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In Vivo Antagonism between a Strain of Shigella Flexneri and Microorganisms Found in the Normal Intestinal Flora

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In Vivo Antagonism Between a Strain of *Shigella flexneri* and Microorganisms Found in the Normal Intestinal Flora

by

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LIFE

David John Hentges was born in LeMars, Iowa, September 18, 1928.

He was graduated from Le Mars High School, Le Mars, Iowa, in May, 1946 and from the University of Notre Dame in August, 1953 with the degree of Bachelor of Science.

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I PURPOSE

The purpose of this thesis is to investigate the relationship between some microorganisms which are normal inhabitants of the intestine of man and a typical human enteric pathogen, Shigella flexneri. It had been suggested that some component of the normal intestinal flora of man may play an important role in preventing or retarding the establishment of intestinal pathogens. This work represents a survey of the relationships in the intestine of mice of several microorganisms and a strain of Shigella flexneri. Organisms displaying an in vivo antagonism to Shigella flexneri are demonstrated.

II HISTORY

Several studies have been undertaken in the past in relation to antagonism between microorganisms. Most of these studies were made by testing the relationships between the organisms using *in vitro* culture methods. Some recent work has been done, however, employing *in vivo* methods.

Nissle (1916) observed in mixed broth cultures, that certain strains of *Escherichia coli* were capable of depressing the growth of other microorganisms, including some enteric pathogens. He suggested that such strains of *E. coli* might play a role in preventing the establishment of enteric pathogens in the intestine and used such strains in the treatment of intestinal infections.

Gratia (1932) described a substance produced by a strain of *E. coli* which was toxic to another strain of *E. coli*. The substance was heat resistant, withstanding temperatures up to 120°C. Since it was not self-reproducing in the presence of susceptible bacteria, it was not considered to be a bacteriophage.

Fulton (1937) studied the growth of mixed cultures of *E. coli* and *Salmonella schottmuelleri* in a synthetic medium. The growth curves were followed for 72 hours by means of periodic plate counts. During early growth of the mixtures, *Salmonella* was strongly inhibited and the population consisted largely of *E. coli*. After *E. coli* reached its maximum concentration,

Salmonella increased in number rapidly until at 72 hours the concentrations of Salmonella and E. coli were approximately equal. In comparing the growth of mixed cultures with pure culture controls, Fulton concluded that there was only a slight inhibition of Salmonella schottmuelleri by E. coli.

Heatley and Florey (1946) demonstrated an antibiotic substance produced by a strain of E. coli isolated from cat feces. The inhibitory substance was produced on glucose agar and was active against strains of Staphlococcus, Escherichia coli, Bacillus, and Pseudomonas. The substance was also formed in aerated beef heart infusion broth, but not in any of the synthetic media which were studied. The inhibiting substance would kill a small inoculum of sensitive organisms even at high dilutions but did not cause lysis of the bacterial cells. It was extremely specific in its action. Some strains of E. coli were highly sensitive while others were unaffected. The substance was soluble in water and reacted chemically like a peptide. It was only moderately toxic for mice and almost non-toxic for leucocytes.

Gratia and Fredericq (1946) described inhibiting compounds common to the coliform group. These compounds, called "colicines", were active in vitro against other coliform organisms and against strains of Shigella and Salmonella as well. Gratia and Fredericq postulated the presence of a number of "colicines", differing in physical properties. Fredericq (1948) detected seventeen active principles in "colicines" differing in range of activity and type of inhibition zone produced. Fredericq and Levine (1947) showed that a considerable number of enteric organisms produced substances active against an equally diverse range of other enteric organisms. In these experiments, agar plates were stabbed with the strains to be tested for antibiotic activity and were

incubated overnight. The following day the plates were exposed to chloroform vapors for one hour prior to inoculation with the strains to be tested for antibiotic sensitivity. After inoculation the plates were again incubated overnight. The appearance of inhibition zones around the "stabbed areas" indicated the presence of an antibiotic substance.

Fredericq and Levine (1947) found that the "colicines" produced by various strains of Escherichia coli differed in their antibiotic spectra and in their physiochemical properties. Some strains produced several "colicines" with different antibiotic spectra. Practically every member of the Enterobacteriaceae was sensitive to one or more "colicines" produced by some other member of the Enterobacteriaceae. Of all strains tested for antibiotic sensitivity, the Shigella strains were frequently sensitive while Aerobacter strains were seldom sensitive.

Wynne (1947) tested a single strain of Aerobacter against twenty-one common bacterial species. Nearly equal numbers of Aerobacter and the test species were inoculated into tubes containing about 4 ml of nutrient broth. After incubation of the mixed cultures for varying time intervals, each tube was thoroughly agitated and a loopful of broth transferred to sterile saline. This saline suspension was diluted to give approximately 200 to 300 colonies, and was streaked on agar plates. The ratio of the colonies of the test species to Aerobacter colonies growing out on test plates was then determined. The Aerobacter strain was found to be more or less antagonistic toward 14 of the 21 bacterial species tested. Comparison of 8 Aerobacter strains against three test species showed only minor differences in antagonistic effects among the Aerobacter strains. Filtrates of cultures of the Aerobacter strain appeared to

have no antagonistic activity. Seventy-two-hour cultures were filtered through a Berkefeld W candle and the filtrates were added to nutrient broth. After incubation to test for sterility, organisms were inoculated into the broth. In all cases, the growth obtained equalled that in nutrient broth controls. From these results, Wynne concluded that the antagonistic action of Aerobacter strains may involve some direct action of living cells rather than the production of a toxic substance.

Some years later, Bowling and Wynne (1951) reported additional studies concerning the mechanism of the antagonistic action displayed by Aerobacter strains. Fourteen Aerobacter strains were tested against seven common bacterial species by a "staled" agar technique. All seven of these organisms were among the species found susceptible to inhibition by Aerobacter. For the preparation of "staled" agar, Aerobacter broth cultures were incubated for three days and then mixed with an equal volume of double strength nutrient agar. The temperature of the two components was adjusted before mixing to give a final temperature of the "staled" agar between 45°C and 50°C. Aliquots of the mixture were poured into petri dishes. Each plate was inoculated shortly after solidification with two test organisms by means of single massive streaks from 24 hour broth cultures. All seven of the test organisms were inhibited, to varying degrees, by each of the fourteen Aerobacter strains. No inhibition of the test strain could be observed when Aerobacter culture filtrates, culture dialysates, heat or chloroform-killed suspensions were employed in the preparation of "staled" agar. Furthermore, live, washed cells gave inhibition equal to that obtained with whole cultures. If an antibiotic substance were involved in antagonism by Aerobacter, the degree of inhibition might be expected to increase

with the age of the Aerobacter culture. However, 12 hour cultures were found to be equal to 72 hour cultures in this respect. From this evidence, Wynne concluded that antagonism by Aerobacter strains may depend upon the presence of living cells in physical proximity with the cells of the inhibited species. This phenomenon was termed "direct antagonism".

The phenomenon of direct antagonism was also observed by Frankel and Wynne (1951) who studied several strains of Gaffkya tetragena which inhibited the growth of seventeen test organisms. The "staled" agar technique was used in this study.

Wynne and Norman (1951) further investigated the concept of "direct antagonism". Test species streaked on "staled" agar plates prepared from cultures of Aerobacter aerogenes or Gaffkya tetragena showed marked inhibition, as was previously described. Layers of sterile agar approximately 3 mm in thickness were cut into radial sectors and placed on the surface of "staled" agar plates. After preliminary incubation of the "staled" plates, several test species were streaked on the radial sectors. Complete inhibition of all species occurred on layers overlying plates "staled" with the Aerobacter strains, and most strains were also inhibited on sterile agar layers covering plates "staled" with Gaffkya strains. Sterility test showed no living Gaffkya or Aerobacter cells in the upper portion of the agar sectors. A layer of sterile cellophane between the "staled" agar and the overlying radial sectors did not materially affect the results. After inhibition of test species had been demonstrated, the radial sectors were removed, melted together, and allowed to solidify. On reinoculation, all test species grew. This finding appeared to rule out depletion of nutrients as an explanation of the observed antagonism.

In the same study, Wynne and Norman (1951) inoculated nutrient broth with a strain of Aerobacter aerogenes. Cellophane dialysis bags containing sterile broth were immersed in the inoculated broth and the entire apparatus was incubated. After incubation the cellophane bags were inoculated with various test organisms. On further incubation little or no growth occurred inside the bags. However, when this inoculated broth was removed from the bag and incubated further, there was only a transient inhibition, and, after 24 hours, growth of the test organisms was roughly equivalent to that in untreated nutrient broth. Wynne concluded from these findings that the concept of "direct antagonism" was inadequate to explain the antagonism exerted by Aerobacter and Gaffkya. Furthermore, it seemed unlikely that depletion of nutrients was involved. The experimental findings, however, were compatible with a theory postulating the production of very unstable antibiotic substances capable of diffusing through agar and through cellophane membranes.

