-Hour Pre-incubation.

Procedure: A 24-hour brain heart infusion broth culture of *E. coli* was diluted 1-2,000 and 0.5 ml was added to 50 ml of YBP medium with 0.25 percent lactose. After a pre-incubation time of six hours at 37 C, 200 units of penicillin per ml were added. At hourly intervals thereafter, five-ml quantities were removed and 35,000 "units" of penase added. After two hours at 37 C, duplicate one-ml quantities were

plated in brain heart infusion agar. Counts after 24 hours at 37 C are recorded in Table 9.

Discussion: From this set of data it is evident that a pre-incubation time of six hours, followed by a penicillin exposure time of five hours, results in a 99.3 percent reduction of E. coli in YBP medium. Also an exposure as short as one hour results in a 95.7 percent loss in viability. This also indicates that pre-incubation time, and not exposure time is of most critical importance.

The next logical step in the investigation was to determine whether S. pullorum would survive these conditions just established for optimum kill of E. coli.

EXPERIMENT X.

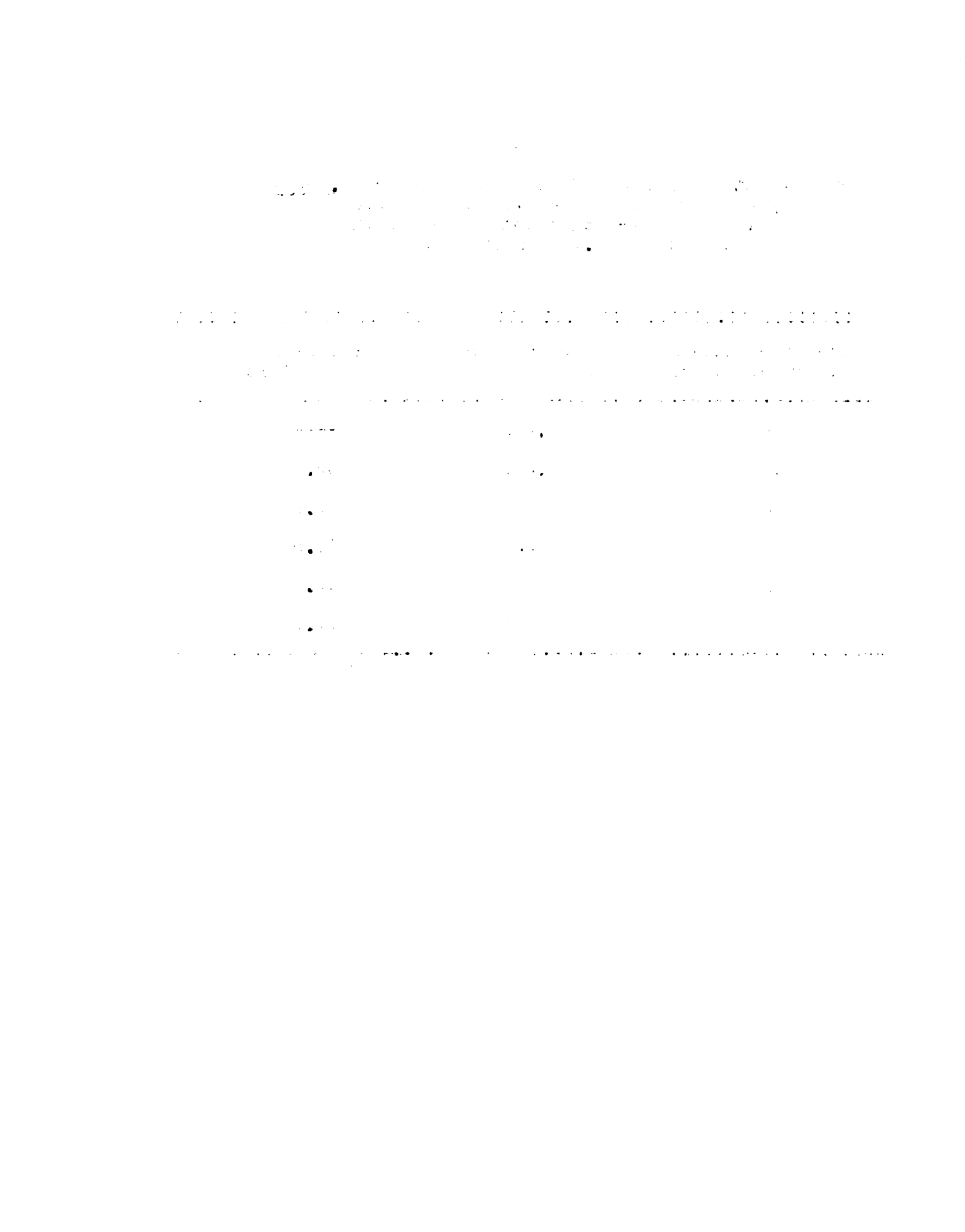
The Determination of the Recovery of S. pullorum from Exposure to 200 Units of Penicillin per ml in YBP Medium.

Procedure: A 24-hour brain heart infusion broth culture of S. pullorum was diluted 1-100,000. Five-tenths ml of this dilution was added to 50 ml of YBP medium plus 0.25 percent lactose. The flask was incubated at 37 C for six hours, at which time 200 units of penicillin per ml were added. After an additional four-hour incubation, five ml were removed and placed with 35,000 "units" of penase for two hours at 37 C. Duplicate one-ml quantities were then plated in brain heart infusion agar and incubated for 24

TABLE 9

The Effect of Exposure Time on the Kill of *E. coli* by 200 Units of Penicillin per ml after a Six Hour Preincubation in YBP Medium Containing 0.25 Percent Lactose

Hours of exposure to 200 units per ml	Bacteria per ml surviving	Percent loss in viability
0	25,000	----
1	1,060	95.7
2	570	97.7
3	350	98.5
4	238	99.0
5	181	99.3



hours at 37 C. The resulting counts are recorded in Table 10.

Discussion: The results show that under these conditions S. pullorum can survive the effects of penicillin exposure to the extent of about 70 percent.

Thus, under identical conditions of exposure, E. coli, which is normally considered very resistant to penicillin, is killed to the extent of more than 99 percent while the more sensitive S. pullorum survives to the extent of about 70 percent.

The reason that S. pullorum was not quantitatively recovered is not clear. The control tube (Table 10) shows that the medium itself was not sufficiently toxic to account for the decrease in numbers. A possible explanation is that the six-hour pre-incubation period was not sufficient to render all the cells in an inactive metabolic state.

In order to determine whether or not this is the case, the following was done:

EXPERIMENT XI.

Determination of the Recovery of S. pullorum after a 12-Hour Pre-incubation in 0.85 Percent Saline and an Exposure to 200 Units of Penicillin per ml.

Procedure: A 24-hour culture of S. pullorum was diluted 1-100,000 and 0.5 ml was added to 50 ml of 0.85 per-

TABLE 10

The Recovery of S. pullorum from Exposure to 200 Units of Penicillin per ml at 37 C in YBP Medium Containing 0.25 Percent Lactose

Exposure time	Penicillin	Control - no penicillin
0 hrs.	250*	230
4 hrs.	162	209

* average count per ml

cent saline. The flask was incubated for 12 hours at 37 C to rid the cells of stored nutrients. Two hundred units of penicillin per ml were then added, and at hourly intervals five-ml samples were removed and placed in test tubes containing 35,000 "units" of penase. These tubes were then incubated at 37 C for two hours, at the end of which time duplicate one-ml quantities were plated in brain heart infusion agar and incubated at 37 C for 24 hours. The results are given in Table 11.

Discussion: These findings show that an exposure as long as eight hours to 200 units of penicillin per ml will cause no significant decrease in numbers of S. pullorum when in a "resting" condition in saline. The most logical explanation for the results observed in Table 10 would then be as follows: The conditions established for optimum kill of E. coli will not allow for simultaneous quantitative survival of S. pullorum due to the fact that some of the cells are still able to metabolize sufficiently to establish the penicillin effect. This metabolism may well be due to nutrients stored in the cell from the previous rich medium in which they were grown.

One possible way to obtain a greater survival of S. pullorum would be to pre-incubate for a longer period of time before adding penicillin to allow the cells to assume an in-

TABLE 11

Recovery of *S. pullorum* from Exposure to 200 Units of Penicillin after a Preliminary 12-Hour Incubation in 0.85 Percent Saline at 37 C

Penicillin exposure in hours	Count per ml after penicillin inactivation
0	208
1	221
2	206
4	219
6	218
8	204

active state. However, this would not be feasible in a procedure designed to obtain selective kill of the coliforms in a mixed population. As was seen in Table 1, a pre-incubation of more than six hours allows E. coli to multiply. This increase in numbers of E. coli would tend to offset the advantage of the pre-incubation for S. pullorum and would decrease the efficiency of the selective process. See also Table 8.

A similar difficulty was anticipated when attempts to obtain selective kill of the coliforms from fecal material were to be made. The fecal material would very likely contribute growth substances to the medium which would enable the salmonellae to initiate growth also, and, hence, be killed.

In order to determine whether or not this anticipated difficulty is a real one, the following experiment was performed:

EXPERIMENT XII.

Determination Whether the Survival of S. pullorum to Penicillin Exposure in YBP Medium Would be Adversely Affected in the Presence of Fecal Material.

Procedure: A 24-hour culture of S. pullorum was diluted 1-100,000 in 0.85 percent saline. Five-tenths ml of this dilution was added to 50 ml of YBP medium with 0.25 percent lactose and approximately 0.5 grams of sterilized

human fecal material. After a six-hour pre-incubation period at 37 C, 200 units of penicillin per ml were added. The flask was further incubated for four hours at 37 C, after which time five ml was removed and mixed with 35,000 "units" of penase. After two more hours at 37 C, duplicate one-ml portions were plated in brain heart infusion agar and incubated for 24 hours.

Discussion: The results given in Table 12 reveal that under these conditions S. pullorum does not survive exposure to 200 units of penicillin per ml. However, the control tube containing no penicillin shows that no appreciable multiplication of S. pullorum takes place under these conditions.

The interpretation given these results is that the fecal material contributes substances to the medium which enable S. pullorum to metabolize sufficiently to establish the penicillin effect, but which do not allow for appreciable multiplication in the time interval used.

A possible means to obviate this difficulty is suggested by the studies previously cited. Bigger (1944), Hobby and Dawson (1944), Schwartzman (1944), Chain and Duthie (1945). These authors all noted that certain bacteriostatic substances render bacteria resistant to penicillin by virtue of the fact that they inhibit growth.

For this phenomenon to be of use here, a bacteriostat would have to be employed which would selectively inhibit,

TABLE 12

The Effect of One Percent Sterile Feces on the Survival of *S. pullorum* after Exposure to 200 Units of Penicillin per ml in YBP Medium

	Count before exposure	Count after exposure
YBP medium plus 0.25% lactose	126	120
YBP medium plus 0.25% lactose plus 1% sterile feces	132	0

but not kill the salmonellae while allowing the coliforms to grow.

McCulloch (1945) stated that "Small amounts of arsenous acid are toxic to many pathogenic organisms, such as E. typhosa, V. comma and many streptococci, while the closely related E. coli, P. vulgaris, and many staphylococci actually are stimulated by such amounts."

Dubos (1947) indicated that it is now considered that the arsenicals, whether tervalent or quinquevalent, are first oxidized or reduced to their corresponding arsenoxides, which in turn react with some reduced sulfhydryl groups of the cell. He pointed out that the simultaneous injection of an arsenical and a compound containing an SH group slows the rate of disappearance of trypanosomes from an infected animal. This lends support to the idea that arsenicals are active due to their ability to block SH groups of enzyme systems. This also suggests that arsenic may be bacteriostatic rather than bactericidal. If indeed this is the case, then an arsenic compound could be of use here as a selective bacteriostat.

