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Morphological and Physiological Effects of 2,4-D on Gram-Negative and Gram-Positive Bacteria

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MORPHOLOGICAL AND PHYSIOLOGICAL EFFECTS OF 2,4-D ON GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

(TITLE)

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JOHN H. RUPNOW

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ABSTRACT

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Morphological and physiological effects of 2,4-D on Gram-negative and Gram-positive bacteria

Major Professor: Dr. William Keppler

Manometric, spectrophotometric, photomicrographic and quantitative biochemical techniques were employed to determine the morphological and physiological effects of 2,4-D on Gram-positive and Gram-negative bacteria.

The growth and motility of Gram-positive bacteria appeared to be more adversely affected than the growth and motility of Gram-negative species. The terminal oxidation, lysis, and chain length of both Gram-positive and Gram-negative bacteria did not appear to be affected by the concentrations used. The average cell size of both Gram-positive and Gram-negative bacteria was observed to increase after exposure to 1000 ppm 2,4-D. The normal leakage of amino acid and ultraviolet light absorbing cell constituents appeared to be retarded by 1000 ppm 2,4-D.

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INTRODUCTION

Apart from the desired toxic effects of a given herbicide, there are a number of possible not-so-desirable effects on non-target organisms. One possibility is a direct effect on one or more components of the complex community of micro-organisms that inhabit the soil, the fertility of which depends, in part, on the equilibrium that exists within the community. Another possible detrimental effect is that herbicides may be leached out of the soil into drainage waters which find their way into canals, rivers and reservoirs, possibly affecting nonsoil micro-organisms. It is not sufficient to consider only the effectiveness of a given herbicide on a specific plant. Rather, the overall effect of the application of the herbicide must also be considered. It is the purpose of this study to examine some of the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on several species of bacteria.

REVIEW OF LITERATURE

THE EFFECTS OF 2,4-D ON EUCARYOTIC CELLS

2,4-D and Cell Division

An analysis of 2,4-D mediated growth responses of multicellular organisms should include a consideration of changes in cell behavior. The cell is the site of all fundamental processes which finally lead to growth. Cell division, cell elongation, and cell differentiation can be distinguished as three consecutive phases of cell growth.

The phase of cell division is characterized by nuclear division, followed by the division of protoplasm. One of the main characteristics of an auxin herbicide is its ability to influence cell division (Kiermayer, 1964), although its influence differs from tissue to tissue. Therefore, the various responses will be discussed in relation to the various plant tissues.

Primary meristems are the centers of the production of all other plant tissues. Disturbances in the normal function of primary meristems will lead to changes in the development of the whole plant body. Ball (1944) revealed that shoot meristems of Tropaeolum majus are only slightly affected by 2,4-D and recover shortly after the application. However, 2,4-D has been found to strongly inhibit the apices of tomato (Kiermayer, 1964). Applications of .25% solutions of 2,4-D have been shown to result in the inhibition of cell division in the terminal buds of dicotyledonous plants, such as sugar beet, radish, grape, kidney bean, red clover, and buckwheat (Kraus and Mitchell, 1947).

Studies on the direct effect of 2,4-D on the nuclear aspects of cell division are limited. Croker (1953) studies Allium cepa and observed that 2,4-D caused stickiness, condensation of chromosomes, and delayed spindle formation. He further observed breaks in the chromosomes and chromatids. Croker stated that concentrations as low as 25 ppm are effective, and a concentration of 3500 ppm halts mitosis. Ryland (1948) reported that 2,4-D causes a general inhibition of cell division and the appearance of c-mitosis in root tip meristems, but does not cause polyploidy.

Meiosis has also been shown to be influenced by 2,4-D treatment. Unrau and Larter (1952) reported that the microsporocyte and megasporocyte aberrations included univalent chromosomes, bridges, fragments, chains and rings, and sticky chromosomes. The authors stated that the univalent chromosomes could result in different types of aneuploid progeny. The bridges were thought to have resulted from tardiness of disjunction, stickiness of individual anaphase chromosomes, or dicentric chromosomes. Fragmentations were attributed to sticky chromosomes breaking at anaphase. The formation of rings and chains was attributed to reciprocal translocations occurring in mitosis preceding reduction division. Stickiness of chromosomes was thought to be caused by changes in the chemical or electrostatic properties of the nucleic acids.

The sensitivity of secondary meristems to auxin herbicides depends on the degree of differentiation. In general, tissues with little differentiation are more sensitive to auxins than are highly differentiated tissues (Beal, 1951). The following is a descending order of sensitivity to auxin action: cambium, pericycle (in roots), endodermis, ray and phloem parenchyma, pith, young phloem, xylem and mature phloem (Leopold,

1955). In addition to decreasing auxin sensitivity, it should be noted that the above also show a decrease in meristematic activity. Harrison (1937) stated that auxin sensitivity depends largely on genetically controlled factors which vary between different plant species.

In an effort to explain the different histological effects of 2,4-D on monocotyledonous and dicotyledonous plants, Struckmeyer (1951) suggested that the presence of less differentiated cells around the phloem of dicotyledonous plants might be an important factor. In monocotyledonous plants the phloem is surrounded by highly differentiated, fibrous cells which are incapable of proliferation. Kiermayer (1964) suggests that the presence of cambium between the phloem and xylem of dicotyledonous plants might also contribute to their pronounced sensitivity to 2,4-D. Kiermayer (1964) further stated that the destruction of phloem leads to severe disturbances of the normal distribution of food by vascular elements and is one of the most important facets of the weed killing action of auxin herbicides.

The extent of histological modifications is closely dependent on the concentration of the auxin. Hypocotyls of bean plants treated with 2,4-D were observed to undergo closure and exhibited strong proliferations in tissues close to the point of application. However, tissues further away from the point of application, those receiving less 2,4-D, experienced only minor effects in the cambium and endodermis (Kiermayer, 1964).

Leaves treated with 2,4-D show considerable modifications of form. These modifications are the result of the influence of 2,4-D on cell division during leaf formation. Burton (1947) reported that

the formation of leaf intercellular spaces was prevented by the application of 2,4-D. He hypothesized that the lack of intercellular spaces was caused by a growth inhibition of epidermal cells or by the prevention of mesophyll cell separation. Watson (1948) reported changes of cell division frequency in developing leaves treated with 2,4-D. Instead of the normal chlorophyll bearing mesophyll, he found a tissue composed of thick walled cells without intercellular spaces. The vulnerability of leaf cells depends on the stage of development. 2,4-D has little influence on the young cells in the leaf initiating region, but does influence their derivatives (Kiermayer, 1964).

2,4-D and Cell Enlargement

The enlargement of plants depends on the elongation of cells derived from the shoot and root apical meristems. The process of cell elongation results, primarily, from an enlargement of the vacuoles by water uptake. According to Kiermayer (1964), auxin herbicides have a strong influence on cell elongation. He reported that the degree of promotion of cell elongation by 2,4-D is dependent on the concentration used. A 0.1% concentration of 2,4-D was observed to cause a 20% promotion, while a 0.5% concentration caused a 30% promotion in Avena sp. coleoptiles. Concentrations above 1% were shown to inhibit cell elongation.

The effect of auxins on root cells is very different from that on stem cells. The optimum auxin concentration for elongation varies in different roots; however, it is generally 1/100,000 of the optimum concentration for shoots (Audus, 1959). Similarly, moderate concentrations of 2,4-D will lead to cell elongation in coleoptiles (stems), the same concentrations are very inhibitory to cell elongation in roots.

Curvatures, swellings, and bendings are termed epinastic responses and are caused by the enlargement of cells or cell complexes. 2,4-D has been shown to cause swelling of cells at the base of leaves (Kiermayer, 1964). Freiberg and Clark (1952) found that 2,4-D caused twisting of stem tips, and swelling of stems and petiole bases in soybean plants. The degree of epinastic effect varies in different plant species. Zimmerman (1951) demonstrated that 2,4-D caused strong epinastic effects in tomato, while tobacco was less affected. Kiermayer (1964) stated that the degree to which epinastic responses are induced is directly related to the concentration of 2,4-D.

Plants normally possess the power to react to the direction of incident light (phototropism) and the force of gravity (geotropism). Unlike many other auxin herbicides, 2,4-D was not found to affect geotropism. However, 2,4-D was found to have a marked inhibitory effect on phototropism (Kiermayer, 1964).

2,4-D and Cell Differentiation and Physiology

Cell differentiation represents the last stage of cell growth. During this phase, the processes occur which determine final cellular morphology and physiology. Although other related auxin herbicides have been shown to affect cell differentiation, the activity of 2,4-D appears to be restricted to the process of cell division and cell elongation (Kiermayer, 1964).

Most of the auxin herbicides inhibit seed germination (Kiermayer, 1964). Hamner et al. (1946) have shown that the seeds of both dicotyledonous and monocotyledonous plants are inhibited by 2,4-D, although seeds of grasses respond somewhat less. At very low concentrations, 2,4-D was found to promote germination in citrus fruits (Kiermayer, 1964).

Changes in water uptake and alterations in water content of tissues are effects of 2,4-D application to some plants. Brown (1946) found that the total amount of water absorbed by 2,4-D sprayed bean plants was less than that of the control. He observed that the rate of accumulation of water in leaves was depressed, whereas in stem tissue it was increased. Brown further found that transpiration was reduced by 34%. Cellular fluid content differed between species (Wort, 1964). Aqueous sprays of 15% 2,4-D resulted in an increased viscosity in the protoplasm of oats and a decreased viscosity in sunflowers.

Protoplasmic streaming is the name given to the movement of the fluid portions of the cytoplasm. Protoplasmic streaming is influenced by factors that modify the amount of energy supplied for the process and by factors that alter the viscosity of the medium. Low concentrations of 2,4-D have been shown to increase streaming in Tradescantia virginiana, while high concentrations depressed the rate, and intermediate concentrations were without effect (Brian, 1964).

Reduction in water loss might be the result of stomatal closure, according to Bradbury and Ennis (1952). A 10% solution of 2,4-D applied to the tops of bean plants or to the soil brought about a partial stomatal closure. An increase in 2,4-D concentration was observed to increase the degree of closure. The stomatal closure was determined not to be the result of decreased water absorption by the roots.

Wort (1964) stated that stomatal closure might be the indirect result of the supression of photosynthesis by 2,4-D. Carbon dioxide concentration in the leaf appears to occupy a key position in the events leading to stomatal opening and closure. A reduction in the ability to use carbon dioxide in photosynthesis could result in the accumulation of the gas, and a resultant closure of the stomate.

Wort (1964) stated that 2,4-D either depresses or has little effect on the intake of some fourteen ions by plants. Following intake, 2,4-D was observed to alter the distribution of minerals within the plant, causing a decrease of minerals in the leaves and in increase in the stems. Metallic ions participate in enzymatic actions and hence a change in ion content is reflected by an altered metabolic pattern or rate.

Freiberg and Clark (1952) found that the ability to absorb sodium, phosphorous, and nitrate was decreased in 2,4-D treated bean plants. Cooke (1957) found that a 200 ppm application of 2,4-D caused an initial increased uptake of Ca, Cl, and K ions. After twenty-four hours, however, an inhibition of uptake was observed. Stahler and Whitehead (1950) reported that applications of 2,4-D to sugar beets caused a twenty-fold increase of nitrate in the tops.