Halbert (1947) tested more than one thousand strains of coliform organisms for their antagonistic properties on solid medium against a strain of Shigella paradysenteriae. Each of the coliform organisms was inoculated on a small area of an agar plate previously seeded with Shigella paradysenteriae. After incubation the appearance of zones of inhibition surrounding the colonies of coliform suggested the presence of an antibiotic substance. About 18% of the coliform organisms tested exhibited zones of inhibition. The mechanism of inhibition was shown to be due to the production of an antibiotic substance. The active substance was obtained from the agar by a variety of methods, including centrifugation of the mashed medium, freezing and thawing, or extraction of the agar with water. In most cases, supernates thus prepared from cultures of

the active strains were capable of inhibiting the test Shigella strain in dilutions of from 1:500 to 1:20,000. Supernates similarly prepared from non-antagonistic strains were completely inactive, even in dilutions as low as 1:2.

Halbert presented the following evidence indicating that the inhibition zones observed in his experiments were not due to bacteriophage. The edges of the zones of inhibition revealed minute stunted colonies when examined at 24 hours. Had these zones been due to bacteriophage, it would seem likely that differention between inhibition zone and areas of growth would have been more distinct. Inocula from the zones of inhibition, when mixed with Shigella cultures and seeded on agar plates, failed to reveal areas lacking growth which would indicate the presence of bacteriophage.

The zones of inhibition observed by Halbert were probably not due to quantitative differences in the coliform and Shigella inoculum with the subsequent, more rapid utilization of local nutrients. This was indicated by the following evidence:

1. Antagonistic and non-antagonistic coliform organisms grew equally well in the survey tests.

2. Tests were performed in which highly antagonistic strains of E. coli were serially diluted and small drops of appropriate dilutions were inoculated on the surface of agar which had previously been seeded with Shigella. Zones of inhibition were produced even around isolated colonies of Escherichia coli.

Further studies conducted by Halbert and Magnuson (1948) with antibiotic producing strains of Escherichia coli showed that although these strains produced antibiotic when grown of the surface of agar media, little or no anti-

biotic developed when the antibiotic producing strains were grown in broth cultures. The antibiotics studied by Halbert and Magnuson were very stable. Extended storage at ice box temperature or immersion of crude preparations in boiling water resulted in no change of activity.

Halbert (1948a) conducted a survey for the presence or absence in institutionalized individuals of coliform organisms antagonistic to Shigella. Rectal swab cultures were streaked on Salmonella-Shigella agar, and in many instances, also on MacConkey agar. Coliform bacteria isolated from lactose-fermenting colonies were tested for their antagonistic properties on Proteose No. 3 agar against a strain of Shigella flexneri type III. Halbert found that 30% of the individuals harbored antagonistic coliform organisms and that many of these individuals were infected with Shigella. The subjects who were infected with Shigella had a significantly higher incidence of antagonistic coliform organisms than did the non-infected individuals. This tendency was especially marked in individuals whose cultures yielded only a small number of Shigella colonies.

In another study by Halbert (1948b), the antagonistic properties of the human intestinal coliform flora were investigated during the course of Shigella infections. Coliform organisms were obtained from patients of a mental hospital in the same manner as described above. Tests for antagonism were performed on Proteose No. 3 agar against one or more strains of Shigella isolated from the patients as well as against a stock strain of Shigella flexneri type III, known to be highly susceptible to the antibiotic substances. During the course of acute infections cultures were taken at regular internals. In some instances, initial rectal swab cultures were obtained from individuals

immediately after admission to the mental hospital and repeatedly thereafter. It was thus possible to accurately date the onset of their infection and to determine the character of the intestinal flora before, during and after their illness.

Halbert found that organisms active against Shigella tended to appear and to increase soon after the onset of infection and then to decrease after several weeks, while the patient was still infected. In some cases, antagonistic coliform strains were active against Shigella strains with which the patient was infected. There was little correlation between the presence of these strains and recovery from infection. The composition of the coliform flora in several instances showed striking changes in the percentage of antagonists over periods as short as two days.

The antagonism of E. coli strains isolated from patients infected with Shigella was shown by the agar plating technique to be due to the production of antibiotic substances. The ability of these E. coli strains to produce antibiotic substances was found to be stable over many months. Halbert concluded therefore that the observed shifts in the intestinal flora represent real changes in the bacterial populations and not just teapory non-hereditable variations of bacterial cells already present in the intestinal tract.

Halbert and Gravatt (1948) found that susceptibility of Shigella to a group of antibiotic producing E. coli strains was related to type specificity. Shigella sonnei strains proved to be the most susceptible, and Shigella paradysenteriae type VI proved to be the most resistant. From his observations, Halbert (1948b) concluded that the role of antibiotic-producing coliform organisms in Shigella infections was not clear. He summarized the following

evidence as suggesting that antibiotic-producing enteric organisms may be related to Shigella infections.

1. Antagonistic strains are prevalent in the intestinal flora and are widely distributed among human beings.

2. The antibacterial spectrum of these organisms is limited to the enteric group of bacteria.

3. Production of antibiotic substance is a stable characteristic of a given strain.

4. Survey observations showed that individuals with positive rectal cultures for Shigella had a greater proportion of antagonistic E. coli strains than did individuals negative for Shigella.

Robbins and Parr (1957) also demonstrated that individuals with shigellosis may harbor antagonistic coliform organisms. They found, however, no positive correlation between the presence of antagonistic organisms and protection against infection by Shigella. Perhaps organisms may display in vitro inhibition but not in vivo inhibition.

Ludford and Lederer (1953) devised a method of comparing the electrophoretic patterns of the antibiotic substances produced by various strains of E. coli. The E. coli strain under test was inoculated in the center of a nutrient agar strip contained in a petri plate. The plates were incubated overnight. The organisms were killed the following day by exposure to chloroform. The plate, with lid removed, was inverted on two blocks of heart infusion agar standing in petri dishes containing a phosphate buffer of pH 7.4. Carbon electrides connected to a 45 volt battery were then placed in the petri dishes. The strip was seeded with a strain of Shigella sonnei and was again incubated

overnight. With this technique, the position of antibiotic substances on the agar strip could be recognized as an area free of Shigella growth. Four electropheretically different antibiotic substances could be detected in the strains of E. coli tested. Most strains produced more than one substance. Serological grouping of the E. coli strains was found to be independent of antibiotic activity.

The studies described so far employed in vitro methods for the demonstration of antagonism between micro-organisms. In vivo studies were hampered by the lack of a suitable laboratory animal in which enteric infections could be reproduced. For this reason, the parenteral inoculation of various organisms was employed by some investigators as an experimental model.

Halbert and Swick (1950) inoculated mice, either intraperitoneally or subcutaneously with coliform organisms which were highly antagonistic in vitro. After five or six hours the animals were bled from the heart and the blood was centrifuged after coagulation. The contents of the peritoneal cavities or the local subcutaneous sites of infection were washed with sterile saline. The blood sera and cavity washings were assayed for inhibitory activity against strain of Shigella flexneri, type III, using agar plates. The plates were first seeded with Shigella and then inoculated with either sera or washings of the peritoneal cavity. Zones of inhibition surrounding inocula were considered indicative of antibiotic activity. Inhibitory substances were usually found in high concentrations in both the washings and the sera. Injection of E. coli strains producing no antibiotic in vitro yielded washings and sera devoid of inhibitory activity.

The significance of antibiotic producing strains in the ecology of

the flora of skin, mouth, intestine, outer ear, eyes, etc. has been a matter of great interest. The rapidity with which changes in concentrations of antibiotic producing strains occur in the ocular and enteric flora of individuals and the high proportion of antagonistic strains in total bacterial populations observed by Halbert (1948, 1948a) suggest a potential significance of bacterial antagonism as a protective mechanism against disease.

In view of these observations, Halbert, Sonn, and Swick (1954) conducted a study of the in vivo relationship between antibiotic producing Micrococcus pyogenes strains isolated from the human ocular microflora and a strain of Clostridium septicum. Clostridium septicum spores and washed micrococci were mixed in appropriate dilutions, prior to intramuscular injection into white mice. The Clostridial strains alone were extremely virulent for mice, yet a high degree of protection was conferred by adding micrococci to the inoculum. The protection was assumed to be associated with in vivo secretion of antibiotics by the micrococci. A strong correlation between in vitro and in vivo results with micrococcus strains of varying antibiotic properties supported this assumption. In vitro studies were carried out on agar plates. The data presented indicated that the human bacterial flora may represent a source of antibiotics potentially valuable in resistance to certain infections.

Prior to 1954, in vivo studies of enteric pathogens could not be related to conditions in an actual enteric infection since there was no laboratory animal readily available in which essential features of human enteric infections could be reproduced. Freter (1954, 1955) devised a method of producing asymptomatic enteric infections in mice and guinea pigs and presented another line of evidence for the possible protective effect of normal enteric

flora. Assuming that a protective action by the normal enteric flora is responsible for the natural resistance of laboratory animals to enteric infections, streptomycin was administered orally to guinea pigs to inhibit the intestinal flora. It was subsequently possible to infect the drug-treated animals with streptomycin-resistant strains of Vibrio cholerae. Untreated animals could not be infected.

At about the same time, Bohnhoff, Drake, and Miller (1954) reported that the susceptibility of mice to oral infection with Salmonella enteritidis was enhanced 100,000 fold after the animals had received a large oral dose of streptomycin.

While these results suggested an antagonistic action of the part of the normal flora, there were two alternate explanations:

1. Streptomycin might directly affect the intestinal tract in such a way as to predispose it to the action of enteric pathogens.

