Responses of yeast to 2,4-D

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RESPONSES OF YEAST TO 2,4-D

by

Charles Richard Swanson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major Subject: Plant Physiology

Approved:

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INTRODUCTION

The importance of 2,4-dichlorophenoxyacetic acid (2,4-D) as a selective herbicide has become firmly established in recent years. Its advantages and limitations in the control of weeds in crops have been well clarified. However, the basic mechanism by which 2,4-D kills plants remains unsolved, and the basis for selectivity has not been clearly established.

Changes in the chemical composition of plants treated with 2,4-D have been widely studied. Stimulation and inhibition of respiration have been noted in a variety of plants and plant tissues. Inhibition of enzyme activity in vitro has been demonstrated. In almost all instances the investigations have been conducted with whole plants or isolated plant tissues.

In the present investigations the cellular approach to the physiological action of 2,4-D was adopted. Higher plants exhibit a complex of reactions to 2,4-D complicated by translocation of the herbicide and differential response of the tissues. Use of excised tissue of homogeneous composition should avoid some of these objections. However, isolation of a part of an integrated entity may so alter normal functions as to cast doubt on the applicability of the results to the whole organism.

In order to study the effects of 2,4-D on cellular metabolism, independent of the interactions involved in multicellular organisms,
bakers' yeast (Saccharomyces cerevisiae) was chosen. Yeast has the advantages of ready accessibility and ease of handling. Furthermore, a large amount of work has been done in establishing the normal metabolism of this organism.

The purposes of the present investigations were:

(1) to determine the growth response of yeast and its recovery or death after treatment with 2,4-D,

(2) to study the respiratory response and its relationship to growth,

(3) to determine the effects of 2,4-D on endogenous and exogenous respiration and fermentation,

(4) to determine the effect of 2,4-D on glucose utilization and polysaccharide synthesis or degradation and the relationship to respiration,

(5) to study the effect of pH on 2,4-D activity,

(6) to determine the response of yeast to several 2,4-D compounds.
REVIEW OF LITERATURE

The field of herbicides, and particularly the utilization of 2,4-D, has been reviewed by Norman et al. (67), Crafts (19), and Blackman et al. (10). The literature reviewed here will be limited to that concerned with growth and metabolic effects of 2,4-D.

Chemical Composition

Early work by Mitchell and Brown (59) with 2,4-D on morning glory plants showed that sugars in treated plants increased initially and then decreased. Starch and dextrin were depleted in three weeks. Rasmussen (71) noted a similar trend for reducing sugars, an initial rise followed by a steady decrease. The reserve carbohydrates in dandelion roots (71) decreased steadily when treated with 2,4-D, more than making up for the increase in reducing sugars. According to Rasmussen the loss could be accounted for by the increase in respiration induced by 2,4-D, indicating that little or no sugar was used in the growth response. Smith et al. (87) and Smith (85) confirmed the effect of 2,4-D on carbohydrate reaction in bindweed and in bean plants. They noted further that there was an increase in total nitrogen of the stems of treated plants and a decrease in the nitrogen content of treated bindweed leaves.

A histochemical study by Tukey et al. (98) on bindweed treated with 2,4-D, showed a disappearance of starch from the flowers and from the
endodermis of the stem as well as the inner cortex of the root and rhizome. Erkle and Dunlap (24) found that sucrose, hemicellulose, and cellulose increased in cotton leaves treated with 2,4-D, although reducing sugars and organic acids decreased. Gall (32) was able to show, by growing bean stem tissue on nutrient agar containing 2,4-D, that starch was degraded faster than by untreated tissue.

Waller et al. (109), Wort (115), and Sell et al. (80) found that 2,4-D produced a continuous decrease of total sugars in roots, but Waller et al. (109) found no change in reducing sugars, starch, or polysaccharides in bean roots. Wolf et al. (114) found total and reducing sugars to be higher in treated plants. Hofmann and Schmeling (41) noted an increase in the simple sugars and a decrease of polysaccharides in the stems and leaves of several species treated with 2,4-D.

Total and protein nitrogen increased in stems of plants treated with 2,4-D (31,80,85,87,115), but protein decreased (31,87,109) or remained unchanged (115) in the leaves. Sell et al. (80) noted an increase in amino acid content of bean stem tissue and Waller et al. (109) observed a decrease of amino acids in bean roots and leaves. In potato tuber tissue, 2,4-D produced an increase of glutamic acid but all the other amino acids tested decreased (68). Sell et al. (80) and Rebstok et al. (72) postulated that the degraded carbohydrate in treated plants is utilized in the formation of protein and that this protein is involved in the proliferation of stems of plants treated with 2,4-D. Wolf et al. (114) grew soybean plants at three levels of nitrogen, in sand cultures containing 2,4-D. They found that injury due to 2,4-D increased with increasing nitrogen in the growth medium.
Studies by Nance (60) on excised wheat roots showed that 2,4-D inhibited nitrate accumulation, and that this inhibition could be counteracted by citrate. Using the same plant tissue, Nance and Cunningham (61) were able to show that 2,4-D increased acetaldehyde production. Lueckes et al. (54) found that thiamine, riboflavin, and nicotinic acid decreased in the leaves and increased in the stems, but carotene content was lower in both stems and leaves, of red kidney bean plants treated with 2,4-D.

Enzymes

The marked alteration of protein and carbohydrate content, as well as the stimulation and inhibition of growth and respiration exhibited by plants treated with 2,4-D, has led a number of investigators to consider the activity of various enzymes as influenced by 2,4-D.

Alpha and beta amylase of red kidney bean have been shown by Neely et al. (62) to be inhibited by 2,4-D. The inhibition was apparent in the stem but not in the leaves. Correlative evidence for the inhibitory effect of 2,4-D on amylolytic activity has been reported by Volker (105), using salivary amylase. Hofmann and Schmeling (41) found that 2,4-D increased amylase and invertase activity in the roots, stems, and leaves of Brassica napus, Vicia faba, Taraxacum officinale, and Plantago lanceolata.

