A STUDY ON THE ANTIBACTERIAL ACTIVITY OF 2, 4-DICHLOROPHENOXYACETIC ACID

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A STUDY ON THE ANTIBACTERIAL ACTIVITY OF 2,4-DICHLOROPHENOXYACETIC ACID

By

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INTRODUCTION

The chemical 2,4-dichlorophenoxyacetic acid (2,4-D) was first introduced as a plant-growth regulator sometime during 1940 and 1941 both in this country and in England by various groups of chemists and botanists who were testing a long list of chemicals in an effort to determine which of them had growth regulating properties. In the United States, E. J. Kraus, then at the University of Chicago, is given credit for first suggesting the use of plant-growth regulators as herbicides. It is claimed that he made the suggestion as early as 1941.

Although their work was not published until 1945, the English investigators Slade, Templeman and Sexton in 1942 offered knowledge of selective weed killers to the Agricultural Research Council and cooperated with the Council in testing their findings. They listed 2,4-dichlorophenoxyacetic acid or "chloroxone" as one of the two most active plant-growth regulators which they had discovered. The other compound was 2-methyl-4-chlorophenoxyacetic acid which came to be known as "methoxone" or "agroxone".

Zimmerman and Hitchcock (1942) published results of their work with plant-growth regulators and were given credit for being the first investigators in the United States to demonstrate the physiological properties of

substituted phenoxy acids, which include 2,4-D.

Literature concerning the use of 2,4-D as a herbicide dates back to early 1944 when Beal (as cited by Akamine, 1948), at the University of Chicago, published results of laboratory tests which suggested its herbicidal possibilities. Mitchell and Hamner (1944) indicated the use of carbowax, a polyethylene glycol, as a carrier of 2,4-D and similar compounds. Later in the same year Hamner and Tukey (as cited by Akamine, 1948) recorded successful experiments in which 2,4-D and 2,4,5-trichlorophenoxyacetic acids were used as herbicides. Numerous investigators began testing the ability of these related compounds as herbicides as a result of Hamner's and Tukey's findings.

Tukey (1947) published a summary of the information available on 2,4-D at that time. He described 2,4-D as being a halogenated phenoxy compound containing two chlorine atoms substituted in the 2 and 4 positions and having an acetic acid side chain. It is manufactured by the chlorination of phenol and is easily converted to its water soluble sodium salt. It is stable to autoclaving, is non-corrosive and apparently non-toxic to animals in the concentrations commonly used for weed killing (one part per one thousand weight in a water spray).

According to Tukey,

> "Field work in 1945 and 1946 from widely separated parts of the country and from Europe indicated great success with 2,4-D as a herbicide. The material seemed to move rapidly into the plant, to be transported in the direction of movement of

> synthesized material, and to be favored in its effectiveness by photosynthetic activity. Accordingly, greatest responses were secured from plants in sunshine. The action was the arrest in development of some parts of the plant and the stimulation of cell division and cell enlargement in other parts, especially in those regions which are young, active, and meristematic such as cambium. Respiration was markedly increased, and starch reserves were depleted. The plants became bent and twisted and otherwise showed growth responses which were finally associated with inhibition and death. In some instances, plants were killed without apparent acceleration of growth in any part. The below-ground parts of bindweed and sow thistle became proliferated, split, and decayed in the course of a week to 10 days. The pollen of flowers shriveled and became nonfunctional, leading to the use of 2,4-D to destroy ragweed pollen in an attempt to reduce hay fever. Many other plants, such as lamb's-quarters and pigweed, were killed."

2,4-D was proved to be selective in action. Grasses, with the exception of Bermuda grass, and cereals are not generally affected by the concentrations of 2,4-D which are toxic to broad-leaved plants.

Among other uses found for 2,4-D are its employment in the retardation of preharvest drop of fruit, in the promotion of rooting of cuttings of plants which are difficult to root, in the setting of tomato flowers as fruit, and in the reduction of scald of apples in storage.

Since the time that Tukey's summary appeared in the literature, a number of investigators have completed experiments testing the results of 2,4-D action. Several of these authors have reported essential chemical as well as anatomical alterations in plant tissue. Hagen, Clagett,

and Helgeson (1949) noted that 2,4-D in the acid form effectively inhibited the activity of castor bean lipase at very low concentrations. Luecke, Hamner, and Sell (1949) found that the quantity of certain vitamins in the leaves and stems of the red kidney bean plant was altered considerably by 2,4-D treatment. The thiamine, riboflavin, nicotinic acid and carotene content was less in leaves of treated than untreated plants. Pantothenic acid was found in greater quantities in leaves of treated than non-treated plants. The amount of thiamine, riboflavin, nicotinic acid was much greater in stems of plants treated with 2,4-D than in stems of non-treated plants. The carotene content was approximately two-thirds as great in stems of treated plants as in stems of non-treated plants. Sell, Luecke, Taylor, and Hamner (1949) published their findings with regard to the changes in chemical composition in the stem of red kidney bean plants treated with 2,4-D. They found that both protein and amino acids accumulated in greater quantities in the stems of plants treated with 2,4-D than in the stems of the non-treated plants; the reducing and non-reducing sugars were depleted in the 2,4-D treated plants. The decrease in sugar content and the increase in total protein seemed to indicate that a large portion of the carbohydrates was utilized in protein synthesis; the amounts of ether extract, unsaponifiable material and fatty acids of the ether extract were slightly greater in the stems of the treated plants than in those of the non-treated

tissue; the amount of crude fiber decreased in the stems of 2,4-D treated plants. Weller, Luecke, Hamner, and Sell (1950) analyzed the leaves and roots of red kidney bean plants treated with 2,4-D. They found a lower percentage of protein and of most of the amino acids in the sample and in the crude protein than in the controls. They observed a depletion of non-reducing sugar in both leaves and roots of treated plants. No significant change was found in the percentage of reducing sugar. Neely, Ball, Hamner, and Sell (1950) reported that 2,4-D lowered considerably the activity of both the alpha and beta amylase in the stems of bean plants. In the same year the same authors published findings which indicated that pectin methoxylase activity increased in both the proliferated stem and leaf tissue of the treated plants; phosphorylase activity decreased in both the proliferated stem and leaf tissue of the 2,4-D treated plants; invertase activity was absent in both test and control tissue and there was no indication of the presence of a phosphatase acting on D-glucose-1-phosphate.

The findings of these authors on the chemical changes in 2,4-D treated plant tissue stimulated an interest in determining the type of effect which 2,4-D might have on bacteria.

A survey of the literature reveals publications with regard to growth regulators (auxins) and their activity on microorganisms which date back as far as 1938 when

Ball indicated that heteroauxin stimulated cell division of Escherichia coli in a manner analogous to the effect of the auxin of higher plants on cambial growth. Burkholder (1939) published the results of a study of the production of a growth substance by E. coli and Aerobacter aerogenes in media containing specific organic and inorganic nitrogenous compounds, and he postulated that it was probable that different substances having physiological properties of auxins might be synthesized by microorganisms in ways other than by the conversion of tryptophane. Brannon and Bartsch (1939) summarized a study of the influence of growth substances on growth and cell division in green algae. Fildes (1941) presented a paper on the inhibition of bacterial growth by indoleacrylic acid and its relation to tryptophane. Smith, Dawson, and Wenzel (1945) found the herbicides varied greatly in their effect on the various groups of soil microorganisms. In some cases they were definitely toxic, in others stimulatory. Stevenson and Mitchell (1945) found that 0.02 per cent of 2,4-D or its sodium salt had a decided retarding effect on the growth of some of the more common bacteria while even 0.08 per cent of the chemical did not noticeably affect two species of fungi. Culler (1946) confirmed the work of Stevenson and Mitchell and noted a greater sensitivity of gram-positive than of gram-negative organisms to 2,4-D. Martin (1946) published experiments which showed that the effect of 2,4-D

on soil microorganisms was much more pronounced at an acid pH than at neutrality. Dubos (1946) indicated that, although in the case of most organisms bacteriostatic activity of all substances increases as the medium becomes more acidic, inhibition of growth of tubercle bacilli by auxins appears to be almost independent of the reaction of the medium. His study determined the bacteriostatic activity of six auxins on Mycobacterium tuberculosis (human strain), Streptococcus homolyticus and salivarius, and Shigella paradysenteriae (Sonne). The activity of the different auxins varied greatly from one species to another each substance seeming to exhibit a selective inhibitory effect against one or several groups of organisms. Indole-3-acetic acid was the most active substance against the gram-negative bacilli, beta indole-3-propionic acid against mycobacteria, and triiodobenzoic acid against certain strains of streptococci. The inhibitory concentration of auxin was not markedly affected by the size of the inoculum and was independent of the addition of serum albumin to the medium. Peptone and tryptophane were found to reverse, in part or completely, the bacteriostatic effect of the auxins, tryptophane being by far the more active in this respect. Reversal of bacteriostasis usually required concentrations of tryptophane approximately 10 times higher than those of auxin added to the medium. Dubos concluded that:

> "When the concentration of auxin in the medium is insufficient to produce complete

> inhibition of growth, bacterial multiplication proceeds at first at a rate similar to that in the untreated culture. However, the presence of auxin results in an interruption of multiplication before maximal development has occurred; the density of the final culture is inversely related to the concentration of auxin. In other words, the antibacterial mechanism under consideration affects the total amount of bacterial protoplasm synthesized rather than the rate of growth of the culture."

Lewis and Hamner (1946), while testing the effect of 2,4-D on microorganisms, found that under normal rates of application for the killing of weeds the amount of 2,4-D which reaches the soil will have no important effect on the soil microorganisms or on plant pathogens present in the soil. Fultz and Payne (1947) stated that the effect of 8,000 ppm of 2,4-D on Rhizobium leguminosarium Frank of common beans was to decrease the percentage of long and markedly increase the percentage of short rods. Carlyle and Thorpe (1947) made a study on some of the effects of ammonium and sodium salts of 2,4-D on legumes and Rhizobium and found that either of the salts present in soil solution at the rate of 0.5 ppm (0.21 lb/acre) would seriously restrict germination, limit growth and practically inhibit nodulation of beans, peas, red clover, and alfalfa but had little effect on the rhizobia living free in the soil. It appeared that the deleterious effect on the symbiotic relationship was apparent by a reduction or inhibition of nodulation caused largely by the action of the herbicide through the medium of the plant.