Two arsenic compounds were tested to determine whether a selective bacteriostasis of the salmonellae could be achieved.

EXPERIMENT XIII.

Determination of the Selectively Inhibitory Property of Sodium Arsenate and Sodium Arsenite on S. pullorum and E. coli.

Procedure: Ten-ml quantities of tryptose-lactose broth, (Darby and Mallmann 1939), containing various concentrations of sodium arsenate and sodium arsenite were prepared. One set of tubes containing each of the two compounds was seeded with 0.1 ml of a 1-1000 dilution of a 24-hour culture of E. coli. A similar set of tubes was seeded with 0.1 ml of a 1-1000 dilution of a 24-hour culture of S. pullorum.

Growth, as determined by visual turbidity, was recorded after 24 hours. These results are given in Table 13.

Discussion: As can be seen from this table, E. coli grows well in much higher concentrations of both chemicals than does S. pullorum. However, it is seen that sodium arsenite inhibits S. pullorum over a much wider range than does sodium arsenate. For this reason the effect of sodium arsenite on E. coli and S. pullorum was further investigated.

EXPERIMENT XIV.

Determination of the Influence of Various Concentrations of Sodium Arsenite on Viability of S. pullorum and Growth of E. coli in Tryptose-lactose Broth.

Procedure: Fifty-ml quantities of tryptose-lactose

TABLE 13

The Selectively Inhibitory Property of Sodium Arsenate and Sodium Arsenite on *E. coli* and *S. pullorum* in Tryptose-lactose Broth

Organism	E. coli	S. pullorum
Percent of sodium arsenate		
0.06	3+*	-
0.05	4+	-
0.04	4+	-
0.03	4+	-
0.02	4+	-
0.01	4+	1+
Control		
0	4+	4+
Percent of sodium arsenite		
0.06	3+	-
0.05	4+	-
0.03	4+	-
0.01	4+	-
0.008	4+	-
0.006	4+	-
0.004	4+	-
Control		
0	4+	4+

* = degree of turbidity compared to control at 4+.

broth were prepared containing various concentrations of sodium arsenite. Five-tenths ml of a 1-600,000 dilution of a 24-hour culture of E. coli was seeded into each flask of one series. In a similar manner a 1-100,000 dilution of a 24-hour culture of S. pullorum was seeded into another series. At hourly intervals one-ml quantities were plated in duplicate in brain heart infusion agar. After 24 hours incubation at 37 C, the colonies were counted and recorded as in Table 14a and 14b.

Discussion: From these results it is seen that in tryptose-lactose broth a concentration of sodium arsenite as high as 0.01 percent has no inhibitory effect on the growth of E. coli. Also, a concentration as low as 0.006 percent is bacteriostatic for S. pullorum.

At this point the effect of 0.01 percent sodium arsenite on several other enteric organisms was also determined.

EXPERIMENT XV.

The Effect of 0.01 Percent Sodium Arsenite on the Growth of Several Members of the Enterobacteriaceae in Tryptose-lactose Broth.

Procedure: Ten-ml quantities of tryptose-lactose broth containing 0.01 percent sodium arsenite were prepared and sterilized by autoclaving for ten minutes at 121 C.

TABLE 14a

The Effect of Various Concentrations of Sodium Arsenite on Viability and Growth of *E. coli* in Tryptose-lactose Broth at 37 C

Incubation time in hours	0	2	4
Sodium arsenite concentration (%)			
0	28*	118	2,400
0.0004	21	125	3,900
0.0006	21	120	2,400
0.0008	28	125	3,600
0.001	25	115	3,700
0.002	31	128	3,400
0.004	28	121	2,700
0.006	27	118	2,400
0.008	24	122	2,600
0.01	20	132	2,800

* average count per ml

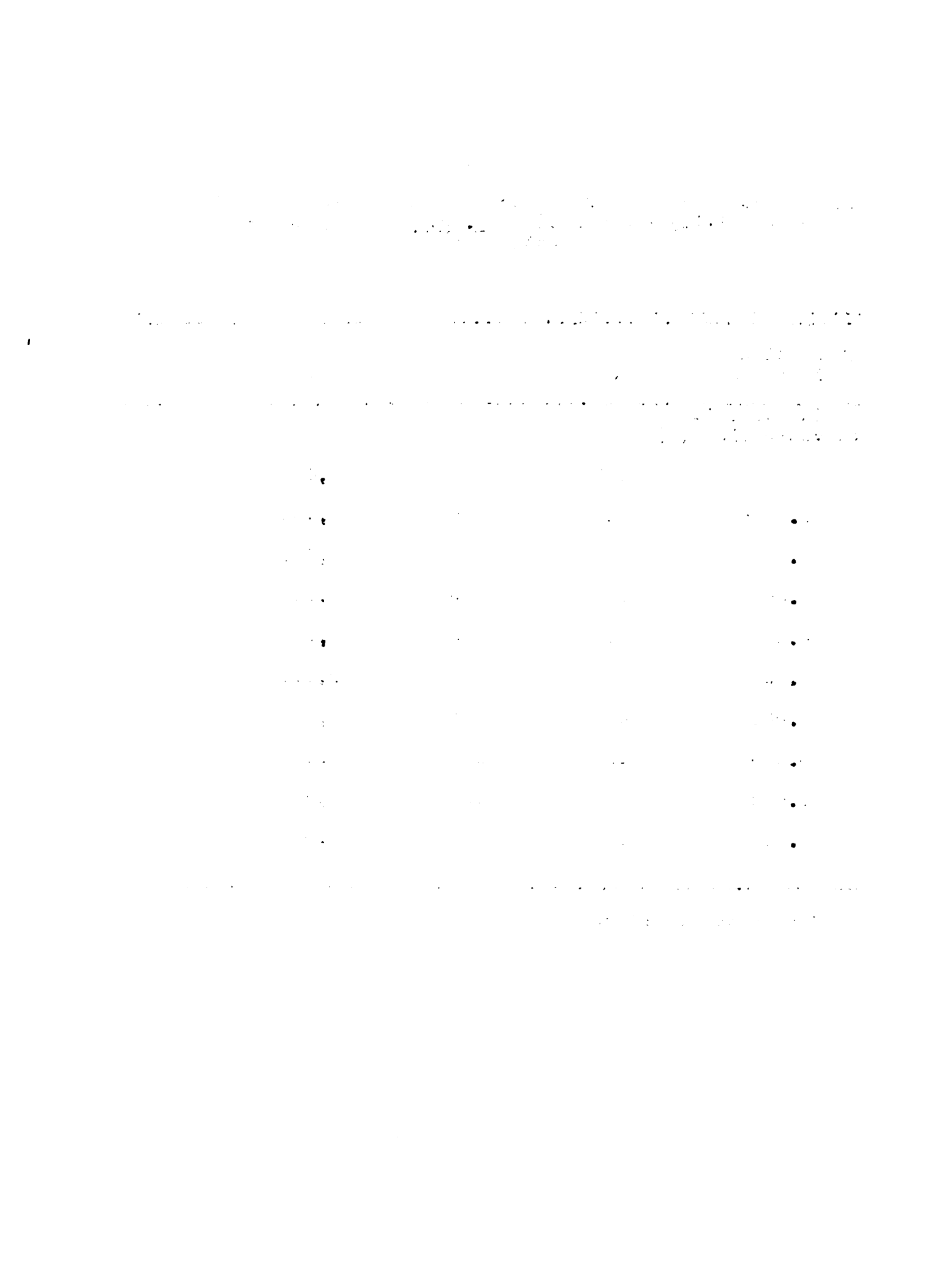


TABLE 14b

The Effect of Various Concentrations of Sodium Arsenite on Viability and Growth of *S. pullorum* in Tryptose-lactose Broth at 37 C

Incubation time in hours	0	2	4	6
Sodium arsenite concentration (%)				
0	123*	390	6,200	TNTC
0.0004	107	152	300	950
0.0006	117	141	147	160
0.0008	129	110	132	140
0.001	107	110	107	105
0.002	124	147	125	120
0.004	130	126	122	105
0.006	113	137	124	103
0.008	118	117	129	109
0.01	107	120	125	111

* average count per ml

These tubes were seeded with 0.1 ml of 1-100 dilutions of 24-hour brain heart infusion broth cultures of several members of the Enterobacteriaceae. Growth, as evidenced by visual turbidity, was determined after 24 and 48 hours and recorded in Table 15.

Discussion: It is evident from this table that all the members of the genus Salmonella tested were inhibited. However, P. vulgaris, Alkaligenes fecalis, Pseudomonas aeruginosa, E. coli, A. aerogenes and the Shigellas did show turbidity within 24 hours.

In the light of these results, and the hypothesis that penicillin affects only growing cells, it seemed advisable to determine whether a selective kill of E. coli and simultaneous survival of S. pullorum could be obtained by use of penicillin in this tryptose-lactose medium containing 0.01 percent sodium arsenite.

EXPERIMENT XVI.

Determination of the Effect of 0.01 Percent Sodium Arsenite on Penicillin Action against S. pullorum and E. coli in Tryptose-lactose Broth.

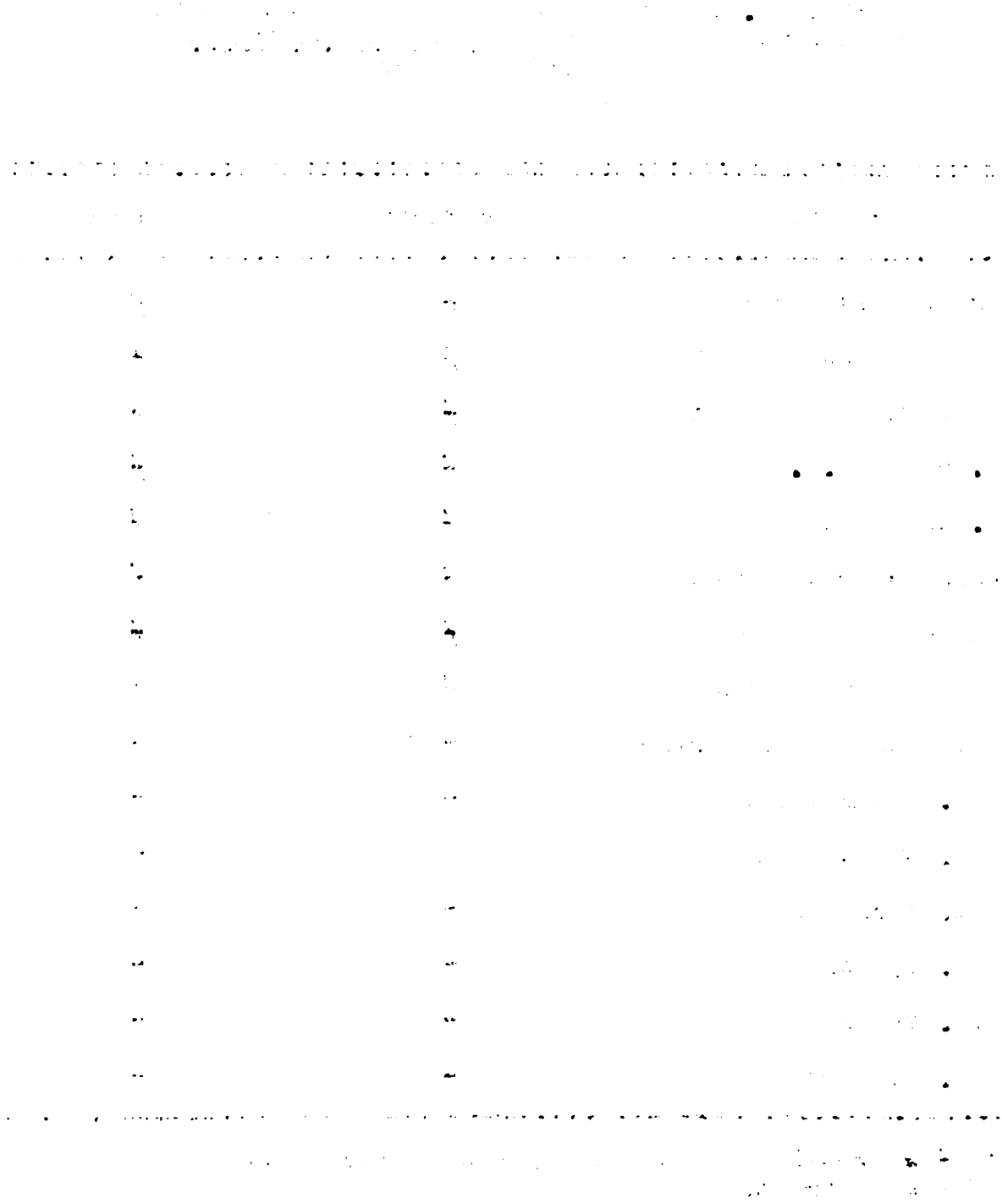
Procedure: Fifty-ml quantities of tryptose-lactose broth with 0.01 percent sodium arsenite were prepared. One of these flasks was seeded with 0.5 ml of a 1-100,000 dilution of a brain heart infusion broth culture of S. pullorum.