The treatment of bean and buckwheat plants with 2,4-D has been shown to decrease the vitamin content of the stems. Luecke et al. (1949) reported that treatment of the red kidney bean reduced the amount of riboflavin, thiamine, and nicotinic acid in the leaves, but increased the content of these vitamins in the stems. Wort (1964) reported that buckwheat, treated with 2000 ppm 2,4-D showed an initial increase of vitamin C in the leaves, followed by a pronounced decrease. However, stem ascorbic acid was observed to increase.

A plant's chemical components depend, either directly or indirectly, on the products of photosynthesis for the raw materials and energy requirements. Therefore, changes in photosynthetic rates have important ramifications. High concentrations of 2,4-D appear to have an adverse effect on the rate of photosynthesis. Freeland (1949) found that a concentration of 100 ppm 2,4-D reduced the photosynthetic rate

by 20% in bean leaves. Wort (1964) stated that a .15% spray of 2,4-D on oats caused a 64% decrease in the photosynthetic rate one day after treatment. However, one week after the application, the normal rate was almost completely restored.

The relation of color of light and photosynthetic rates was investigated by Williams and Dunn (1961). Brassica juncea plants were sprayed with 1000 ppm solution of 2,4-D and were grown at an intensity of 300 foot-candles of light of different colors. Blue light was most effective in promoting interference with carbon dioxide utilization in photosynthesis. Red light caused the greatest reduction in the chlorophyll content of 2,4-D treated plants.

The depletion of starch and sugar is a result of 2,4-D application to a wide variety of plants. Sell et al. (1949) treated the red kidney plant with a 2,4-D concentration of 1000 ppm. Six days after application, he observed the following:

Stems:	Depletion of reducing and nonreducing sugars; Reduction in starch, crude fiber, and hydrolyzable polysaccharides;
Roots and Leaves:	Depletion of nonreducing sugars; No change in reducing sugars, starch, polysaccharide and crude fiber.

An application of a 1000 ppm solution of 2,4-D to buckwheat plants produces an increase-decrease pattern in carbohydrate content (Wort, 1951). The total sugar content of stems and leaves increased within one day of treatment, but decreased to 48% by the eighth day.

High concentrations of 2,4-D have been found to decrease respiration rates, while low concentrations appear to be a stimulant. Smith (1951) stated that 2,4-D exerts its toxicity during the aerobic phase of respiration. Increases in respiratory rates are affected by the

influence of 2,4-D on phosphate metabolism. Therefore, 2,4-D affects phosphorylation and the production of adenosine triphosphate, determining the availability of energy for a large number of reactions. Black and Humphreys (1962) found that corn seedlings treated with 2,4-D showed a general increase in the activity of enzymes associated with the pentose phosphate cycle. The increase was evidenced by an increased utilization of ribose-5-phosphate, an increased formation of heptulose and hexoses from ribose-5-phosphate, and an increased rate of oxidation of glucose-6-phosphate and 6-phosphogluconate.

2,4-D was found to bring about an increase in protein and free amino acids in the stem and, simultaneously, a decrease in these compounds in the leaves and roots. Sell et al. (1949) found that when a 1000 ppm solution of 2,4-D was applied to the primary leaves of beans, the protein content of the stems was double that of the untreated plants. The amounts of arginine, histidine, isoleucine, leucine, phenylalanine, valine, lysine and methionine were increased. There was evidence, based on the percentages of specific amino acids, that the character of the protein was changed. Sell et al. (1949) further observed that sugars, carbohydrate reserves, and acid hydrolyzable polysaccharides decreased, and they concluded that the increase in protein was at the expense of the carbohydrates. Weller et al. (1957) also observed that treatment of bean plants with 2,4-D resulted in a reduction in amino acids and protein in the roots and leaves. He hypothesized that the increase in stem proteins was the result of a transfer of nitrogenous material to the stem from the leaves and roots.

Phosphorous is important in the plant because it takes part in a multitude of metabolic processes: photosynthesis, nucleotide formation, respiration, protein synthesis, to mention a few. 2,4-D has the

ability to alter the availability of this element and, therefore, has far-reaching effects. Loustalot and Muzik (1953) observed an increase in phosphorous absorption in white bean plants following 2,4-D application. However, Fang and Butts (1954) and Rebstock et al. (1954) found that a 2,4-D application decreased phosphorous absorption.

Rebstock et al. (1954) found that stem nucleic acids were doubled as a result of treatment of bean plants with 1000 ppm 2,4-D. Similarly, West et al. (1960) found that ribonucleic acid and protein increased in the cucumber following a 2,4-D application. Basler and Nakazawa (1960) cultured young cotton seedling cotyledons in various concentrations of 2,4-D. A concentration of 10^{-2}M resulted in an increase in soluble and particulate protein. Ribonucleic acid synthesis was increased and the location of the nucleic acid within the cell components was altered. Key and Hanson (1961) sprayed soybean seedlings with a 5×10^{-4}M solution of 2,4-D. After three and one-half days of growth, the RNA content doubled over that of an untreated control.

Plant metabolic processes are dependent on enzymes, implying that changes in metabolic processes resulting from the application of 2,4-D may be the result of altered enzymatic activity. Wort (1964) has drawn a comparison between sensitivity of castor bean and wheat to 2,4-D, and the effect of this herbicide on the lipase of the two plants. 2,4-D was much more effective as an inhibitor of castor bean lipase than it was on wheat lipase. Wheat is also relatively insensitive to 2,4-D, while castor bean is very sensitive. Freed et al. (1961) has shown that 2,4-D can cause both stimulation and inhibition of an enzyme, depending on the concentration. A marked stimulation of glyceraldehyde-3-phosphate dehydrogenase was obtained at 100 ppm 2,4-D,

whereas an inhibition resulted at 1000 ppm. Freed et al. (1961) postulated that the stimulation caused by low concentrations of 2,4-D may result from the participation of the herbicide in a substrate-regulator-enzyme complex. The accumulation of larger amounts of 2,4-D may saturate both the enzyme and substrate separately. Therefore, the possible sites of enzyme-substrate combination are decreased, and the reaction is retarded.

Wort (1964) suggests that the activity of enzymes may be altered by 2,4-D in a more indirect way, probably through its effects on the conditions under which the enzymatic reaction progresses (pH, hydration, etc.), on the supply of materials for apo-enzyme and coenzyme formation, or on the supply of energy for endergonic reactions.

It is evident that 2,4-D has a myriad of effects on eucaryotic organisms. However, the actual effect of the herbicide is at the cellular level. Since many of the metabolic processes found in eucaryotic organisms are also found in bacteria, several investigators have examined the effect of 2,4-D on procaryotic cells.

THE EFFECTS OF 2,4-D ON PROCARYOTIC CELLS

2,4-D Degradation By Soil Microorganisms

In an effort to demonstrate that 2,4-D degradation is due to microbial action, Brown and Mitchell (1948) showed that the disappearance of 2,4-D was favored by soil conditions that also favored bacterial growth. Soil temperatures below 50°F retarded the rate of 2,4-D deactivation. Prolonged toxic effects of 2,4-D were observed where low (below 10%) soil moisture prevailed. Maximum rates of 2,4-D deactivation occurred in soil that was rich in organic nutrients. Akamine (1951) related the disappearance of 2,4-D to the total numbers of bacteria in the soil and found that soils where 2,4-D persisted the longest also had the smaller number of bacteria. Brown and Mitchell (1948) demonstrated further indirect evidence of microbial breakdown by autoclaving soil and observing a decreased detoxication of 2,4-D. Audus (1949) reported that the time progress curve of 2,4-D detoxication had a considerable lag followed by an exponential rise, both of which are characteristic of bacteria in pure culture. Audus (1951) demonstrated that a 100 ppm solution of sodium azide, a cytochrome oxidase inhibitor, will completely prevent the breakdown, suggesting that aerobic organisms are responsible for the biodegradation of 2,4-D.

Audus (1950) was the first to isolate an organism able to use 2,4-D as its sole source of carbon. In his technique, an aerated 0.01 M sodium dichlorophenoxyacetate solution was percolated through garden soil for from fourteen to twenty-eight days. The perfusate was then spread on agar plates containing 0.1% sodium dichlorophenoxyacetate as the sole source of carbon. In a following study Audus (1951) found that the bacterium was a short non-motile rod measuring 4 X 8 m. Its

morphology suggested a close relationship with the corynebacteria. Growth and fermentation reactions on differentiating media placed it in the Bacterium globiform group. The organism differed from the typical Bacterium globiform in that, after continuous growth on the same plate for a month, no cocci were formed.

According to Audus (1951), the ability of these bacteria to metabolize chlorinated organic compounds is one of the most striking features of the biodegradation of 2,4-D. There are very few chlorinated organic molecules found in nature and bacteria must, therefore, have evolved under conditions in which they have seldom or never been exposed to such molecules. However, they are able to adapt themselves to the chlorinated compounds and avail themselves of the carbon they contain for use in metabolism and growth.

The mechanism by which soils become enriched with bacteria capable of using 2,4-D as a source of energy has not been elucidated. One theory suggests that from time to time a species would be produced possessing the enzymes capable of utilizing 2,4-D and thus, when supplied with the herbicide would have an advantage over nonadapted members. Such mutants would proliferate rapidly in the absence of competition. However, Audus (1950) suggests that it is possible that adaptive enzymes are induced by herbicides in all or in a large fraction of the cells of certain responsive species.

In an effort to discriminate between these two theories, Audus (1964) stopped bacterial growth by the application of sulphanilamide which does not affect metabolic activities. Soil was perfused with 2,4-D and sulphanilamide for a period of time. Multiplication was prevented but adaptive enzyme formation was not affected by this treatment. Upon removal of the sulphanilamide, the rate of 2,4-D disappearance

was measured. If the mutation theory is correct, the lag in 2,4-D disappearance should be equal to the normal lag, since the mutants had no opportunity to proliferate. Audus found that the lag in 2,4-D utilization was shorter and concluded that this supported the adaptation theory of soil enrichment.

Audus (1952) found that bacteria capable of utilizing 2,4-D as the sole source of carbon were also capable of utilizing 2,4-dichlorophenol. He suggested that this phenol was an intermediate in the biodegradation of 2,4-D. Audus (1964) demonstrated that very low concentrations of 2,4-dichlorophenol are very toxic to adapted organisms. He hypothesized that a feed back mechanism involving the toxicity of the phenol intermediate may be the factor limiting the rate of 2,4-D breakdown. Audus concluded that the ability to oxidize the phenol determines if the organism is adapted. Steenson and Walker (1957) demonstrated that an Achromobacter sp. adapted to 2,4-D will rapidly destroy 4-chlorocatecol, suggesting that this too is an intermediate in 2,4-D degradation derived by the substitution of an hydroxy for an ortho chlorine in 2,4-dichlorophenol.

Rogoff and Reid (1956) found that a Corynebacterium sp. was able to decompose relatively large amounts of 2,4-D (1000 ppm) within 3 to 5 days. These authors stated that the molecule was attacked as a whole, the ring was ruptured and complete destruction followed. This was evidenced by measurements where CO_2 equivalent to the 2,4-D carbon was recovered and organic chlorine was released from the molecule equivalent to the amount of 2,4-D decomposed by the culture.

The relationship between the chemical structure of the herbicide and its ease of attack by micro-organisms has been examined by several

investigators. DeRose and Newman (1947) showed that the rates of disappearance of 2,4-D, methylchlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were in the order of 18:8:1 respectively. Similarly, Audus (1951) found in his soil perfusion experiments that the rates of adaptation to the same compounds fell in the order of 20:5:1. Therefore, the substitution of a methyl for a chloro group in the ortho position in 2,4-D or the introduction of a third chlorine in the ring results in an increase in its resistance to bacterial attack.