Trigg and Stahly (1948) published the results of an extensive study of the effects of plant-growth regulating substances on microorganisms (bacteria, molds, and actinomycetes). West and Henderson (1948) found that concentrations of 2,4-D ranging from 10 to 1,000 ppm had an antagonistic effect upon the growth of yeast as determined by a turbidimetric method. Worth and McCabe (1948) subjected small groups of aerobic, facultative anaerobic, and anaerobic organisms to concentrations of 2,4-D varying from 0.0002 per cent to 2.0 per cent and found that the aerobes were inhibited especially by the higher concentrations; the facultative anaerobes were not inhibited at all, and both groups of organisms seemed to be stimulated by the lower concentrations. Of the anaerobes tested, Clostridium tetani behaved similarly to the facultative anaerobes while Clostridium welchii and Clostridium botulinum varied so much in their response that no definite conclusions could be reached. Richards (1949) noted the responses of representative fungi to certain growth regulating substances and found that, in general, inhibition occurred within the concentration range 10^{-2} to 10^{-3} molar; stimulation within the range 10^{-3} to 10^{-4} molar.

Although all of the organisms used in the study made by Worth and McCabe were maintained at the same pH in the same concentrations of 2,4-D, they were not all grown on the same media.

Since preliminary work done with 2,4-D in this laboratory has shown that the kind of medium may make a difference in the growth-inhibition range of an organism subjected to the chemical, this study was set up to compare the effects of different concentrations of 2,4-D on a group of bacterial species, all of which can be grown on the same media. Therefore the organisms used in the study have been so chosen as to be of interest to the departments concerned with the study and also to be applicable to the same kinds of media.

This study includes a survey of the response of several types of bacteria to 2,4-D in four different liquid media and in one solid medium. It also includes an attempt to develop a 2,4-D resistant organism and a study of the morphological changes occurring in the organisms as a result of the action of 2,4-D.

EXPERIMENTAL

Organisms. The organisms included in this study were: a stock strain of Bacillus cereus var. mycoides, Salmonella paratyphi, Salmonella pullorum, Salmonella schottmuelleri and of Xanthomonas phaseoli and an American type culture strain of Bacillus subtilis (9466), Escherichia coli (9637), Micrococcus pyogenes var. aureus (6538p), and Mycobacterium phlei (355). All were maintained on nutrient agar slants.

Materials and Methods. Difco's dehydrated media were used throughout the entire study. Media included were: nutrient agar, nutrient broth, tryptose, brain heart infusion, and Wolf's casamino acids broth. The media were prepared and sterilized according to Difco directions. All broth media, except the brain heart infusion, were adjusted to a pH of 7.0 $\pm$ 0.2. The brain heart infusion medium was used without pH adjustment but was found to lie in that same pH range.

The 2,4-dichlorophenoxyacetic acid was used in the form of its sodium salt. The chemical was supplied by the Dow Chemical Company and recrystallized by Dr. H. M. Sell of the Department of Agricultural Chemistry, Michigan State College. The desired quantities of sodium 2,4-dichlorophenoxyacetate were added to the respective broth

media as needed and the resultant 2,4-D media were sterilized by autoclaving. The 2,4-D was dissolved in the hot broths prior to the first sterilization. Each broth medium was then tubed and sterilized, thus avoiding repeated autoclaving.

With the nutrient agar it was found to be more practical to autoclave the medium first to insure thorough melting of the agar. The hot agar was then measured, added to weighed quantities of 2,4-D and sterilized.

Both plain agar and plain broth were prepared for use as controls.

The preliminary experiment was done on 2,4-D in nutrient broth. The nine organisms used were transferred from stock cultures to nutrient broth and three consecutive transfers (24 hours) were made in order to eliminate any effect of the stock media. Thereafter, for the duration of the study, the cultures were maintained in nutrient broth.

A rack containing eleven tubes which included a 2,4-D free control (referred to in the tables as 0 per cent 2,4-D) and 2,4-D concentrations ranging from 0.05 to 3.0 per cent 2,4-D in nutrient broth, set up in duplicate, was prepared for each organism. Each tube contained 5 ml of broth.

The standard amount of inoculum in this study was 0.1 ml of a 24 hour broth culture (48 hour culture for M. phlei). Incubation temperature and time were 37°C and 24 hours for all organisms except M. phlei which was incubated for 48 hours and X. phaseoli which required 27°C. Readings, in

all cases, were based on obvious turbidity of the broth as compared to control (2,4-D free) broth tubes.

At the end of the incubation period 0.5 ml broth was transferred from each 2,4-D tube to plain nutrient broth (5 ml nutrient broth per tube) to determine the highest concentration of the chemical in which the organism had survived. These tubes were incubated in the same manner as the 2,4-D tubes. Results were recorded after 24 hours (48 hours for M. phlei).

In all other broth experiments 0.2 ml of a 24 hour broth culture of the organisms, grown in the desired medium, was the standard inoculum (48 hour culture for M. phlei), and the tubes were incubated as were those of the preliminary study except that M. phlei tubes in the remaining survival experiments were incubated 96 hours to obtain sharper end points. The volume of broth medium in the survival experiments was increased to 10 ml to make negligible the final concentration of 2,4-D in those tubes.

The experiments in which solid medium was employed were set up in two sections:

In one section the surface of each of two control plates (plain nutrient agar) and each of two 2,4-D plates of eight different concentrations (0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 per cent) was streaked using a loop and a heavy inoculum taken from a fresh nutrient agar slant culture of the organism in question. This made a total of eighteen plates for each organism since all plates,

including the control, were set up in duplicate. The plates were incubated for 24 hours at 37°C (27°C for X. phaseoli, 48 hours for M. phlei) and observed for the presence or absence of growth. Plates were held and observed daily with and without the aid of a disection microscope until the agar began to dry and/or the 2,4-D began to crystallize out.

In the other section of the nutrient agar experiment, small Erlenmeyer flasks, containing 40 ml volumes of sterile nutrient agar of the same concentrations used in the first section, were melted and cooled to between 42° and 46°C. Each of these flasks was then inoculated with 0.2 ml of nutrient broth culture of the desired organism and the flasks rotated well to insure thorough mixing of agar and culture. Two plates were poured from each flask. The plates were incubated for 48 hours at 27°C. These plates were read and observed in the same manner as the streaked plates.

Two experiments were set up to attempt to develop strains resistant to 2,4-D:

1. A 24 hour nutrient agar slant culture of E. coli was used as the original material for the first of these experiments.

   a. The surfaces of two plain nutrient agar plates and two 2,4-D plates of three different concentrations (0.5, 1.0, 1.5 per cent) were streaked with a 3 mm loopful of the E. coli culture. These plates were

incubated for 24 hours at 37°C.

b. The plates were read and the culture from the highest 2,4-D concentration showing growth was transferred by the streak method to plates of that same concentration, to plates containing 0.5 per cent greater 2,4-D concentration, and also to plain nutrient agar plates.

c. This set of subcultures was incubated for 24 hours at 37°C, read and subcultured just as were the original plates.

d. Subcultures from (1-c) were further subcultured as indicated under (1-b). With these new subcultures as stock, steps (1-b) and (1-c) were repeated and cultures from each new 2,4-D end-point were subcultured similarly at 24 and 48 hours until a concentration of 2,4-D was reached at which a subculture could not be maintained; that is, until a concentration of 2,4-D was found on which E. coli would grow in 24 or 48 hours but from which subcultures in medium with the same or higher concentrations would not grow.

2. A 24 hour nutrient broth culture of E. coli was used as starting material for the second experiment.

a. This culture was transferred to plain nutrient broth and to four different concentrations of 2,4-D

broth (0.5, 0.8, 1.0, and 1.5 per cent) so chosen that the organism's growth end-point and at least one concentration 0.5 per cent above and below it were included. The broth series was set up in duplicate and incubated at 37°C for 24 hours.

b. The positive broth cultures from (2-a) representing the two highest 2,4-D concentrations in which growth was observed were subcultured to broth tubes of the same 2,4-D concentration, to concentrations higher and lower by 0.5, and by 1.0 per cent and to plain nutrient broth. Both the seed and subcultures were incubated for 24 hours at 37°C.

c. The 2,4-D broth cultures from (2-a) were subcultured in plain nutrient agar plates and to 2,4-D plates of four different concentrations (0.1, 0.3, 0.5, and 1.0 per cent) so chosen that E. coli's growth end-point on 2,4-D agar and at least one concentration above and below it were included. The plates were incubated for 24 hours at 37°C.

d. Colonies were picked from the positive plates and cultured in broth having a 2,4-D concentration equal to and greater than that from which the plates were inoculated (greater by 0.5 per cent 2,4-D).

e. At the end of 24 hours' incubation at 37°C the broths from (2-d) were further subcultured as described

under (2-b).

f. Steps (2-b) through (2-e) were repeated several times stepping up the 2,4-D concentration (at which the culture would grow) to 4.0 per cent.

Two experiments were set up in order to observe whether or not any morphological changes occurred in the organisms while under the influence of 2,4-D in nutrient broth and on nutrient agar. The first experiment was set up by inoculating series of nutrient broth tubes containing various concentrations of 2,4-D with 0.2 ml of 24 hour broth cultures (48 hour culture of M. phlei) of the respective organisms just as had been done in previous experiments to determine growth end-points in broth. The lowest 2,4-D concentration used was 0.3 per cent. A concentration of 1.3 per cent was included in the series to serve as an intermediate between 1.0 and 1.5 per cent since the growth end-point for several of the organisms had previously been found to lie in the range 1.0 to 1.5 per cent 2,4-D in nutrient broth. The 2,4-D broth tubes and controls were incubated at 37°C for 24 hours with X. phaseoli and M. phlei being the usual exceptions.

All broth tubes which were turbid at the end of this incubation period were centrifuged and the supernatant broth was discarded. Duplicate slides were made from the sediment from each tube.

The second of the two experiments used to observe

morphological changes in the organisms was set up on nutrient agar containing various concentrations of 2,4-D (0.05, 0.1, 0.3, 0.5, 0.7, and 1.0 per cent). The streaked plate technique was employed in order to obtain single colonies. All plates were incubated for 24 hours at 37°C. X. phaseoli and M. phlei were incubated as previously.

Slides were made from each plate on which visible growth occurred. In the case of B. cereus var. mycoides, smears were made from both plates on which growth occurred and from the plates representing the two next higher concentrations of 2,4-D, transfers from these concentrations to plain agar and broth having shown that the organisms were alive but not exhibiting any visible evidence of growth.