TABLE 15

The Effect of 0.01 Percent Sodium Arsenite on the Growth of Several Members of the Enterobacteriaceae in Tryptose-lactose Broth

Organism	24 hours	48 hours
Proteus vulgaris	≠*	≠
Alkaligenes fecalis	≠	≠
Pseudomonas aeruginosa	≠	≠
E. coli D.B.	≠	≠
E. coli #22	≠	≠
Aerobacter aerogenes	≠	≠
Shigella ambigua	≠	≠
Shigella alkalescens	≠	≠
Salmonella typhimurium	-**	-
Sal. enteritidis	-	-
Sal. typhosa #1	-	-
Sal. paratyphi B	-	-
Sal. typhosa #2	-	-
Sal. typhosa #3	-	-
Sal. pullorum	-	-

* ≠ = growth as evidenced by visual turbidity
** - = no growth



The other was seeded with 0.5 ml of a 1-1000 dilution of a similar culture of E. coli. Control flasks containing no sodium arsenite were also prepared. After a one hour pre-incubation, 200 units of penicillin per ml were added. After four more hours at 37 C, five ml amounts were removed and placed in test tubes containing 35,000 "units" of penase. These tubes were incubated for two hours at 37 C and plated in duplicate in brain heart infusion agar. After 24 hours at 37 C, the plates were counted. The results are given in Table 16.

Discussion: As can be seen here, the viability of E. coli was reduced about 99.8 percent by this penicillin exposure. The recovery of S. pullorum was about 20 percent. However, S. pullorum in tryptose-lactose broth without sodium arsenite showed no recovery at all. This is evidence for the fact that a bacteriostatic concentration of sodium arsenite does inhibit penicillin action to some extent. However, under the conditions of this experiment, a quantitative recovery of S. pullorum from exposure to 200 units of penicillin per ml was not attained.

The inhibition of penicillin action by sodium arsenite must be considered as a secondary effect. The primary effect appears to be that of bacteriostasis. This hypothesis is substantiated by the fact that 0.01 percent sodium arsenite

TABLE 16

The Effect of 0.01 Percent Sodium Arsenite on Penicillin Action against *S. pullorum* and *E. coli* in Tryptose-lactose Broth

	S. pullorum	E. coli
Before penicillin exposure	115*	19,200
After penicillin exposure	21	38
Control - no sodium arsenite	0	34

* average count per ml

does not inhibit the action of penicillin against E. coli, but does inhibit the penicillin effect against S. pullorum to some extent. It is significant that this sodium arsenite concentration is bacteriostatic for S. pullorum, but not for E. coli.

It is a more difficult matter to demonstrate the bacteriostatic effect of sodium arsenite on S. pullorum in YBP medium containing lactose. The obvious reason for this is that the medium itself will not support the growth of the organism. However, the highest concentration of sodium arsenite which will not cause a kill of S. pullorum in YBP medium with 0.25 percent lactose was next determined.

EXPERIMENT XVII.

Determination of the Effect of Various Concentrations of Sodium Arsenite on Survival of S. pullorum in YBP Medium with 0.25 Percent Lactose.

Procedure: Fifty-ml quantities of YBP medium were prepared with various concentrations of sodium arsenite. After autoclaving, 0.25 percent lactose was added to each flask. The flasks were then seeded with 0.5 ml of a 1-100,000 dilution of a 24-hour culture of S. pullorum and incubated at 37 C. After hourly intervals, one ml quantities were plated in duplicate in brain heart infusion agar. Counts obtained after 24 hours at 37 C are recorded in Table 17.

TABLE 17

The Effect of Various Concentrations of Sodium Arsenite on Growth and Survival of *S. pullorum* in YBP Medium plus 0.25 Percent Lactose

Sodium arsenite concentration (%)	0.001	0.002	0.004	0.006
Time (Hours)				
0	430*	430	430	42**
5	430	420	320	31
10	440	420	290	18

* count per ml
** due to inhibition, not low inoculum

Discussion: As is seen from Table 17, a concentration of sodium arsenite as high as 0.002 percent causes no appreciable decrease of S. pullorum after as long as 11 hours at 37 C. This concentration proved to be bacteriostatic even in tryptose-lactose broth, Table 14, and on this basis, it was assumed to be bacteriostatic in YBP medium also.

It was next determined whether 0.002 percent sodium arsenite in YBP medium containing 0.25 percent lactose would protect S. pullorum from exposure to 200 units of penicillin per ml in the presence of approximately one percent fecal material.

EXPERIMENT XVIII.

Determination of the Effect of 0.002 Percent Sodium Arsenite on the Recovery of S. pullorum from Exposure to Penicillin in YBP Medium Containing One Percent Sterile Feces.

Procedure: A 50 ml quantity of YBP medium containing 0.25 percent lactose was prepared. A similar quantity of YBP medium was prepared but 0.002 percent sodium arsenite and approximately one percent sterile human fecal material were added. Each flask was seeded with 0.5 ml of a 1-100,000 dilution of a 24-hour brain heart infusion culture of S. pullorum. After a six-hour incubation at 37 C, 10-ml portions were removed from each flask to serve as controls on

the effect of the medium itself on the organism. Penicillin was then added to the original flasks to give a level of 200 units per ml. After an additional four-hour incubation, five ml amounts were removed and mixed with 35,000 "units" of penase. Two hours later, duplicate one-ml quantities were plated in brain heart infusion agar pour plates. The results are given in Table 18.

Discussion: It can be seen here that in YBP medium with 0.25 percent lactose, 0.002 percent sodium arsenite and one percent fecal material, S. pullorum can survive penicillin exposure to the extent of about 83 percent. Under identical conditions, except for the absence of sodium arsenite, no survival occurred. This also confirms the finding in Experiment XVI that sodium arsenite in bacteriostatic concentrations does tend to inhibit the action of penicillin.

It now became necessary to determine whether this concentration of sodium arsenite will also have an inhibitory effect on the growth of E. coli in YBP medium plus 0.25 percent lactose, and a consequent effect on the penicillin action against this organism. For this determination the following was done:

TABLE 18

The Influence of 0.002 Percent Sodium Arsenite on Recovery of *S. pullorum* from Exposure to 200 Units of Penicillin per ml in YBP Medium with 0.25 Percent Lactose and Approximately 1.0 Percent Sterile Feces

	0.002% Sodium Arsenite and 1.0% feces	No Sodium Arsenite plus 1.0% feces
Control - no penicillin	153*	162
After penicillin exposure	128	0

* average count per ml

EXPERIMENT XIX.

Determination of the Effect of Two Concentrations of Sodium Arsenite on the Growth of *E. coli* in YBP Medium.

Procedure: Fifty-ml quantities of YBP medium were prepared containing 0.001 and 0.002 percent sodium arsenite. Each flask was seeded with 0.5 ml of a 1-200,000 dilution of a 24-hour brain heart infusion broth culture of *E. coli*. At hourly intervals, appropriate dilutions were made and plated in brain heart infusion agar. The resulting counts made after 24 hours at 37 C are given in Table 19.

Discussion: By comparing these counts with those of the control flask, it becomes evident that 0.001 and 0.002 percent sodium arsenite in YBP medium caused no appreciable inhibition of growth of *E. coli*. Since penicillin action has been shown to be positively correlated with growth, these concentrations of arsenite should also show no inhibition of penicillin action against *E. coli*. This will be further confirmed in subsequent experiments.

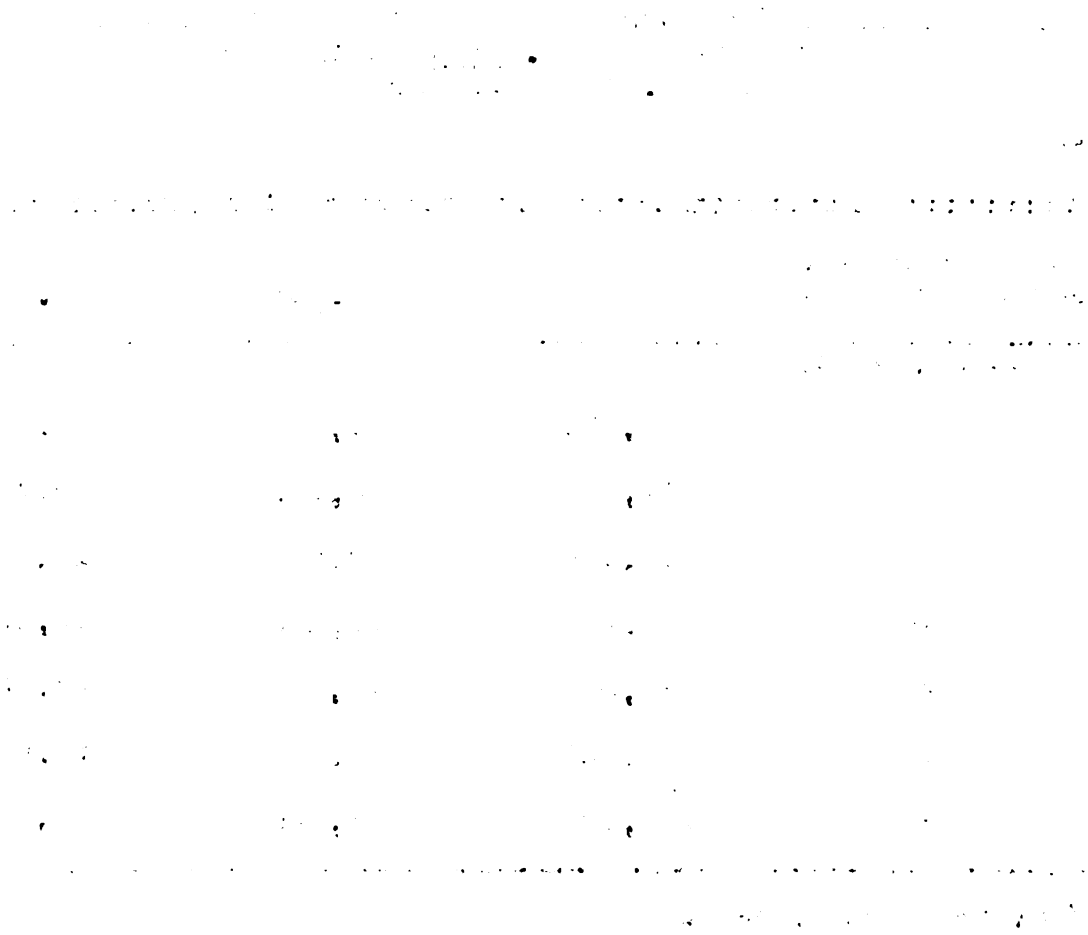
The experiments done thus far have established the optimum conditions of time and medium for kill of *E. coli* by penicillin, and simultaneous survival of *S. pullorum*. The next step deals with determining the optimum penase concentration and time to use for maximum recovery of *S. pullorum* from exposure to penicillin in YBP medium under the conditions established above.