2. The drug might directly increase the virulence of the bacteria studied.

The problem was further investigated by Freter (1956). Streptomycin and erythromycin were administered orally to several groups of white mice in order to inhibit the normal intestinal flora. Two days later, the animals were infected with mixtures of a streptomycin-resistant Shigella flexneri and an Escherichia coli strain isolated from a human being. One day after infection, stool samples from the majority of the mice studied were positive for Shigella. Two days after infection the majority of the stool samples showed abundant growth of the E. coli only, while Shigella could be recovered from only a few of the animals. A control group of mice which had received only Shigella

organisms gave positive stool cultures on all three days. On later days, all of the mice studied excreted E. coli only while the control mice continued to give stool cultures abundant with Shigella growth. No diffusible antibiotic substance could be demonstrated in vitro in plate cultures of the E. coli strain studied. Freter suggested that the in vivo antagonism of the E. coli strain might be due to the production of an extremely unstable antibiotic substance, a competition for essential nutrients, or merely a difference in growth rate.

To summarize, a great deal of evidence has been presented suggesting antagonism between microorganisms. This evidence is, for the most part, the result of surveys conducted with various in vitro culture methods. A correlation could not be made, until recently, between the antagonism exhibited by microorganisms in vitro and the in vivo inhibitory activity of microorganisms in the intestine because of the lack of a suitable laboratory animal. Freter (1954, 1955) devised a method for inhibiting the normal flora and for producing asymptomatic enteric infections in mice and guinea pigs. In 1956, he demonstrated in vivo antagonism to Shigella flexneri by a strain of E. coli. Although antagonism between microorganisms could be demonstrated in vivo, the exact mechanism of this antagonistic activity was not studied.

It is the purpose of this thesis, therefore, to investigate the in vivo antagonism between several microorganisms which are normal inhabitants of the intestine of man and a typical human enteric pathogen, Shigella flexneri, and to develop an in vitro culture technique in which the results of the in vivo study can be reproduced. In correlating the in vitro antagonism displayed by the microorganisms with their in vivo antagonistic properties, a system may

be developed in which the mechanisms of antagonism could be readily investigated.

III MATERIALS AND METHODS

Organisms

The organisms used in this study were collected from various sources. Most of the strains were taken from stock cultures maintained at the Stritch School of Medicine. All five strains of Escherichia coli and Aerobacter, strains 4 and 23, however, were collected from infant dysentery cases at the Hospital Infantil in Mexico City during the summer of 1955. Frozen stool specimens sent from Mexico through the courtesy of Doctor Samuel Formal, were streaked on desoxycholate agar plates (Difco) and organisms from colonies resembling coliform organisms were isolated and subjected to biochemical tests to establish their identity.

Streptomycin resistant mutants of all strains were selected by the gradient plate technique for use in the study. The organisms were streaked first on veal infusion agar (Difco) gradient plates containing twenty-five micrograms of streptomycin per milliliter of agar in the bottom layer. The resistant mutants were subsequently streaked on gradient plates containing higher streptomycin concentrations. The process was continued five times until mutants were obtained resistant to a concentration of one milligram streptomycin per milliliter of agar.

The streptomycin-resistant organisms were then subjected to morphological examination and biochemical testing to establish their identity, as presented in Table I.

The following method was developed to facilitate a rapid quantitative estimation of the organisms in an inoculum.

Organisms were streaked on a veal-infusion agar plate (Difco) and incubated at 37 C for 24 hours. Growth was washed from the surface of the plate with 5 cc saline and a two fold dilution series was made of this suspension. Klett readings were then taken of each dilution using a blue filter, No. 42. In addition, a tenfold dilution series was made from the original suspension and 0.1 ml. of each dilution was plated on the surface of veal infusion agar. From the colony count it was possible to determine the number of organisms in each dilution in the series. With the Klett readings thus obtained, graphs were constructed correlating the bacterial count with the Klett reading.

Culture Media

The differentiation of Shigella colonies from the colonies of test organisms in stool cultures of mice infected with both strains or in mixed broth cultures was accomplished by plating on either desoxycholate agar (Difco) or on veal infusion agar modified by the addition of 1% mannitol, 0.1% sodium desoxycholate and 40 mg. neutral red. Both agars contained 1 mg./ml. streptomycin. The lactose utilizers, Escherichia coli and Aerobacter, produced red colonies on desoxycholate agar while the non-lactose utilizing Shigella strain produced white colonies. 1% mannitol, 40 mg./liter of neutral red and 0.1% sodium desoxycholate added to veal infusion agar provided an easy device for the differentiation of Shigella colonies from colonies of Pseudomonas, Proteus or Alcaligenes. Since the Shigella strain attacked mannitol it produced red colonies. Pseudomonas, Proteus, and Alcaligenes strains produced white colonies.

The addition of sodium desoxycholate to the agar controlled the characteristic spreading of Proteus colonies and of some of the Pseudomonas colonies.

Plates of Protease #3 or veal infusion agar (Difco), containing 1 mg./ml. streptomycin were used to observe the production of antibiotic substances by various organisms. In studies involving broth cultures, veal infusion broth (Difco), brain heart infusion broth (Difco), or nutrient broth (Difco), each containing 0.5 mg. streptomycin and 0.01 mg./ml. erythromycin, were used.

In Vivo Methods

Infection of Mice:

Swiss mice approximately 25 to 30 grams in weight were used in the study. The technique of Freter (1956) was employed in infecting the mice. Two days before infection, 1 mg. erythromycin and 5 mg. streptomycin in 1 ml. of boiled water were given by stomach tube. The mice were not fed on this day. Boiled tap water containing 0.1 mg. erythromycin, 4 mg. streptomycin, and 400 units nystatin (mycostatin, Squibb) per milliliter was supplied to the mice throughout the experiment. Food was given the day before infection but withdrawn at night. On the day of infection, known numbers of viable test organisms from 24 hour veal infusion agar cultures were suspended in veal infusion broth containing 5 mg. of streptomycin and 50 mg. calcium carbonate per ml. Each mouse was given 1 ml. of the broth suspension containing a mixture of approximately equal numbers of viable Shigella organisms and viable test organisms. Control mice were each given 1 ml. of the broth suspension containing viable Shigella organisms or 1 ml. of the broth suspension containing viable

test organisms. Plate counts were made on all suspensions prior to the infection of the mice to quantitatively estimate the organisms contained in the infecting suspensions.

To determine the proportion of Shigella organisms to test organisms in the feces, one stool pellet was collected from each mouse in one milliliter of sterile saline. The pellet was emulsified and streaked on either desoxycholate agar plates, or on modified veal infusion agar plates as described above. After streaking, the plates were incubated at 37 C for 24 hours. In all studies, slide agglutination tests with Shigella flexneri O-antiserum were made to confirm the identity of Shigella recovered from stool samples.

pH Values and Plate Counts of Intestinal Contents:

Bacterial counts and the pH of the intestinal contents were determined in untreated mice, in animals whose normal flora was inhibited with antibiotics, and in mice infected with equal numbers of viable E. coli and Shigella flexneri. The normal intestinal flora was inhibited and the mice were infected as described above. One section of the upper small intestine about 2 cm long and the caecum was removed from each untreated mouse and from each mouse whose normal flora had been inhibited 96 hours previously. The intestinal contents were suspended in a drop or two of boiled distilled water and the pH values of the suspensions were determined with a Beckman pH meter.

Two sections of the upper small intestine, about 2 cm long, and the caecum were removed from each infected mouse with sterilised instruments 48 hours after infection (i.e. 96 hours after inhibition of the normal flora). Each section of intestine was incised longitudinally. The contents of one

section of small intestine and one half the contents of the caecum each were suspended in a few drops of boiled distilled water and the pH values of the suspensions were determined with a Beckman pH meter. The contents of the other section of small intestine and the remaining caecal contents each were suspended in 5 ml. of veal infusion broth containing 0.5 mg./ml. streptomycin. Ten-fold dilutions were made of the veal infusion suspensions. These dilutions were plated on desoxycholate agar and counts were made of the colonies.

In Vitro Methods

Cellophane dialysis bags were introduced into the center opening of two liter three neck flasks. Both the bag and the flask were filled with broth and were sterilized at 15 pounds steam pressure for 15 minutes. The broth within the dialysis bag was inoculated either with single strains or with suspensions of approximately equal numbers of Shigella and test organisms. The apparatus was incubated at 37°C for 48 hours. At 24 hours, the sterile broth outside the bag was replaced. At 24 and 48 hour intervals, aliquots of the broth within the bag were streaked on agar plates. The plates were incubated for 24 hours at 37°C.

Another in vitro test was carried out with test tubes containing ten ml. of broth which were inoculated with single strains of viable organisms or with suspensions of approximately equal numbers of Shigella and test organisms. After 24 hours incubation at 37°C, a loopful of the culture was transferred to a fresh tube of broth and re-incubated. A loopful of culture was removed from each tube after 24 hours incubation and streaked on either desoxycholate agar plates or modified veal infusion agar plates.