In Audus' techniques (1951), soils enriched with one phenoxy herbicide were then perfused with a solution of another whose subsequent detoxication was compared with its detoxication in fresh soil columns. These experiments demonstrated that a microflora adapted to 2,4-D could rapidly detoxicate methylchlorophenoxyacetic acid and vice-versa. However, in both cases the rates of degradation of the second molecule was slower than in those soils directly enriched with that molecule. Soils enriched with 2,4-D did not detoxicate 2,4,5-trichlorophenoxyacetic acid although some activity was temporarily induced by an intermediate perfusion with methylchlorophenoxyacetic acid. Audus concluded that it was likely that both 2,4-D and methylchlorophenoxyacetic acid encouraged the growth of the same organism by inducing the specific degradation enzyme systems which possessed the power to degrade the other molecule, although less efficiently.

2,4-D Toxicity and the Soil Ecosystem

2,4-D has been found to be toxic to several soil micro-organisms, but susceptibility varies from type to type. Worth and McCabe (1948) found that high concentrations (1%, 2%) inhibited growth of aerobic bacteria. At the 48 and 72 hr. readings it was reported that lower

concentrations (less than 0.02%) increased the amount of growth. Facultative anaerobes were not affected and anaerobic bacteria were inhibited only slightly by high concentrations of 2,4-D. Newman and Downing (1958) reported that gram-positive bacteria were more sensitive to 2,4-D than were gram-negative bacteria.

Soil fertility depends, in part, on the complex community of micro-organisms that inhabit the soil. Several investigators have examined the effect of 2,4-D on the microflora processes that contribute to soil fertility.

Ammonification represents the first stage in the degradation of proteins and other complex nitrogenous compounds of plant and animal origins and is carried out by putrifying bacteria of the soil. It is an essential part of the nitrogen cycle. The effect of 2,4-D on these organisms was investigated by Johnson and Colmer (1955a, 1955b). These investigators found that ammonia production was inhibited when Bacillus cereus was exposed to 900 ppm 2,4-D and when Pseudomonas fluorescens was exposed to 20,000 ppm. It was concluded that concentrations normally applied to the field have little effect on the ammonification activities of these two organisms.

The next stage of the nitrogen cycle is the oxidation of ammonia to nitrates and nitrites which is carried out by nitrifying bacteria. Slepecky and Beck (1950) found the conversion of ammonia nitrogen to nitrate nitrogen was completely inhibited by solutions containing 50 ppm 2,4-D. However, continuous percolation of the 2,4-D solution through the soil resulted in a reappearance of nitrification. The rates of nitrification after recovery were as high as the initial rates. It was concluded that the recovery of nitrification follows the disappearance of 2,4-D.

Jones (1948) found that the application of 2,4-D to prairie soil had no detrimental influence upon nitrate production in soil to which no nitrogen had been added. However, when nitrogen in the form of urea and sodium nitrite was added there was an indication that 15 pounds per acre of 2,4-D was sufficient to temporarily inhibit the formation of nitrates.

METHODS AND MATERIALS

Bacterial Cultures

A total of six bacterial species was used in this study. The gram-negative species were *Proteus vulgaris*, *Escherichia coli B* and *Escherichia coli AS12*. The gram-positive species were *Bacillus megaterium*, *Bacillus cereus* and *Micrococcus lysodeikticus*. The sources and characteristics of these strains are summarized in Appendix A.

The cultures were grown on Plate Count Agar (PCA) (Appendix B) at the optimal temperature for each species and maintained at 9°C. New stock cultures were prepared at four month intervals.

General

Glassware

Manometric flasks and spectrophotometric tubes were cleaned by immersion in chromic acid (Appendix B) for five hours, followed by thorough, successive rinses in cold water, deionized water, and distilled water. All other glassware was washed in detergent followed by thorough rinsing in cold water, deionized water, and distilled water.

Sterilization

When sterilization was necessary, non-heat labile materials were autoclaved in a Castle autoclave at 15 psi for 20 minutes.

Reagents

Reagents (Appendix B) were prepared in distilled water and stored at 9°C. All but heat labile reagents were sterilized by autoclaving.

Centrifugation

The Sorvall Model SS-4 Manual Superspeed Centrifuge, equipped with a SS-34 rotor, was used for all centrifugation procedures.

Gyrotory Shaker

Bacteria grown in Plate Count Broth (PCB) (Appendix B) were incubated at the species' optimal temperature and agitated at 140 RPM in a Gyrotory Water Bath Shaker, New Brunswick Model G76.

Spectrophotometry

Visible spectrum spectrophotometric techniques were performed with a Bausch and Lomb Spectronic 20 in matched spectrophotometric flasks and tubes. All spectrophotometric readings were made at 540 nm.

The Beckman DB-G Grating Spectrophotometer equipped with a Beckman 10 inch recorder and matched quartz cuvettes were used for ultraviolet spectrophotometric procedures.

Photomicrography

Photomicrographic techniques were done with an Exakta 35 mm camera and a Bausch and Lomb Flat Field Phase Contrast Microscope. Kodak Panatomic X film (ASA 32), Microdol X film developer, Medalist F-3 paper and Dektol paper developer were used in all photomicrographic procedures.

Manometry

Manometric studies were conducted with a Precision Scientific Warburg Apparatus according to methods outlined in Manometric Techniques

(Umbreit et al., 1959). Brodie's solution (Appendix B) was used as the manometric fluid. The species were incubated at optimal temperatures and agitated at 90 strokes per minute. Flask volumes were obtained from Dr. L. Stephen Whitley, Eastern Illinois University, Charleston, Illinois.

Microscopy

All microscopic techniques were performed with a Bausch and Lomb Flat Field Phase Contrast Microscope.

Standard Culture Work-up

A loop of each species was transferred from the stock cultures to 250 ml Erlenmeyer flasks containing 100 ml PCB. The cultures were grown to mid-exponential phase (.60 optical density).

Effect of Ethanol on Growth Rates

Since 2,4-D is not readily soluble in water, a suitable carrier was needed. Ethanol was found to be an adequate solvent, but its effects on bacterial growth were unknown at the concentration used. Five ml of each culture grown by the standard work-up, was aseptically transferred to a flask containing 100 ml PCB and a flask containing 99 ml PCB and 1 ml 95% ethanol. The growth of each species was monitored spectrophotometrically and the growth in the ethanol supplemented medium was compared to control flasks.

Effect of 2,4-D on Growth Rates

Five ml of each culture was aseptically transferred to 250 ml Erlenmeyer flasks containing 100 ml PCB and various concentrations of 2,4-D (in 1 ml 95% ethanol) ranging from 0 to 1000 ppm final concentration. The growth of each species was monitored spectrophotometrically.

Effect of 2,4-D on Aerobic Respiration

The effect of 2,4-D on the aerobic respiration of *Escherichia coli B*, *Escherichia coli AS12*, *Bacillus megaterium* and *Proteus vulgaris* was examined. The cells were harvested by centrifugation at 10,000 RPM for 10 minutes, washed once in Sorenson's phosphate buffer (pH 7.0) (Appendix B) followed by centrifugation. The pellet of cells was resuspended in 40 ml of Sorenson's phosphate buffer (pH 7.0) and allowed to stabilize for 30 minutes to minimize endogenous oxygen uptake. Then, 2.3 ml of the cell-buffer suspension was transferred into manometric flasks containing .5 ml of 1% glucose solution. Two tenths ml of 20% KOH was placed on a wick in the center well and various concentrations of 2,4-D in 0.5 ml 95% ethanol, ranging from 0 to 500 ppm final concentration, were placed in the side arms. The flasks were fitted on the manometers, sealed, and incubated at the optimal temperature for each species. The manometers and flasks were allowed to incubate for 15 minutes to allow for temperature and gas solubility to stabilize. Oxygen uptake was monitored at 15 minute intervals for 45 minutes. 2,4-D was carefully decanted from the side arm to the flask proper and oxygen uptake was monitored at 15 minute intervals for an additional 2 hours. Using the appropriate conversion factors, ul of oxygen taken up was calculated for each flask (Appendix C).

Effect of 2,4-D on the Aerobic Respiration of Sonicated Cells

The effect of 2,4-D on the aerobic respiration of sonicated *Bacillus megaterium* cells was examined. The cells were harvested by centrifugation at 10,000 RPM for 10 minutes, washed once in Sorenson's phosphate buffer (pH 7.0) and centrifuged. The pellet of cells was resuspended in 20 ml of Sorenson's phosphate buffer (pH 7.0) and sonicated at setting 70 for

15 seconds. The resultant cell fragment suspension was diluted with 20 ml of Sorenson's phosphate buffer (pH 7.0). The same manometric procedure used in determining the effect of 2,4-D on the aerobic respiration of whole cells was employed in determining the effect of 2,4-D on the aerobic respiration of sonicated cells.

Effect of 2,4-D on Heterolysis

The lytic effect of 2,4-D on *Escherichia coli B*, *Bacillus megaterium*, *Bacillus cereus*, *Micrococcus lysodeikticus* and *Proteus vulgaris* was examined. The cells were harvested by centrifugation at 10,000 RPM for 10 minutes, washed once in Sorenson's phosphate buffer (pH 7.0) and centrifuged. The pellet of cells was resuspended in 40 ml of Sorenson's phosphate buffer (pH 7.0) and allowed to stabilize for 30 minutes. Two ml of the cell-buffer suspension was transferred to triplicate spectrophotometric tubes containing the following:

Set A ... 2 ml Sorenson's phosphate buffer (pH 7.0)

Set B ... 2 ml of 20 µg/ml lysozyme

Set C ... 2 ml of 1000 ppm 2,4-D

Set D ... 1 ml of 40 µg/ml lysozyme
1 ml of 2000 ppm 2,4-D

Lysis was monitored spectrophotometrically.

Effects of 2,4-D on Leakage of Ultraviolet Light Absorbing Cell Constituents

The effect of 2,4-D on the leakage of nucleic acids and proteins from *Bacillus cereus* was examined. The cells were harvested by centrifugation at 10,000 RPM for 10 minutes, washed once in Sorenson's phosphate buffer (pH 7.0) and centrifuged. The pellet of cells was resuspended in 50 ml of Sorenson's phosphate buffer. Five ml of this suspension

was transferred to replicate flasks containing 0, 250, 500, and 1000 ppm concentrations of 2,4-D. Ten ml of each suspension was withdrawn from each flask at 0 hr, 1 hr, 2 hr and 4 hr. The 10 ml suspension was centrifuged at 10,000 RPM for 15 minutes and the supernatant was retained for ultraviolet spectophotometric analysis. Standard ultraviolet spectrophotometric techniques were employed to scan absorbance from 320 nm to 220 nm. The area under the curve from 280 nm to 240 nm was determined by cutting the chart paper along the curve and weighing it.