TABLE 19

The Effect of Two Concentrations of Sodium Arsenite on the Growth of *E. coli* in YBP Medium plus 0.25 Percent Lactose

Sodium arsenite concentration (%)	0	0.001	0.002
Time (hours)			
0	15,800*	16,200	15,900
1	17,300	15,700	16,700
2	16,200	15,800	15,800
3	19,400	21,100	20,000
4	41,000	42,000	36,000
5	84,000	91,000	96,000
6	123,000	131,000	127,000

* average count per ml



EXPERIMENT XX.

Determination of the Optimum Penase Concentration and Incubation Time for Maximum Recovery of S. pullorum from Exposure to 200 Units of Penicillin per ml in YBP Medium.

Procedure: Fifty ml of YBP medium containing 0.002 percent sodium arsenite, 0.25 percent lactose and one percent sterile fecal material was prepared. This was seeded with 0.5 ml of a 1-100,000 dilution of a 24-hour culture of S. pullorum and incubated at 37 C for six hours. Two hundred units of penicillin per ml were then added. After four hours, five-ml quantities were removed and mixed in test tubes with various amounts of penase. After hourly intervals, duplicate one-ml quantities were plated in brain heart infusion agar. The colonies which developed were counted after 24 hours at 37 C. These results are found in Table 20.

Discussion: It is clearly seen from these data that under these conditions 3,500 and 17,500 "units" of penase are inadequate for recovery of S. pullorum from the 1,000 units of penicillin contained in the five ml of medium. It is further evident that 35,000 "units" of penase did permit a recovery of about 80 percent. The percent of recovery was not appreciably influenced by as much as 70,000 "units" of penase. It is also evident here that a penase exposure time of more than one hour at 37 C does not further enhance the

TABLE 20

The Effect of Varying Penase Concentrations and Incubation Times on Recovery of S. pullorum from Exposure to 1,000 Units of Penicillin Contained in Five ml of YBP Medium

Hours of penase exposure	1	2	3
Units of penase			
3,500	0*	0	0
17,500	0	0	0
35,000	121	110	110
70,000	116	94	112
Control - no penicillin	149		

* average count per ml

recovery of S. pullorum. Thus, a one hour incubation at 37 C in the presence of 35,000 "units" of penase is considered adequate for recovery of S. pullorum from 1,000 units of penicillin contained in five ml of YBP medium with 0.25 percent lactose and one percent sterile fecal material. The reason for the failure to attain a quantitative recovery of the organisms is to be sought elsewhere.

There are several possible explanations for failure to attain a quantitative recovery. These possibilities are considered below:

1. The fairly long period of bacteriostasis necessary here may have been lethal to some of the organisms. However, the results in Table 17 show that this is not responsible for the reduction in numbers. Here it is shown that a 10-hour incubation period at 37 C in YBP medium, containing 0.002 percent sodium arsenite and 0.25 percent lactose, does not cause a significant decrease in numbers of S. pullorum.

2. Another possibility is that not all the cells are in a completely static state, and therefore, do not resist the penicillin effect. This is a rather difficult point to determine with certainty. However, the results of Table 17 show that S. pullorum does not multiply in YBP medium with 0.002 percent sodium arsenite. This is evidence for, but not proof of, the completely static state of the organism.

It is possible that some of the organisms are able, under these conditions, to metabolize sufficiently to become irreversibly injured by penicillin without actually undergoing multiplication.

3. The third possibility is that the plating medium used may not provide optimum conditions for recovery from penicillin exposure. This third possibility was considered worthy of investigation in the light of the findings of Wainwright and Mullaney (1954). These authors showed that the growth of *E. coli* exposed to penicillin in a synthetic medium was profoundly influenced by the availability of the carbon source in a synthetic medium.

Curran and Evans (1937) also showed that the composition of media used for recovery of bacterial spores and vegetative cells previously exposed to lethal agents greatly influenced the viable count. They conclude that organisms which survive killing factors are more fastidious in their food requirements than the same culture before exposure.

An attempt was now made to determine whether the composition of the plating medium actually does influence the amount of recovery after penicillin exposure.

EXPERIMENT XXI.

Determination of the Influence of the Final Plating Medium on Recovery of S. pullorum from Penicillin Exposure.

Procedure: Five-tenths ml of a 1-100,000 dilutions of a 24-hour culture of S. pullorum was added to 50 ml of YBP medium containing 0.25 percent lactose and 0.002 percent sodium arsenite. After a six-hour pre-incubation at 37 C, 200 units of penicillin per ml were added. Four hours later a five-ml quantity was removed and mixed with 35,000 "units" of penase. After one hour at 37 C, one-ml quantities were plated in duplicate in each of two media. These media were plain nutrient agar and brain heart infusion agar. As can be seen from the results in Table 21, the nature of the medium has a very marked effect on recovery. Brain heart infusion agar again gave approximately an 81 percent recovery, while nutrient agar gave no recovery at all. Control plates show that when not previously exposed to penicillin, essentially the same number of S. pullorum colonies develop on both media, although it was noted that the colonies formed on brain heart infusion agar are somewhat larger than those on nutrient agar.

The fact that a greater recovery is obtained when using brain heart infusion agar rather than plain nutrient agar indicates that after penicillin exposure in a static state, S. pullorum is still viable. However, the percent of via-

bility is a function of the plating medium used for evaluation of this viability.

Brain heart infusion agar is generally considered a very rich medium; and, by comparison, plain nutrient agar may be considered relatively poor. However, the difference in survival of S. pullorum after penicillin exposure, when plated in these two media, must be due to a factor or factors other than this general "richness". Evidence for this is the fact that equal numbers of organisms develop on each medium when S. pullorum is plated without a previous penicillin exposure (See Table 21 - controls).

At this point in the investigation it was desired to determine to what extent other Salmonella species would survive this penicillin enrichment technique.

EXPERIMENT XXII.

Determination of the Survival of Several Salmonella Strains from Exposure to Penicillin in YBP Medium.

Procedure: Twenty-four hour brain heart infusion broth cultures of five Salmonella species were diluted 1-1,000,000 in saline. Ten-ml quantities of YBP medium with 0.002 percent sodium arsenite, 0.25 percent lactose and 1.0 percent sterile fecal material were seeded with one ml of the above dilutions. After a six-hour incubation at 37 C, five-ml quantities were removed and 200 units of penicillin per ml

TABLE 21

The Influence of the Nature of the Plating Medium on the Recovery of *S. pullorum* from Penicillin Exposure

Plating medium	Nutrient agar	Brain heart infusion agar
Before exposure	145*	149
After exposure	0	121

* average count per ml

were added. The remainder from each tube was held until the end of the experiment and plated as a control on the toxicity of the medium. After four more hours at 37 C, 35,000 "units" of penase were added and mixed thoroughly by shaking. One hour later, duplicate one ml quantities were plated in brain heart infusion agar. The results are given in Table 22.

Discussion: From these results, it is evident that these five Salmonella strains also survive penicillin exposure in YBP medium but to varying degrees; S. typhimurium, S. paratyphi B, S. choleresuis and S. typhosa show a survival of from 80 to 90 percent, while S. enteritidis survived only about 14 percent. It is not clear why S. enteritidis did not survive better. The control tubes show that this sharp reduction was not due to the toxicity of the medium. Table 15 also shows that sodium arsenite is bacteristatic to the organism. A repeated determination with the same organism showed essentially the same result.

TABLE 22

The Recovery of Several Salmonella Species from Exposure to Penicillin in YBP Medium

Organism	Before exposure	Control on medium toxicity	After exposure	Percent recovery
S. typhimurium	321*	318	260	82
S. paratyphi B	187	139	124	89
S. choleraesuis	242	190	187	99
S. typhosa	226	213	195	91
S. enteritidis	73	79	16	13

* average count per ml

DEVELOPMENT OF A DIFFERENTIAL PLATING MEDIUM FOR DISTINGUISHING THE SALMONELLAE FROM COLIFORM BACTERIA

In this investigation under certain definite conditions of incubation in the proper medium, E. coli was selectively killed by penicillin. Under these same conditions several Salmonella species were shown to survive at least to the extent of about 80 percent.

In order to determine whether this selectivity would apply to other coliforms and salmonellae in naturally infected fecal material, it became evident that a good, nontoxic differential medium which would distinguish the salmonellae from the coliforms must be employed.

It was desired to be able to plate relatively large volumes of YEP medium in which the organisms had been exposed to penicillin. This would enhance the statistical probability of recovering the salmonellae, especially where they occur in relatively small numbers. On the basis of these considerations then, a medium which could be used as a pour plate medium would be the one of choice.

A relatively nontoxic medium such as MacConkey agar is unsuitable because only surface colonies are differentiated.

Bismuth sulfite agar is satisfactory as a poured plate medium. This was considered unsatisfactory for use here because of the presence of the dye, brilliant green. A medium containing no substances inhibitory for the gram negative organisms was desired in order that all the salmonellae surviving penicillin exposure would have the maximum opportunity to initiate growth and form colonies.

Lederberg (1948) employed 0.005 percent triphenyltetrazolium chloride in nutrient agar plus one percent lactose as a medium for the detection of fermentative variants of E. coli. He observed that the biological reduction of the tetrazolium salt to the highly colored, insoluble formazan is probably pH dependent. The parent type of E. coli, which is capable of producing acid from lactose in the medium, does not reduce the tetrazolium. Therefore, colonies of the parent type which develop remain colorless. On the other hand, occasional variants which do not produce acid from lactose do reduce the tetrazolium salt and take on a very intense red color. This is also advantageous in that the color remains confined to the colony and does not obscure the true nature of nearby colonies by diffusion throughout the medium, as often occurs with ordinary pH indicators.

Lederberg (1948) also suggested the possible value of tetrazolium in this respect in a differential medium for

the salmonellae.

Various tetrazolium salts are being used in the field of bacteriology for diagnostic purposes. Chapman (1951) incorporated triphenyltetrazolium chloride in his tergitol-7 medium for the differentiation of various types of coliforms. Reinbold, Swern and Hussong (1953) also describe the use of a tetrazolium salt in a medium for the isolation and enumeration of the enterococci.

Preliminary work using nutrient agar with 0.5 percent lactose and 0.005 percent triphenyltetrazolium chloride revealed that S. pullorum produced rather small, very intensely red colonies in poured plates as well as on streak plates. With the same medium, E. coli produced colorless or very faint pink colonies, somewhat larger in size. It thus appeared that tetrazolium could be used as an indicator to differentiate lactose fermenters from lactose non-fermenters.

However, the results of Experiment XXI show that nutrient agar is not a satisfactory medium for recovery of S. pullorum after penicillin exposure. Therefore, some other medium must be used as a base to insure adequate recovery. It was also shown in Experiment XXI that brain heart infusion agar enabled a relatively high recovery rate of S. pullorum.

One main difference between brain heart infusion agar and nutrient agar is that a higher concentration of peptone

is present in the former.

The following experiment was run in order to determine whether this higher peptone concentration could be at least partially responsible for the enhanced recovery of S. pullorum from penicillin exposure. Bacto-tryptose was the peptone used.

EXPERIMENT XXIII.

The Effect of Varying the Tryptose Concentration in the Plating Medium on the Recovery of S. pullorum from Penicillin Exposure in YBP Medium.