The effect of 2,4-D on lipase activity has been studied by Hagen et al. (36) in castor beans, and by Kvams et al. (48) in wheat germ.
In both cases lipase activity was reduced, but sodium 2,4-D was 400 times as effective in reducing castor bean lipase activity as wheat germ lipase activity (48). Hagen et al. (36) noted that the butyl ester of 2,4-D was inactive on castor bean lipase until hydrolyzed to the acid.

Corns (18) found catalase activity in bean plants to be stimulated or inhibited by 2,4-D, depending upon soil moisture, concentration of 2,4-D, and time of sampling after treatment.

Studies by Miller and Burris (57) showed that 2,4-D inhibited oxidation of glycolic and ascorbic acid in crude barley juice. Their results support the hypothesis that 2,4-D acts as an herbicide through inhibition of respiratory enzymes. However, Wagenknecht et al. (106) were not able to find any activity of 2,4-D on cell-free glycolic acid dehydrogenase and ascorbic acid oxidase at physiological concentrations of the herbicide. Only at high concentrations of 2,4-D were these enzymes inhibited.

Freiberg (30) studied the proteinase and polypeptidase activity of soybeans grown in nutrient solutions containing 2,4-D. He reported that the activity of these enzymes decreased in the leaves but increased in the stems and roots. Pectin methoxylase activity increased and phosphorylase activity decreased in stem and leaf tissue proliferated by 2,4-D treatment, according to Neely et al. (63). Felber (26) introduced hydrogen peroxide and guaiacol into bean tissues proliferated by 2,4-D and noted an increase in peroxidase activity.

Using lyophilized yeast cells, El-Azzazi (23) found that 2,4-D stimulated pyrophosphatase, adenosinetriphosphatase, and oxidative
phosphorylation activity in the absence of divalent cations. He concluded that some divalent cations necessary for the activity of energy transferring enzymes might be liberated upon addition of 2,4-D. The change in amount of divalent cations may influence the activity of the energy transferring enzymes in growth and respiration.

Goldacre (34) found that 2,4-D increased the inactivation of indoleacetic acid by indoleacetic acid oxidase, and that boiled onion juice counteracted the effect of 2,4-D. He suggested that the function of the enzyme is controlled by a natural heat-stable inhibitor in the tissue. Addition of 2,4-D opposed the action of the inhibitor, permitting accelerated inactivation of indoleacetic acid.

Respiration and Photosynthesis

Smith (86) has summarized the data available on the effect of indoleacetic acid and 2,4-D on respiratory changes. He compared the action of growth regulators with that of known respiratory inhibitors and stated that (86, p. 118):

These comparisons are not meant to imply that IAA or 2,4-D act necessarily by the same mechanism as any of the respiratory inhibitors but only to indicate that the various types of growth and respiratory effects shown by growth substances have parallels in the action of metabolic inhibitors whose action is more or less well known. This is merely a further justification for the useful working principle that growth substances act, either in a physiological or in a toxic way, by some effect on respiratory metabolism.
Early work by Brown (16) on bean seedlings and morning glory plants showed that 1000 ppm of 2,4-D increased carbon dioxide evolution significantly. Rasmussen (71) sprayed dandelions with 120, 480 and 1920 ppm of 2,4-D and determined the respiration of the roots at intervals after spraying. The light and heavy concentrations caused a rapid stimulation of carbon dioxide evolution followed by a return to normal. The medium concentration of 2,4-D produced a rapid stimulation of respiration which was maintained at a high level as long as the roots lived. Rhizome and root tissue slices from bindweed plants treated with 2,4-D were shown by Smith et al. (87) to have stimulated rates of respiration which reached a maximum by the tenth day after spraying and declined by the fourteenth day. Further work by Smith (85), with stem slices from bean plants treated with 2,4-D, showed a stimulated oxygen uptake and anaerobic carbon dioxide evolution. Nance (60) found stimulated oxygen uptake in excised wheat roots treated with 2,4-D.

Inhibited respiration in root and stem tissue, upon treatment with 0.002 molar 2,4-D, was noted by Mitchell et al. (58). They found that the inhibition was greater at pH 5.0 than at pH 6.0. Comparing the respiratory sensitivity of pea stem tissue and oat coleoptiles to 2,4-D, Kelly and Avery (45) showed that 1000 times as much 2,4-D was required to produce an 18 to 20 per cent stimulation of respiration in oat tissue as in pea stem tissue. They found that 2,4-D stimulated starved more than non-starved oat tissue. In a subsequent publication, Kelly and Avery (46) demonstrated that maximum stimulation of pea stem respiration was obtained with 0.1 mg of 2,4-D per liter and that a concentration of
100 mg per liter was inhibitory. They noted greater stimulation of respiration in elongating than in non-elongating pea stem tissue as well as increased stimulation of starved tissue.

Taylor (97) treated day-old seedlings of wheat and mustard with 2,4-D at concentrations of 0.25 to 10.0 ppm and determined their respiratory rates manometrically. Carbon dioxide evolution and oxygen uptake were reduced in both species, but carbon dioxide was affected less than oxygen in wheat, producing a slight increase in the respiratory quotient. Oxygen uptake was slowly inhibited in blue lupine roots by 0.01 molar 2,4-D, according to West and Henderson (111). The lowest concentration at which inhibition could be detected was 0.001 molar.

Respiration of barley and rice seeds treated with 2,4-D has been studied by Hsush and Lou (43). They determined oxygen uptake and aerobic and anaerobic carbon dioxide evolution, and found that treated seeds were unable to use the oxygen of the air efficiently in respiration and therefore energy must come from anaerobic respiration. According to these investigators the greater tolerance of rice than barley to 2,4-D may be explained on the basis that rice is better able to carry on normal metabolism under conditions where oxygen is limiting.