Effect of 2,4-D on the Leakage of Amino Acids

The effect of 2,4-D on the leakage of amino acids from *Bacillus cereus* was examined. The cells were harvested by centrifugation at 10,000 RPM for 15 minutes, washed twice in Sorenson's phosphate buffer, and centrifuged. The pellet of cells was resuspended in 50 ml of Sorenson's phosphate buffer. Five ml of this suspension was transferred to replicate flasks containing 45 ml of 0, 250, 500 and 1000 ppm concentrations of 2,4-D. Ten ml of each suspension was withdrawn from each flask at 0 hrs, 1 hr, 2 hrs and 4 hrs. The suspension was centrifuged at 10,000 RPM for 15 minutes and the supernatant was retained for amino acid assay. A standard curve for amino acid assay was prepared by adding 0.0, 0.1, 0.2, 0.4, and 0.5 ml of .001 M glycine to test tubes. The volume of each was brought to 0.5 ml with water. One and one-half ml ninhydrin was added to each tube. The contents of each tube was mixed thoroughly and placed in boiling water for 20 minutes. After heating, the tubes were cooled in an ice bath for 5 minutes and 8 ml of 50% aqueous n-propanol was added to each tube, followed by thorough mixing. The tubes

were allowed to stand for 10 minutes to develop full color, and the optical densities were read at 570 nm. A standard curve was constructed.

The above technique and derived standard curve were used to determine the concentrations of amino acids in 0.5 ml portions of cell-free supernatants.

Effect of 2,4-D on Motility

The effect of 2,4-D on the motility of Bacillus megaterium and Escherichia coli B was examined. Five ml of each species was transferred to PCB containing 200 ppm 2,4-D. The percent motility was approximated by viewing 100 cells in different fields at various time intervals.

Effect of 2,4-D on the Growth of Log Phase Cells

The effect of 2,4-D on the growth of metabolically active Bacillus megaterium and Escherichia coli B cells was examined. Five ml of each species was transferred to two spectrophotometric flasks containing 100 ml PCB. Growth was monitored spectrophotometrically until mid-exponential phase (0.7 optical density). 2,4-D (final concentration, 1000 ppm) was added to one of the flasks. Growth was monitored spectrophotometrically for an additional 4 hours.

Effect of 2,4-D on the Size of Cells

The effect of 2,4-D on the size of Bacillus megaterium and Escherichia coli B was examined. Five ml of each species was transferred to two spectrophotometric flasks containing 100 ml PCB. Growth was monitored spectrophotometrically until mid-exponential phase (0.7 optical density). 2,4-D (final concentration, 1000 ppm) was added to one of the flasks. At one-half hour intervals crystal violet stained slides were prepared. Average cell size was determined by measuring 200 cells per slide.

Effect of 2,4-D on Chain Length

The effect of 2,4-D on the length of chains of *Bacillus megaterium* and *Escherichia coli B* was examined. Five ml of each species was transferred to two spectrophotometric flasks containing 100 ml PCB. Growth was monitored spectrophotometrically until mid-exponential phase (0.7 optical density). 2,4-D (final concentration, 1000 ppm) was added to one of the flasks. At one-half hour intervals, crystal violet stained slides were prepared. Average chain length was determined by examining 200 cells per slide.

RESULTS

Effect of Ethanol on Growth Rates

Due to the insolubility of 2,4-D in water, it was necessary to locate a carrier. Examination of the effect of ethanol on the growth of the selected bacteria was conducted to determine if ethanol could be used as the 2,4-D carrier. No difference was observed between bacteria grown in 100 ml PCB and bacteria grown in 100 ml PCB containing 1 ml of 95% ethanol.

Effect of 2,4-D on Growth Rates and Terminal Oxidation

The results of the growth rate experiments indicate that Gram-positive species are more sensitive to 2,4-D than are Gram-negative bacteria (Figs. 1 - 6). A total inhibition of the growth of Gram-positive species was observed at concentrations of 500 ppm and above. Typical Gram-negative species, Escherichia coli B and Proteus vulgaris, were observed to grow at 1000 ppm. In all species examined, an increase in the 2,4-D concentration tended to increase the lag phase and decrease the stationary phase population density. The concentration of 2,4-D did not appear to affect the generation time.

The examination of the effect of 2,4-D on the growth of phase cells indicated that 1000 ppm prevented further growth of both Bacillus megaterium (Fig. 7) and Escherichia coli B (Fig. 8). The population density remained constant after the addition of the 2,4-D, demonstrating that although growth was prevented, lysis did not occur.

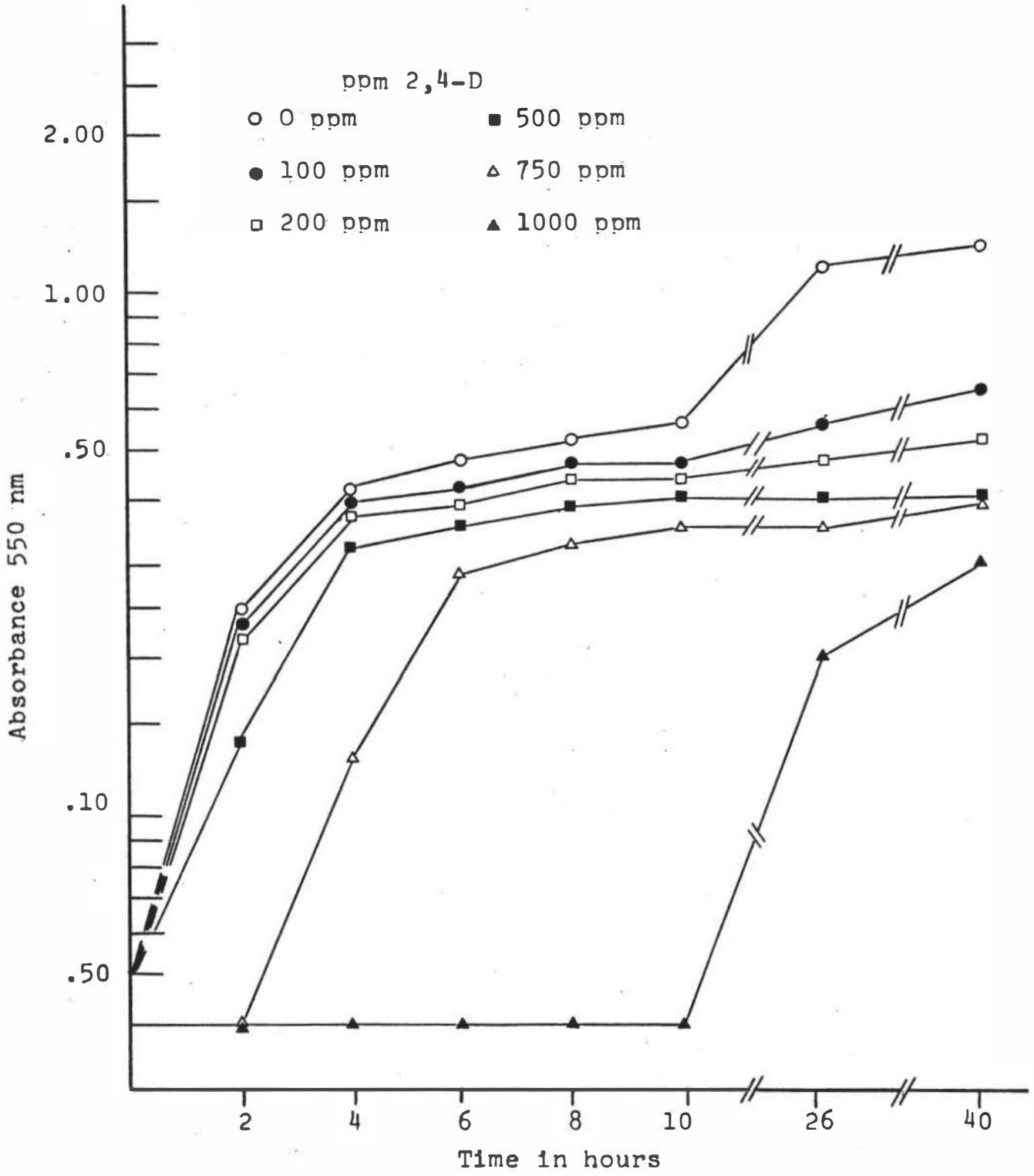

FIG. 1. Growth of Escherichia coli B in PCB at various concentration of 2,4-D.

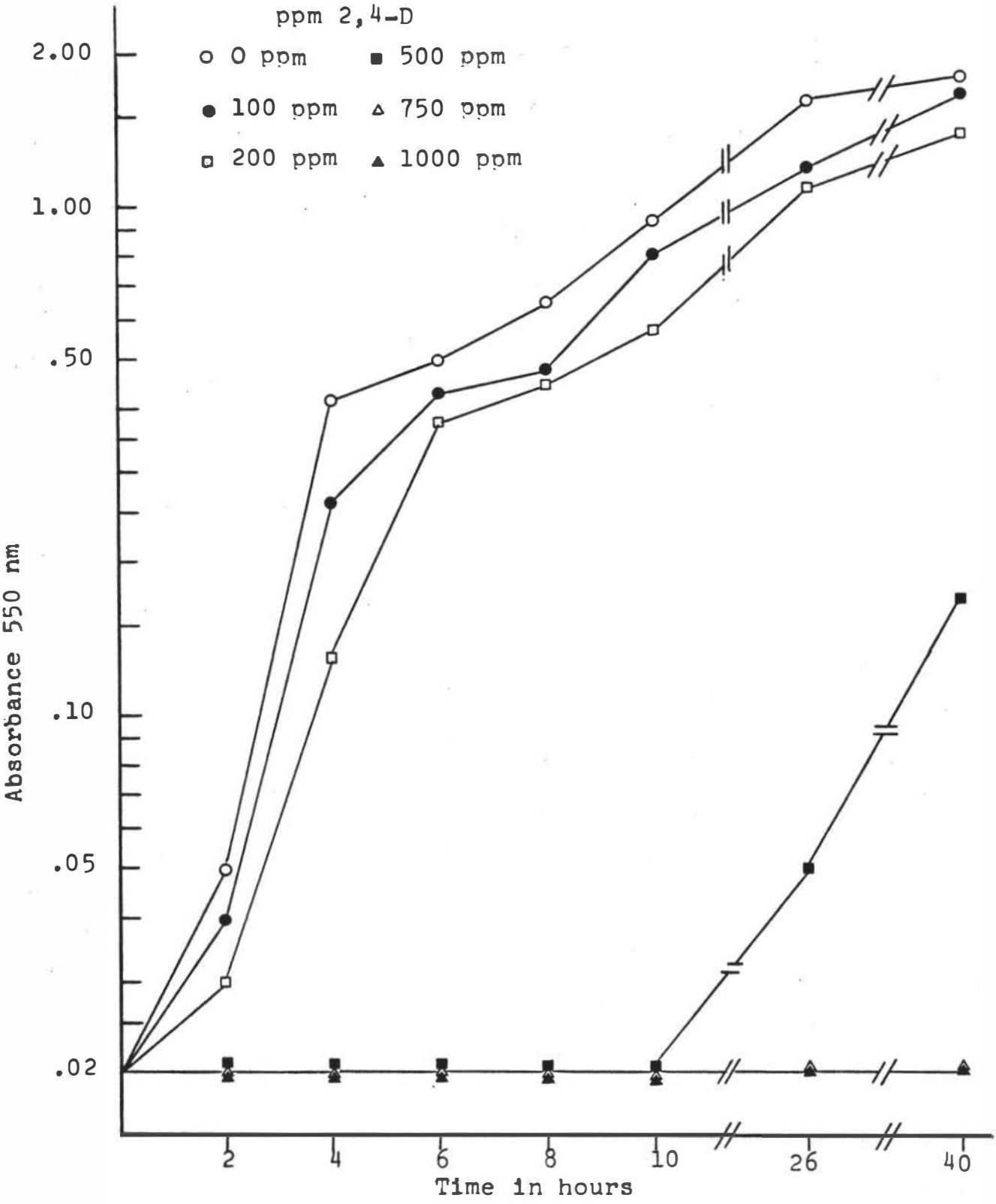

FIG. 2. Growth of Escherichia coli AS12 in PCB at various concentrations of 2,4-D.