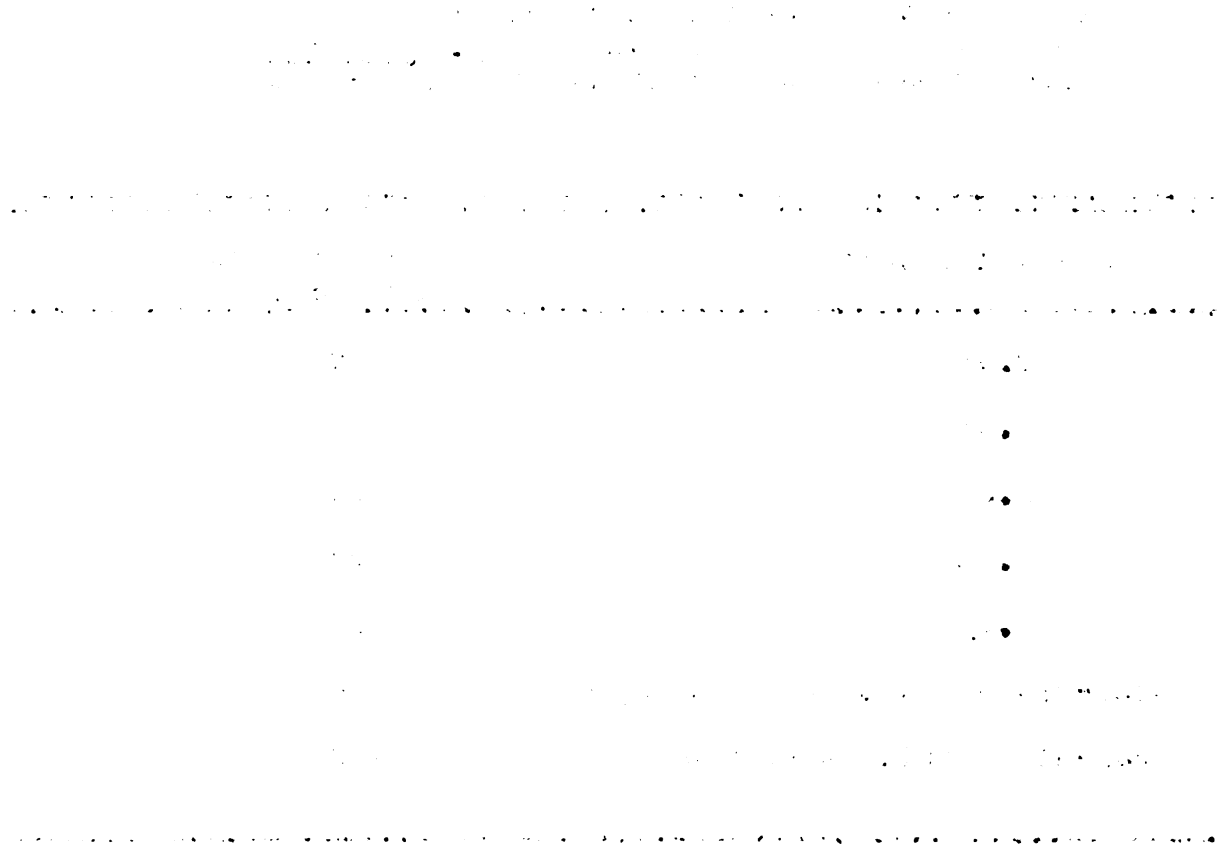
Procedure: A 50-ml quantity of YBP medium with 0.25 percent lactose and 0.002 percent sodium arsenite was seeded with 0.5 ml of a 1-100,000 dilution of a 24-hour brain heart infusion broth culture of S. pullorum. After six hours at 37 C, 200 units of penicillin per ml were added. After a further four-hour incubation, a 10-ml quantity was removed to a test tube containing 70,000 "units" of penase. After one hour at 37 C, one-ml amounts were plated in duplicates in media containing 0.5 percent sodium chloride, 1.5 percent agar and various concentrations of tryptose. After 24-hours incubation at 37 C, the colonies were counted. The results are shown in Table 23.

Discussion: It can be seen from these results that the amount of peptone in the form of bacto-tryptose in the plating

TABLE 23

The Effect of Tryptose Concentration in the Plating Medium on Recovery of S. pullorum from Exposure to Penicillin in YBP Medium

Tryptose Concentration	Count per ml after exposure
0.5%	0
1.0%	42
1.5%	70
2.0%	92
2.5%	89
Control-Brain heart infusion agar	160
Control-no penicillin exposure	190



medium does have a pronounced effect on the recovery of S. pullorum from exposure to penicillin in YBP medium. A two percent concentration of tryptose appears to be optimum. Lower concentrations tested, appreciably decreased the recovery, while a higher concentration did not increase recovery to any extent. The recovery of S. pullorum using this optimum concentration of tryptose was approximately 50 percent. In comparing this result with that obtained using brain heart infusion agar as the plating medium, it is as evident that some surviving cells still failed to develop in 2.0 percent tryptose agar.

Woodruff and Foster (1945), while studying bacterial penicillinase, made the observation that cysteine is capable of inactivating penicillin. Also other SH containing compounds were found to enhance penicillinase activity.

It was next deemed advisable to determine whether the addition of such SH containing substances to the plating medium would increase the rate of recovery of S. pullorum from exposure to penicillin.

EXPERIMENT XXIV.

Determination of the Effect of Cysteine and Sodium Thioglycollate on the Recovery of S. pullorum from Penicillin Exposure.

Procedure: A 50-ml quantity of YBP medium with 0.25

percent lactose and 0.002 percent sodium arsenite was prepared and seeded with 0.5 ml of a 1-100,000 dilution of a 24-hour culture of S. pullorum. After six hours at 37 C, 200 units of penicillin per ml were added. After a further four-hour incubation at 37 C, a twenty-ml quantity was mixed with 140,000 "units" of penase. After one hour, duplicate one-ml quantities were plated in two percent tryptose agar containing various concentrations of cysteine and sodium thioglycollate. These results may be found in Table 24.

Discussion: It is evident from these data that the addition of 0.02 percent cysteine to 2.0 percent tryptose agar increases the recovery of S. pullorum from penicillin exposure to about 82 percent. This is comparable to the recovery experienced using brain heart infusion agar. Any further increase in cysteine concentration does not appear to enhance recovery additionally.

Also, it can be seen that the addition of sodium thioglycollate does enhance the recovery to some extent, but not to the extent experienced with cysteine.

From the information gained in the previous experiments, the following was formulated as a possible differential medium for the detection of members of the genus Salmonella in the presence of other fecal bacteria:

Tryptose 2.0%

Sodium Chloride 0.5%

Cysteine 0.002%

Triphenyltetrazolium chloride 0.005%

Lactose 0.5%

Mixtures of E. coli and S. pullorum plated in this medium gave a very clear cut differentiation. The S. pullorum colonies were a very intensely red, while the E. coli colonies were a light pink and somewhat larger.

The next experiment was designed to determine the effectiveness of this tryptose lactose tetrazolium agar in differentiating S. pullorum from other fecal organisms which survive the penicillin exposure in YBP medium.

EXPERIMENT XXV.

Determination of the Ability of Tryptose Lactose Tetrazolium Agar to Differentiate S. pullorum from Other Fecal Organisms which Survive Penicillin Exposure in YBP Medium.

Procedure: A 50-ml quantity of YBP medium with 0.002 percent sodium arsenite and 0.25 percent lactose was prepared. Approximately 0.5 grams of fresh human feces and 0.5 ml of a 1-100,000 dilution of a 24-hour culture of S. pullorum were added. This was incubated for six hours at 37 C. Penicillin was added to a level of 200 units per ml, and further incubated for four hours. A five-ml quantity was then mixed

TABLE 24

The Effect of Cysteine and Sodium Thioglycollate on the Recovery of *S. pullorum* from Penicillin Exposure

Concentration (%)	Average count per ml
Cysteine	
0	126
0.0025	140
0.005	138
0.01	148
0.02	186
0.03	190
Sodium thioglycollate	
0	126
0.01	142
0.025	145
0.05	151
0.1	143
Control - before penicillin	230

with 35,000 "units" of penase and incubated further for one hour. One-ml quantities were then plated in tryptose lactose tetrazolium agar. After 24 hours, the plates were examined with the following result:

S. pullorum colonies which developed were deeply dark red. The relatively few coliform colonies (lactose fermenting gram negative rods) varied from nearly colorless to pink. These were also considerably larger than the S. pullorum colonies. A fairly large proportion of the small intensely red colonies proved to be gram positive cocci, and cocci in short chains or pairs characteristic of the enterococci.

Discussion: This medium showed a sharp distinction between S. pullorum and the other gram negative fecal forms encountered in this specimen. However, the development of the gram positive cocci made it apparent that some means of eliminating these forms was necessary if the differential value of triphenyl tetrazolium chloride was to be used here.

Several chemical substances might be added to this agar to inhibit selectively the gram positive organisms. Among these are the aniline dyes, various wetting agents, and certain bis-phenols.

Litsky, Mallmann and Fifield (1952) showed that in sufficient concentrations all the basic dyes which they tested

showed some inhibition of the gram negative as well as a marked inhibition of the gram positive organisms. Litsky, Mallmann, and Fifield (1953) worked with ethyl violet, which is the least toxic of these dyes. These authors show that the enterococci are intermediate in sensitivity between the gram positive and gram negative organisms. The results of these studies indicate that a concentration of ethyl violet, which is sufficient to inhibit the enterococci, could also show considerable toxicity toward gram negative organisms. Since the enterococci are among the organisms which it is desired to inhibit, these dyes were not considered further.

Mallmann and Darby (1941) showed that the anionic wetting agent sodium lauryl sulfate in a concentration of 0.01 percent is effective in inhibiting several gram positive organisms in tryptose broth. This same concentration proved completely nontoxic to E. coli, the gram negative organism tested.

Bordt (1951), working with the bisphenol 2,2' methylenebis-4-chloro-6-isopropylphenol (K-7643), demonstrated a marked selective property of this compound also. A concentration as low as 0.000001 percent inhibited the growth of the gram positive organisms tested, while the highest concentration tested, 0.001 percent, showed no toxicity toward E. coli.

These two compounds were next considered as possible agents to use for the elimination of the gram positive forms

which interfere with the diagnostic efficiency of tryptose lactose tetrazolium agar.

EXPERIMENT XXVI.

Determination of the Efficiency of 0.01 Percent Sodium Lauryl Sulfate and 0.001 Percent 2,2'Methylenebis-4-chloro-6-isopropylphenol in Suppressing the Growth of Gram Positive Fecal Organisms Which Survive Penicillin Exposure in YBP Medium.

Procedure: Ten gram positive organisms isolated from plates in Experiment XXIV were grown in brain heart infusion broth. Five of these were morphologically typical of micrococci, and five were large cocci in short chains or pairs morphologically typical of the enterococci. One drop of a 1-10,000 dilution of a 24-hour culture of each of these organisms was plated in tryptose lactose tetrazolium agar containing 0.01 percent sodium lauryl sulfate. Identical plates were poured using tryptose lactose tetrazolium agar with 0.001 percent of 2,2'methylenebis-4-chloro-6-isopropylphenol. Duplicate plates of each medium, as well as tryptose lactose tetrazolium agar base, were also seeded with one ml of a 1-1,000,000 dilution of a 24-hour culture of S. pullorum. These were controls on the toxicity of the two selective agents for S. pullorum. The plates were examined after 24 hours at 37 C, and recorded in Table 25.

TABLE 25

The Development of *S. pullorum* in the Presence of Sodium Lauryl Sulfate and K-7643

Inhibitor	Average count per ml
None	264
Sodium lauryl sulfate 0.01%	259
K-7643 0.001%	266

Discussion: The results of this study show that sodium lauryl sulfate was effective in completely inhibiting three of the five micrococcus types, while two did grow and produce dark red colonies. All five of the enterococcus types developed dark red colonies.

The plates containing the bisphenol, however, showed no growth of either type of gram positive organism.

An examination of Table 25 shows that neither sodium lauryl sulfate nor 2,2'methylenebis-4-chloro-6-isopropylphenol exhibit an appreciable toxicity toward S. pullorum as far as numbers of colonies developing is concerned.