Nickell (66) found maximum stimulation of growth at 0.001 ppm of 2,4-D in virus tumor tissue, and maximum stimulation of respiration at 1.0 ppm.

Apparent photosynthesis was retarded by 100 ppm of 2,4-D in Freeland's (28) experiments with bean plants. Respiration was initially suppressed, followed by an increase and finally a decrease. Freeland
(29) treated Anacharis with 30 and 100 ppm of 2,4-D and noted a retardation of both photosynthesis and respiration. At the higher concentration the plants began to lose their chlorophyll after 48 hours, but respiration recovered considerably.

Weintraub et al. (108) found that radioactive carbon dioxide was produced by plants treated with 2,4-D which contained C\(^{14}\) in the carboxyl or methylene groups. Brody (15) found that 2,4-D stimulated respiration and acted as an uncoupling agent for oxidative phosphorylation in rat liver mitochondria in a phosphate-deficient system.

Microorganisms

The effect of plant growth regulators has been studied on a wide variety of microorganisms. The literature concerned with responses of soil microorganisms to plant growth regulators has been reviewed by Newman (65) and Schmidt (79).

Turfitt (99) found yeast growth responsive to a number of growth regulators, and noted their stimulatory effect on fermentation. Grace (35) also noted a stimulation of fermentation in yeast treated with small amounts of naphthaleneacetic and indoleacetic acids, but fermentation was decreased at high concentrations of the growth regulators. Bain and Rusch (5) studied the effect of two phenylenediamines and two quinones on yeast metabolism and found that those systems which oxidize pyruvate were most sensitive to the inhibitors. Those enzymes participating in
the oxidation of glucose were less sensitive and those involved in anaerobic utilization of glucose were least sensitive.

Early work with microorganisms by Stevenson and Mitchell (92) demonstrated that 0.02 per cent 2,4-D retarded the growth of Bacillus, Aerobacter, Staphylococcus and Phytomonas, but had no effect on Fusarium and Penicillium. Smith et al. (88) found no effect of 2,4-D on general soil microorganisms within the concentration range of 0.5 to 100 ppm. Kratochvil (47) confirmed this lack of effect of 2,4-D and 2,4,5-T on the soil microorganisms at rates up to 4 pounds per acre. Lewis and Hammer (50) found no effect of 1000 ppm of 2,4-D on Alternaria, Sclerotinia, Fusarium, Penicillium, Escherichia, Staphylococcus, Phytomonas, Bacillus, and Rhizobium. The lack of effect of 2,4-D on Bacillus subtilis and Staphylococcus aureus was reported by Culler et al. (20). These investigators also noted that cell division in Aerobacter aerogenes and Bacillus subtilis was unaffected. Phytomonas was inhibited to a greater degree than Erwinia by high concentrations of 2,4-D.

Other workers (22, 65, 70, 75, 92, 116) have found notable effects of 2,4-D on growth and metabolism of microorganisms. Newman (65) studied the effect of 2,4-D on the growth of bacteria, actinomycetes, and several fungi. At 500 ppm the growth of soil bacteria and actinomycetes was greatly reduced, particularly at pH 6.4 and 5.6. The diameter growth of Cunninghamella, Trichoderma, Aspergillus, and Fusarium at pH 4.6 was noticeably reduced at 125 ppm 2,4-D, and greatly reduced at 500 ppm. Certain human pathogens have been found to be sensitive to 2,4-D; Dubos (22) determined that approximately 0.01 per cent is necessary to produce
a 50 per cent growth inhibition of *Streptococcus* and *Shigella* species, and the same inhibition of *Mycobacterium* is obtained with 0.003 per cent. Inhibition of vegetative growth and spore germination by $10^{-3}$ to $10^{-2}$ molar 2,4-D has been recorded by Richards (75) in several filamentous fungi.

Worth and McCabe (116) studied the effects of 2,4-D on microorganisms which vary in their oxygen requirements. The organisms were grown on agar containing 2,4-D in concentrations ranging from 0.0002 to 2.0 per cent. The amount of growth was estimated daily for three days. Aerobic organisms were inhibited by 2,4-D but there was no apparent inhibition of the facultative anaerobes. Inhibition of the anaerobes was slight.

A rapid and simple method for the determination of the effect of 2,4-D upon the growth of yeast has been developed by West and Henderson (110). The test was based on the assumption that growth is directly proportional to turbidity. The method consists of inoculating Czapek's nutrient culture solutions containing the desired concentrations of 2,4-D with yeast previously grown on wort agar. After incubation for seven days, the percentage light transmission is determined with a photoelectric colorimeter. Concentrations of 10 ppm and higher were found to have the greatest effect on yeast growth.

Pool (70) grew yeast in nutrient culture containing various concentrations of 2,4-D and determined growth turbidimetrically. He found 0.001 molar 2,4-D to be the maximum concentration which did not reduce yeast growth. No stimulation of growth was noted.
Effect of pH

The effect of the pH of the solution on the biological activity of weak acids and bases has been recognized by a number of investigators (4, 7, 38, 52, 53, 58, 65). Hammer et al. (38), Lucas and Hammer (53), and Lucas et al. (54) have reported an increased effect of 2,4-D applied to bean plants at low pH. Greater inhibition of root and stem tissue respiration at pH 5.0 than 6.0 has been noted by Mitchell et al. (58). Newman (65) found 2,4-D to be increasingly inhibitory to bacteria, actinomycetes, and fungi as the pH was lowered from 8.2 to 5.6.

It was noted further (52) that within the pH range of 4 to 7, buffered solutions of 2,4-D caused more injury than unbuffered solutions, but at pH 2, 3, and 8 there were no real differences between buffered and unbuffered solutions. Hammer et al. (38) suggested that the increased effect of 2,4-D at low pH may be attributed more to the titratable acidity than to the pH of the solution.