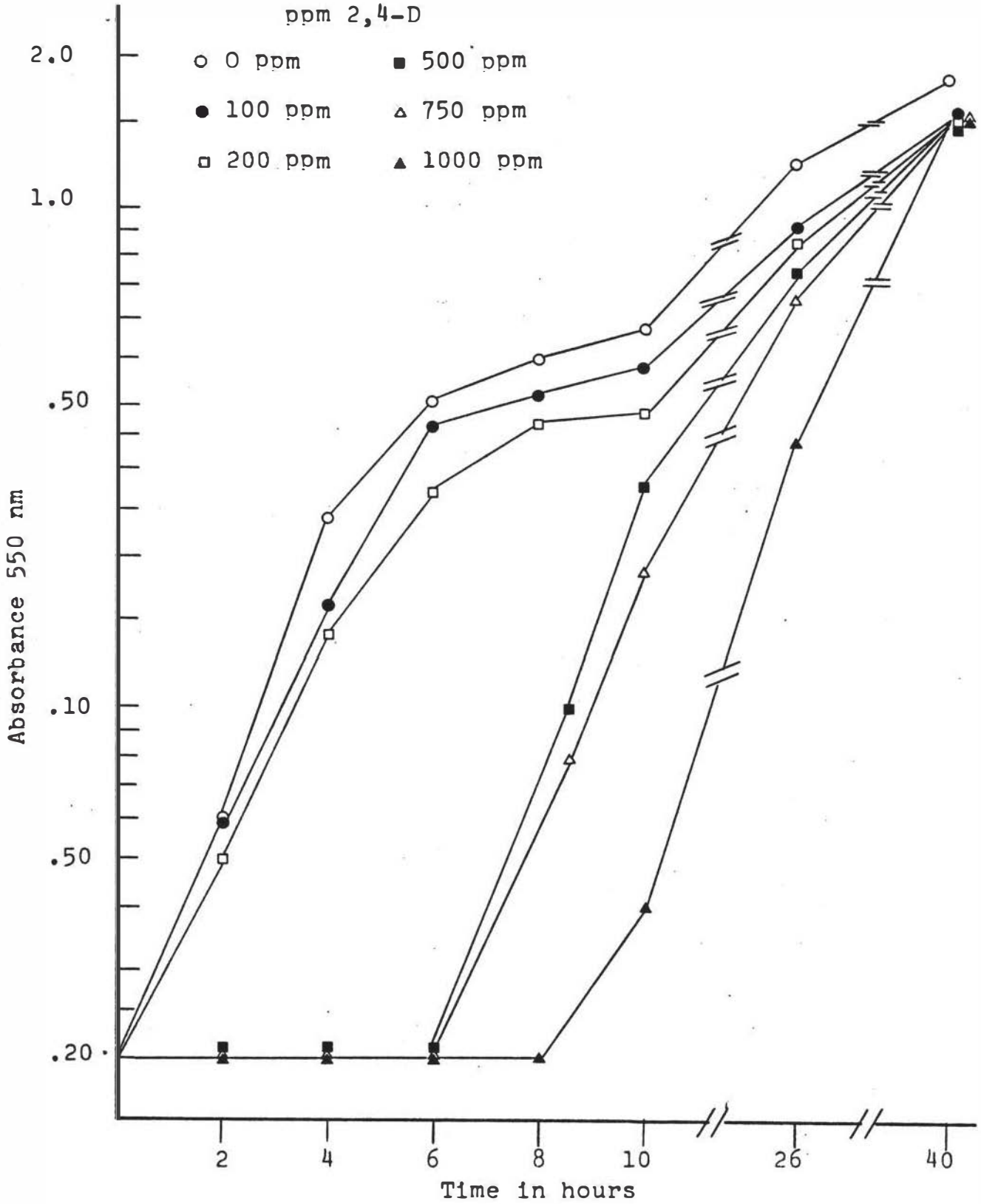

FIG. 3. Growth of Proteus vulgaris in PCB at various concentrations of 2,4-D.

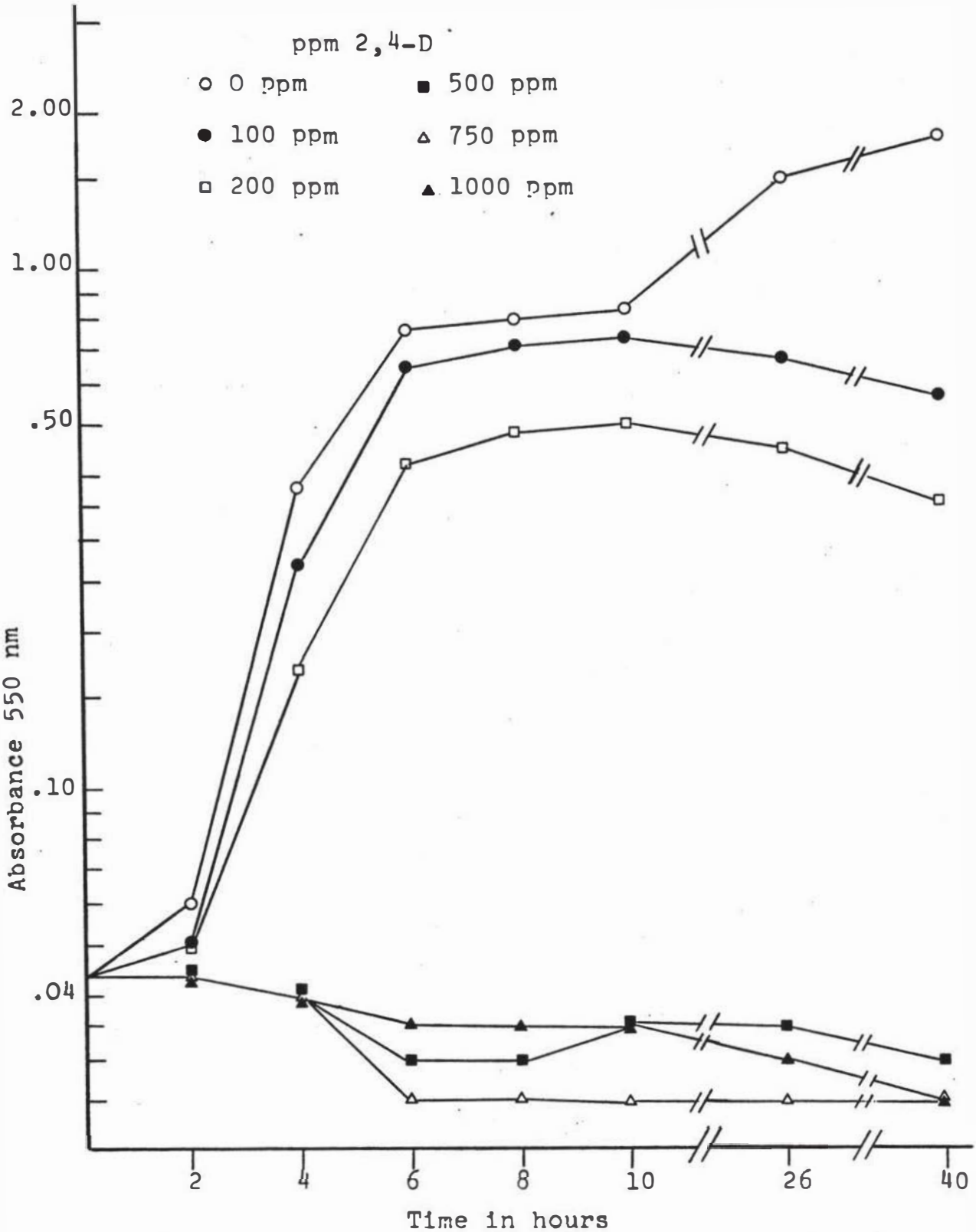

FIG. 4. Growth of Bacillus cereus in PCB at various concentrations of 2,4-D

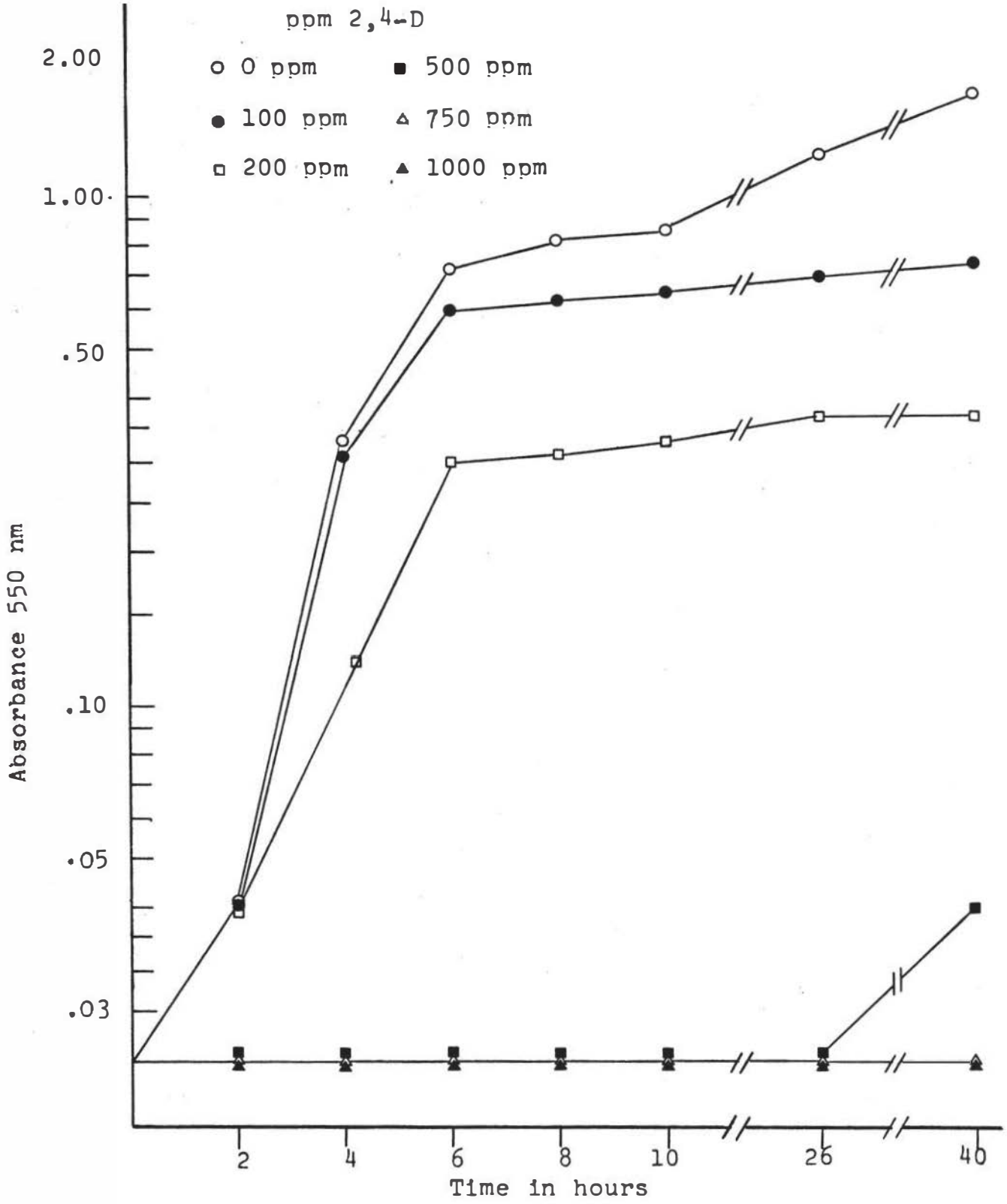

FIG. 5. Growth of Bacillus megaterium in PCB at various concentrations of 2,4-D.