On the basis of these results then, it was decided to use the following as a differential plating medium for the detection of salmonellae in subsequent studies:

Tryptose	2.0%
Lactose	0.5%
Sodium chloride	0.5%
Cysteine	0.02%
Triphenyltetrazolium chloride	0.005%
2,2'Methylenebis-4-chloro-6-isopropylphenol	0.0001%

Discussion: The results of this study show that sodium lauryl sulfate was effective in completely inhibiting three of the five micrococcus types, while two did grow and produce dark red colonies. All five of the enterococcus types developed dark red colonies.

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Sodium chloride	0.5%
Cysteine	0.02%
Triphenyltetrazolium chloride	0.005%
2,2'Methylenebis-4-chloro-6-isopropylphenol	0.0001%

USE OF THE PENICILLIN ENRICHMENT TECHNIQUE FOR THE ISOLATION OF SALMONELLAE FROM NATURALLY INFECTED HUMAN FECAL SPECIMENS

The final portion of this work was concerned with determining whether or not this "penicillin enrichment" procedure could be used for the isolation of members of the genus Salmonella from naturally infected human fecal material.

Source of specimens: The specimens used here were obtained from the diagnostic laboratory of the Michigan Department of Health, Lansing, Michigan. They consisted of fecal samples from known carriers, suspected carriers, suspected active cases of Salmonella infections, and from persons in contact with carriers or active cases. The specimens were usually obtained at weekly intervals, and varied in age from three to twelve days after the date of receipt at the laboratory.

Procedure for examination: Approximately two gm of the specimen was suspended by shaking in ten ml of sterile physiological saline in a large test tube. This suspension was then freed of gross particles by slowly forcing a loosely packed cotton plug down through the suspension to the bottom

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Procedure for examination: Approximately two gm of the specimen was suspended by shaking in ten ml of sterile physiological saline in a large test tube. This suspension was then freed of gross particles by slowly forcing a loosely packed cotton plug down through the suspension to the bottom

of the tube with a sterile pipette. One-and 0.1 ml-quantities of the supernatant were then placed in five-ml quantities of YBP medium containing 0.25 percent lactose and 0.002 percent sodium arsenite. After thorough mixing, these tubes were placed in a water bath at 37 C for six hours. Penicillin was then added to the level of 200 units per ml. After four hours at the same temperature, 35,000 "units" of penase were added to each tube. One hour later, 0.1-and five-ml amounts were plated in the differential tryptose, lactose tetrazolium agar described on page 85. The plates were examined after 24 and 48 hours at 37 C, and the very intensely red colonies characteristic of the salmonellae were picked and inoculated into tubes of Bacto-Kligler Iron Agar. Those tubes showing an alkaline slant, and acid butt with or without gas or hydrogen sulfide were further studied. The following media were used for a partial identification of the organisms isolated: Bacto-Purple Broth base containing 0.5 percent concentrations of dextrose, lactose, maltose, and sucrose. Also seeded were one percent tryptose solution for determination of indole production from tryptophane, semisolid motility test agar for determination of motility, Simmons' citrate agar for citrate utilization, and urea broth to detect the organisms ability to hydrolyze urea. Each culture was also gram-stained to check for purity and gm reaction.

In order to establish some standard to indicate the relative efficiency of this penicillin enrichment procedure, the specimens were also examined by using four of the several diagnostic media designed for salmonella isolation. In this parallel procedure, two ml of the original saline suspension was placed in test tubes containing ten-ml quantities of Bacto-Selenite broth, and incubated for 24 hours. After this preliminary enrichment, 0.1-ml quantities were spread on the surface of Bacto-MacConkey agar, Bacto-SS agar, and Baeto-Bismuth Sulfite agar plates. Also five-ml and one drop quantities of the original saline suspension were plated in bismuth sulfite agar pour plates. After 24 and 48 hours, these plates were examined and suspected salmonella colonies were picked and partially identified by the procedure outlined above.

Pure cultures showing reactions typical of the genus Salmonella were sent to the Salmonella Typing Station of the Michigan Department of Health for positive identification. The results obtained by the two methods of isolation are given in Table 26.

Discussion: The results of this limited number of examinations indicate that the efficiency of the penicillin enrichment procedure compares quite favorably with that of the combination of currently employed media. A total of 103

TABLE 26

Results of the Examination of Fecal Specimens for Salmonellae by the Penicillin Enrichment Technique and by Four Currently Employed Diagnostic Media

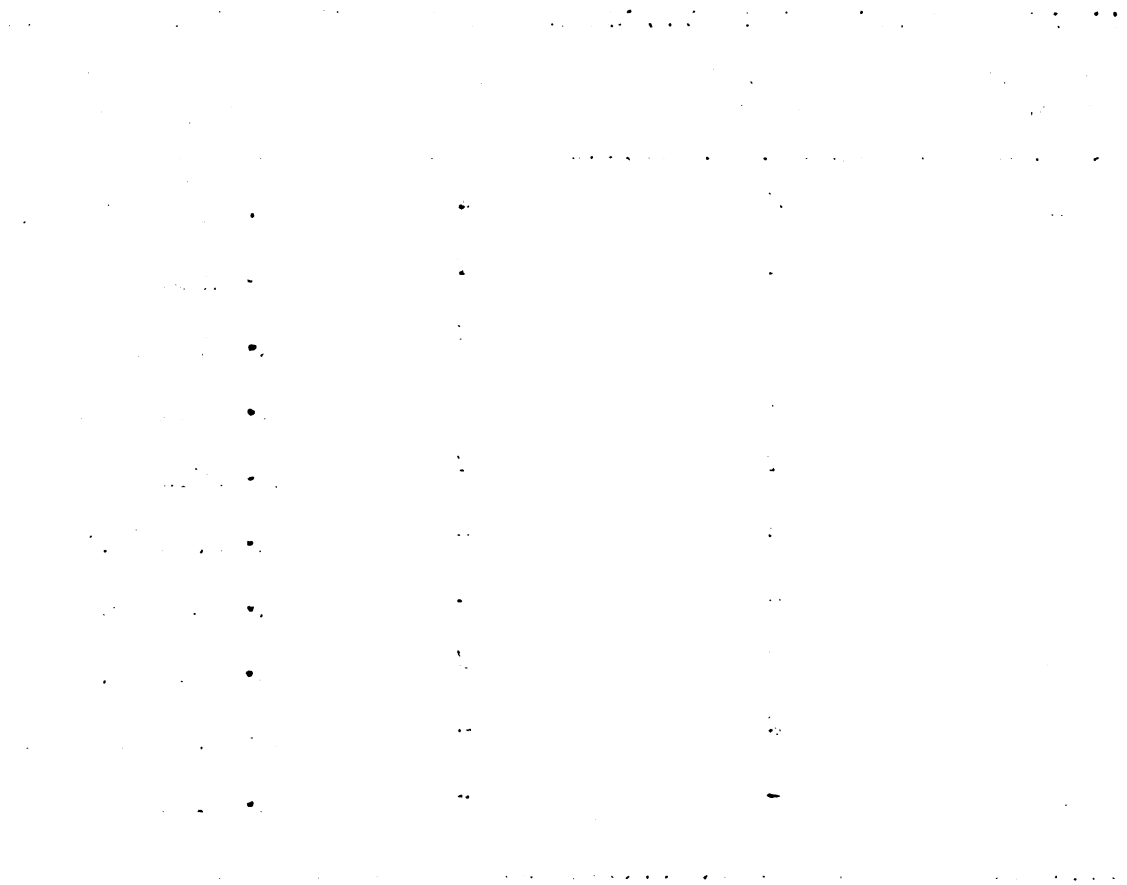
Specimen number	Current media	Penicillin technique	Organism isolated
42	+	-	S. typhosa
50	+	+	S. typhosa
57	-	+	S. typhosa
53	+	+	S. typhosa
62	+	+	S. montivedeo
64	+	+	S. tennessee
66	+	+	S. typhosa
123	+	+	S. typhimurium
129	+	+	S. typhosa
295	+	+	S. meunchen
405	+	+	S. oranienburg
731	+	+	S. typhosa
811	+	+	S. typhosa
835	+	-	S. montevideo
847	+	+	S. montevideo
868	+	+	S. typhimurium
899	-	+	S. typhosa

TABLE 26 (Continued)

Specimen number	Current media	Penicillin technique	Organism isolated
761	+	+	S. typhimurium
1153	+	+	S. typhosa
1168	+	+	S. oranienburg
1289	+	-	S. montevideo
1299	+	+	S. typhosa
1314	+	+	S. typhosa
1315	+	-	S. typhosa
1331	+	+	S. typhosa
1470	+	+	S. worthington
1611	-	+	S. typhosa

TABLE 26 (Continued)

Specimen number	Current media	Penicillin technique	Organism isolated
761	+	+	S. typhimurium
1153	+	+	S. typhosa
1168	+	+	S. oranienburg
1289	+	-	S. montevideo
1299	+	+	S. typhosa
1314	+	+	S. typhosa
1315	+	-	S. typhosa
1331	+	+	S. typhosa
1470	+	+	S. worthington
1611	-	+	S. typhosa



specimens were examined. From 22 of these, Salmonella species were isolated by the penicillin enrichment method, while 23 were positive by the commonly employed media. As is seen from the tables, in three cases salmonellae were isolated by the penicillin enrichment procedure but missed by the currently used media, while in four cases the penicillin technique failed, but the currently used media did not.

During the examination of these several specimens, it was noted that in the majority of the cases the non-salmonella population of the fecal specimen was reduced sufficiently to allow for a ready isolation of discrete colonies. However, in a few cases the resulting plates were very heavily populated, making differentiation and isolation of discrete colonies impossible. Upon closer examination of these overcrowded plates it was discovered that the organisms involved were most often those of the genus Pseudomonas. These colonies were dark red, but where not too abundant, they could be distinguished from the salmonellae by the presence of a greenish-white halo surrounding the colony.

Also in a few specimens the resulting plates were overcrowded with light pink colonies characteristic of the coliforms. Isolates from such colonies produced acid and gas in lactose broth and gave other typical coliform reactions. The reason for their surviving the penicillin exposure in

large numbers was not immediately apparent. However, when these organisms were placed in YBP medium containing 0.25 percent lactose, but no sodium arsenite, about 90 percent of them failed to show growth after 24 hours at 37 C. A number of such isolates are listed in Table 27 along with their IMViC reactions. Evidently these are intermediate coliform strains which do not possess the nutritional efficiency characteristic of the tribe Escherichieae. The survival of these types immediately suggests the possible use of a penicillin enrichment technique for the isolation of naturally occurring biochemically deficient forms of bacteria in much the same manner that artificially induced mutant forms are isolated.

Roepke, Libby and Small (1944) also reported the natural occurrence of nutritionally deficient strains of E. coli. These workers investigated the added requirements of some of these strains and found them to be one of the following: methionine, thiamine, nicotinic acid or its amide, lysine, cystine, arginine, threonine, or tryptophane.