In experiments involving the adjustment of pH values of broth the Beckman pH meter was used. If a buffer was required, 1 cc. of one of the following was added to 9 cc. of broth:

pH value							
6.5	50%	1N	KH_2PO_4	and	15.20%	1N	NaOH
6.8	50%	"	"	"	23.65%	"	"
7.4	50%	"	"	"	39.50%	"	"
7.8	50%	"	"	"	45.20%	"	"

Studies of Inhibition Zones

The antibiotic relationships of several test organisms and a strain of Shigella flexneri were studied using techniques developed by Fredericq (1946) and Halbert (1948).

The following is a description of Fredicq's method. A small loopful of broth culture of a strain to be tested for the production of an antibiotic substance was stabbed on a previously dried agar plate. After incubation for 48 hours at 37 C, the culture was killed by exposure to chloroform vapors for a period of about one hour after which the chloroform was allowed to evaporate. The entire surface of the agar plate was then inoculated with Shigella flexneri and the plate was incubated for 48 hours at 37 C. The presence of areas of inhibition around the test organisms indicated the presence of a diffusible antibiotic substance.

The following is a description of Halbert's method. The growth from a 24 hour culture of the Shigella strain was suspended insterile saline and was flooded onto the surface of an agar plate. The plate was thoroughly dried by

inverting it without its cover in an incubator at 37 C for 30 minutes. The strain to be tested for the production of an antibiotic substance was then inoculated onto a small area of the previously seeded agar plate. The plate was incubated for 48 hours at 37 C and was checked for the presence of inhibition zones as described above.

IV RESULTS

1. In Vivo Studies

A. The measurement of the infective dose of Shigella flexneri.

The object of this experiment was to determine the number of organisms required to produce an asymptomatic shigellosis in mice. Groups of approximately ten mice each were infected with graded doses containing from 10 to 10,000 Shigella organisms. A control group was given broth only. Stool cultures were made as described above at 24 and 48 hours after infection. The results of this experiment (Table II) indicate that infection could be induced with doses of only ten organisms.

B. Surveys of in vivo antagonism toward Shigella.

Six surveys of in vivo antagonism were carried out with the organisms listed in Table III through IX. Groups of from seven to ten mice were infected with approximately 1×10^5 viable Shigella and 1×10^5 viable test organisms. Control groups of approximately 8 mice were included, which received pure cultures of each organism involved in the study. One million organisms were used in the final survey in an attempt to decrease the number of sterile stool samples.

Stool samples, taken after administration of antibiotics, but prior to infection, showed no growth on veal infusion agar plates containing or not containing antibiotics.

The following results are illustrated in Tables III through IX:

Throughout the entire series of experiments, the Aerobacter strains

displayed a marked *in vivo* antagonism toward the *Shigella* strain. In only two cases could *Shigella* be recovered from the feces of mice infected with a mixture of *Shigella* and *Aerobacter* strains.

Escherichia coli strains displayed an antagonism toward the *Shigella* which was less marked than that shown by *Aerobacter*. Generally, *Shigella* could be recovered from the feces twenty-four hours after mixed infection but 48 hours after infection the majority of the mice excreted only *E. coli*.

None of the *Alcaligenes* strains survived in the mice. Only *Shigella* organisms were recovered from mice infected with a mixture of *Shigella* and *Alcaligenes*. Plate cultures made of the infecting broth indicated that the organisms were viable at the time of infection.

The *Pseudomonas* and *Proteus* strains displayed varying degrees of antagonism toward the *Shigella* strain. In the majority of cases, both *Shigella* and the test organisms, *Proteus* and *Pseudomonas*, could be recovered from the stools of the mice after 24 hours. *Pseudomonas* 2681 and *Proteus* 492, however, completely inhibited the *Shigella* strains in all mice tested at 96 hours and 48 hours respectively. *Pseudomonas* 2803, 2820, and 2698 showed antagonism toward *Shigella* at 48 hours. *Proteus* 856 and *Proteus* 235 displayed no antagonistic action toward the *Shigella* strain 48 hours after mixed infection. Most of these stools contained mixtures of both *Shigella* and test strains. *Proteus* 172 failed to grow at all in the mice. Therefore, only *Shigella* could be recovered from the stools of these mice. A summary of *in vivo* results can be found in Table IX.

I. Measurements of the pH of the Intestinal Contents of Mice.

Organisms recovered directly from the Intestinal Contents of

Infected Mice.

Table X illustrates the results of pH measurements of the contents of the caecum and upper small intestine of infected and non-infected mice. The table also presents results of plate counts made of the contents of the caecum and upper small intestine of infected mice. Several trends are indicated by the results. Contents of the upper small intestine had consistently lower pH values than the contents of the caecum in the same mouse. The difference in pH between the contents of the small intestine and the caecum was nearly constant for each group. Oral administration of antibiotics significantly raised the pH of the contents of both the small intestine and the caecum. Subsequent infection of the mice with Shigella alone or with a Shigella, E. coli mixture, signficantly reduced the pH of the intestinal contents but not to the level of the untreated mice. Shigella or E. coli were recovered from the intestinal contents of all infected mice, except one, by the method described above. These organisms could usually be found both in the upper small intestine and in the caecum. In two cases, however, the organisms could be recovered only from the caecum. (See Table X). The results in Table X represent the approximate numbers of organisms recovered from the contents of each section of intestine. Since the volume of caecal material greatly exceeded the volume of material from the upper small intestine in each case, one might expect that the number of organisms isolated from the caecum would greatly exceed the number isolated from the section of small intestine. This, however, was not the case, indicating that much of the growth occurred in the small intestine.

In Vitro Measurements

Several studies were carried out to duplicate the in vivo antagonism between the organisms tested with an in vitro culture technique.

In the first experiment, nutrient broth, without antibiotics, at pH 6.8 was inoculated with mixed cultures. The results are listed in Table XI. Tubes containing the broth were inoculated with equal numbers of Shigella and test organisms, and were incubated at 37°C. A loopful of culture was transferred to fresh broth after 24 hours incubation. After 48 hours incubation only Aerobacter strains survived in mixed cultures. This situation had occurred in vivo, as is shown in Table IX. Both Shigella and Pseudomonas strains survived 48 hours in mixed broth cultures, which is in contrast to the in vivo results obtained (Table XI). Shigella organisms did not survive after 48 hours in mixed Proteus and Shigella culture. This latter result was again contrary to the in vivo relationship between the organisms. When brain heart infusion broth was substituted for nutrient broth to sustain mixed cultures, Shigella as well as the test organisms, could be recovered after 48 hours incubation. The results of this study are shown in Table XII. These results, except in the case of Proteus 235, are contrary to the in vivo relationship between the organisms.

Several studies were attempted to simulate the conditions which exist within the lumen of the intestine. In the first study, sterile paraffin oil was used to cover the surface of the inoculated broth in order to reduce oxygen tension to a level closer to that presumably existing within the intestine. Table XIII shows that the addition of paraffin oil had little effect on the relationship between the organisms in vitro. The results in Table XIII are,

therefore, similar to the results in Table XII. The second study involved the use of a broth-filled cellophane bag which was inoculated with mixed cultures and was suspended in a flask filled with sterile broth. The dialysis bag was used to allow a continuous diffusion of media into the bag and the diffusion of metabolites of the growing cells out of the bag. This action would provide the growing cells with a large reservoir of fresh nutrient material. It was thought that such a constant supply of nutrient material might simulate, to a degree, the existing conditions in the intestinal tract where enteric organisms continually encounter new food material. The results in Table XIV a, however, show a discrepency between the antagonistic relationship of organisms in mixed infections in the dialysis bag and the relationship of organisms in vivo. This discrepency disappeared when the pH value of the brain heart infusion broth was adjusted to 6.8. Table XIV b and c shows that the adjustment of the broth altered the results so that the relationships between Shigella and test organisms were in this case the same as the in vivo relationships.

An extensive survey was therefore made of the organisms which had been included in the in vivo experiments. Each of the test organisms was mixed with Shigella and inoculated into brain heart infusion broth at pH 6.8. One cc of 1.6% brom-thymol blue, in alcohol, was added to one liter of the broth. A good correlation was found between the in vitro broth results and previous in vivo results (Table XV). The in vivo, in vitro correlation was most striking with organisms which had displayed strong in vivo antagonism to Shigella such as Aerobacter and the E. coli, strains 36, 58, and 132. The correlation was pronounced also with Alcaligenes, 2045, and 2258 and Proteus 172, which failed to survive with Shigella in broth culture at pH 6.8. Controls showed that the

strains did survive, but grew very poorly alone in broth culture at pH 6.8.

Brom-thymol blue, in the broth, indicated a decided drop in the pH (to 6.0 or less) of the inoculated broth after 24 hours incubation. The possibility existed that this pH drop might be responsible for the particular patterns of *in vitro* relationships. To investigate this possibility, equal numbers of viable test organisms and viable *Shigella* organisms were inoculated into buffered veal infusion broth of pH 6.8 to which brom-thymol blue indicator had been added. The pH 6.8 buffer described in "Materials and Methods" was used. Veal infusion broth replaced brain heart infusion broth because of the difficulty in effectively buffering the brain heart infusion broth. Table XVI illustrates that the buffering had no effect on the final outcome.