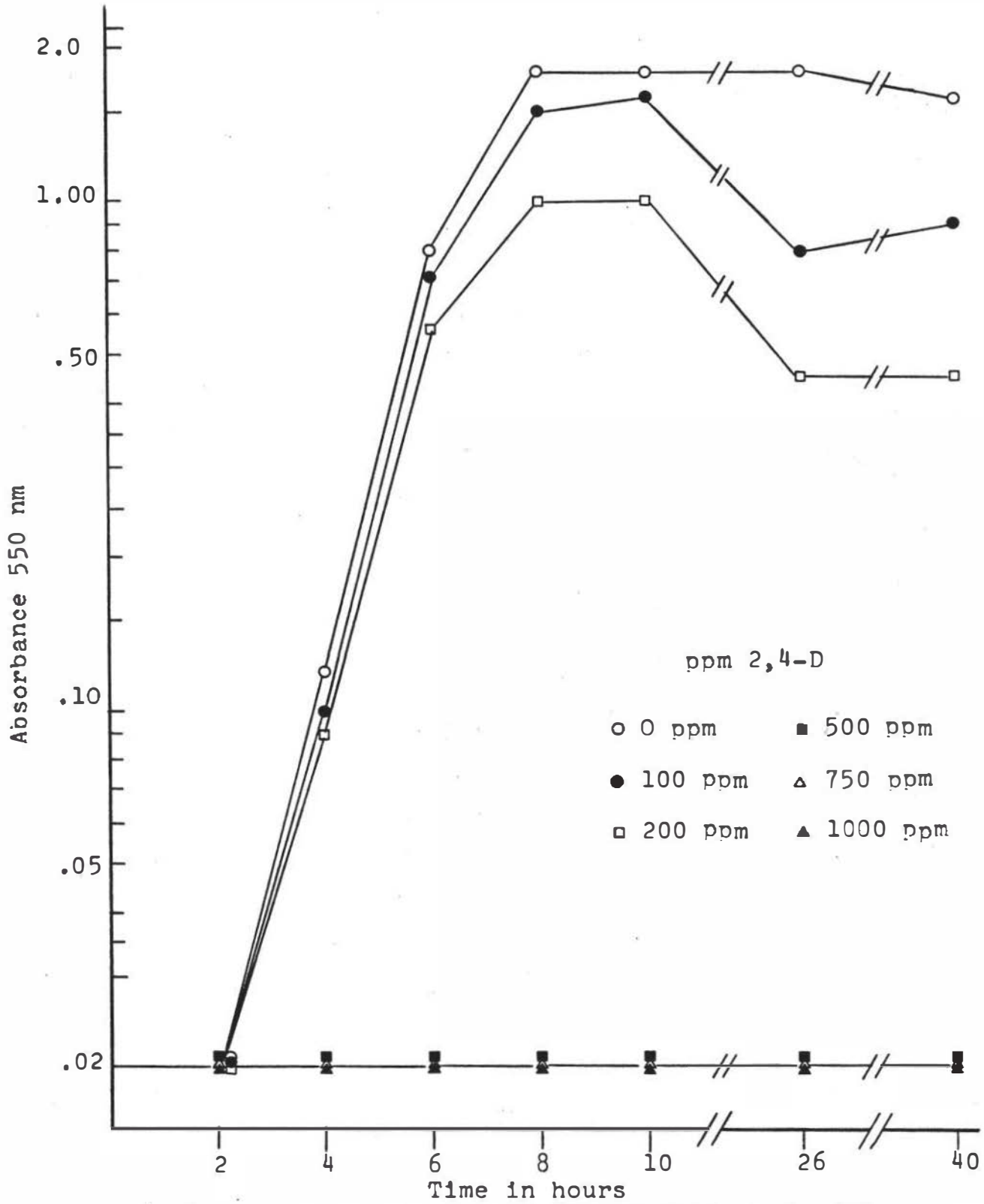

FIG. 6. Growth of Micrococcus lysodeikticus in PCB at various concentrations of 2,4-D

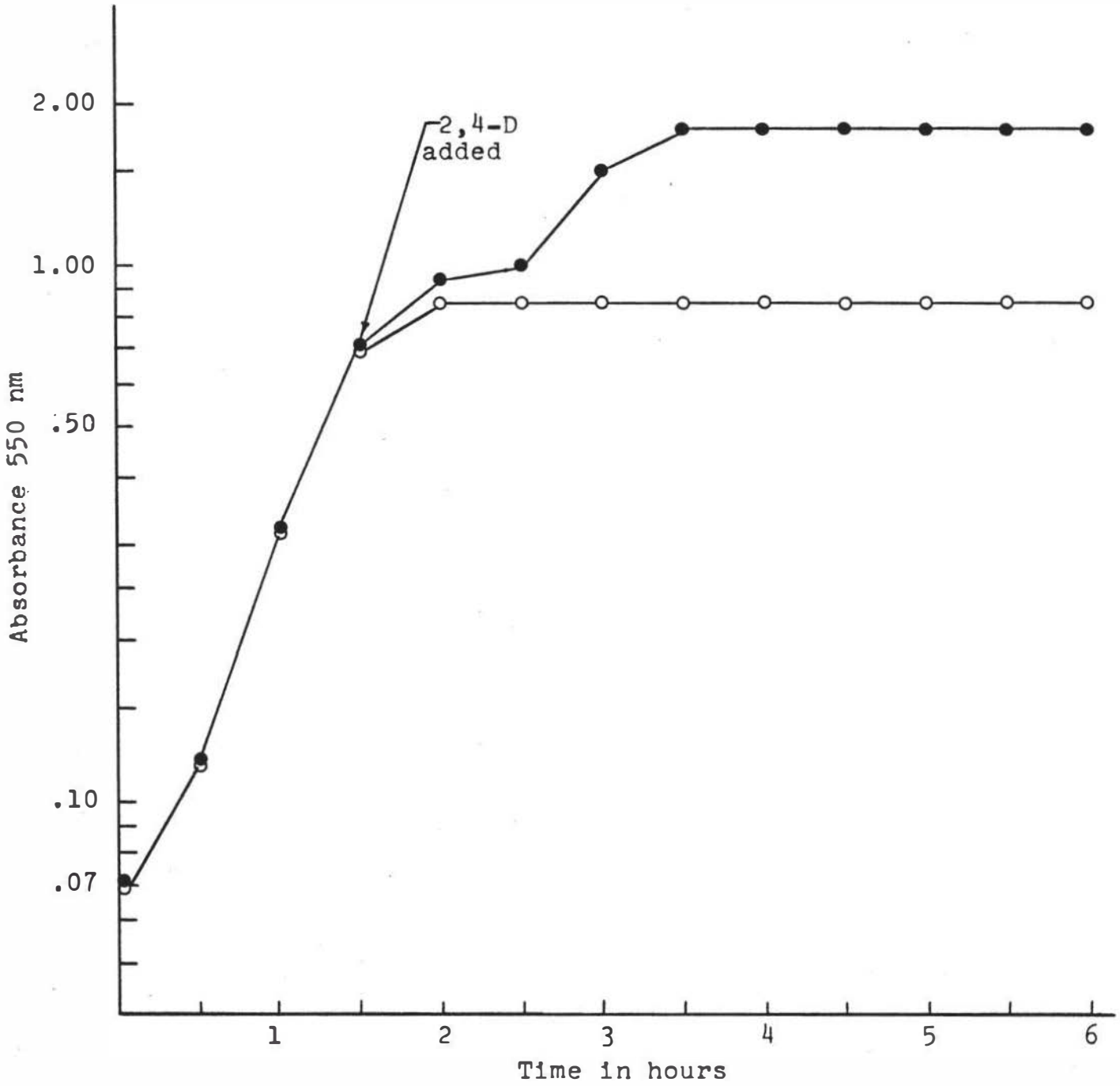

FIG. 7 . Effect of a 1000 ppm concentration of 2,4-D on actively growing Bacillus megaterium cells.

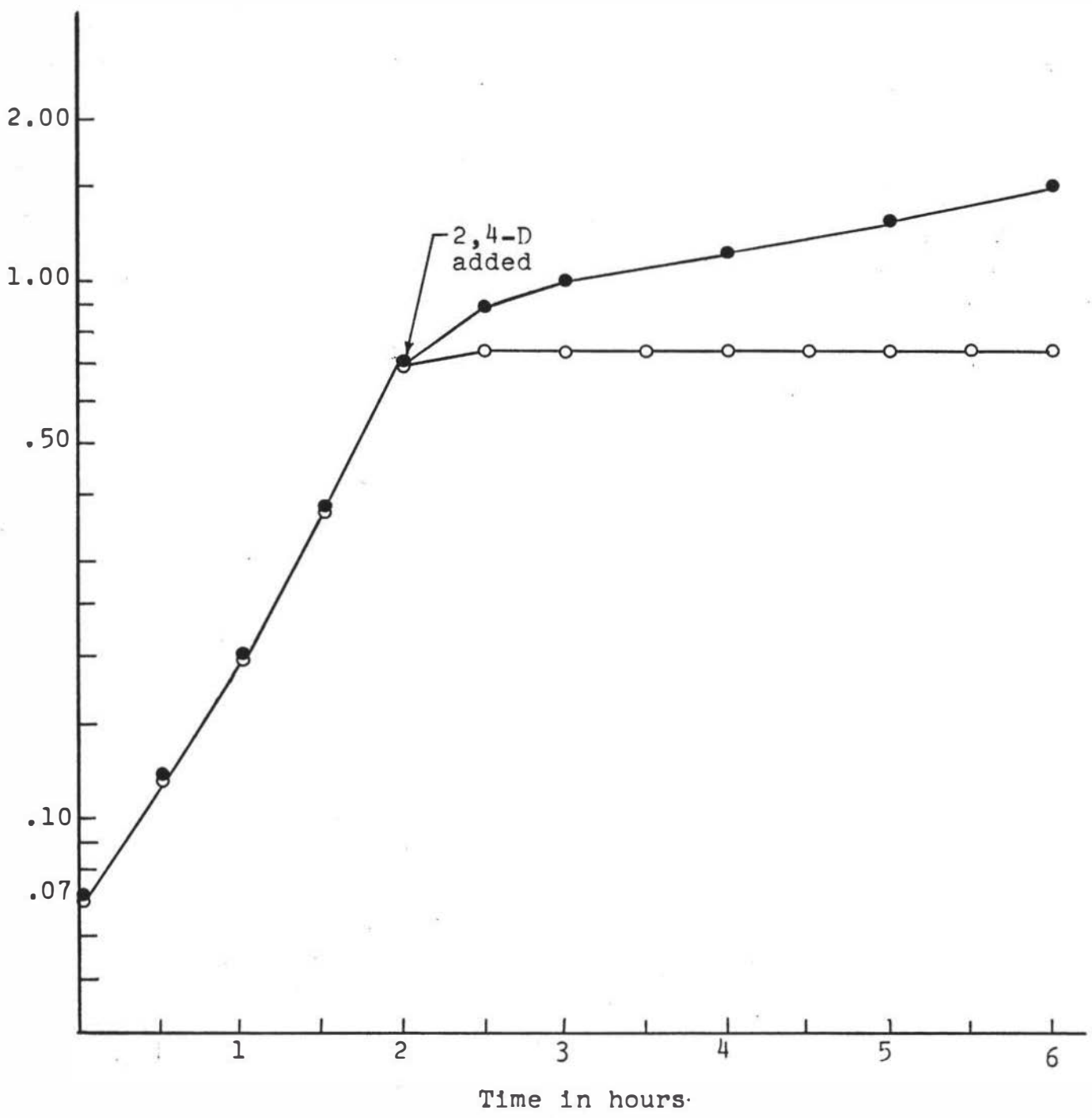

FIG. 8. Effect of a 1000 ppm concentration of 2,4-D on actively growing Escherichia coli B cells.