These slides which were prepared from both broth and agar preparations and included both broth and agar controls were stained by Gram's method and examined carefully for any obvious morphological changes in the organisms. Then, for each of the organisms which showed any conspicuous change in morphology, the control smears and the smears from both the lowest and the highest concentration at which growth had occurred were photographed.

The photomicrographs were taken from fields which represented typical morphology at given 2,4-D concentrations and do not pretend to represent population numbers. The organisms shown are magnified 1500 times.

RESULTS

The results of the preliminary study with nutrient broth are recorded in Tables I and II. Table I shows the comparative ability of the nine organisms concerned to grow in the presence of 2,4-D in a concentration range of 0.05 to 3.0 per cent in nutrient broth.

These results indicate that the growth end points of E. coli, S. pullorum, S. paratyphi, and M. pyogenes var. aureus all lay within the same range, 1.0 to 1.5 per cent. Those of B. cereus var. mycoides and X. phaseoli lay between 0.5 and 0.8 per cent while B. subtilis, S. schottmuelleri, and M. phlei did not show any growth above the range, 0.3 to 0.5 per cent 2,4-D.

Table II indicates the viability of the nine organisms studied under the conditions described for Table I. A survey of Table II will, however, reveal a somewhat different perspective than that observed in Table I. B. subtilis and M. pyogenes var. aureus remained viable in the highest concentration of 2,4-D used in the experiment while M. phlei survived in 2.5 but not 3.0 per cent 2,4-D. X. phaseoli had a survival end-point between 2.0 and 2.5 per cent. E. coli and S. paratyphi lay between 1.5 and 2.0 per cent; S. pullorum and S. schottmuelleri between 1.0 and 1.5 per cent. B. cereus var. mycoides survived in

0.5 per cent with the organisms from one tube surviving in 1.0 per cent. No survival of B. cereus var. mycoides was noted above 1.0 per cent under the conditions of this experiment.

All other broth experiments (Tables III - X) done in this study differed in procedure from the preliminary experiment (Tables I and II) by the amount of inoculum (0.2 ml instead of 0.1), by the length of incubation time given M. phlei (96 hours instead of 48), and by the assortment of 2,4-D concentrations used (0.7 and 0.9 per cent 2,4-D were substituted for 0.8 per cent used in the preliminary experiment).

Tables III and IV show the growth and survival end-points, of the organisms studied, in nutrient broth under the conditions stated above. A brief study of these two tables will show that the growth end-point of M. pyogenes var. aureus lay between 2.0 and 2.5 per cent. E. coli and S. pullorum grew in the presence of 1.0 per cent of the chemical but not in 1.5 per cent. S. paratyphi grew in 0.9 per cent but not in 1.0 per cent; X. phaseoli in 0.7 but not in 0.9 per cent; B. cereus var. mycoides and M. phlei in 0.5 per cent; B. subtilis in 0.3 but not in 0.5 per cent 2,4-D in nutrient broth. B. cereus var. mycoides, B. subtilis, M. phlei, M. pyogenes var. aureus, and X. phaseoli were all viable in 3.0 per cent of the chemical. E. coli and B. subtilis survived 1.5 per cent but not 2.0 per cent 2,4-D. S. schottmuelleri, S. pullorum, and S.

paratyphi survived 1.0 but not 1.5 per cent.

Tables V and VI show the growth and survival end-points of the organisms in Wolf's casamino acids broth medium. Table V indicates that E. coli grew in 0.9 per cent but not in 1.0 per cent 2,4-D in casamino acids broth; M. pyogenes var. aureus in 0.7 but not in 0.9 per cent; S. schottmuelleri in 0.5 but not in 0.7 per cent; S. paratyphi and S. pullorum in 0.3 but not in 0.5 per cent; B. cereus var. mycoides, B. subtilis, X. phaseoli and M. phlei in 0.1 but not in 0.3 per cent. Table VI shows that B. cereus var. mycoides and M. pyogenes var. aureus were viable in 1.5 but not in 2.0 per cent; E. coli, B. subtilis, X. phaseoli, and M. phlei in 1.0 but not in 1.5 per cent; S. paratyphi and S. pullorum in 0.7 but not in 0.9 per cent; S. schottmuelleri in 0.5 but not in 0.7 per cent 2,4-D in casamino acids broth.

Tables VII and VIII show the comparative ability of the organisms used to grow and to remain viable in 2,4-D incorporated in tryptose broth. Results tabulated in Table VII show that M. pyogenes var. aureus grew in 2.0 per cent but not in 2.5 per cent 2,4-D; E. coli grew in 1.5 but not in 2.0 per cent; S. paratyphi, S. pullorum, and S. schottmuelleri in 0.7 but not in 0.9 per cent; X. phaseoli in 0.5 but not in 0.7 per cent; B. cereus var. mycoides in 0.3 per cent. B. subtilis showed no growth at all in any concentration of 2,4-D used. Table VIII indicates the viability of the organisms in plain tryptose broth after having been exposed to 2,4-D. M. pyogenes var.

aureus, B. cereus var. mycoides, B. subtilis, and X. phaseoli survived 3.0 per cent 2,4-D. M. phlei was viable in 2.5 per cent but not in 3.0 per cent; E. coli was viable in 1.5 but not in 2.0 per cent; S. paratyphi and S. pullorum in 1.0 but not in 1.5 per cent; S. schottmuelleri in 0.9 but not in 1.0 per cent 2,4-D.

Tables IX and X show the comparative growth and viability of the organisms in the presence of 2,4-D incorporated in brain heart infusion broth under the conditions of the study. Table IX indicates that M. pyogenes var. aureus grew in 3.0 per cent 2,4-D; E. coli in 1.5 but not in 2.0 per cent; S. pullorum in 1.0 but not in 1.5 per cent; S. paratyphi in 0.9 but not in 1.0 per cent; S. schottmuelleri in 0.7 but not in 0.9 per cent; B. cereus var. mycoides in 0.5 but not in 0.7 per cent; X. phaseoli in 0.3 but not in 0.5 per cent; M. phlei in 0.1 but not in 0.3 per cent. B. subtilis showed no growth in any concentration used in brain heart infusion broth. Table X shows that B. cereus var. mycoides, M. pyogenes var. aureus, and X. phaseoli were viable in 3.0 per cent 2,4-D; B. subtilis and M. phlei in 2.5 but not in 3.0 per cent; E. coli and S. paratyphi in 1.5 but not in 2.0 per cent; S. schottmuelleri in 0.7 but not in 0.9 per cent.

Table XIII is a composite chart which shows the growth and survival end-points of the organisms in each of the four broth media used.

Tables XI and XII indicate the growth end points of

the organisms on and in nutrient agar containing 2,4-D, streaked and poured plates. A survey of the results from the streaked plates (Table XI) shows that E. coli grew on 0.5 per cent 2,4-D in 24 hours and on 1.0 per cent in 96 hours. M. pyogenes var. aureus, S. paratyphi, S. pullorum, and S. schottmuelleri grew on plates containing 0.5 per cent of the chemical in 24 hours and showed no growth on a higher concentration even after very prolonged incubation. B. subtilis grew on plates containing 0.3 per cent 2,4-D. X. phaseoli grew on 0.1 per cent 2,4-D in 24 hours and on 0.3 per cent in 10 days. The record of the growth of the organisms in poured plates is shown in Table XII. In this experiment E. coli, S. paratyphi, S. pullorum, S. schottmuelleri and M. pyogenes var. aureus all grew in 0.5 per cent 2,4-D in nutrient agar in 48 hours but showed no growth above that concentration after prolonged incubation. B. subtilis, M. phlei and X. phaseoli showed no growth in any concentration higher than 0.1 per cent either at 48 hours or at the end of the experiment. B. cereus var. mycoides did not grow on or in any concentration of 2,4-D used in these two experiments.

The results of the two experiments set up in an attempt to develop a 2,4-D resistant strain of E. coli were as follows:

In the first experiment growth of E. coli was obtained by the streak method on agar containing a concentration of 2,4-D as high as 2.5 per cent, but attempts to sub-culture

the organism from 2.5 to 2.5 per cent or to higher concentrations were unsuccessful.

In the second experiment growth of E. coli was obtained in broth and on agar at a concentration of 3.0 per cent 2,4-D. Some growth was obtained at 3.5 and even 4.0 per cent on agar plates, but attempts to maintain the culture and make subcultures from 4.0 per cent were unsuccessful. Subcultures were made successfully from 3.0 to 3.0 and to 3.5 per cent 2,4-D in both media and maintained as long as three days.

Plates I and II show the changes which occurred in the morphology of E. coli which had been subjected to various concentrations of 2,4-D in broth and in agar respectively. Figure 1, Plate I, shows typical normal morphology in nutrient broth. In Figure 2 (o.3 per cent) the organisms are slightly larger and longer than normal with an increased number of club-shaped forms while in Figure 3 (1.3 per cent 2,4-D) cells are so markedly elongated that, in some cases, they might be called filamentous. Figure 1, Plate II, illustrates normal E. coli organisms from nutrient agar. Figure 2 (0.05 per cent 2,4-D) demonstrates no conspicuous change in morphology. It can be seen in Figure 3, however, that some of the cells which had been exposed to 0.7 per cent 2,4-D are definitely enlarged, while some are diplobacilli-like, and some are extremely long or filamentous.

Plates III and IV indicate the morphological changes which occurred in B. cereus var. mycoides as a result of exposure to various concentrations of 2,4-D. Figures 1 - 3, Plate III, show the morphology in 2,4-D free broth and broth containing 0.3 and 0.5 per cent 2,4-D. All cells observed on the control slide were in the vegetative form (Figure 1). The organisms seen in Figure 2 (0.3 per cent 2,4-D) are both larger and longer than normal and a few spores are apparent. Figure 3 (0.5 per cent 2,4-D) shows cells which are not as wide as the normal cells but are approximately the same length. Groups of the cells are clumped and a few spores are present. Figures 1 - 3, Plate IV, reveal the morphology of the organisms taken from 2,4-D free agar and from agar containing 0.05 and 0.7 per cent 2,4-D. Figure 1 shows normal morphology on plain nutrient agar. A few extra-cellular and intra-cellular spores were seen. Agar containing 0.05 per cent 2,4-D had the effect of cell enlargement and elongation together with marked spore formation (Figure 2). B. cereus var. mycoides showed no evident signs of growth but subcultures from the site of the original inoculum to 2,4-D free broth and agar produced visible growth in 24 hours. Microscopic examination of a smear taken from 0.5 per cent 2,4-D agar (Figure 3) revealed a predominance of spores which were larger than those apparent at 0.05 per cent.