Occasionally dark red colonies appeared on the plates which were indistinguishable from those of the salmonellae except for their slightly greater size. In Kligler's iron agar these gave acid and abundant gas in the butt, and an alkaline slant. Further biochemical studies revealed that

TABLE 27

IMViC Reactions of Coliform Strains which do not Grow in YBP Medium Plus Lactose and which Survived Penicillin Exposure in this Medium

Number	24 hour reaction in lactose broth	IMViC formula
1	AG	- ≠ - ≠
2	AG	- ≠ - ≠
3	AG	- ≠ - ≠
4	AG	- ≠ - -
5	AG	- ≠ - -
6	AG	- ≠ - ≠
7	AG	- ≠ - ≠
9	AG	- ≠ - -
10	AG	- ≠ - ≠
11	AG	- ≠ - ≠
12	AG	- ≠ - -
13	AG	- ≠ - ≠
15	AG	- ≠ - ≠
16	AG	- ≠ - ≠
17	AG	- ≠ - -
18	AG	- ≠ - ≠
19	AG	- ≠ - ≠
22	AG	- ≠ - -

TABLE 27

IMViC Reactions of Coliform Strains which do not Grow in YBP Medium Plus Lactose and which Survived Penicillin Exposure in this Medium

Number	24 hour reaction in lactose broth	IMViC formula
1	AG	- ≠ - ≠
2	AG	- ≠ - ≠
3	AG	- ≠ - ≠
4	AG	- ≠ - -
5	AG	- ≠ - -
6	AG	- ≠ - ≠
7	AG	- ≠ - ≠
9	AG	- ≠ - -
10	AG	- ≠ - ≠
11	AG	- ≠ - ≠
12	AG	- ≠ - -
13	AG	- ≠ - ≠
15	AG	- ≠ - ≠
16	AG	- ≠ - ≠
17	AG	- ≠ - -
18	AG	- ≠ - ≠
19	AG	- ≠ - ≠
22	AG	- ≠ - -

TABLE 27 (Continued)

Number	24 hour reaction in lactose broth	IMViC formula
23	AG	- ≠ - ≠
24	AG	- ≠ - ≠
26	AG	- ≠ - ≠
28	AG	- ≠ - -
30	AG	- ≠ - -
31	AG	- ≠ - ≠
32	AG	- ≠ - -
33	AG	- ≠ - -

in general these were very slow lactose fermenters or non-fermenters otherwise typical of members of the coliform group. These, according to Kauffmann (1951), are probably best called slow lactose fermenting or nonfermenting coliforms.

It is postulated that these interfering types survived exposure to penicillin because they were unable to grow in the synthetic YBP medium. The members of the Pseudomonas group probably survive due to their inability to utilize lactose as a carbon source. This also may be true of the lactose nonfermenting coliforms. The majority of the lactose fermenting types which survive probably do so because of some other nutritional deficiency which they possess. Proteus species would be expected to interfere here also. However, these were only very rarely encountered and caused no serious difficulty in the procedure.

One of the chief limitations to the penicillin enrichment technique is the relatively small sample which can be ordinarily examined. As can be seen from the procedure on page 87, the maximum amount of fecal material plated in any one plate is about 0.2 grams. This is in contrast to about one gram which can be plated in bismuth sulfite agar.

Another limitation, of course, is the fact that a hundred percent survival of the salmonella is not obtained. In most cases, 80-90 percent survival is experienced, but

with one organism it was as low as 13 percent. This is most serious, of course, when dealing with specimens containing relatively small numbers of salmonellae.

with one organism it was as low as 13 percent. This is most serious, of course, when dealing with specimens containing relatively small numbers of salmonellae.

SUMMARY

Various factors were investigated to determine their influence on the action of penicillin against selected members of the Enterobacteriaceae. These factors were composition of the medium, pre-incubation time, penicillin exposure time, methods of inactivation of penicillin prior to plating, and composition of the plating medium.

Two hundred units of penicillin per ml was bactericidal for E. coli in a synthetic medium with lactose as a carbon source. Also penicillin was found to be most actively bactericidal when exposure took place after the lag phase of growth had been passed and the organisms had begun to divide.

Dilution was not an effective means for reduction of penicillin concentration where recovery of previously exposed organisms was desired. Penicillinase was found to destroy penicillin effectively and allow recovery of previously exposed cells. However, a much higher concentration of the enzyme was required for recovery of cells exposed to penicillin than was necessary to inactivate completely the same amount of penicillin under identical conditions of time and temperature. S. pullorum, when pre-incubated for a sufficient time in a medium unfavorable for growth and then exposed to 800 units of peni-

cillin per ml, survived this exposure. This was presumably due to the fact that penicillin is effective only against growing cells.

Under identical conditions in YBP medium with lactose as the sole carbon source, 200 units of penicillin was highly bactericidal to E. coli, but showed very little action against S. pullorum. The addition of approximately one percent sterile fecal material resulted in a bactericidal action against S. pullorum as well. This was presumably due to the fact that the fecal material contributed substances to the medium which were utilizable for growth by S. pullorum, and thus established the penicillin effect.

Sodium arsenite in proper concentration was found to be selectively bacteriostatic against members of the genus Salmonella, but not against other Enterobacteriaceae studied. Sodium arsenite in a concentration of 0.002 percent in YBP medium protected members of the genus Salmonella from penicillin exposure even in the presence of one percent fecal material.

The presence of a two percent concentration of tryptose as well as 0.02 percent cysteine in the plating medium materially enhanced the recovery of S. pullorum from exposure to penicillin.

Also, the addition of 0.5 percent lactose and 0.005 per-

cent triphenyltetrazolium chloride to the plating medium resulted in colonies of diagnostic significance. Lactose non-fermenting organisms reduced the tetrazolium salt and developed intensely red colonies. Those organisms which produced acid from lactose failed to reduce the salt or did so only very slightly and gave rise to colorless or faint pink colonies.

In early attempts to utilize this penicillin enrichment technique for isolating salmonellae from fecal material, it was noted that certain gram positive bacteria also survived the exposure and interfered with the efficiency of the differential medium by forming colonies indistinguishable from those of the salmonellae. The addition of 0.001 percent of a bisphenol, 2,2'methylenebis-4-chloro-6-isopropylphenol, to the plating medium successfully inhibited these forms.

A series of human fecal samples was examined to determine whether this penicillin enrichment technique could be used for the isolation of salmonellae from feces. Comparative results obtained indicate that the technique is workable. The result of this limited series of examinations reveals a favorable comparison with several media presently used in routine diagnostic procedures. Among the limitations of the method is the fact that certain organisms other than the salmonellae survive the penicillin exposure, and when

initially present in very large numbers, these result in overcrowded plates which makes isolation of typical colonies difficult or impossible. The organisms involved in this type of interference are (1) Pseudomonas species, (2) nutritionally deficient coliforms, (3) lactose non-fermenting or slow fermenting coliforms. Other limitations of the method are the lack of quantitative recovery of the salmonellae and the necessarily relatively small inoculum used.

In addition to its use in isolating salmonellae from fecal material, this penicillin enrichment technique with modifications might also be used for the isolation of biochemically deficient forms of bacteria from nature.

CONCLUSIONS

1. 200 units of penicillin per ml was bactericidal for *E. coli* in a synthetic medium with lactose as a carbon source.
2. Penicillin was most actively bactericidal when exposure of the cells took place after the lag phase of growth had been passed and the organisms had begun to divide.
3. Dilution was not an effective means for reduction of penicillin concentration where recovery of previously exposed cells was desired.
4. A proper concentration of penicillinase did destroy penicillin effectively and allowed subsequent recovery and growth of cells previously exposed to the antibiotic.
5. *S. pullorum* was found to survive exposure to 200 units of penicillin per ml when in a synthetic medium where growth was nutritionally impossible.
6. Sodium arsenite in a concentration of 0.002 percent was found to be selectively bacteriostatic against various salmonellae. This bacteriostatic action of sodium arsenite also tended to protect salmonellae from the action of penicillin.
7. Members of the genus *Salmonella* were successfully isolated from infected fecal material by this selective action of peni-

cillin against growing cells.

8. The addition of two percent tryptose and 0.02 percent cysteine to the final plating medium increased the percent recovery of S. pullorum from penicillin action.

9. The presence of 0.5 percent lactose and 0.005 percent triphenyltetrazolium chloride in the plating medium resulted in the development of dark red salmonella colonies and light pink or colorless coliform colonies.

10. A concentration of 0.01 percent 2,2'methylenebis-4-chloro-6-isopropylphenol in the plating medium inhibited gram positive fecal organisms which survived penicillin exposure.

11. This "penicillin enrichment" technique compared favorably in efficiency with media currently employed for isolation of salmonellae from fecal material.

MATERIALS USED

Ammonium Sulfate $(NH_4)_2SO_4$ C.P.	Baker
Bacto Lactose	Difco
Bacto Penase Concentrate, Control Number 424049	Difco
Bacto Tryptose	Difco
Ferrous Sulfate $FeSO_4 \cdot 7H_2O$ C.P.	Merk
Magnesium Sulfate $MgSO_4 \cdot 7H_2O$ C.P.	Eimer & Amend
2,2'methylenebis-4-chloro-6-isopropylphenol	Dow
Penicillin G Potassium, Control Number 3A58827	Squibb
Potassium Phosphate monobasic KH_2PO_4 C.P.	Baker
Sodium Arsenate dibasic $Na_2HAsO_4 \cdot 7H_2O$ reagent	Merk
Sodium Arsenite $NaAsO_2$ anal. reagent	Mallinckrodt
Sodium Chloride NaCl C.P.	Baker
Sodium Phosphate dibasic Na_2HPO_4 C.P.	Baker
2,3,5, Triphenyl-2H-Tetrazolium Chloride	Distillation Products

LITERATURE CITED

Abraham, E.P. and E. Chain. An enzyme from bacteria able to destroy penicillin. Nature. 146:837. 1940.

Bigger, J.W. Treatment of staphylococcal infections with penicillin. Lancet. 142:497-500. 1944.

Bordt, D.E. A study of chemical agents for selective growth of the coliform organisms. Unpublished thesis for the degree of M.S. Michigan State College. 1951.

Burnet, F.M., J.D. Stone and J.G. Anderson. An epidemic of influenza B in Australia. Lancet. 250:807-811. 1946.

Cavallito, C.J. and J.H. Baily. Preliminary note on the inactivation of antibiotics. Science. 100:390. 1944.

Chain, E. and E.S. Duthie. Bactericidal and bacteriolytic action of penicillin on the staphylococcus. Lancet. 248: 652-657. 1945.

Chapman, G.H. A culture medium for detecting and confirming Escherichia coli in ten hours. Am. Jour. of Pub. Health. 41:1381. 1951.