The results of the experiments measuring the effect of 2,4-D on terminal oxidation suggest that 2,4-D has no effect at the concentrations used (Fig. 9). Due to the high initial concentrations necessary to achieve final concentrations above 500 ppm, the effect of concentrations above 500 ppm could not be examined.

In an effort to determine if 2,4-D would affect terminal oxidation once in contact with respiratory membranes, cells were first sonicated, then examined for terminal oxidation activity. A total inhibition of respiration was observed. Apparently, sonication altered the membrane systems necessary for terminal oxidation.

Effect of 2,4-D on Heterolysis and Leakage

The ability of 2,4-D to alter the cell wall-membrane complex was examined by observing heterolysis and leakage. The results of the heterolysis experiments indicate that 2,4-D does not inhibit bacterial growth by dissolving the murein, lipopolysaccharide or lipoprotein layers of the bacterial cell wall (Table 1).

Examination of the effects of 2,4-D on leakage of ultraviolet light absorbing cell constituents suggests that 2,4-D does not promote leakage of these materials. Rather, all 2,4-D containing supernatants had less ultraviolet light absorbing material than did the control (Table 2.)

The experiments measuring the effects of 2,4-D on the leakage of amino acids from Bacillus megaterium suggests that 2,4-D does not promote leakage of amino acids. Rather, all 2,4-D containing supernatants had quantitatively less amino acid than did the control (Table 3). An increase in the amounts of amino acid was observed over time at 1, 500 and 1000 ppm. At 250 ppm 2,4-D, there was an initial increase in

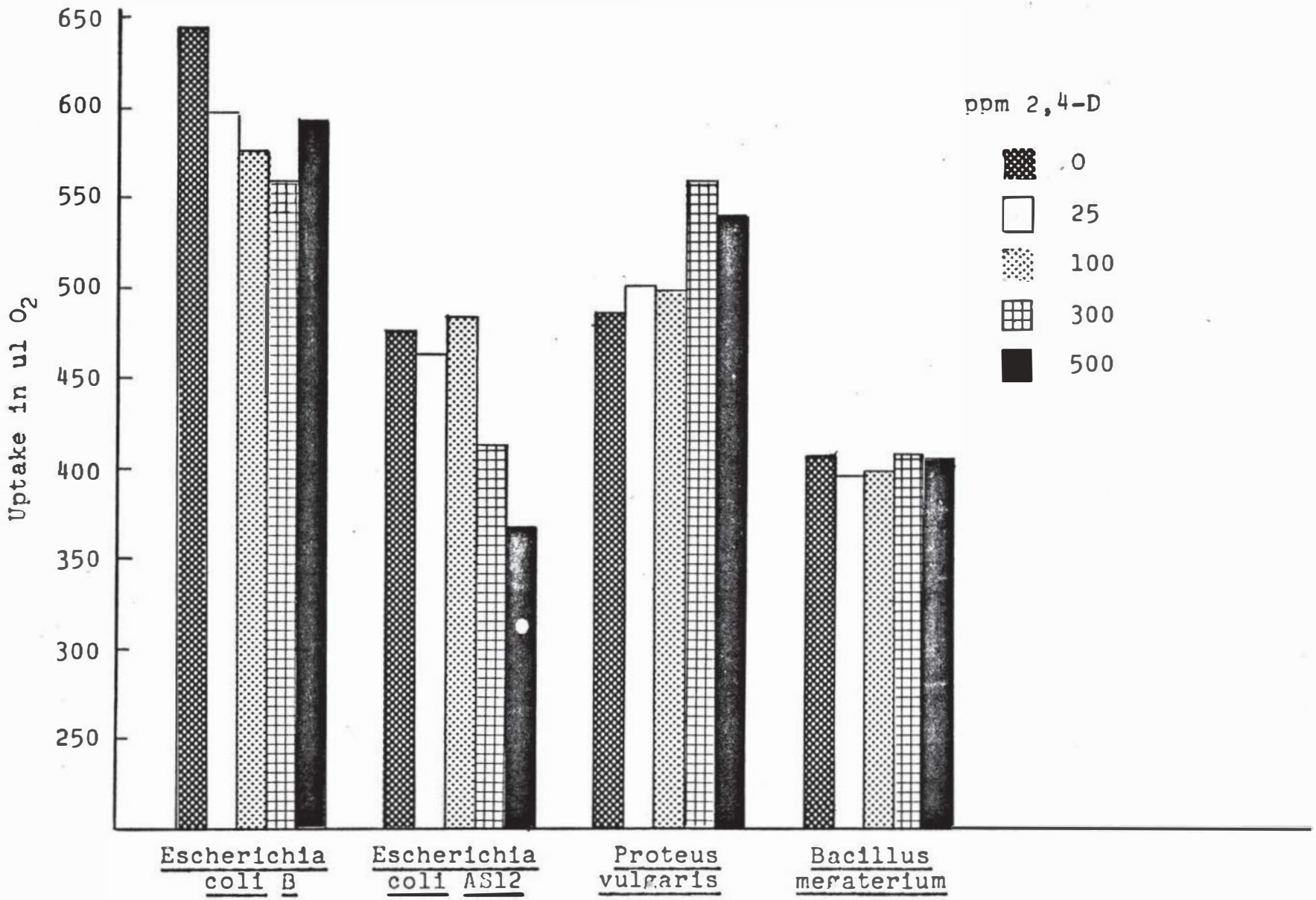

FIG. 9. Effect of 2,4-D on terminal oxidation by four bacterial species

TABLE 1. The effect of 500 ppm 2,4-D and 10 µg/ml lysozyme on the lysis of five bacterial species.

1a. *Escherichia coli B*

1b. *Proteus vulgaris*

1c. *Micrococcus lysodeikticus*

Table 1a. Escherichia coli B

Time	Control	Lysozyme	2,4-D	Lysozyme + 2,4-D
0 hr	.9	.9	.9	.9
1 hr	.9	.83	.86	.82
2 hr	.85	.72	.81	.71
4 hr	.75	.60	.71	.56

Table 1b. Proteus vulgaris

0 hr	.7	.7	.7	.7
1 hr	.7	.7	.7	.7
2 hr	.66	.66	.66	.66
4 hr	.60	.59	.61	.59

Table 1c. Micrococcus lysodeikticus

0 hr	.65	.65	.65	.65
30 sec	.65	.32	.62	.32
1 min	.65	.11	.62	.11
5 min	.62	.02	.60	.02

1d. Bacillus cereus

1e. Bacillus megaterium

Table 1d. Bacillus cereus

Time	Control	Lysozyme	2,4-D	Lysozyme + 2,4-D
0 hr	.75	.75	.75	.75
3 min	.75	.61	.75	.61
20 min	.69	.40	.62	.39
1 hr	.52	.12	.49	.13

Table 1e. Bacillus megaterium

0 hr	.45	.45	.45	.45
3 min	.45	.19	.43	.18
20 min	.45	.07	.41	.06
1 hr	.43	.05	.38	.05

TABLE 2. Effect of various concentrations of 2,4-D on the leakage of ultraviolet absorbing material from Bacillus megaterium.

Time (hrs)	Concentration (ppm) 0	250	500	1000
0	18.71 [a]	2.81	8.91	2.63
1	24.69	9.07	12.08	7.92
2	28.43	9.29	14.84	11.71
5	28.78	28.55	4.99	15.51

a Relative amounts of ultraviolet absorbing material.

TABLE 3. Effect of various concentrations of 2,4-D on the leakage of amino acids from Bacillus megaterium.

Time (hrs)	Concentration (ppm) 0	250	500	1000
0	0.60 [a]	0.35	0.35	0.40
1	0.85	0.85	0.55	0.45
2	0.95	0.50	0.65	0.85
4.	1.05	0.35	0.85	0.95

(a) mM X 10^{-4} glycine equivalents

amino acid release followed by a substantial decrease of amino acids. Apparently either the cells reabsorbed the amino acids or they precipitated with the cells.

Effect of 2,4-D on Motility, Cell Size and Chain Length

Two hundred ppm 2,4-D was found to inhibit the motility of Bacillus megaterium 2 minutes after application. However, Escherichia coli B was still motile 10 hrs after the addition of the 200 ppm 2,4-D.

Cell size appears to be increased by the addition of 2,4-D. Both Bacillus megaterium (Fig. 10) and Excherichia coli B (Fig. 11) experienced an increase in cell length following the addition of 1000 ppm 2,4-D (Table 4). The increase in cell size was found to occur rapidly. Immediately after the addition of the 2,4-D the cells were examined. Escherichia coli B experienced an average 20% increase in size and Bacillus megaterium averaged a 25% increase in size.

The examination of the effects of 2,4-D on chain length indicates that 2,4-D does not affect chain length to any significant degree (Table 4).

FIG. 10. Effect of 2,4-D on cell size: Bacillus megaterium.

10a. Size of Bacillus megaterium cells 3 hrs after the addition of 1 ml ethanol (2,4-D carrier).

10b. Size of Bacillus megaterium cells 3 hrs after the addition of 1000 ppm 2,4-D.

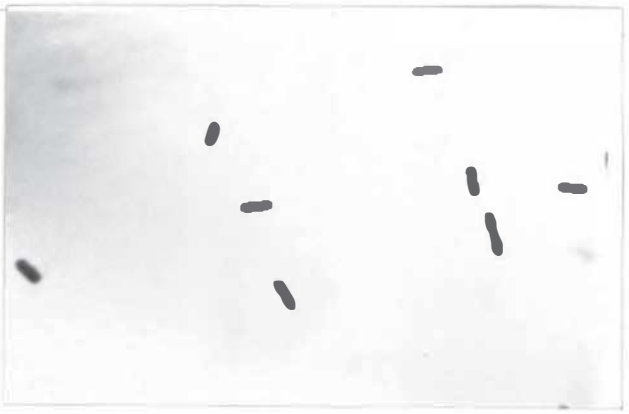

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Table 10a. -2,4-D

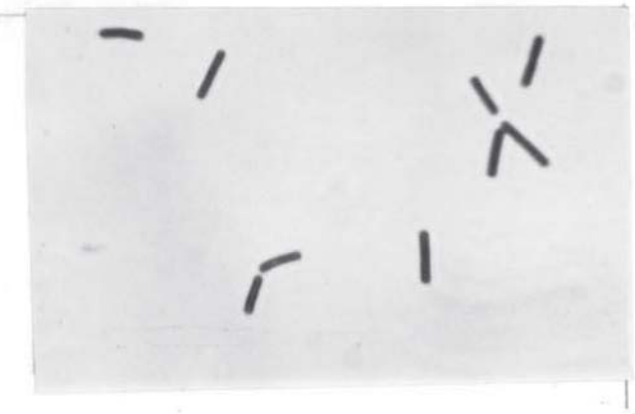

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Table 10b. +2,4-D

FIG. 11. Effect of 2,4-D on cell size: Escherichia coli B.