The effect of pH on the activity of weak acids and bases has been intensively studied by Simon and Beavers (81, 82, 83), Simon et al. (84), and Beavers and Simon (7). It was pointed out (81) that the pK of a weak acid or base determines the effect of pH on the activity of a compound. Below the pK, pH changes make little or no difference in the activity of weak acids but above the pK, activity decreases with increase in pH. They recognized that the pH effect may be masked in certain instances where a small amount of chemical is applied to a large bulk of tissue, such as in herbicide applications. Simon and Beavers (81, p. 125)
state that:

Masking results because the pH of the applied solution affects the penetration of weak acids and bases only into those cells which are bathed by it. The degree of such masking, then, will be determined by the proportion of cells with which the external solution comes into contact; in bulky tissues this proportion may be very small, and the active substance will reach cells remote from the surface only after passage through other cells or intercellular fluids.

Simon and Beevers (62) used the Warburg manometric technique to study the effect of pH on the activity of weak acids on yeast respiration. They noted little or no effect of alteration of pH alone on the rate of oxygen uptake by yeast between pH 2.4 and 6.9.

Hagen et al. (36) noted that the butyl ester of 2,4-D was inactive in the inhibition of castor bean lipase activity until hydrolyzed to the acid. However, Newman (65) found that the ethyl ester of 2,4-D was equally active on several fungi at all pH values between 4.6 and 7.6. He suggested, therefore, that this compound is inhibitory only in the undissociated form. Simon and Beevers (81,82) stated that the effect of pH on degree of dissociation plays a large part in determining the activity of an inhibitor, but they noted that the ions are also active. Beevers et al. (8) compared the effectiveness of malonic acid, diethyl malonate, and ethyl alcohol in inhibiting respiration of excised corn root tips. They found that malonic acid and ethyl alcohol alone had no inhibitory effect at pH 8, but at pH 4 malonic acid was inhibitory. By using the diethyl ester of malonic acid they were able to show inhibition of respiration at pH 8. They suggested that the penetration of biologically weak acids could be enhanced by using the esters.
Bentley and Housley (9) studied the activity of 2,4-D acid and 2,4-D nitrile in the *Avena* straight-growth test, and found that the nitrile has greater growth promoting activity than the acid at low, and greater inhibitory activity at high concentrations. They explained these results as a greater penetration of the nitrile than the acid form of 2,4-D.
The stock medium employed was an adaptation of that of Mitina and Sendersky (192) used by Holst and Josefson (47) in which the Ringer's solution was distilled water to make one liter.

20 gms glucose
2.5 gms yeast extract (Difco)
0.25 gms magnesium sulfate
0.25 gms calcium chloride (CaCl₂·2H₂O)
2 gms potassium nitrate
3 gms ammonium nitrate

20 gms Eagle's medium of the following composition was grown on an agar nutrient plate of Sabouraud's glucose agar at 24°C on an excothecial colony of Saccharomyces cerevisiae on an agar nutrient plate of Eagle's medium of the following composition was grown on an agar nutrient plate of Sabouraud's glucose agar at 24°C on an excothecial colony of Saccharomyces cerevisiae to determine the effect of 2,4-D on growth.

MATERIALS AND METHODS
The experimental yeast cultures were subcultured three times in a liquid nutrient medium before use in the growth studies. A double strength medium was prepared having the following composition:

20 gm glucose
3 gm ammonium sulfate
2 gm potassium acid phosphate
0.25 gm calcium chloride (CaCl₂·2H₂O)
0.25 gm magnesium sulfate
2.5 gm yeast extract (Difco)
distilled water to make 500 ml.

For subculturating, 25 ml of the double strength nutrient solution and 25 ml of distilled water were placed in 125 ml Erlenmeyer flasks. The flasks were plugged and the contents autoclaved for 20 minutes at 15 pounds pressure. When cool, the flasks, now containing a single strength nutrient solution, were inoculated with yeast cells from an agar slant. Growth was allowed to proceed for 24 hours with frequent shaking. Subsequent subcultures were made by transferring yeast cells to freshly autoclaved nutrient medium with a sterile inoculating loop, and incubating at 34°C.

Growth experiments were carried out in 18x150 mm Pyrex test tubes calibrated according to the method of Atkin et al. (3). The test tubes were calibrated in a Lumetron photoelectric colorimeter, model 402-E, fitted with a 17 to 22 mm test tube holder. No filter was used in the colorimeter but the light intensity was cut down to the minimum with a number 3 stop on the bottom and a number 2 stop on the top. A test tube
was selected at random from a stock of new tubes, cleaned, and filled with distilled water. The light transmission of this tube was set at 90 per cent. Additional test tubes were cleaned, filled with distilled water, and their percentage light transmission was read. Test tubes deviating more than one per cent from the standard were rejected. For uniformity, all test tubes were so placed in the colorimeter holder that the Pyrex label faced in one direction. It was noted that rotation of the tubes in the holder changed the light transmission slightly. Three dozen test tubes were selected for use.

Five ml of the double strength nutrient solution and five ml of a double strength solution of monohydrate sodium salt of 2,4-D were pipetted into each of the calibrated test tubes. In this manner (70) a single strength nutrient and 2,4-D solution was obtained. The stock solutions were previously adjusted to pH 6.0 with HCl or NaOH. The test tubes were plugged and the contents autoclaved prior to inoculation.

The stock solutions of 2,4-D were made by dissolving 12.152 grams of the monohydrate sodium salt of 2,4-dichlorophenoxyacetic acid (Bakers lot no. 62549) in distilled water and making to 250 ml. A double strength solution was produced which was $2 \times 10^{-1}$ M. After 1 to 1 dilution with double strength nutrient medium, a $10^{-1}$ M solution of 2,4-D was obtained. A range of concentrations was prepared by serial dilution of the stock solution. Application of heat was necessary to effect solution of the 2,4-D in the stock solution.