Plate V demonstrates the morphological changes which occurred in S. pullorum while in contact with 2,4-D.

Figures 1 - 3, Plate V, portray the normal morphology in 2,4-D free broth and the morphological changes occurring in 0.3 and 1.0 per cent 2,4-D broth cultures. The phenomenon observed in Figure 2 varies from normal in that it shows marked elongation and beading of the cells many of which appear to be transparent. A few distinctly coccoid forms may also be seen at this concentration. 1.0 per cent 2,4-D (Figure 3) seemed to cause a shrinking of the cells. It is obvious that the organisms in Figure 3 are smaller than normal and resemble diplobacilli. The smears of S. pullorum which were made from 0.1 and 0.7 per cent 2,4-D agar cultures revealed no conspicuous morphological change except that in many cases (0.7 per cent 2,4-D culture only) cell division seemed to be incomplete and the organisms had the diplo-like appearance seen in Figure 3.

The morphological changes which occurred in M. pyogenes var. aureus when it was subjected to various concentrations of 2,4-D in agar were not conspicuous. However, the smears from agar containing 0.7 per cent 2,4-D showed some large forms and some empty or "ghost" cells. Plate VI illustrates the morphology of M. pyogenes var. aureus when cultured in nutrient broth containing 2,4-D. Figure 1 shows normal morphology with some of the cells occurring singly, some in short chains, but most of the cells in small, loosely clumped groups. Figure 2 (aureus in 0.3 per cent 2,4-D) varies from normal in that the individual cells are larger

and the clumps are more compact. In 1.0 per cent 2,4-D broth the cells are still larger and are even more conspicuously aggregated into dense clumps as is shown in Figure 3.

Although no photomicrographs were taken of S. paratyphi and S. schottmuelleri, the type of morphological change exhibited by these two organisms was very similar to that shown by S. pullorum. B. subtilis resembled B. cereus var. mycoides in spore formation but failed to show conspicuous elongation of vegetative cells. No distinct morphological change was observed in either M. phlei or X. phaseoli under the conditions of these experiments.

Only one conspicuous change in gross morphology was noted during the course of the experiments. B. cereus var. mycoides, which normally grows luxuriantly and spreads over the entire surface of the inoculated plate on suitable media, did not progress more than a distance of two or three millimeters from the original site of inoculation on an agar plate containing 0.05 per cent 2,4-D.

TABLE I

GROWTH OF MICROORGANISMS IN NUTRIENT BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium									
		0.05	0.1	0.3	0.5	0.8	1.0	1.5	2.0	2.5	3.0
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ ±	- -	- -	- -	- -
X. phasecli	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ ±	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phasecli - 27°C)

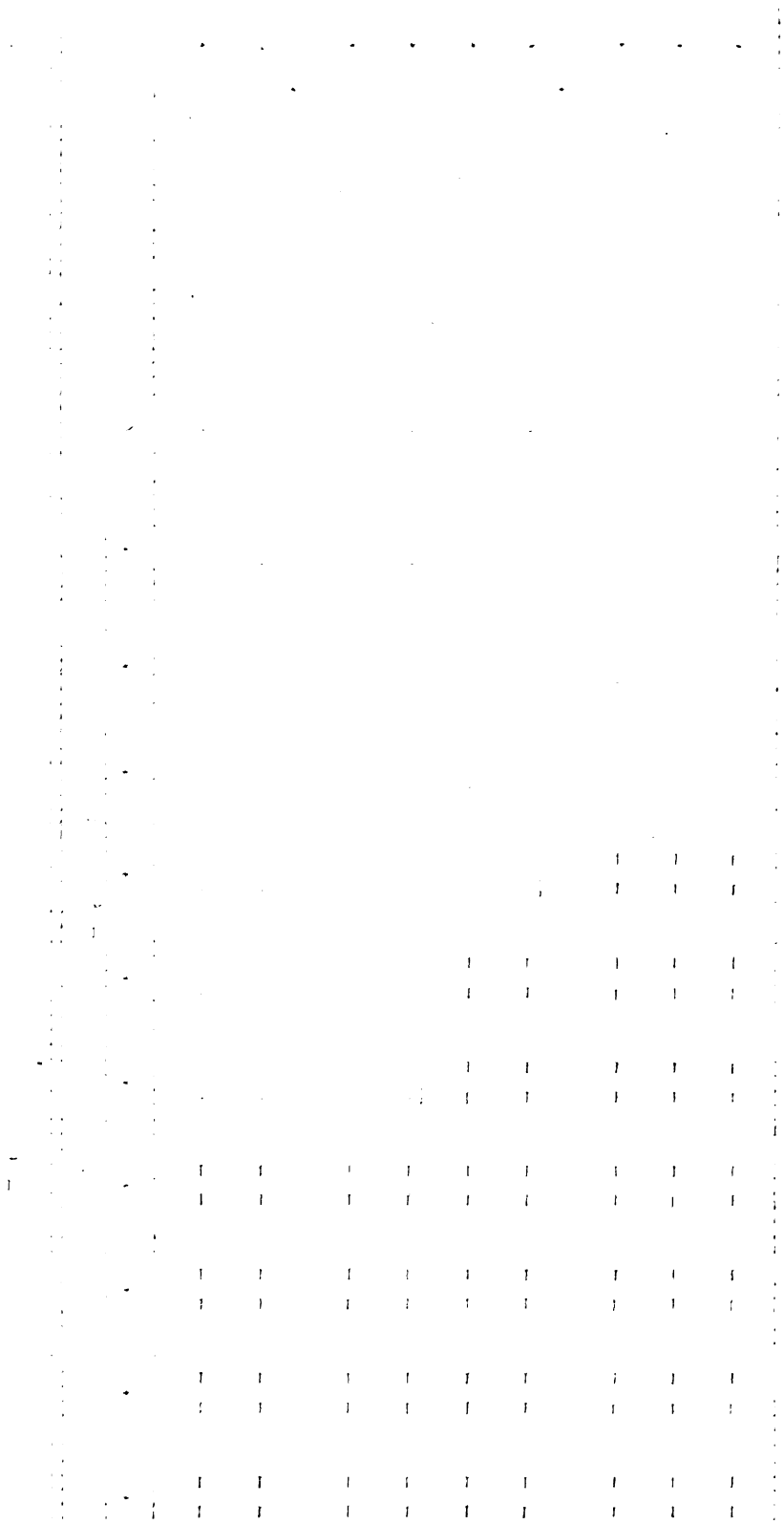


TABLE II

SURVIVAL OF MICROORGANISMS IN NUTRIENT BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium									
		0.05	0.1	0.3	0.5	0.8	1.0	1.5	2.0	2.5	3.0
B. subtilis	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. phlei	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ +	- -	+ -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE III

GROWTH OF MICROORGANISMS IN NUTRIENT BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	± ±	- -	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ ±	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	+ +	± ±	- -	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

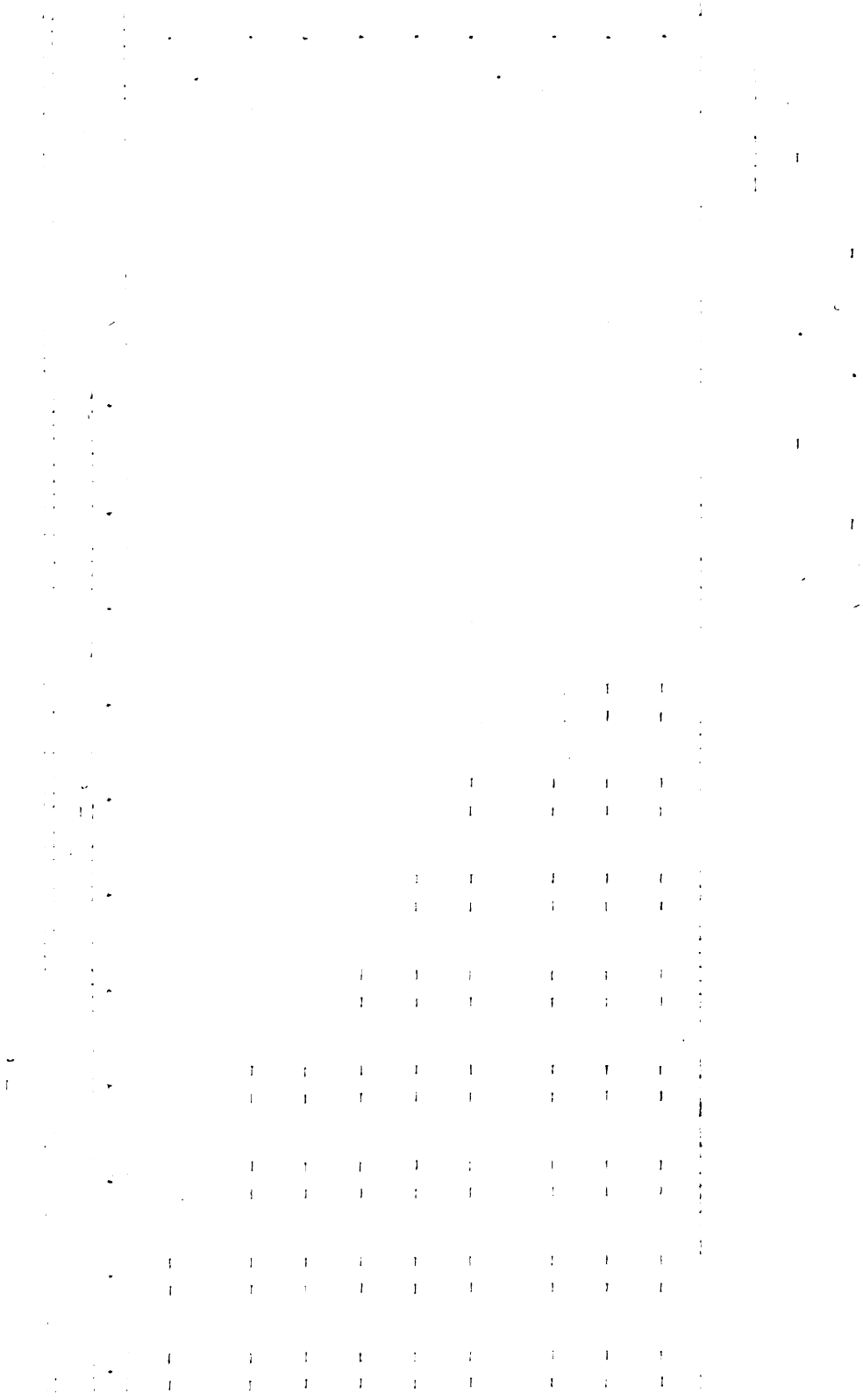


TABLE IV

SURVIVAL OF MICROORGANISMS IN NUTRIENT BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. subtilis	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. phlei	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 96 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE V