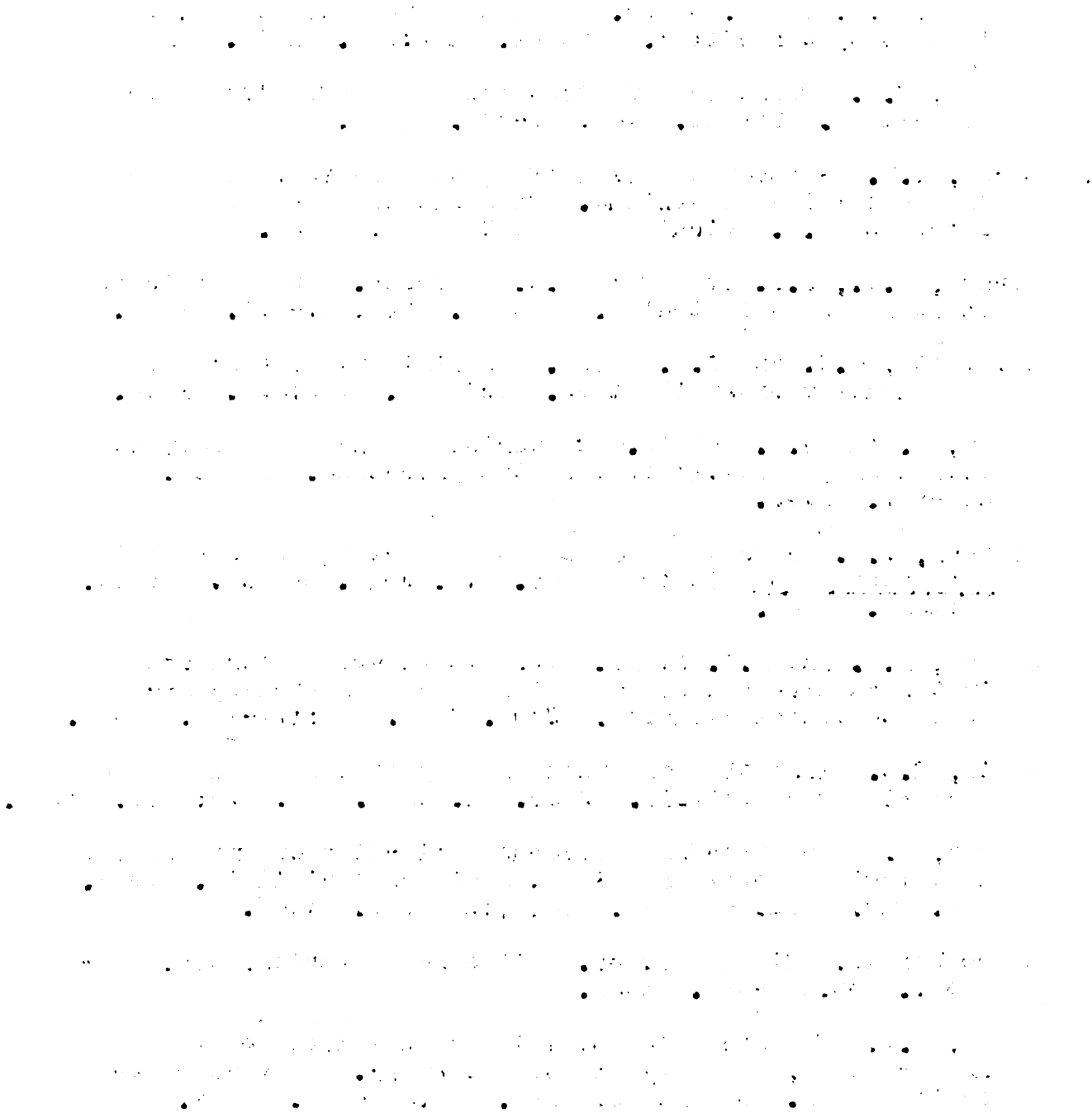
Curran, H.R. and F.R. Evans. The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. Jour. Bact. 34:179-189. 1937.

Davis, B.D. Isolation of biochemically deficient mutants of bacteria by penicillin. Jour. Am. Chem. Soc. 70:4267. 1948.

Dienes, L. Isolation of pleuropneumonia-like organisms from pathological specimens with the aid of penicillin. Proc. Soc. Exp. Biol. and Med. 64(2):165-166. 1947.

Difco Manual. Ninth Edition. Difco Laboratories, Inc. Detroit. pp.284-285. 1953.

Dubos, R.J. The Bacterial Cell in its Relation to Problems of Virulence, Immunity and Chemotherapy. Harvard University Press. Cambridge, Mass. pp.309-311. 1947.



Dufrenoy, S., L.A. Strait and R. Pratt. IV Comparative responses of gram-positive and gram-negative bacteria to penicillin. Jour. Bact. 54:719-730. 1947.

Eagle, H. and A. D. Musselman. The slow recovery of bacteria from the toxic effects of penicillin. Jour. Bact. 58: 475-490. 1949.

Fleming, A. On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenzae. Brit. Jour. Exp. Path. 10: 226. 1929.

Foster, J.W. and B.L. Wilker. Microbiological aspects of Penicillin. II Turbidometric studies on penicillin inhibition. Jour. Bact. 46:377-389. 1943.

Hawking, F. Growth of protozoa in tissue culture. II Plasmodium relictum, exoerythrocytic forms. Trans. Roy. Soc. Trop. Med. and Hyg. 40(2):2183-2188. 1946.

Hobby, G.L. and M.H. Dawson. Bacteriostatic action of penicillin on hemolytic streptococcus in vitro. Proc. Soc. Exp. Biol. Med. 56:178-181. 1944a.

Hobby, G.L. and M.H. Dawson. Effect of rate of growth of bacteria on action of penicillin. Proc. Soc. Exp. Biol. Med. 56:181-184. 1944b.

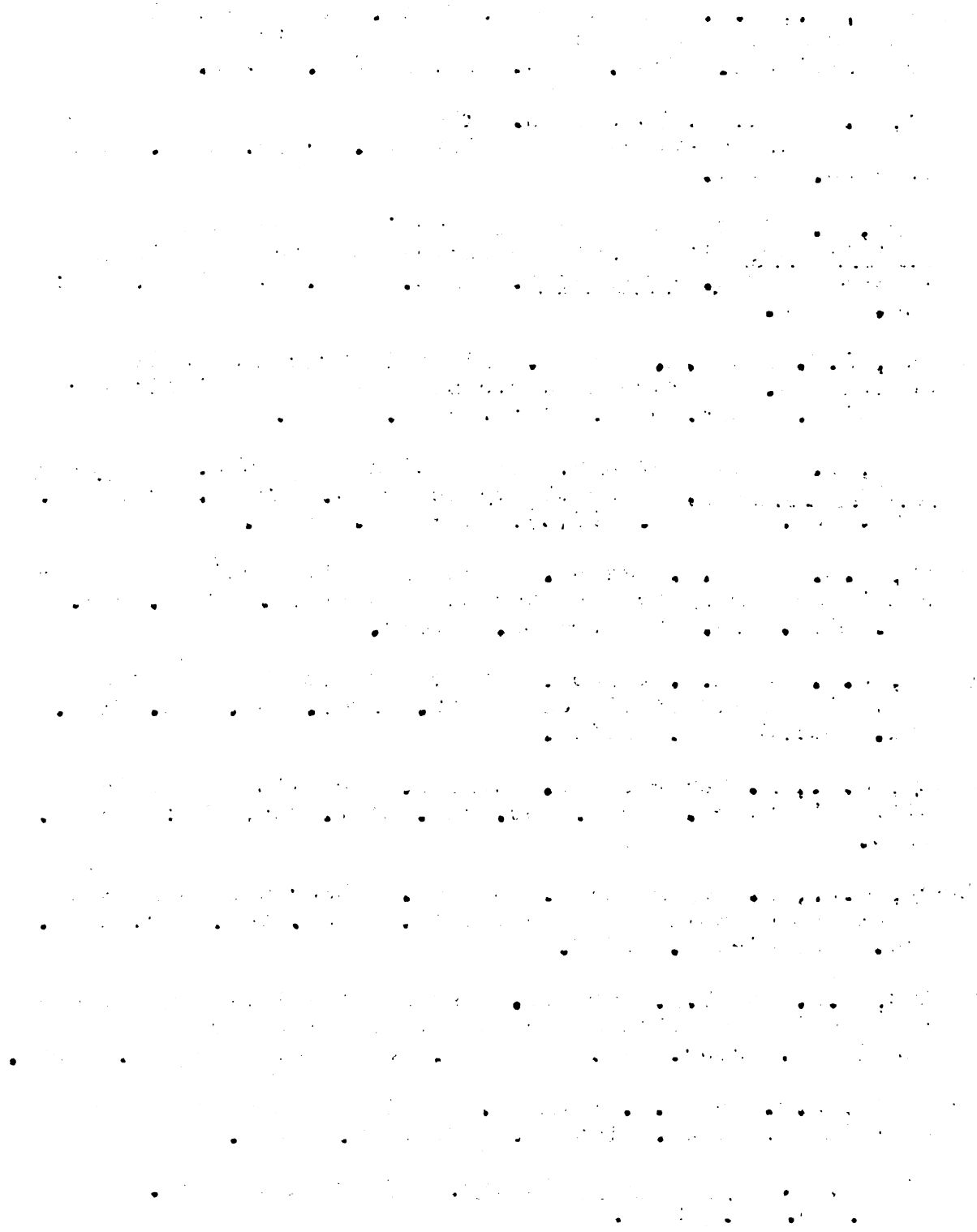
Hobby, G.L., K. Meyer and E. Chaffee. Activity of Penicillin in vitro. Proc. Soc. Exp. Biol. Med. 50:277-280. 1942.

Hobby, G.L., K. Meyer and E. Chaffee. Observations on the mechanism of action of penicillin. Proc. Soc. Exp. Biol. Med. 50:281-285. 1942.

Howitt, B.F. and V.H. Barnett. Use of penicillin and streptomycin in isolation of poliomyelitis virus from fecal specimens. Jour. Lab. and Clin. Med. 33:1402-1409. 1948.

Krampitz, L.O. and C.H. Werkman. On the mode of action of penicillin. Arch. Biochem. 12:57-67. 1947.

Kauffmann, F. Enterobacteriaceae. Ejnar Munksgaard. Copenhagen. p.150. 1951.



Lawrence, C.A. Sterility test for penicillin. Science. 98: 413-414. 1943.

Lawrence, C.A. Action of clarase upon penicillin. Science. 99:15-16. 1944.

Lederberg, J. Detection of fermentative variants with tetrazolium. Jour. Bact. 56:695. 1948.

Lederberg, J. and N. Zinder. Concentration of biochemical mutants of bacteria with penicillin. Jour. Am. Chem. Soc. 70:4267. 1948.

Lee, S.W., E.J. Foley and J.A. Epstein. Mode of action of penicillin. I Bacterial growth and penicillin activity. Jour. Bact. 48:393-399. 1944.

Litsky, W., W.L. Mallmann and C.W. Fifield. Ethyl violet, A selective dye for the isolation of gram-negative bacteria. Stain Techn. 27:229-232. 1952.

Litsky, W., W.L. Mallmann and C.W. Fifield. A new medium for the detection of enterococci in water. Amer. Jour. Pub. Health. 43:873-879. 1953.

MacLean, I.H. A modification of the cough plate method of diagnosis in whooping cough. Jour. of Path. and Bact. 2:472. 1937.

Mallmann, W.L. and C.W. Darby. Uses of lauryl sulfate tryptose broth for the detection of coliform organisms. Am. Jour. of Pub. Health. 31:127-134. 1941.

McCulloch, E.C. Disinfection and Sterilization. Second Ed. Lea and Fabiger. Philadelphia.

Morin, J.E. and H. Turcotte. The biological purification of vaccine emulsions by penicillin. Canadian Jour. Res. Sect. E. Med. Sci. 24:149-154. 1946.

Pizzi, T. Penicilina en medios de cultivo para Trypanosoma cruzi. Biologica (Santiago, Chile). 3:107-109. 1945.

Pramer, D., H. Houhannes and R.A. Ragotzkie. Survival of tubercle bacilli in various sewage treatment processes. Pub. Health Reports. 65(27):851-859. 1950.

Rantz, L.A. and W.M. Kirby. The action of penicillin on staphylococcus in vitro. Jour. Immunol. 48:335-343. 1944.

Reinbold, G.W., M. Swern, and R.V. Hussong. A plating medium for the isolation and enumeration of enterococci. Jour. Dairy Sci. 36:1-6. 1953.

Roepke, R.R., R.L. Libby and M.H. Small. Mutation or variation of E. coli with respect to growth requirements. Jour. Bact. 48:401-412. 1944.

Schwartzman, G. Inhibition of E. coli by penicillin. Science. 100:477-478. 1944.

Spicer, S. and D. Blitz. A study of the response of bacterial populations to the action of penicillin; a quantitative determination of its effect on the organisms. Jour. Lab. and Clin. Med. 33(4):417-429. 1948.

Thomas, A.R. Jr. and M. Levine. Some effects of penicillin on intestinal bacteria. Jour. Bact. 49:623-625. 1945.

Wainwright, S.D. and J. Mullaney. An influence of carbon source upon the penicillin sensitivity of Escherichia coli in solid medium. Jour. Bact. 67:504. 1954.

Woodruff, H.B. and J.W. Foster. Microbiological aspects of penicillin. VII Bacterial penicillinase. Jour. Bact. 49:7-17. 1945.

Young, E.G., R.W. Begg and E.I. Pentz. Inorganic nutrient requirements of Escherichia coli. Arch. Biochem. 5:121-136. 1944.