To determine the effect on growth, the test tubes containing single strength nutrient and 2,4-D were inoculated with yeast cells from the
third subculture, using a sterile inoculating loop. The test tubes were
then placed in an incubator maintained at 30°C, and shaken frequently
during the 20 hour incubation period. At the end of the incubation
period, the outside of the test tubes was cleaned carefully before tur-
bidity measurements were taken. Light transmission of the yeast suspen-
sions was read in the colorimeter as a percentage of the blank, with a
test tube containing nutrient but no yeast as the blank.

Experiments on the recovery or death of yeast treated with 2,4-D
were initiated in the same manner as the growth studies. However, after
the 20 hour growth readings were taken, the yeast suspensions were
centrifuged for 15 minutes at 1500 revolutions per minute. The super-
natant nutrient, or nutrient plus 2,4-D, was decanted and the residual
yeast cells were washed with fresh, single strength nutrient. Finally,
10 ml of fresh single strength nutrient was added to each tube. The
original inoculum was resuspended, and growth measurements were made
after an additional 20 and 44 hours of incubation.

Respiration

Pound cakes of bakers' yeast (Fleischmann's) were used as a source
of plant material for the respiration experiments. The yeast was stored
at 5°C, and used for no more than three experiments on consecutive days.
Experimental samples were isolated from the interior of each cake.
Moisture determinations on approximately 10 gm samples from four sep-
arate cakes of yeast showed marked uniformity of moisture content,
averaging 70.8 per cent, with less than 1 per cent variation between individual samples.

The moist yeast cells were weighed out and suspended in distilled water immediately before starting each experiment. In the early experiments the yeast cells were dispersed in water by vigorous shaking. In later experiments a magnetic stirrer was used. The latter method had the advantage of permitting the removal of aliquots of the suspension with a pipette while the suspension was being agitated.

A sufficient weight of yeast cells was used so that a concentration of 20 mg of yeast per flask was obtained for endogenous experiments when an aliquot of the suspension was diluted in the Warburg flasks. Five mg of yeast per flask was used in the exogenous experiments.

Solutions of 2,4-D were added directly to the flasks and allowed to equilibrate with the yeast, or added to the side arm and tipped in after equilibration. Concentration of the 2,4-D was adjusted in the stock solution to produce a solution of the desired molarity when diluted in the Warburg flasks. The buffers used were either potassium acid phosphate or potassium acid phthalate to produce a final molarity of M/40.

A standard, rectangular model Warburg respirometer was used in the early experiments, and a rotary Warburg respirometer in the later experiments. A bath temperature of 30.0 ± 0.05°C. was maintained throughout. The manometers were shaken at a frequency of 100 to 120 oscillations per minute, and an equilibration period of 20 minutes was employed.

Measurements of respiration were made with the direct method of Warburg as described by Umbreit et al. (100). Flasks with side arms were
used. The total fluid volume in the flasks was 3.0 ml unless otherwise indicated. When oxygen uptake was to be measured, 0.5 ml of 20 percent potassium hydroxide was added to the center well. An accordion-folded filter paper was placed in the center well with the alkali to increase the absorbing surface. In the experiments on the effect of concentration of 2,4-D on the respiratory quotient, paired flasks containing 0.5 ml of M HCl in the sidearms were used. In one flask the acid was tipped in at zero time, and the carbon dioxide evolved was a resultant of the carbon dioxide bound by the medium and that taken up during preparation and equilibration of the flasks. Acid was tipped into the other flask at the end of each experiment. The difference between the two was the carbon dioxide produced by the yeast during the experimental period.

Measurements of oxygen uptake and carbon dioxide evolution were taken at five minute intervals in the experiments of short duration, and at 10 minute intervals in the longer experiments. The values for gas exchange were calculated on the basis of the rate per hour and expressed in terms of the fresh weight of yeast.

The basic medium for endogenous experiments consisted of a buffer solution, with or without 2,4-D, and distilled water to make the proper liquid volume in the Warburg flasks. The volume of the yeast cells in the flasks was considered negligible. In the exogenous experiments, 0.5 per cent glucose was used as the source of carbohydrate. The proportion of the various constituents in the flasks varied with the conditions of each experiment, but the proper concentrations of the components were maintained.
Anaerobic conditions were obtained by flushing nitrogen gas through the flasks. The nitrogen was bubbled through two bottles of alkaline pyrogallol to remove oxygen, and the flasks were flushed for 15 minutes at a positive pressure of 1.5 cm on the manometers.

Carbohydrates

Glucose utilization by bakers' yeast treated with 2,4-D was determined in nutrient medium and in distilled water. One gram samples of yeast were incubated in 100 ml of solution for one hour. The yeast was centrifuged at 1500 rpm for 20 minutes and the supernatant was used for determination of glucose uptake. The ceric sulfate reducing sugar method of Hassid (40) was employed for glucose determinations.

After incubation for one hour and removal of the medium containing the treatments, the yeast was washed with distilled water, centrifuged, and the supernatant was discarded. HCl solution, containing 5 ml concentrated HCl per 100 ml distilled water, was added to the yeast, and hydrolysis of the polysaccharides was carried out in an autoclave at 15 pounds pressure for 20 minutes. The yeast was centrifuged and the supernatant was decanted into 100 ml volumetric flasks. The acid was neutralized with NaOH and the solution was made to volume with distilled water. Polysaccharide synthesis and degradation were calculated from glucose determinations made on 5 ml samples using the ceric sulfate method.
managed statistically according to the method of group comparisons (69).