GROWTH OF MICROORGANISMS IN MOLE'S CASAMINO ACIDS MEDIUM CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ -	- -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ -	- -	- -	- -	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -
X. phaseoli	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE VI

SURVIVAL OF MICROORGANISMS IN WOLF'S CASAMINO ACIDS MEDIUM CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ -	- -	- -	- -
B. subtilis	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ -	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 96 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE VII

GROWTH OF MICROORGANISMS IN TRYPTOSE BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
M. phaseoli	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE VIII

SURVIVAL OF MICROORGANISMS IN TRYPTOSE BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. subtilis	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. phlei	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 96 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE IX

GROWTH OF MICROORGANISMS IN BRAIN HEART INFUSION BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	± ±	± ±	- -	- -	- -	- -
S. paratyphi	+ +	- +	+ +	+ +	+ +	+ +	± -	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	± ±	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	+ +	+ +	+ +	± -	- -	- -	- -	- -	- -	- -	- -
X. phaseoli	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE X

SURVIVAL OF MICROORGANISMS IN BRAIN HEART INFUSION BROTH CONTAINING 2,4-D

Organism	Control (0%)	Per cent 2,4-D incorporated in medium										
		0.05	0.1	0.3	0.5	0.7	0.9	1.0	1.5	2.0	2.5	3.0
B. cereus var. mycoides	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
X. phaseoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ ±
B. subtilis	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -
M. phlei	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ ±	+ +	- -
E. coli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -

Incubation

Time - 24 hours (M. phlei - 96 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE XI

GROWTH OF MICROORGANISMS ON NUTRIENT AGAR PLATES CONTAINING 2,4-D

(SURFACED PLATES)

Organism	Control (0%)	Per cent 2,4-D incorporated in medium							
		0.1	0.3	0.5	1.0	1.5	2.0	2.5	3.0
E. coli	+ +	+ +	+ +	+ +	- -*	- -	- -	- -	- -
M. pyogenes var. aureus	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
B. subtilis	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
X. phaseoli	+ +	+ +	- -**	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	- -	- -	- -	- -	- -	- -	- -	- -

* E. coli showed growth at 1.0% after 4 days incubation
** X. phaseoli showed growth at 0.3% after 10 days incubation

Incubation

Time - 24 hours (M. phlei - 48 hours)

Temperature - 37°C (X. phaseoli - 27°C)

TABLE XII

GROWTH OF MICROORGANISMS IN NUTRIENT AGAR PLATES CONTAINING 2,4-D

(POURED PLATES)

Organism	Control (0%)	Per cent 2,4-D incorporated in medium							
		0.1	0.3	0.5	1.0	1.5	2.0	2.5	3.0
E. coli	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. paratyphi	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. pullorum	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
S. schottmuelleri	+ +	+ +	+ +	+ +	- -	- -	- -	- -	- -
M. pyogenes var. aureus	+ +	+ +	+ +	- -	- -	- -	- -	- -	- -
B. subtilis	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
M. phlei	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
X. phaseoli	+ +	+ +	- -	- -	- -	- -	- -	- -	- -
B. cereus var. mycoides	+ +	- -	- -	- -	- -	- -	- -	- -	- -

Incubation

Time - 48 hours (M. phlei - 96 hours)

Temperature - 37°C (X. phaseoli - 27°C)

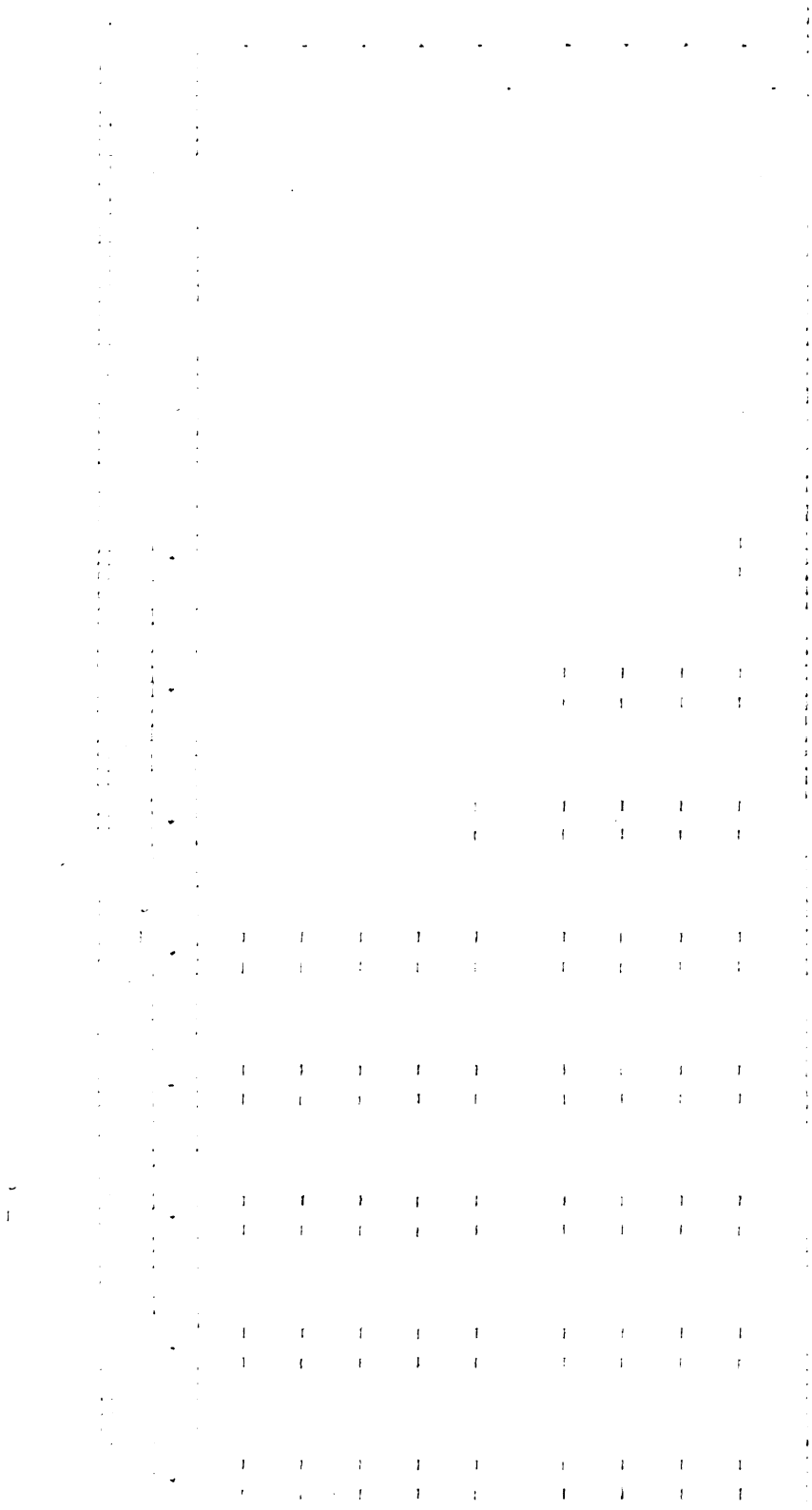
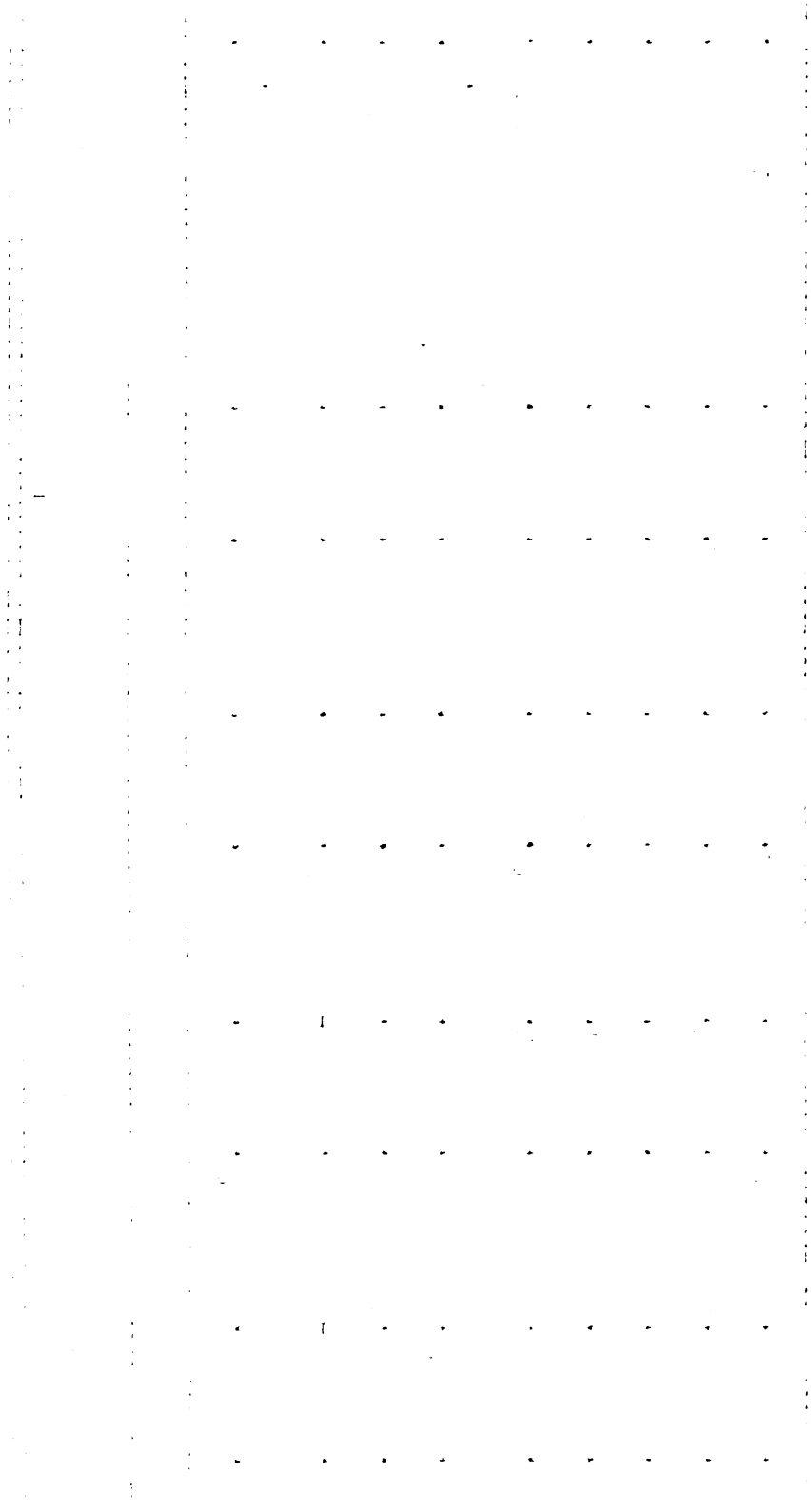