To further investigate the importance of the pH value of the broth, several veal infusion broth samples were adjusted to different pH values ranging from pH 6.5 to pH 7.8. Brom-thymol blue indicator and buffers were added to the broth samples. The results shown in Table XVII indicate changes in relationship patterns as the pH values of the broth changed. The *Shigella* strain grew best in media of lower pH. In mixed cultures, the *Pseudomonas* and the *Proteus* test strains were apparently better able to survive in media of high pH values than the *Shigella* strain. This probably accounted for the predominance of these two test organisms in broth cultures of high pH. Table XVII shows that *Escherichia coli* and *Aerobacter* strains survived alone, with one exception, in mixed cultures with *Shigella* regardless of the pH values or the length of incubation. The one exception, a *Shigella*, *Aerobacter* mixture, revealed foru *Shigella* colonies after 24 hours incubation at a pH of 6.8.

In the great majority of cases, only *Aerobacter* or *E. coli* could be

recovered from the stools of mice 48 hours after mixed infection and only Aerobacter or E. coli survived alone in mixed broth cultures. It seemed feasible that an antibiotic substance might be responsible for this antagonism toward Shigella. The methods of both Halbert (194') and Fredericq (1946) were employed to test this theory. All the organisms included in the in vivo studies were tested by the method of Fredericq described above. A circular zone of inhibition around the test organism was considered indicative of the production of a diffusible antibiotic substance by the test organisms. Small zones of inhibition were observed around two typically antagonistic organisms when Halbert's method was employed. No visible zone of inhibition could be detected using Fredericq' method with a small inocula, of any of the test organisms. Zones of inhibition could be detected, however, when large inocula were used as is illustrated in Table XVIII. These zones of inhibition were observed with Proteose #3 agar but were not observable in two cases when veal infusion agar was substituted. All test organisms produced zones on Proteose #3 agar whehter or not they displayed in vivo antagonism. Furthermore, there was no correlation between the size of the zones of inhibition and the degree of in vivo antagonism. Shigella flexneri, Aerobacter 2718 and E. coli 58 were tested by Fredericq's method to determine the possible effect of auto-inhibition on the results. No auto-inhibitory action could be demonstrated with these three organisms.

TABLE I

ORGANISM		Adonitol	Arginin	Glucose	Levulose	Lactose	Maltose	Mannitol	Salicin	Sucrose	Xylose	Urea	Indole	Methyl-Red	Voges-Proskauer	Citrate	Gelatin	Motility	Sulfide	Flagella	Green Pigment
Aerobacter aerogenes	4		+	+		+	+	+	+		+		-	-	+	+	-		-		
Aerobacter aerogenes	23		+	+		+	+	+	+		+		-	-	+	+	-		-		
Aerobacter aerogenes	2451	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-		
Aerobacter aerogenes	2718	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-		
Aerobacter aerogenes	2822	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-		
Alcaligenes fecalis	2120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	P	
Alcaligenes fecalis	2255	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	P	
Alcaligenes fecalis	2258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	P	
Alcaligenes fecalis	2445	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	P	
Escherichia coli	5			+		+	+	+	-		+		+	+	-	-			-		
Escherichia coli	22			+		+	+	+	-		+		+	+	-	-			-		
Escherichia coli	36			+		+	+	+	-		+		+	+	-	-			-		
Escherichia coli	58			+		+	+	+	-		+		+	+	-	-			-		
Escherichia coli	132			+		+	+	+	-		+		+	+	-	-			-		
Proteus mirabilis	172	-	-	+	-	-	-	-	+	-	-	+	-	+	-	+	-	+	-	P	
Proteus mirabilis	856	-	-	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	P	
Proteus vulgaris	235	-	+	+	-	+	+	-	+	+	+	+	+	+	-	-	-	+	+	P	
Proteus vulgaris	492	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+	P	
Pseudomonas convexa	2681	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	M	+
Pseudomonas convexa	2803	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	M	+
Pseudomonas cruciviae	2689	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	M	+
Pseudomonas cruciviae	2820	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	M	+
Shigella flexneri				+	-	-	-	+			-						-	-			

+ ... Acid produced, pigment produced, motility.

M ... primarily monotrichous

P ... primarily peritrichous

TABLE II

ESTIMATION OF THE MINIMUM INFECTIVE DOSE FOR SHIGELLA FLEXNERI

	Group	No. of Organisms in the Infecting Dose	Infection
24 hr. Results	1	1.15×10	1/10*
	2	1.15×10^2	6/11
	3	1.15×10^3	3/10
	4	1.15×10^4	7/9
	Control	0	0/10
48 hr. Results	1	1.15×10	4/10
	2	1.15×10^2	7/11
	3	1.15×10^3	3/10
	4	1.15×10^4	7/9
	Control	0	0/10

* 1/10, the stool from one of ten mice was positive for Shigella.

TABLE III

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 1

Infecting dose: 10^5 organisms each of *Shigella* and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
48 hr. results				
Shigella	8/8	-	-	0/8
Pseudomonas 2681	-	6/7	-	1/7
Alcaligenes 2120	-	0/8	-	8/8
Aerobacter 2451	-	6/6	-	0/8
Proteus 235	-	3/8	-	5/8
Sh. Pseudomonas 2681	0/8	4/8	4/8	0/8
Sh. Alcaligenes 2120	6/8	0/8	0/8	2/8
Sh. Aerobacter 2451	0/8	7/8	1/8	0/8
Sh. Proteus 235	1/8	1/8	1/8	5/8

1 Fractions indicate the number of cultures positive for *Shigella* only over the total number tested.

2 Fractions indicate the number of cultures positive for the test organism only over the total number tested.

3 Fractions indicate the number of mixed cultures over the total number tested.

4 Fractions indicate the number of sterile cultures over the total number tested.

TABLE III (con't)

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
96 hr. results				
Shigella	8/8	-	-	0/8
Psuedomonas 2681	-	6/7	-	1/8
Alcaligenes 2120	-	0/8	-	8/8
Aerobacter 2451	-	6/6	-	0/6
Proteus 235	-	5/8	-	3/8
Sh. Pseudomonas 2681	0/8	8/8	0/8	0/8
Sh. Alcaligenes 2120	4/6	0/6	0/6	2/6
Sh. Aerobacter 2451	0/8	8/8	0/8	0/8
Sh. Proteus 235	2/8	0/8	6/8	0/8

1 Fractions indicate the number of cultures positive for Shigella only over the total number tested.

2 Fractions indicate the number of cultures positive for the test organism only over the total number tested.

3 Fractions indicate the number of mixed cultures over the total number tested.

4 Fractions indicate the number of sterile cultures over the total number tested.

TABLE IV

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 2

Infecting dose: 10^6 organisms each of Shigella and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
24 hr. results				
Shigella	5/9	-	-	4/9
Alcaligenes 2255	-	0/9	-	9/9
Aerobacter 2718	-	7/8	-	1/8
Proteus 235	-	2/8	-	6/8
Sh. Alcaligenes 2255	9/9	0/9	0/9	0/9
Sh. Aerobacter 2718	0/7	7/7	0/7	0/7
Sh. Proteus 235	0/8	1/8	0/8	7/8
48 hr. results				
Shigella	6/9	-	-	3/9
Alcaligenes 2255	-	0/9	-	9/9
Aerobacter 2718	-	7/8	-	1/8
Proteus 235	-	5/5	-	0/5
Sh. Alcaligenes 2255	9/9	0/9	0/9	0/9
Sh. Aerobacter 2718	0/4	4/4	0/4	0/4
Sh. Proteus 235	0/8	2/8	5/8	1/8

1,2,3,4,: See Table III

TABLE V

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 3

Infecting Dose: 10^5 organisms each of Shigella and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
24 hr. results				
Shigella	5/8	-	-	3/8
Pseudomonas 2803	-	4/7	-	3/7
Alcaligenes 2445	-	0/8	-	8/8
Aerobacter 4	-	4/7	-	3/7
Proteus 856	-	5/7	-	2/7
E. coli 5	-	4/7	-	3/7
Sh. Pseudomonas 2803	0/9	2/9	3/9	4/9
Sh. Alcaligenes 2445	8/8	0/8	0/8	0/8
Sh. Aerobacter 4	0/9	5/9	0/9	4/9
Sh. Proteus 856	0/9	5/9	2/9	2/9
Sh. E. coli 5	0/9	0/9	3/9	6/9

1,2,3,4, See Table III

TABLE V (con't)

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
48 hr. results				
Shigella	7/8	-	-	1/8
Pseudomonas 2803	-	5/7	-	2/7
Alcaligenes 2445	-	0/8	-	8/8
Aerobacter 4	-	5/6	-	1/6
Proteus 856	-	6/7	-	1/7
E. coli 5	-	5/7	-	2/7
Sh. Pseudomonas 2803	0/9	7/9	1/9	1/9
Sh. Alcaligenes 2445	8/8	0/8	0/8	0/8
Sh. Aerobacter 4	0/9	7/9	0/9	2/9
Sh. Proteus 856	0/9	4/9	5/9	0/9
Sh. E. coli 5	0/9	6/9	1/9	2/9