The nutrient medium used in these investigations was the same as

The results were

mete just before the beginning of each experiment. The results were

at 60°C and cooled in a desiccator. Glucose was added to the treat-

that employed in the growth studies. C. P. Glucose was directed overni-
EXPERIMENTAL RESULTS

Effect of 2,4-D on Growth

A turbidimetric method was used to determine the effects of 2,4-D on growth of yeast in a nutrient medium. The details of the method have been described previously. It was noted early in these investigations that inoculation of the nutrient medium with a loopful of cells from a 24 hour subculture produced a dense suspension of yeast cells in 20 hours when incubated at 34°C. Although the variability common to biological systems was apparent between experiments run at different times, measurements on duplicate treatments within each experiment agreed well. In addition, the effects of 2,4-D on growth of yeast were found to follow closely the same trend in each of the several repetitions of any experiment.

The effect of a wide range of concentrations of 2,4-D on yeast growth, when incubated with the chemical for 20 hours, is presented in Table 1. There was no effect of the monohydrate sodium salt of 2,4-D at any concentration up to 10⁻⁶ M. From 10⁻⁶ to 10⁻³ M a slight inhibition of growth, as measured by light transmission of the turbid suspension, became apparent. At 10⁻² M yeast growth was reduced sharply, and at 10⁻¹ M there was almost complete inhibition of growth.
Table 1. Growth of yeast in a range of 2,4-D concentrations.
(Growth measured as percentage transmission of light by turbid yeast suspensions after 20 hours incubation.)

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Growth</th>
<th>1</th>
<th>2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>17.0</td>
<td>17.0</td>
<td>17.0</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td></td>
<td>17.7</td>
<td>17.0</td>
<td>17.4</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td></td>
<td>17.3</td>
<td>16.7</td>
<td>17.0</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td></td>
<td>18.2</td>
<td>16.7</td>
<td>17.5</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td></td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td></td>
<td>20.0</td>
<td>20.6</td>
<td>20.3</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td></td>
<td>21.8</td>
<td>20.0</td>
<td>20.9</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td></td>
<td>22.8</td>
<td>21.0</td>
<td>21.9</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td></td>
<td>56.6</td>
<td>49.8</td>
<td>53.2</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td></td>
<td>100.0</td>
<td>98.9</td>
<td>99.5</td>
</tr>
</tbody>
</table>
In Table 2 are presented results typical of a group of experiments intended to characterize more clearly the effect of 2,4-D in the region of maximum effect. Growth was inhibited only slightly at $10^{-3}$ M 2,4-D. Inhibition of growth increased progressively with increase in concentration. Total inhibition of growth was noted at concentrations of $3 \times 10^{-2}$ M and higher. The complete series of 2,4-D concentrations and yeast growth is plotted in Figure 1 as percentage growth in terms of the control. It was noted that there was no stimulation of yeast growth at any concentration of 2,4-D within the range investigated. The sigmoid nature of the inhibitory phase of the growth curve was apparent from measurements of transmission of light in yeast suspensions treated with several severely inhibitory concentrations of 2,4-D. Greatest changes in growth were produced in the range of concentrations from $10^{-3}$ to $2 \times 10^{-2}$ M. Further increase in concentration produced smaller increases in inhibition.

In Figure 2 are plotted the mean growth measurements of yeast derived from five experiments. By interpolation it was possible to obtain the 50 per cent effective dose ($ED_{50}$) for 2,4-D. A perpendicular to the abscissa was erected at the point where the curve intersected 50 per cent growth. The concentration which would produce 50 per cent inhibition of growth was found to be approximately $6.5 \times 10^{-3}$ M. The $ED_{50}$ for each experiment was found to lie between $6 \times 10^{-3}$ and $7 \times 10^{-3}$ M.
Table 2. Effect of 20 hours contact with 2,4-D on the growth of yeast. (Growth measured as percentage transmission of light by turbid yeast suspensions.)

<table>
<thead>
<tr>
<th>Date</th>
<th>Concentration of 2,4-D (moles)</th>
<th>Growth 1 (percentage light transmission)</th>
<th>Growth 2 (percentage light transmission)</th>
<th>Mean (percentage light transmission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-16-49</td>
<td>Control</td>
<td>11.8</td>
<td>10.0</td>
<td>10.9</td>
</tr>
<tr>
<td>9-28-49</td>
<td>Control</td>
<td>11.9</td>
<td>11.4</td>
<td>11.7</td>
</tr>
<tr>
<td>9-16-49</td>
<td>$1 \times 10^{-3}$</td>
<td>13.0</td>
<td>13.2</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>36.0</td>
<td>34.9</td>
<td>35.5</td>
</tr>
<tr>
<td>9-28-49</td>
<td>$1 \times 10^{-2}$</td>
<td>47.3</td>
<td>43.5</td>
<td>45.4</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-2}$</td>
<td>88.3</td>
<td>87.2</td>
<td>87.8</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-2}$</td>
<td>99.0</td>
<td>98.8</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>$4 \times 10^{-2}$</td>
<td>99.7</td>
<td>98.8</td>
<td>99.3</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-2}$</td>
<td>98.8</td>
<td>98.2</td>
<td>98.5</td>
</tr>
<tr>
<td>9-16-49</td>
<td>$1 \times 10^{-1}$</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Fig. 1 The growth of yeast in a range of concentrations of 2,4-D.
Fig. 2 The 50 per cent effective dose (ED$_{50}$) of 2,4-D on the growth of yeast.
Recovery of treated cultures

A narrow range of concentrations over which 2,4-D was severely or completely inhibitory to yeast growth in 20 hours, was chosen for studies of recovery. Numerous investigations have shown the inhibitory effect of 2,4-D on growth of microorganisms, but information is not available on the reversibility of such inhibitions. In the present work yeast was grown for 20 hours in 2,4-D, and then the 2,4-D solution was removed by centrifugation. The yeast cells were washed, resuspended in fresh nutrient, and growth was measured for two additional days. By this method it was possible to learn something of the ability of yeast to recover from treatment, as well as to determine the concentration of 2,4-D at which death of the original inoculum occurred.