TABLE XIII

GROWTH AND SURVIVAL END POINTS OF ORGANISMS IN BROTH MEDIA

Organism	Wolf's Casamino Acids		Nutrient Broth		Tryptose Broth		Brain Heart Infusion	
	G	S	G	S	G	S	G	S
B. cereus var. mycoides	0.1	1.5	0.5	3.0+	0.7	3.0+	0.5	3.0+
B. subtilis	0.1	1.0	0.3	3.0+	-	3.0+	-	2.5
E. coli	0.9	1.0	1.0	1.5	1.5	1.5	1.5	1.5
M. pyogenes var. aureus	0.7	1.5	2.0	3.0+	2.0	3.0+	3.0+	3.0+
M. phlei	0.1	1.0	0.5	3.0+	0.1	2.5	0.1	2.5
S. paratyphi	0.3	0.7	0.9	1.0	0.7	1.0	0.9	1.5
S. pullorum	0.3	0.7	1.0	1.0	0.7	1.0	1.0	1.0
S. schottmelleri	0.5	0.5	0.3	1.0	0.7	0.9	0.7	0.7
X. phaseoli	0.1	1.0	0.7	3.0+	0.5	3.0+	0.3	3.0+

G indicates growth end point
S indicates survival end point
+ indicates growth in the highest concentration used, hence not necessarily an end point



LEGEND FOR PLATES

Plate I: *E. coli* in nutrient broth

Figure 1. 2,4-D free nutrient broth
Figure 2. 0.3 per cent 2,4-D
Figure 3. 1.3 per cent 2,4-D

Plate II: *E. coli* from cultures grown on nutrient agar

Figure 1. 2,4-D free nutrient agar
Figure 2. 0.05 per cent 2,4-D
Figure 3. 0.70 per cent 2,4-D

Plate III: *B. cereus* var. *mycoides* in nutrient broth

Figure 1. 2,4-D free nutrient broth
Figure 2. 0.3 per cent 2,4-D
Figure 3. 0.5 per cent 2,4-D

Plate IV: *B. cereus* var. *mycoides* from cultures grown on nutrient agar.

Figure 1. 2,4-D free nutrient agar
Figure 2. 0.05 per cent 2,4-D
Figure 3. 0.5 per cent 2,4-D

Plate V: *S. pullorum* in nutrient broth

Figure 1. 2,4-D free nutrient broth
Figure 2. 0.3 per cent 2,4-D
Figure 3. 1.0 per cent 2,4-D

Plate VI: *M. pyogenes* var. *aureus* in nutrient broth

Figure 1. 2,4-D free nutrient broth
Figure 2. 0.3 per cent 2,4-D
Figure 3. 0.7 per cent 2,4-D

Magnification: All of these photomicrographs were magnified 1500 times.
Scale: 15 millimeters are equivalent to 10 microns.