1,2,3,4, See Table III

TABLE VI

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 4

Infecting dose: 10^5 organisms each of Shigella and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
24 hr. results				
Shigella	6/8	-	-	2/8
E. coli 22	-	0/6	-	6/6
Proteus 172	-	0/8	-	8/8
Aerobacter 23	-	7/7	-	0/7
Sh. E. coli 22	4/8	0/8	1/8	3/8
Sh. Proteus 172	10/10	0/10	0/10	0/10
Sh. Aerobacter 23	0/8	8/8	0/8	0/8
48 hr. results				
Shigella	7/8	-	-	1/8
E. coli 22	-	4/6	-	2/6
Proteus 172	-	0/8	-	8/8
Aerobacter 23	-	7/7	-	0/7
Sh. E. coli	2/8	4/8	1/8	1/8
Sh. Proteus 172	10/10	0/10	0/10	0/10
Sh. Aerobacter 23	0/8	8/8	0/8	0/8

1,2,3,4,: See Table III

TABLE VII

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 5

Infecting dose: 10^5 organisms each of Shigella and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
24 hr. results				
Shigella	7/8	-	-	1/8
Pseudomonas 2820	-	4/7	-	3/7
E. coli 132	-	8/8	-	0/8
E. coli 58	-	5/8	-	3/8
Sh. Pseudomonas 2820	0/10	7/10	3/10	0/10
Sh. E. coli 132	0/10	7/10	3/10	0/10
Sh. E. coli 58	1/7	2/7	4/7	0/7
48 hr. results				
Shigella	6/7	-	-	1/7
Pseudomonas 2820	-	5/7	-	2/7
E. coli 132	-	8/8	-	0/8
E. coli 58	-	6/8	-	2/8
Sh. Pseudomonas 2820	0/10	5/10	1/10	4/10
Sh. E. coli 132	0/10	10/10	0/10	0/10
Sh. E. coli 58	0/7	7/7	0/7	0/7

1,2,3,4: See Table III

TABLE VIII

A SURVEY OF IN VIVO ANTAGONISM TOWARD SHIGELLA: EXPERIMENT 6

Infecting Dose: 10^6 organisms each of Shigella and the test strain.

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
24 hr. results				
Shigella	7/9	-	-	2/9
Pseudomonas 2689	-	7/8	-	1/8
E. coli 36	-	6/7	-	1/7
Proteus 492	-	4/8	-	4/8
Aerobacter 2822	-	7/7	-	0/7
Alcaligenes 2258	-	0/8	-	8/8
Sh. Pseudomonas 2689	1/10	1/10	7/10	1/10
Sh. E. coli 36	0/10	9/10	1/10	0/10
Sh. Proteus 492	0/10	3/10	2/10	5/10
Sh. Aerobacter 2822	0/9	8/9	1/9	0/9
Sh. Alcaligenes 2258	8/10	0/10	0/10	2/10

1,2,3,4, See Table III

TABLE VIII (con't)

Infecting Organism	All[1] Shigella	All Test[2] Organism	Mixture[3]	Sterile[4]
48 hr. results				
Shigella	8/9	-	-	1/9
Pseudomonas 2689	-	7/8	-	1/8
E. coli 36	-	8/8	-	0/8
Proteus 492	-	6/7	-	1/7
Aerobacter 2822	-	7/7	-	0/7
Sh. Pseudomonas 2689	0/10	8/10	1/10	1/10
Sh. E. coli 36	0/10	9/10	1/10	0/10
Sh. Proteus 492	0/10	10/10	0/10	0/10
Sh. Aerobacter 2822	0/9	9/9	0/9	0/9

1,2,3,4, See Table III

TABLE IX

A SUMMARY OF IN VIVO RESULTS

Organism	In Vivo Antagonism to Shigella	Table
Aerobacter 4	S	V
Aerobacter 23	S	VI
Aerobacter 2451	MS	III
Aerobacter 2718	S	IV
Aerobacter 2822	MS	VIII
Alcaligenes 2120	O	III
Alcaligenes 2255	O	IV
Alcaligenes 2258	O	VIII
Alcaligenes 2445	O	V
E. coli 5	M	V
E. coli 22	M	VI
E. coli 36	MS	VIII
E. coli 58	M	VII
E. coli 132	MS	VII
Proteus 172	O	VI
Proteus 235	N	III,IV
Proteus 492	MS	VIII
Proteus 856	N	V
Pseudomonas 2681	MS	III
Pseudomonas 2689	M	VIII
Pseudomonas 2803	M	V
Pseudomonas 2820	MS	VII

S-Total overgrowth of the test organism at 24 and 48 hr.

MS-Predominant overgrowth of the test organims at 24 and 48 hr.

M-Predominant mixture of Shigella and the test organism at 24 hr.; predominant overgrowth of the test organism at 48 hr.

N-Predominant mixture of Shigella and the test organism at 24 and 48 hr.

O-Inability of the test organism to survive in mice.

TABLE X

pH OF THE INTESTINAL CONTENTS OF MICE

Untreated Mice

Mouse	pH-Caecum	pH-Upper Small Intestine
1	7.25	6.70
2	7.00	6.80
3	6.90	6.80
4	7.05	6.80
5	6.95	6.80
	Mean Average = 7.03 ± .13	Mean Average = 6.78 ± .04

Mice with Antibiotic-inhibited Flora

1	7.95	7.50
2	7.60	7.15
3	7.85	7.20
4	8.00	7.40
5	7.90	7.30
	Mean Average = 7.86 ± .15	Mean Average = 7.31 ± .14

All readings made 96 hours after inhibition of the flora.

TABLE X (con't)

pH OF THE INTESTINAL CONTENTS OF MICE[1]

Organisms Recovered from the Intestinal Contents of Infected Mice

Mice infected with Shigella-10^5 organisms

Mouse	pH Reading-Caecum	Shigella Recovered	pH Reading-Upper Small Intestine	Shigella* Recovered
1	7.50	3.15×10^5	7.00	2.1×10^5
2	7.25	1.50×10^5	6.90	1.5×10^5
3	7.65	6.0×10^5	6.95	0
4	7.75	1.20×10^5	7.00	0
5	7.80	1.60×10^5	7.05	2.0×10^4
	Mean 7.59±.23		Mean 6.98±.05	

Mice infected with Shigella, E. coli 36 mixture-10^5 organisms

		E. coli Recovered		E. coli Recovered
1	7.55	5.0×10^6	7.00	4.2×10^5
2	7.40	2.5×10^5	6.50	2.0×10^5
3	7.75	0	7.10	0
4	7.40	1.0×10^5	6.90	2.0×10^6
	Mean 7.53±.16		Mean 6.88±.26	

* Shigella recovered per 2 cm section of small intestine.

1, All readings made 48 hours after infection.

2, No Shigella recovered.

TABLE XI

BROTH CULTURE IN TEST TUBES

Broth: Nutrient
No. of organisms inoculated/ml broth: 10^5
Antibiotics added to broth: None
Indicator added to broth: None
pH of broth: 6.8

Organisms	Original 24 hr.	Subculture 24 hr.	*
Aerobacter 2451	Growth	Growth	
Aerobacter 2718	Growth	Growth	
Pseudomonas 2681	Growth	Growth	
Proteus 235	Growth	Growth	
Shigella	Growth	Growth	
Sh. Aerobacter 2451	All Aero.	All Aero.	MS-III
Sh. Aerobacter 2718	Mixture	Mixture	S-IV
Sh. Pseudomonas 2681	Mixture	Mixture	MS-III
Sh. Proteus 235	Mixture	All Prot.	N-III,IV

* In vivo antagonism to Shigella and Tables where illustrated

TABLE XII

BROTH CULTURE IN TEST TUBES

Broth: Brain heart infusion
No. of organisms inoculated/ml broth: 10^5
Antibiotics added to broth: None
Indicator added to broth: None
pH of broth: 7.4

Organisms	Original 24 hr.	Subculture 24 hr.	*
Aerobacter 2451	Growth	Growth	
Pseudomonas 2681	Growth	Growth	
Proteus 235	Growth	Growth	
Shigella	Growth	Growth	
Sh. Aerobacter 2451	Mixture	Mixture	MS-III
Sh. Pseudomonas 2681	Mixture	Mixture	MS-III
Sh. Proteus 235	Mixture	Mixture	N-III,IX

* In vivo antagonism to Shigella and Tables where illustrated.

TABLE XIII

BROTH CULTURE IN TEST TUBES

Broth: Brain Heart Infusion
No. of Organisms Inoculated/ml Broth: 10^2
Antibiotics added to Broth: None
Indicator added to Broth: None
pH of Broth: 7.4

Organism	Original Culture 24 hr.	Subculture 24 hr.	Second Subculture 24 hr.	*
Broth overlayed with 1 cc of parafin oil.				
Aerobacter 2451	Growth	Growth	- 1	
Pseudomonas 2681	Growth	Growth	- 1	
Proteus 235	Growth	Growth	- 1	
Shigella	Growth	Growth	- 1	
Sh. Aero. 2451	Mixture	Mixture	Mixture	MS-III
Sh. Pseud. 2681	Mixture[2]	Mixture[2]	Mixture[2]	MS-III
Sh. Prot. 235	Mixture	Mixture	Mixture	N-III,IV
Controls without parafin oil.				
Sh. Aero. 2451	Mixture	Mixture	Mixture	MS-III
Sh. Pseud. 2681	Mixture[3]	Mixture[3]	Mixture[3]	MS-III
Sh. Prot. 235	Mixture	Mixture	Mixture	N-III,IV

-1, Not read

2, Predominantly Pseudomonas

3, Predominantly Shigella

* In vivo antagonism to Shigella and Tables where illustrated.