Results typical of three experiments on recovery of yeast from 2,4-D treatment are presented in Table 3. Initially, severe inhibition of yeast growth was obtained by treatment with 2 and 2.5x10^{-2} M 2,4-D. Complete inhibition was produced by concentrations of 3x10^{-2} M and higher. After washing, resuspension in nutrient medium alone, and incubation for 20 hours, growth of yeast previously treated with 2 and 2.5x10^{-2} M 2,4-D equalled that of untreated yeast. The severe inhibition produced at these concentrations was overcome rapidly. After an additional 24 hours of incubation, growth still equalled that of the control. Yeast previously treated with 3x10^{-2} M 2,4-D recovered to a large extent in 20 hours, and by 44 hours growth equalled that of the control. When previously treated with 3.5x10^{-2} M 2,4-D there was partial recovery after 20 hours in fresh nutrient and complete recovery after 44 hours.
Table 3. Recovery of yeast incubated for 20 hours in 2,4-D, then grown in pure nutrient medium. (Growth measured as percentage transmission of light by turbid yeast suspensions.)

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (mols)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 Hours in 2,4-D</td>
</tr>
<tr>
<td>Control</td>
<td>8.9</td>
</tr>
<tr>
<td>2x10^{-2}</td>
<td>92.3</td>
</tr>
<tr>
<td>2.5x10^{-2}</td>
<td>97.3</td>
</tr>
<tr>
<td>3x10^{-2}</td>
<td>99.5</td>
</tr>
<tr>
<td>3.5x10^{-2}</td>
<td>99.6</td>
</tr>
<tr>
<td>4x10^{-2}</td>
<td>100.0</td>
</tr>
<tr>
<td>4.5x10^{-2}</td>
<td>100.0</td>
</tr>
<tr>
<td>5x10^{-2}</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\*No further growth in three days.
Yeast pre-treated with 4x10^{-2} M 2,4-D showed no growth after 20 hours. The depressing effect was not permanent, however. After 44 hours growth was nearly equal to that of the control. Permanent inhibition of growth, considered to be death of all cells, was produced by the initial 20 hour period in 4.5 and 5x10^{-2} M 2,4-D. Measurements after 20 and 44 hours in fresh medium showed no growth of the original inoculum, and no further recovery of growth was noted in three days of additional incubation.

The critical concentrations of sodium 2,4-D on the growth of yeast in the nutrient medium at pH 6.0 were:

(a) 10^{-6} M — no inhibition of growth below this concentration.

(b) 10^{-3} M — slight inhibition below and marked inhibition above this concentration.

(c) 6.5x10^{-3} M — concentration required to produce 50 per cent inhibition of growth.

(d) 3x10^{-2} M — minimum concentration required to produce complete inhibition of growth.

(e) 4.5x10^{-2} M — minimum concentration required to produce irreversible, complete inhibition of growth.

Additional studies of recovery of growth of yeast treated with 2,4-D were conducted in a different manner. Sufficient 2,4-D to produce the desired concentrations was dissolved in single strength nutrient medium and inoculated directly with 0.1 gm yeast isolated from the interior of a pound cake of bakers' yeast. Yeast was withdrawn from the treatment with a filter stick at several intervals after initiating the experiments. A
Within 2 hours after transfer from the
tracer or exposure to \( \text{NH}_3 \) gas, the
growth was recovered with increases in the
amount of growth, and the amount of growth was related to the length of treatment
after 6 hours in fresh medium. Measurable growth was recorded after 16
hours. Growth of yeast pretreated with \( \text{NH}_3 \) gas and subsequently
pretreated with nutrient medium did not noticeably affect subsequent yeast growth.

Preliminary time periods followed by transfers of an inoculum to fresh
medium in untreasted medium for
are means of triplicate determinations. Growth in untreated medium for
treatment \( \text{NH}_3 \) for 60, 72, and 96 hours are presented in Table 4. The data
show that with prior treatment in concen-

Preliminary experiments showed that prior treatment in concen-

with the \( \text{KCN} \) showed that

The fitter paper was dropped into a test tube
paper piece was washed by immersion of the tip of the filter stick in

These experiments with a thread end into which was fitted a small filter
Table 4. Recovery of yeast incubated with 2,4-D for several time intervals, then grown in pure nutrient medium. (Growth measured as percentage transmission of light by turbid yeast suspensions.)

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (mols)</th>
<th>Time after removal from treatment (hours)</th>
<th>Growth Duration of treatment (percentage light transmission)</th>
<th>8 hrs</th>
<th>16 hrs</th>
<th>24 hrs</th>
<th>32 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>59.2 56.4 56.6 58.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>14.6 15.9 16.0 18.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>12.5 13.5 12.9 13.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>11.7 12.4 12.0 13.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>11.4 12.1 11.3 13.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3x10^{-2} M</td>
<td>8</td>
<td>97.4 98.1 98.8 100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>52.0 61.8 81.0 87.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>14.7 13.7 16.7 23.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>13.5 12.3 13.3 13.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>13.0 11.7 12.8 12.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6x10^{-2} M</td>
<td>8</td>
<td>99.3 100.0 100.0 100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>98.5 99.3 100.0 100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>77.6 91.0 92.8 95.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>30.7 75.0 71.0 79.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>13.4 70.4 67.4 70.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
treatments, however, growth equalled that of the controls and was independent of the duration of pretreatment.