11a. Size of Escherichia coli B cells 3 hrs after the addition of 1 ml ethanol (2,4-D carrier).

11b. Size of Escherichia coli B cells 3 hrs after the addition of 1000 ppm 2,4-D.

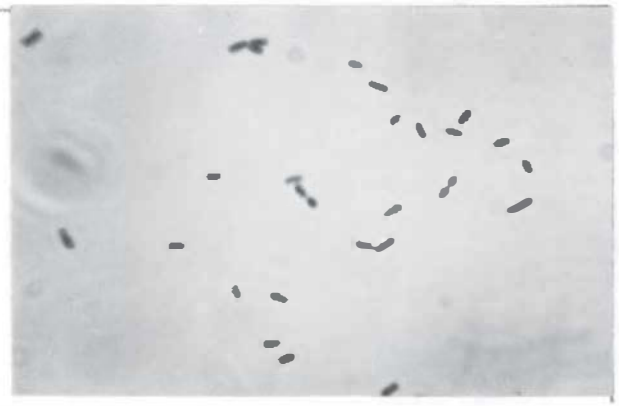

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Table 11a. -2,4-D

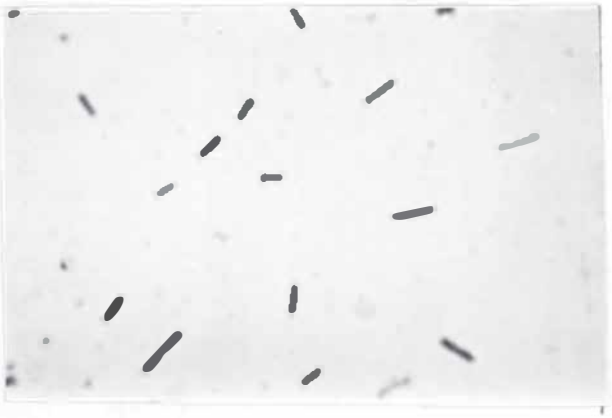

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Table 11b. +2,4-D

TABLE 4. The effect of 1000 ppm 2,4-D on the average cell size and average chain length of Escherichia coli B and Bacillus megaterium.

a. Escherichia coli B

Time (hrs)	Average cell size(a)		Average chain length	
	+2,4-D	-2,4-D	+2,4-D	-2,4-D
2	3.24	2.71	1.04	1.32
3	3.32	2.55	1.28	1.22
5	3.12	1.89	1.10	1.31
7	3.11	1.00	1.11	1.05
12	2.96	1.00	1.14	1.12

b. Bacillus megaterium

2	4.39	3.50	1.51	1.67
3	4.47	3.20	1.41	1.40
5	4.72	3.46	1.37	1.38
7	4.13	3.07	1.41	1.72
12	4.27	3.37	1.45	1.79

(a) cell length measured in microns

DISCUSSION

The literature concerning the effects of 2,4-D on multicellular organisms is abundant. However, investigations concerning the cytological and biochemical effects of 2,4-D on bacteria are limited. For this reason, many of the results of this study will be compared to findings of researchers who dealt with multicellular organisms.

The site of respiration in bacteria is not located in the mitochondrion. Rather, respiratory activity in bacteria is located in the mesosomes, membraneous structures formed by invaginations of the plasma membrane. Although the morphology of the respiratory surfaces differ, many of the biochemical pathways are similar. Techniques employed in studying respiration in cells of multicellular organisms are often applicable to the study of bacterial respiration.

Although this investigator found neither stimulatory nor inhibitory effects as a result of 2,4-D application, several authors did report results. Carns and Addicott (1964) reported that Euonymus areas treated with 2,4-D maintained an abnormally high rate of respiration, accumulated nitrogen compounds and retained greater amounts of photosynthates than untreated areas. Lotlikar (1960) tested the effects of 2,4-D on the mitochondria from cabbage. He reported that 2,4-D has a greater inhibitory effect on phosphorylation than on respiration. This could explain the lack of inhibition of oxygen uptake by the bacterial species used in these studies.

All bacterial cell walls have in common a structural component called the murein layer. This layer provides the rigidity necessary to maintain the integrity of the cell. Murein is a large molecule made up of a polymer of N-acetylmuramic acid and N-acetylglucosamine. To each molecule of N-acetylmuramic acid is attached a pentapeptide or a tetrapeptide. To provide additional strength to this molecule, bridges of amino acids cross link the peptides attached to the N-acetylmuramic acid moiety.

The cell walls of Gram-positive organisms consist of 60 to 100% murein. The cell walls of Gram-negative bacteria are chemically more complex. They contain less murein (10 to 20%), but they possess a lipoprotein and lipopolysaccharide layer exterior to their murein layer (Carpenter, 1972).

The primary function of the cell wall is to provide a strong, rigid, structural component. Without a cell wall a bacterium, under normal environmental conditions, would burst. Several methods in this study have been employed to determine if 2,4-D affects the cell wall complex. If cell walls are dissolved by 2,4-D, the treatment of actively growing cells should cause lysis. Or, if 2,4-D preferentially dissolves only the murein layer, Gram-positive species should be more adversely affected (lysed) than Gram-negative species owing to the exterior lipopolysaccharide-lipoprotein layers on Gram-negative bacteria.

The results of this study indicate that although Gram-positive species are more adversely affected than are Gram-negative species, it is not due to a dissolution of the cell wall complex. The exposure of actively growing bacteria to 2,4-D did halt further growth, but lysis was not induced by 1000 ppm 2,4-D (Figs. 7 and 8). If 2,4-D

affected only the lipopolysaccharide and lipoprotein layers, its application with lysozyme, an enzyme that selectively dissolves the murein layer, would cause lysis of Gram-negative species. The results of the heterolysis experiment (Table 1) suggest that 2,4-D does not affect the lipopolysaccharide or lipoprotein layers.

The protoplasmic membrane, located just inside the murein layer controls the permeability of substances entering and leaving the cell. Like the cell membranes of multicellular organisms, it is semipermeable and chemically composed of lipoprotein. Passive diffusion accounts for much of the passage of materials into and out of the cell. However, permeases, proteins located in the membrane, transport substances through the membrane against the concentration gradient.

If the bacterial cell membrane is weakened by 2,4-D a leakage of cellular components could result. An examination of the supernatant of bacteria treated with 2,4-D (Tables 2 and 3) revealed that leakage of amino acids and ultraviolet 1 light absorbing cell constituents did not occur. Rather, the natural leakage of the above mentioned cellular components appeared to be retarded by treatment with 1000 ppm 2,4-D. Therefore, it is possible that 2,4-D reduces the permeability of bacterial cell membranes. This supposition is supported by the experiments reported by Brian (1964), who stated that high concentrations of indolacetic acid, the plant hormone to which 2,4-D is chemically related, reduced the loss of anthocyanins (water soluble red pigments) from Rhoea epidermal cells placed in water.

Binary fission is the predominant mode of reproduction of bacteria. First, near the middle of the organism the cytoplasmic membrane grows inward, dividing the dytoplasm into two sister cells.

The cytoplasmic membrane then splits and each sister cell then lays down its own cell wall. An increase in turgor of the growing cells eventually forces a complete separation.

If 2,4-D does affect the plasma membrane it is also possible that it would prevent the inward growth of the membrane thus preventing cell division. If this cell continues to assimilate new cell components, a volumetric growth would result. Both of the above possibilities are supported by the results of this study. Growth was prevented by 1000 ppm 2,4-D (Figs. 7 and 8) and an increase in cell size was observed (Figs. 10 and 11).

Alternatively, 2,4-D could stimulate cell elongation while inhibiting cell division. According to Kiermayer (1964) auxin herbicides have a strong influence on cell elongation. He reported that a 0.1% concentration of 2,4-D was observed to cause a 20% promotion, and a 0.5% concentration caused a 30% promotion in Avena sp. coleoptiles.

CONCLUSIONS

1. The growth of Gram-positive bacteria is more inhibited by 2,4-D than is the growth of Gram-negative bacteria.

2. Terminal oxidation of resting cells of both Gram-positive and Gram-negative bacteria does not appear to be affected by the 2,4-D concentrations used.

3. The normal leakage of amino acid and ultraviolet light absorbing cell constituents appears to be retarded by 1000 ppm 2,4-D.

4. Lysis of Gram-positive or Gram-negative bacteria is not induced by 1000 ppm 2,4-D.

5. The motility of Gram-positive cells is more sensitive to 200 ppm 2,4-D than is the motility of Gram-negative cells.

6. 2,4-D at 1000 ppm stimulates an increase in size of cells of both Gram-positive and Gram-negative bacteria.

7. The chain length of both Gram-positive and Gram-negative bacteria does not appear to be altered by 1000 ppm 2,4-D.

APPENDIX A

Source and Characterization of Cultures

1. *Escherichia coli B*

   Source: Purdue University Culture Collection
   West Lafayette Laboratory
   Lafayette, Indiana

2. *Escherichia coli AS12*

   Sekiguchi and Iida (1967) isolate
   Source: Purdue University Culture Collection
   West Lafayette Laboratory
   Lafayette, Indiana

   Characterization: Sensitive to actinomycin

3. *Proteus vulgaris*

   Source: Purdue University Culture Collection
   West Lafayette Laboratory
   West Lafayette, Indiana

4. *Micrococcus lysodeikticus*

   Source: Original Alexander Fleming isolate (1922)
   Purdue University Culture Collection
   West Lafayette Laboratory
   West Lafayette, Indiana

   Characterization: Complete lysis by lysozyme

5. *Bacillus megaterium*

   Source: Purdue University Culture Collection
   West Lafayette Laboratory
   West Lafayette, Indiana

6. *Bacillus cereus*

   Source: Purdue University Culture Collection
   West Lafayette Laboratory
   West Lafayette, Indiana

APPENDIX B

Media, Buffers, and Reagents

Media:

1. Plate Count Agar (PCA)

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose	1.0 g
Bacto-Agar	15.0 g
Distilled Water	1000.0 ml

Autoclaved for 20 minutes, 15 psi

2. Plate Count Broth (PCB)

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose	1.0 g
Distilled Water	1000.0 ml

Autoclaved for 20 minutes, 15 psi

Buffers:

1. Sorenson's Phosphate Buffer (0.067 M)

Solution A: 9.1 g KH_2PO_4 (anhyd.) / liter distilled HOH

Solution B. 9.4 g Na_2HPO_4 (anhyd.) / liter distilled HOH

Mix Formula: 610 ml A + 390 ml B (pH 7.0)

Reagents:

1. Chromic acid: 25 ml Chromerge (Manostate Co.) per liter H_2SO_4 (Fisher Scientific)

2. 95% Ethanol

3. Brodies solution, Sp. Gr. 1.033 at 25°C (Harleco)

4. Dextrose (Difco)
5. Potassium hydroxide
6. 2,4-Dichlorophenoxyacetic acid (J. T. Baker Co.)
7. Ninhydrin (Calbiochem)
8. Glycine (Calbiochem)
9. Lysozyme (Nutritional Biochemicals)

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