TABLE XIV

BROTH CULTURE IN CELLOPHANE BAGS

Broth: Brain Heart Infusion
No. of Organisms Inoculated/ml Broth: 10^2
Antibiotics added to Broth: .005 mg/ml erythromycin
.5 mg/ml streptomycin
Indicator added to Broth: None
pH of Broth: As indicated

A. Organisms	pH	Original Culture 24 hr.	Subculture 24 hr.	*
Sh. E. coli 36	7.4	Mixture	All E. co.	MS-VIII
Sh. Aero. 2822	7.4	All Aero.	All Aero.	MS-VIII
Sh. Proteus 235	7.4	All Pro.	All Pro.	N-III,IV
B. pH of the Broth Adjusted				
Sh. Proteus 235	6.8	Mixture	Mixture	N-III,IV
Sh. Proteus 235	7.5	All Pro.	All Pro.	N-III,IV
C. pH of the Broth Adjusted to 6.8				
Sh. Aero. 2822	6.8	All Aero.	All Aero.	MS-VIII
Sh. E. coli 5	6.8	All E. co.	All E. co.	MS-VIII
Sh. Pseud. 2803	6.8	Mixture	Mixture'	M-V

* In vivo antagonism to Shigella and Tables where illustrated.

' Mostly Pseudomonas

TABLE XV

BROTH CULTURE IN TEST TUBES

Broth: Brain heart infusion
No. of organisms inoculated/ml broth: 10^2
Antibiotics added to broth: .005 mg/ml erythromycin
.5 mg/ml streptomycin
Indicator added to broth: Brom-thymol blue
pH of broth: 6.8

Organisms	Original 24 hr.	Subculture 24 hr.	*
Aerobacter 4	All Aero.	All Aero.	S-V
Aerobacter 23	All Aero.	All Aero.	S-VI
Aerobacter 2451	All Aero.	All Aero.	MS-III
Aerobacter 2718	All Aero.	All Aero.	S-IV
Aerobacter 2822	All Aero.	All Aero.	MS-VIII
Alcaligenes 2120	All Shig.	All Shig.	O-III
Alcaligenes 2258	All Shig.	All Shig.	O-VIII
Escherichia coli 5	-'	All E.co.	M-V
E. coli 22	Mixture	All E.co.	M-V
E. coli 36	All E.co.	All E.co.	MS-VIII
E. coli 58	All E.co.	All E.co.	M-VII
E. coli 132	All E.co.	All E.co.	MS-VII
Proteus 172	All Shig.	All Shig.	O-VI
Proteus 235	-'	Mixture	N-III,IV

* *In vivo* antagonism to *Shigella* and Tables where illustrated.

' Not read.

TABLE XV (con't)

Organisms	Original 24 hr.	Subculture 24 hr.	*
Proteus 492	Mixture	Mixed Mostly Prot.	MS-VIII
Proteus 856	Mixed Mostly Prot.	Mixed Mostly Prot.	N-V
Pseudomonas 2681	Mixture	Mixture	MS-III
Pseudomonas 2689	Mixture	Mixture	M-V
Pseudomonas 2803	-'	Mixed Mostly Pseu.	M-V
Pseudomonas 2820	Mixture	Mixed Mostly Pseu.	MS-VII

* In vivo antagonism to Shigella and Tables where illustrated.

' Not read

TABLE XVI

BROTH CULTURE IN TEST TUBES

Broth: Veal infusion with phosphate buffer
(See Materials and Methods)
No. of organisms inoculated/ml broth: 10^2
Antibiotics added to broth: .005 mg/ml erythromycin
.5 mg/ml streptomycin
Indicator added to broth: Brom-thymol blue
pH of broth: 6.8

Organisms	Original 24 hr.	Subculture 24 hr.	*
Pseudomonas 2681	Growth	-'	
Proteus 235	Growth	-'	
E. coli 36	Growth	-'	
Alcaligenes 2120	Growth	-'	
Aerobacter 23	Growth	-'	
Shigella	Growth	-'	
Sh. Pseudomonas 2681	Mixture	Mixture	MS-III
Sh. Prtoeus 235	Mixture	Mixture	N-III,IV
Sh. E. coli 36	All E.co.	All E.co.	MS-VIII
Sh. Alcaligenes 2120	All Shig.	All Shig.	O-III
Sh. Aerobacter 23	All Aero.	All Aero.	S-IV

* In vivo antagonism to Shigella and Tables where illustrated.

' Not read.

TABLE XVII

BROTH CULTURE IN TEST TUBES

Broth: Veal Infusion with Phosphate Buffers
(See Materials and Methods)
No. of Organisms/ml Broth: 10^2
Antibiotics added to Broth: .005 mg/ml erythromycin
.5 mg/ml streptomycin
Indicator added to Broth: Brom-thymol blue
pH of Broth: As indicated

Organisms 24 hr.	pH 6.5	pH 6.8	pH 7.4	pH 7.8	*
Pseudomonas 2681	Growth	Growth	Growth	Growth	
Proteus 235	Growth	Growth	Growth	Growth	
E. coli 36	Growth	Growth	Growth	Growth	
Aerobacter 23	G rowth	Growth	Growth	Growth	
Alcaligenes 2120	Growth	No Growth	No Growth	No Growth	
Shigella	Growth	Growth	Growth	Growth[1]	
Sh. Pseud. 2681	Mixture	Mixture[2]	Mixture[2]	All Pseu.	MS-III
Sh. Prot. 235	Mixture	Mixture	Mixture[3]	All Prot.	N-III, IV
Sh. E. co. 36	All E.co.	All E.co.	All E.co.	All E.co.	MS-VIII
Sh. Aero. 23	All Aero.	Mixture[4]	All Aero.	All Aero.	S-III
Sh. Alca. 2120	All Shig.	All Shig.	All Shig.	All Shig.	O-III

* In vivo antagonism to Shigella and Tables where illustrated.

1, Very sparse growth of Shigella
2, Mostly Pseudomonas
3, Mostly Proteus
4, Mostly Aerobacter

TABLE XVII (con't)

Subculture
24 hr.

Organisms	pH 6.5	pH 6.8	pH 7.4	pH 7.8	*
Sh. Pseud.2681	Mixture[2]	Mixture[2]	All Pseu.	All Pseu.	MS-III
Sh. Prot. 235	Mixture[3]	All Prot.	All Prot.	All Prot.	N-III IV
Sh. E. co. 36	All E.co.	All E.co.	All E.co.	All E.co.	MS-VIII
Sh. Aero. 23	All Aero.	All Aero.	All Aero.	All Aero.	S-III
Sh. Alcal.2120	All Shig.	All Shig.	All Shig.	All Shig[1]	O-III

* In vivo antagonism to Shigella and Tables where illustrated.

1, Very sparse growth of Shigella

2, Mostly Pseudomonas

3, Mostly Proteus

4, Mostly Aerobacter

TABLE XVIII

INHIBITION ZONE MEASUREMENTS

Fredericq's Method, Large Inocula
Proteose No. 3 Agar

Organisms	Approximate Radius of Inhibition Zone-Centimeters	In Vivo Antagonism to Shigella and Table where illustrated.
Aerobacter 2822	1.50	MS-VIII
Aerobacter 4	1.75	S-V
Aerobacter 2451	1.75	MS-III
Aerobacter 2718	1.50	S-IV
Aerobacter 23	1.25	S-VI
Alcaligenes 2258	1.00	O-VIII
Alcaligenes 2120	1.25	O-III
Alcaligenes 2445	1.25	O-V
E. coli 5	1.50	M-V
E. coli 132	1.25	MS-VII
E. coli 58	1.50	M-VII
E. coli 22	2.00	M-VI
E. coli 36	1.25	MS-VIII
Proteus 235	*	N-III, V
Proteus 856	0.75	N-V
Proteus 492	0.25	MS-VIII
Pseudomonas 2803	2.25	M-V
Pseudomonas 2681	0.75	MS-III
Pseudomonas 2659	1.00	M-VIII

* Growth inhibited on entire surface of plate.

V DISCUSSION AND CONCLUSIONS

This thesis demonstrates that infection can be induced in mice with very small doses of Shigella after the intestinal flora is inhibited with streptomycin and erythromycin. (See Table II) Infection could not be induced in normal mice even with inocula of several million viable organisms (Freter, 1955). Three explanations were offered for these observations (Freter, 1956).

1. Some component of the normal intestinal flora may play a role in retarding the establishment of intestinal pathogens.

2. Streptomycin might directly affect the intestinal tract in such a way as to predispose it to the action of enteric pathogenes.

3. The drug might directly increase the virulence of the pathogen.

If the second and third Postulates are correct, the reduction of the normal enteric flora would be incidental to the increased susceptibility to Shigella. If the first is correct, inhibition of antagonistic compohents in the flora would be a necessary preliminary to the induction of infection.

This thesis tneds to substantiate the first interpretation since strong in vivo antagonism to Shigella was exhibited by all strains of Aerobacter, by two strains each of E. coli and Pseudomonas and by one strain Proteus (Summarized in Table IX). If streptomycin alone were sufficient for predisposing the intestine to invasion by intestinal pathogens or for increasing the virulence of the bacteria, the presence of various organisms, as Aerobacter, would hardly be expected to impede the action of this drug. Inhibition

of Shigella by several organisms which are normal inhabitants of the intestine and the ease with which enteric infection was induced in the absence of certain components of the normal intestinal flora, strongly suggest that the normal flora plays a role in preventing or retarding the establishment of intestinal pathogens.