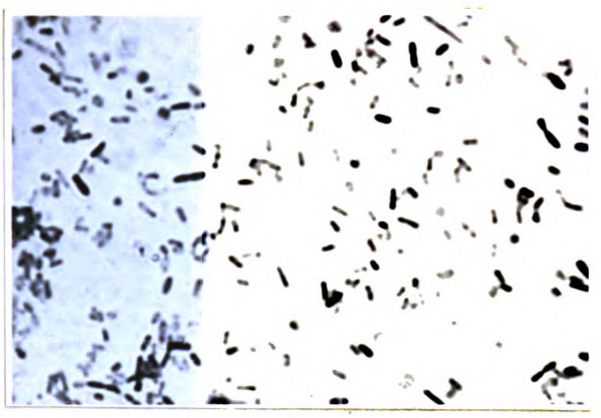


FIG. 1

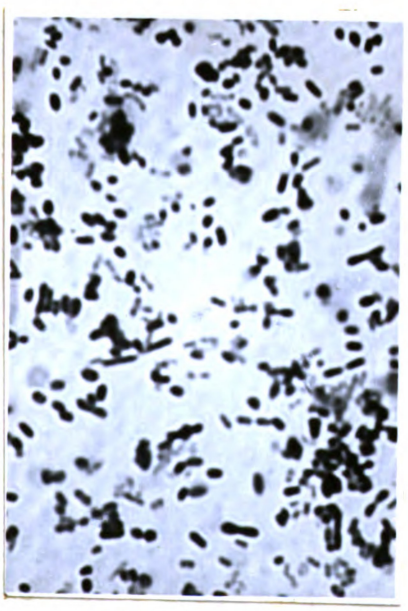


FIG. 2

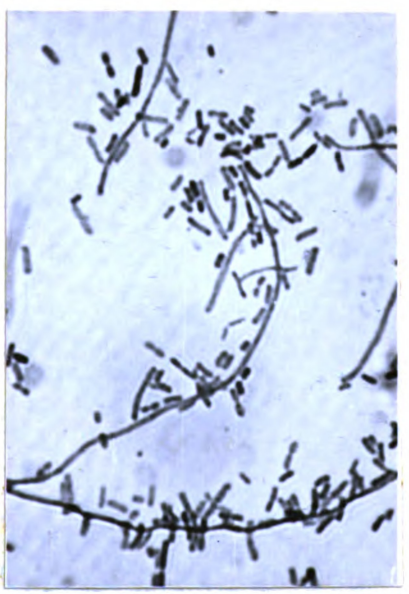


FIG. 3

PLATE I

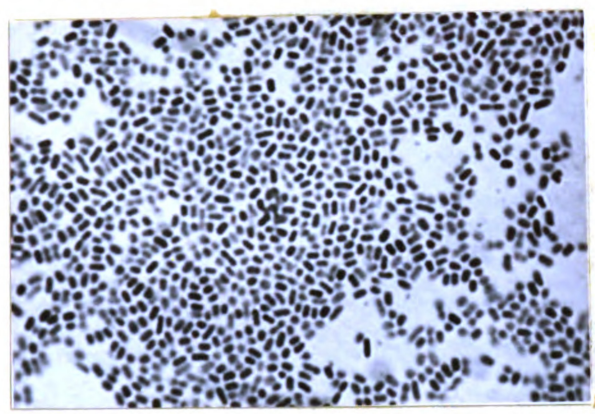


FIG. 1

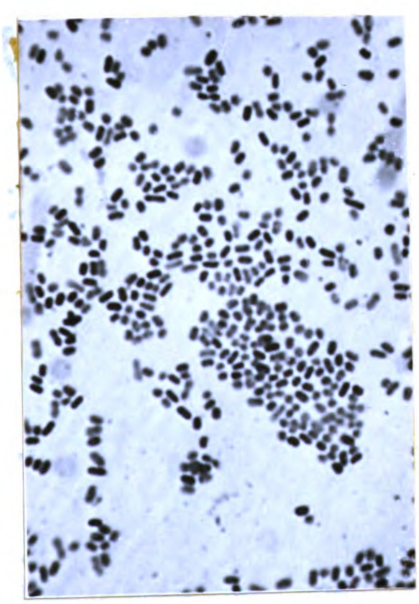


FIG. 2

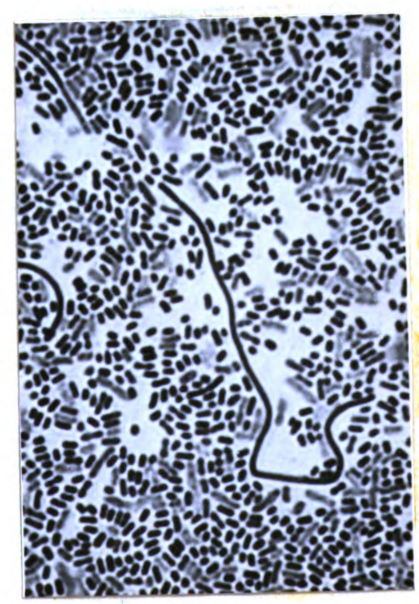


FIG. 3

PLATE II

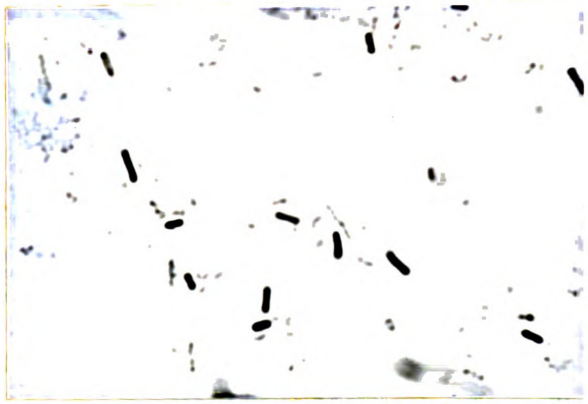


FIG. 1

FIG. 2

FIG. 3

PLATE III

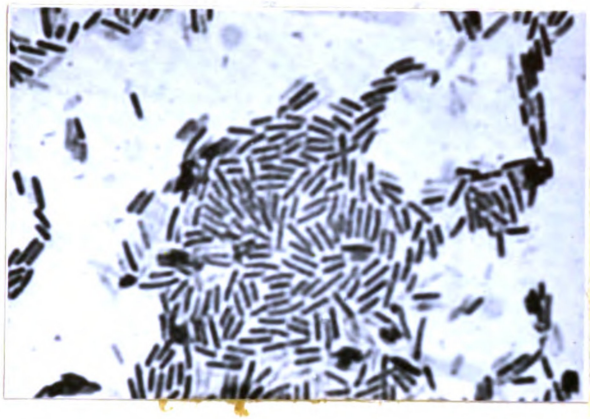


FIG. 1

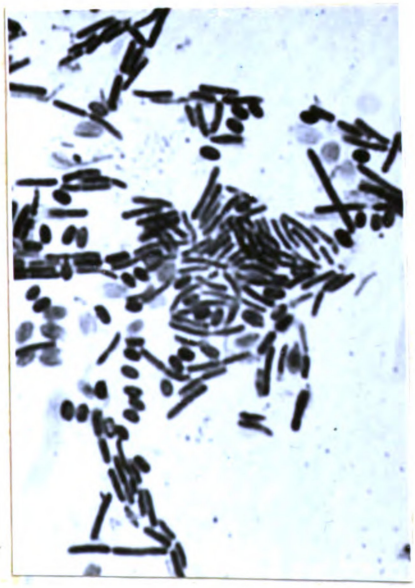


FIG. 2

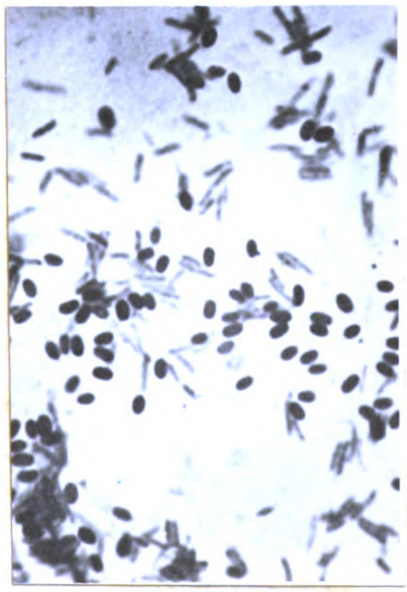


FIG. 3

PLATE III

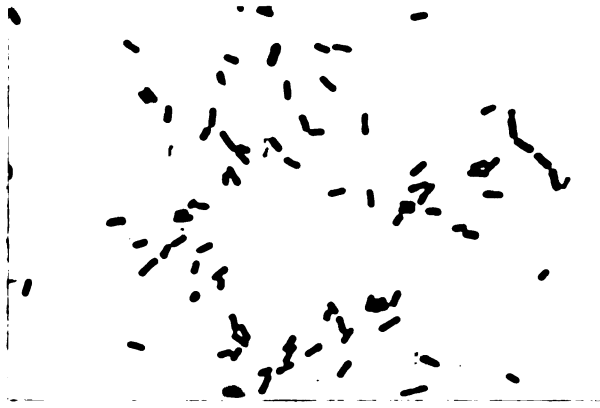


FIG. 1

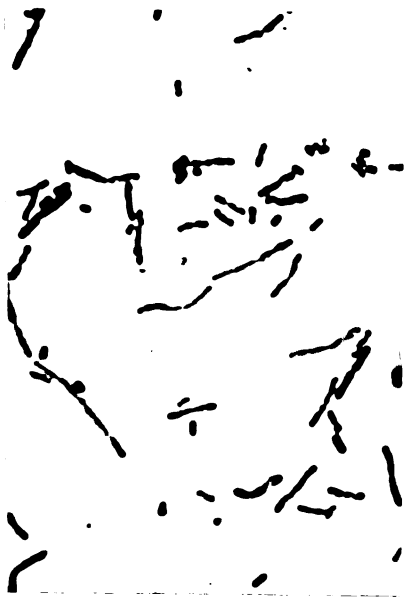


FIG. 2

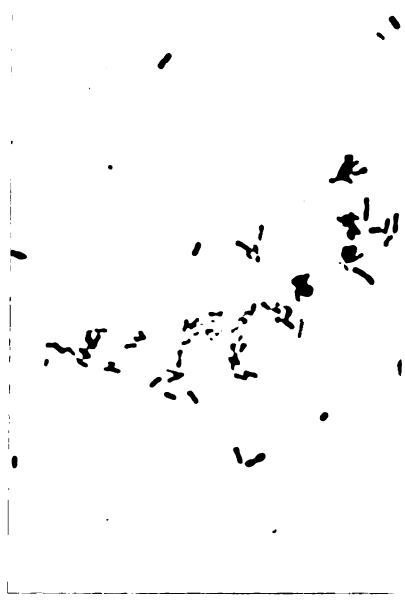


FIG. 3

PLATE V

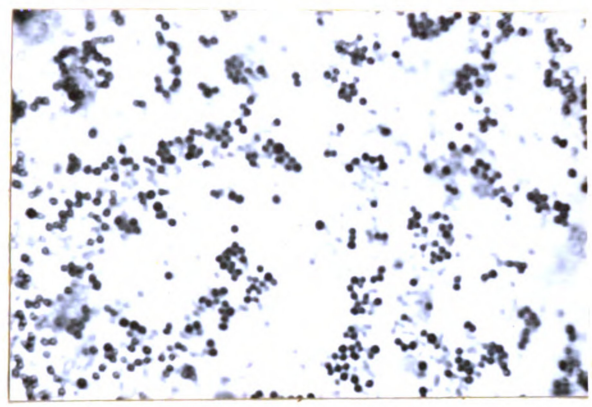


FIG. 1

FIG. 2

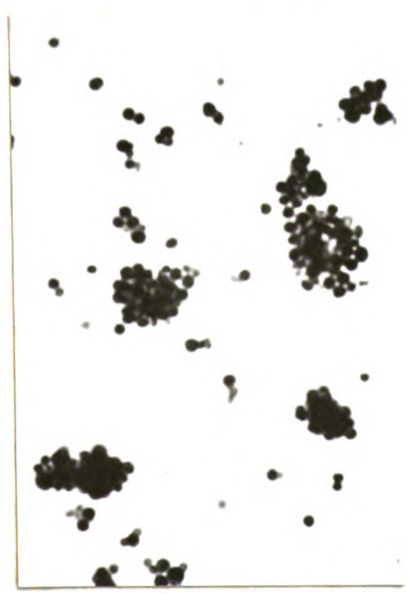


FIG. 3

PLATE VI.

DISCUSSION

A survey of Tables I - XII reveals one pertinent result, namely, that 2,4-D did definitely inhibit bacterial growth under the conditions of this study. The extent and degree of this inhibition varied according to the genus and species of organism used and also according to the type of medium employed.

Results as summarized in Tables I - IV indicate that the organisms can be inhibited when grown in nutrient broth under the usual conditions of time and temperature of incubation. Furthermore Tables I and III indicate that the results were not greatly influenced by amount of inoculum since twice as much inoculum was used for the experiment summarized in Table III as was used for the experiment which Table I represents. In these same experiments it can be observed that the growth of S. schottmuelleri was stopped at a relatively low concentration of 2,4-D while M. pyogenes var. aureus grew in a considerably higher concentration of 2,4-D.

The degree of sensitivity or resistance was not correlated with the gram-staining properties of the organisms. M. pyogenes var. aureus (gram-positive) and E. coli (gram-negative) were both resistant while organisms such as B. subtilis (gram-positive) and X. phaseoli (gram-

negative) were highly sensitive.

Survival experiments showed that absence of turbidity in the original tubes during the time of incubation was not an indication of complete inhibition of growth since it was shown that B. subtilis, M. pyogenes var. aureus, B. cereus var. mycoides, M. phlei, and X. phaseoli were able to produce growth on subculture in 2,4-D free broth after having been subjected to 3.0 per cent of 2,4-D.

The organisms were more sensitive to 2,4-D in Wolf's casamino acids broth medium than they were in nutrient broth with the exception of S. schottmuelleri whose sensitivity remained approximately the same as it was in the nutrient broth. This result may have been due to the fact that Wolf's medium is decidedly less nutritive for the organisms. A survey of the growth end-points of the organisms in tryptose and brain heart infusion broth, as compared to nutrient broth, however, indicates that there was no consistent relationship between increased nutritional value and decreased sensitivity (increased resistance).

The fact that most of the survival end-points were obtained in higher concentration of 2,4-D in the more nutrient media than in the less nutrient, that is, nutrient broth, tryptose, and brain heart infusion broth on the one hand and Wolf's casamino acids broth on the other might be considered to substantiate, at least in part, the findings of Fildes to the effect that the bacteriostatic effect of auxins, including 2,4-D, was reversed by such

constituents of media as peptones and tryptophane.

With regard to sensitivities in liquid media as compared with solid media it was shown that end-points of B. subtilis and E. coli remained fairly constant in nutrient broth and on nutrient agar containing 2,4-D. Most of the organisms however failed to show as high a growth end-point on the solid medium as was shown in the liquid medium.

The attempt which was made to develop a 2,4-D resistant strain of E. coli revealed that that organism acquires resistance to 2,4-D at a fairly rapid rate. This phenomenon is, perhaps, in keeping with the theory proposed by Ball (1938) which indicates that E. coli may be self-activated in its development of resistance to auxins since it produces indole-3-acetic acid from media containing tryptophane. The heteroauxin apparently stimulates the cells to further growth and division which produces more cells and hence more heteroauxin, thus repeating the cycle again and again until the cells literally "grow themselves to death".

The changes observed in the morphology of the organisms during this study appear to be similar, in some respects, to those found in green algae by Brannon and Sell (1945) as a result of the action of indole-3-acetic acid. They also resemble the histological changes observed by Watson (1948 and 1950) and by Eames (1949) in that cell elongation and enlargement was a conspicuous result of 2,4-D treatment. Somewhat similar changes in morphology have been observed by various authors while testing the effects of

such antibiotics as streptomycin (Czarnecki, 1948) and penicillin (Gardner, 1940 and 1945) on bacteria. The appearance of diplobacilli-like and filamentous forms can be explained by Dubos' statement (1946) concerning concentrations of auxin insufficient to produce complete inhibition.

One of the effects of 2,4-D on B. cereus var. mycoides seems to be that of loss of motility as is evident from the small area over which the culture spreads on agar containing 0.05 per cent 2,4-D and by increased spore formation observed on smears taken from this and higher concentrations of 2,4-D.

The results of the experiments on morphology are in disagreement with previous findings recorded in the literature.

The pH at which the experiments in this study were carried on might possibly have accounted for the results observed since it has been stated that 2,4-D is more active in an acid pH range (optimum pH 2 - 3) than in a higher pH range. There are, however, observations recorded in the literature (Lucas et al, 1947) to the effect that 2,4-D, when applied in buffered solutions, is just as active at pH 7 as at a lower pH.

Since these experiments were designed to survey the antibacterial activity of 2,4-D, pH 7.0 was chosen in order to insure growth of all of the organisms studied in the 2,4-D free control media.

The range of concentrations of 2,4-D used in this

study were chosen to lie within the solubility of this salt (3 - 4 per cent in aqueous solution).

The effect of 2,4-D on bacteria must have caused either no injury or very little injury to the cell protoplasm. This is apparent from the fact that the survival experiments showed that the organisms were able to produce visible growth in 2,4-D free media after having been exposed to various concentrations of 2,4-D.

SUMMARY

1. Under the conditions of this study the action of 2,4-D was found to be far more bacteriostatic than bactericidal.
2. Although the type of medium used did exert some effect upon the antibacterial activity of 2,4-D, no consistent pattern of relationship between type of medium used and the resistance of the organisms, in general, to 2,4-D was observed.
3. Resistance of *E. coli* to 2,4-D was induced readily by two different methods.
4. Distinct morphological changes, as a result of the action of 2,4-D, were observed in most of the organisms studied.

REFERENCES

Akamine, Ernest K. 1948 Plant-growth regulators as selective herbicides. Circular 26, University of Hawaii Agricultural Experiment Station.

Ball, Ernest J. 1938 Heteroauxin and the growth of E. coli. Journal of Bacteriology. 36:559-565.

Beal, J. M. 1944 Further observations of the telemorphic effects of certain growth-regulating substances. Botanical Gazette 106:165-178.

Beckwith, T. D. and E. M. Geary. 1940 Effect of indole-3-acetic acid on the multiplication of E. coli and E. typhosa. Journal of Infectious Disease 66:78-79.

Brannon, M. A. and H. M. Sell. 1945 The effect of indole-3-acetic acid on the dry weight of Chlorella pyrenoidosa. American Journal of Botany 32:257-258.

Brannon, M. and A. Bartsch. 1939 Influence of growth substances on growth and cell division in green algae. American Journal of Botany 26:271-279.

Bucher, N. 1946 Effects of 2,4-dichlorophenoxyacetic acid on experimental animals. Proceedings of the Society for Experimental Biology and Medicine 63:204-205.

Burkholder, Paul R. 1939 Production of growth substance by bacteria in media containing specific organic and inorganic nitrogenous compounds. American Journal of Botany 26:423-428.

Czarnecki, R. B. 1948 Studies on the antistreptomycin activity of brain infusion and the changes in morphology of bacteria subjected to streptomycin and the anti-streptomycin factor. Ph.D. Thesis, University of Illinois.

Dubos, Rene J. 1946 Inhibition of bacterial growth by auxins. Proceedings of the Society for Experimental Biology and Medicine 63:317-319.

Eames, Arthur J. 1949 Histological effects of treatments with growth-regulating substances of the 2,4-D group. Science 110:235-236.

Fildes, Paul. 1941 Inhibition of bacterial growth by indoleacrylic acid and its relation to tryptophan; an illustration of the inhibitory action of substances chemically related to an essential metabolite. British Journal of Experimental Pathology 22:293-298.

Fults, J. L. and Merle G. Payne. 1947 Some effects of 2,4-D, D.D.T. and Colorado 9 on the bacteria Rhizobium leguminosarium Frank in the root nodules of the common bean. American Journal of Botany 34:245-248.

Gardner, A. D. 1940 Morphological effects of penicillin on bacteria. Nature 146:837-838.

Gardner, A. D. 1945 Microscopical effect of penicillin on spores and vegetative cells of Bacilli. Lancet I: 658-659.

Gilbert, F. A. 1946 The status of plant-growth substances and herbicides in 1945. Chemical Reviews 39:199-218.

Grigsby, B. H., B. R. Churchill, C. L. Hamner and R. F. Carlson. 1950 Chemical weed control. Circular Bulletin 214, Michigan State College Agricultural Experiment Station.

Hagborg, W. A. F., Gladys M. Warner and N. Agnes Philips. 1950 Use of 2,4-D as an inhibitor of germination in routine examination of beans for seed borne infection. Science 111:91.

Hagen, C. E., C. O. Clagett and E. A. Helgenson. 1949 2,4-Dichlorophenoxyacetic acid inhibition of castor bean lipase. Science 110:116-117.

Haagen-Smit, A. J., W. B. Bandliker, S. H. Wittwer, and A. E. Murneek. 1946 Isolation of 3-indoleacetic acid from immature corn kernels. American Journal of Botany 33:118-120.

Hamner, C. L. and H. B. Tukey. 1944 The herbicidal action of 2,4-dichlorophenoxyacetic acid on bindweed. Science 100:154-155.

Hamner, C. L., E. H. Lucas and H. M. Sell. 1947 The effect of different acidity levels on the herbicidal action of the sodium salt of 2,4-dichlorophenoxyacetic acid. Michigan Agricultural Experiment Station, Quarterly Bulletin 29:337-342.

Hsueh, Y. L. and C. H. Lou. 1947 Effects of 2,4-D on seed germination and respiration. Science 105:283-285.

Klemek, J. W., C. J. Cavallito and J. H. Bailey. 1948 Induced resistance of Staphylococcus aureus to various antibiotics. Journal of Bacteriology 55:139-145.

Kraus, E. J. and J. W. Mitchell. 1947 Growth-regulating substances as herbicides. Botanical Gazette 108:301-350.

Lewis, R. W. and C. L. Hamner. 1946 The effect of 2,4-D on some microorganisms. Michigan State College Agricultural Experiment Station, Quarterly Bulletin 29: 112-114.

Lucas, E. H., I. L. Felber, C. L. Hamner and H. L. Sell. 1948 The effects of buffers on the growth inhibiting properties of sodium 2,4-dichlorophenoxyacetate. Michigan State College Agricultural Experiment Station, Quarterly Bulletin 30:289-297.

Luecke, R., C. L. Hamner and H. L. Sell. 1949 Effects of 2,4-dichlorophenoxyacetic acid on the content of thiamine, riboflavin, nicotinic acid, pantothenic acid and carotene in stems and leaves of red kidney bean plants. Plant Physiology 24:546-548.

Martin, J. P. 1946 The hormone weed killer, 2,4-D. The California Citrograph 31:248, 264-265.

Mitchell, J. W. and P. C. Martin. 1944 Effects of 2,4-D on the ripening of detached fruit. Botanical Gazette 106:199-207.

Morton, Avery A. 1946 The Chemistry of Heterocyclic Compounds. McGraw-Hill Book Company, New York.

Neely, W. B., C. D. Ball, C. L. Hamner and H. L. Sell. 1950 Effect of 2,4-dichlorophenoxyacetic acid on the invertase, phosphorylase and pectin methoxylase activity in the stems and leaves of the red kidney bean plant. Plant Physiology 25:525-528.

Neely, W. B., C. D. Ball, C. L. Hamner and H. L. Sell. 1950 Effect of 2,4-dichlorophenoxyacetic acid on the alpha and beta amylase activity in the stems and leaves of red kidney bean plants. Science 111:118.

Nutman, P. S., H. G. Thorton and J. H. Quastel. 1945 Inhibition of plant growth by 2,4-dichlorophenoxyacetic acid and other plant-growth substances. Nature 155: 498-500.

Richards, R. Ruth. 1949 Responses of representative fungi to certain growth-regulating substances. Botanical Gazette 110:523-549.

Schoene, D. L. and O. L. Hoffmann. 1949 Maleic hydrazide, a unique growth regulant. Science 109:588-590.

Sell, H. M., R. W. Luecke, B. M. Taylor and C. L. Hamner. 1949 Changes in chemical composition of the stems of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiology 24:295-299.

Slade, R. W., W. G. Templeman and W. A. Sexton. 1945 Plant-growth substances as selective weed-killers. Differential effect of plant-growth substances on plant species. Nature 155:497-498.

Smith, N. R., V. Dawson and H. E. Wenzel. 1945 The effect of certain herbicides on soil microorganisms. Soil Science Society of America Proceedings 10:197-201.

Stevenson, E. C. and J. W. Mitchell. 1945 Bacteriostatic and bactericidal properties of 2,4-dichlorophenoxyacetic acid. Science 101:642-644.

Trigg, Carol and Grant L. Stahly. 1948 Some effects of plant growth-regulating substances on microorganisms. Ohio Journal of Science 448:49-55.

Tukey, H. B. 1947 2,4-D, a potent growth regulator of plants. Scientific Monthly 64:93-97.

Tukey, H. B., C. L. Hamner and B. Imhofe. 1945 Histological changes in bindweed and sow thistle following applications of 2,4-dichlorophenoxyacetic acid in herbicidal concentrations. Botanical Gazette 107:62-73.

Watson, Donald P. 1948 An anatomical study of the modification of bean leaves as a result of treatment with 2,4-D. American Journal of Botany 35:543-555.

Watson, Donald P. 1950 Anatomical modification of velvet bent grass (Agrostis Canina L) caused by soil treatment with 2,4-dichlorophenoxyacetic acid. American Journal of Botany 37:424-431.

Weller, L. E., R. W. Luecke, C. L. Hamner and H. M. Sell. 1950 Changes in chemical composition of the leaves and roots of red kidney bean plants treated with 2,4-dichlorophenoxyacetic acid. Plant Physiology 25:289-293.

West, F. W. and J. H. M. Henderson. 1948 A turbidimetric method for determining the effect of 2,4-D upon the growth of yeast. Science 107:604.

West, Fred R. and James H. M. Henderson. 1950 The effects of 2,4-D and various other substances upon the respiration of blue lupine seedling roots. Science 111:579-581.

Worth, W. A. Jr., and A. M. McCabe. 1948 Differential effects of 2,4-D on aerobic, anaerobic and facultative anaerobic microorganisms. Science 108:16-18.

Zimmerman, P. W. and A. E. Hitchcock. 1942 Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Boyce Thompson Institute Contributions 12:321-343.