Until recently, there was no effective method for the investigation of in vivo antagonism by the enteric flora. In vitro observations could only suggest the probability of in vivo antagonism. Various in vitro reactions were interpreted as evidence for the existence of in vivo antagonism. These reactions included the production of inhibition zones by test strains on agar plates, the inhibition of one organism by another in broth culture, and the inhibition of the growth of sensitive strains on staled agar plates (See History).

In contrast to such studies, this thesis represents a survey of the in vivo antagonism to Shigella exhibited by several organisms which are normal inhabitants of the intestine. The procedure used measured the ability of Shigella to establish itself in the intestine of mice. The mouse, in which, only an asymptomatic Shigella infection could be induced, obviously did not reflect the exact conditions of human Shigellosis. The actual disease process, consequently, was not studied in the investigation. It would seem, however, that the ability of a pathogen to establish itself in the intestine of human beings is a necessary preliminary to the occurence of a disease process. It is probable that the Shigella population must reach a certain level in humans before the organisms are able to invade the tissues and cause the symptoms associated with dysentery. The mouse infections, induced in this survey, may

have been analogous to the first stages of infection in human beings, i.e., when it is established whether or not the Shigella population can reach sufficient numbers to cause disease.

The in vivo antagonistic activity of various organisms was compared in the present work with the in vitro antagonism displayed by the same organisms using different in vitro culture techniques. In this manner, the reliability of the in vitro techniques in expressing in vivo results was evaluated, and a satidfactoru in vitro technique for the reproduction of in vivo results could be determined. Experiments were carried out by the author to demonstrate inhibition zones on agar plates. The diameter of the zones of inhibition had no relation to the degree of antagonism to Shigella displayed by the test organism in vivo (See Table XVIII). The largest inhibition zone was produced by Proteus 235 which showed no inhibitory activity to Shigella in vivo. The smallest zone was produced by Proteus 492 which was antagonistic to Shigella in vivo. These results indicated that the production of inhibition zones by test organisms against sensitive strains cannot be used as criteria for in vivo antagonism. This might explain the lack of correlation between resistance to Shigella infection in human beings and the presence of colicine-producing bacteria as shown by Halbert (1940b). It might also explain the presence in individuals with shigellosis of coliform organisms which inhibit the infecting strains of Shigella on agar plates (Robbins and Parr, 1957).

No autoinhibition zones could be demonstrated by the author with Shigella flexneri, Aerobacter 2718, or E. coli 58 when Fredericq's technique was employed with heavy inocula. It seemed unlikely then that a depletion of

nutrients or an accumulation of metabolites was responsible for the zones of inhibtion. It is probable that an antibiotic substance was responsible for the zones of inhibition. Inhibition zones could not be demonstrated, however, when veal infusion agar was substituted for Proteose No. 3 agar or when small inocula were used. Consequently, if inhibition zones are due to an antibiotic substance, the production of this substance must vary considerably depending upon the conditions under which the organisms are grown.

When, in the present experiments, the pH of the broth was adjusted to approximate the pH of the upper small intestine of mice (pH 6.81). A good correlation was found between *in vivo* and *in vitro* antagonism. (Table XIV) It is evident from Tables XI through XV that slight changes in pH altered inhibitory activity of test strains. The ability to reproduce *in vivo* results with the *in vitro* culture techniques appeared to depend primarily upon proper adjustment of the pH of the broth. This would suggest that variations in intestinal pH might have a marked effect on the antagonistic characteristics of the normal enteric flora.

Table X illustrates that the small intestine of mice, which had been infected with a *Shigella*, *E. coli* mixture, had a pH of about 6.8 while the contents of the caecum had a pH of about 7.5. These results suggest that a broth culture of pH 6.8 would reflect with some accuracy the antagonism operating within the small intestine, which has a pH of approximately 6.8. Table XVII shows that the *Proteus* and *Pseudomonas* strains exhibited a stronger antagonism to the *Shigella* strain at pH 7.4 to pH 7.8 than they did at pH 6.8. A broth culture of pH 6.8 might be expected, therefore, to minimize the *in vivo* antagonism of these strains because the final phase of in vivo growth in the

caecum is not taken into account. Experimental results seemed to bear out this assumption (Table IV).

The data presented in this work suggests that appropriate broth cultures may be used as experimental models in the investigation of the mechanisms of *in vivo* antagonism.

VI SUMMARY

A survey was made of the relationship between several microorganisms which are normal inhabitants of the intestine of man and a strain of *Shigella flexneri*. Infection was induced in the intestine of mice. The survey indicated the following:

1. When the normal intestinal flora of mice was inhibited, infection could be induced with *Shigella* with only 10 viable organisms.

2. Five strains of *Aerobacter* displayed strong *in vivo* antagonism to *Shigella flexneri*.

3. Four strains each of *E. coli* and *Pseudomonas* displayed moderate to strong *in vivo* antagonism to *Shigella flexneri*.

4. One of 4 strains of *Proteus* displayed strong antagonism to *Shigella flexneri*.

In vivo relationships between antagonistic organisms and *Shigella* could be reproduced in broth cultures if the pH of the broth was adjusted to approximate the pH (6.8) of the small intestine of mice. Growth of *Shigella* on agar plates was inhibited around colonies of all test organisms even if the test organisms displayed no antagonism to *Shigella in vivo* ~~antagonism displayed by the test organism~~.

VII. BIBLIOGRAPHY

Bohnhoff, M., Drake, B. L., and Miller, C. P., 1954, Effect of Streptomycin on Susceptibility of Intestinal Tract to Experimental Salmonella. Proc. Soc. Exp. Biol. and Med., 86:132.

Bowling, R. E., and Wynne, E. S., 1951, Studies on the Mechanism of Antagonism by Aerobacter Strains. J. Infect. Dis., 89:277.

Frankel, J. J., and Wynne, E. S., 1951, Antagonism by Goffyka Tetragena Strains. J. Infect. Dis., 89:52.

Frederiocq, P., and Levine, M., 1947, Antibiotic Relationships Among the Enteric Group of Bacteria., J. Bact. 54:785.

Freter, R., 1954, Fatal Enteric Cholera Infection in the Guinea Pig. Bact. Proc. 1954, p. 56.

Freter, R., 1955, The Fatal Enteric Cholera Infection in the Guinea Pig, Achieved by Inhibition of Normal Enteric Flora. J. Infect. Dis., 97:57.

Freter, R., 1956, Experimental Enteric Shigella and Vibrio Infections in Mice and Guinea Pigs. J. Exper. Med., 104:411.

Fulton, M., 1937, Antibiosis in the Colon-Typhoid Group. J. Bact., 34:301.

Gratia, A., 1932, Antagonisme Microbien et "bacteriophagie". Ann. Inst. Past., 48:413.

Gratia, A., and Frederiocq, P., 1946, Diversite des souches antibiotiques de B. coli et entendu variable de leur champs d'action. Compt. Revd. Soc. Biol., 140:1032.

Halbert, S. P., 1948a, The Antagonism of Coliform Bacteria Against Shigella. J. Immunol. 58:155.

Halbert, S. P., 1948b, The Relation of Antagonistic Coliform Organisms to Shigella Infections. I. Survey Observations. J. Immunol. 60:23.

Halbert, S. P., 1948c, The Relation of Antagonistic Coliform Organisms to Shigella Infection. II. Observations in Acute Infections. J. Immunol. 60:359.

Halbert, S. P., and Gravatt, M., 1949, The Relation of Shigella Type-Specificity and Suceptibility to Antibiotic-Producing Strains of Escherichia Coli. J. Immunol. 61:271.

Halbert, S. P., and Magnuson, H. J., 1948, Studies With Antibiotic-Producing Strains of Escherichia Coli. J. Immunol., 58:397.

Halbert, S. P., Sonne, C., and Swick, L., 1954, Mixed Bacterial Infections in Relation to Antibiotic Activities. J. Immunol., 73:169.

Halbert, S. P., and Swick, L. S., 1950, In Vivo Antibiotic Production by Escherichia Coli. J. Immunol. 65:675.

Heatley, N. G., and Florey, H. W., 1946, An Antibiotic From Bacterium Coli, Brit. J. Exper. Path., 27:378.

Luderford, C. G., and Lederer, M., 1953, The Antibiotics of Escherichia Coli. Austral. J. Exper. Biol. Med., 31:553.

Nissle, A., 1916, Ueber Die Grundlagen Einer Neuen Ursaechlichen Bekaempfung Der Pathologischer Darmflora. Deutsche Med. Wohnschr., 42:1181.

Wynne, E. S., 1947, Antagonism by Aerobacter Straits. J. Bact., 53:469.

Wynne, E. S., and Norman, J. O., 1953, On the Concept of "Direct Antagonism" in Bacteria. J. Infect. Dis., 93:243.

APPROVAL SHEET

The thesis submitted by David Hentges has been read and approved by three members of the Department of Microbiology.

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

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

January 10, 1958
Date

Elmer Leiyson
Signature of Advisor