Growth of yeast pretreated with $6 \times 10^{-2}$ $\mu$g 2,4-D was not measurable within 16 hours after transfer to fresh nutrient. Growth of the yeast transferred from 8 hours pretreatment increased steadily and reached the control levels within 40 hours. The 16, 24, and 32 hour pretreatments showed slight growth after 24 hours and a further increase after 32 hours. Additional growth was slight as indicated by the 40 hour readings. There was no progressive inhibition of growth due to pretreatments in excess of 16 hours.

In this series of experiments, growth was not permanently inhibited by any of the treatments. The lack of complete inhibition was probably related to the method of preparation of the original yeast and 2,4-D mixtures. In the earlier experiments the yeast was subcultured three times in nutrient medium prior to inoculation of the treatments, but in the later experiments, where a larger source of inoculum from the treatments was required, yeast was added directly from a commercial cake of yeast. The greater tolerance of yeast under the latter conditions may be attributed to the fact that the yeast was not adapted to the nutrient medium prior to its use. This lack of physiological adaptation may have resulted in less sensitivity to 2,4-D.

Growth and respiration

A part of the respiration data from the next section is reproduced in Figure 3 in order to correlate the effects of 2,4-D on growth and
Fig. 3 Growth and respiration of yeast at several concentrations of 2,4-D.
Effect of 7.4-0 on Respiration

Growth and Respiration

Inhibition to Growth at 7 and 6.4-0, 7.4-0 was inhibitory to both respiration occurred at 6.4-0, a concentration which was completely inhibited to a maximum at 7.4-0. Maximum stimulation of growth was observed above 10-0 7.4-0-0.

Inhibition of growth, respiration was stimulated above 10-0 7.4-0-0 on respiration at a concentration which produced a 50 per cent decrease in oxygen uptake within this range. There was no effect on respiration at 7.4-0-0 between 10-0 and 10-0, but there was no apparent concentration of 7.4-0-0 observed with increased

Growth was inhibited gradually but progressively with increased

The concentration of 7.4-0-0

uptake are expressed as percentage of the control and plotted against phosphate buffer rather than nutrient medium. Both growth and oxygen uptake after treatment. The respiration studies were carried out in 0.07 M phosphate buffer. Both growth and respiration experiments were carried out at pH 6.0. The experiments differed in that the growth studies required

-34-
were used routinely in the exogenous experiments and 20 mg in the endogenous experiments.

**Effect of concentration**

The data presented in Table 5 are a summary of the effect of concentration of 2,4-D on oxygen uptake at pH 6.0. The data were derived from six experiments in which each concentration was replicated three times. Figure 4 shows mean oxygen uptake data obtained from an experiment in which several of the critical concentrations were run simultaneously for a two hour period. In these experiments the 2,4-D was added to the yeast suspension from the side arm at zero time.

There was no effect of 2,4-D on the endogenous, aerobic respiration of yeast, up to and including a concentration of $10^{-2}$ M. Oxygen uptake was stimulated markedly above this concentration and reached a maximum at about $4 \times 10^{-2}$ M. The drop in oxygen uptake above $4 \times 10^{-2}$ M was even more precipitous; at $5 \times 10^{-2}$ M 2,4-D respiration was slightly more than half that of the control, and at $6 \times 10^{-2}$ M respiration was less than one tenth that of the control.

Despite the narrow range of concentrations over which 2,4-D affected respiration of yeast at pH 6.0, the data were readily reproducible. Respiration was not completely inhibited at any concentration employed. However, oxygen uptake of yeast in $6 \times 10^{-2}$ M 2,4-D was very low for the first hour and the rate decreased subsequently to a negligible figure.
Table 5. Effect of several concentrations of 2,4-D on the aerobic, endogenous oxygen uptake of yeast.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Oxygen uptake (cmm/20 mg wet wt.xhour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50.1 49.1 48.0 49.1</td>
</tr>
<tr>
<td>1x10^{-7}</td>
<td>49.4 48.8 47.5 48.4</td>
</tr>
<tr>
<td>1x10^{-5}</td>
<td>48.0 48.6 50.0 48.9</td>
</tr>
<tr>
<td>1x10^{-3}</td>
<td>48.8 49.0 49.5 49.1</td>
</tr>
<tr>
<td>1x10^{-2}</td>
<td>51.2 50.5 50.0 50.6</td>
</tr>
<tr>
<td>2x10^{-2}</td>
<td>62.1 60.4 60.0 60.8</td>
</tr>
<tr>
<td>3x10^{-2}</td>
<td>92.2 85.3 91.0 89.5</td>
</tr>
<tr>
<td>4x10^{-2}</td>
<td>112.2 117.4 115.3 114.9</td>
</tr>
<tr>
<td>5x10^{-2}</td>
<td>25.2 30.2 27.2 27.5</td>
</tr>
<tr>
<td>6x10^{-2}</td>
<td>3.4 4.2 3.6 3.7</td>
</tr>
</tbody>
</table>
Fig. 4. The aerobic, endogenous oxygen uptake of yeast treated with several concentrations of 2,4-D.
centrations of 2-'UO and on the exogenous Hg as presented in Table 7.

The effect of control treatments showed a different response to 2-'UO. The results were 0.5 and 0.25 mM concentrations of carbon dioxide evolution than oxazepam uptake. However, there was less incorporation of carbon dioxide evolution and carbon dioxide evolution were inhibited at

*basic respiratory substrate.*

2-'UO and oxazepam with no significant change in the

For this range of concentrations, there was a marked effect of

3' and 5' oxazepam were 0.26, 0.00 and 0.26 respectively. It was apparent
dioxide evolution to the same degree. Thus, the mean Hg values for 2-'UO

2' and 5' oxazepam were 0.99 and 0.99, respectively. The mean Hg values for 2-'UO

The results of Table 6 show the effect of several concentrations of

the effect of control treatments. Only when 2-'UO was incorporated, the

dioxide evolution showed that 2-'UO was significantly inhibited at low

2-'UO on oxazepam uptake of yeast. Simultaneous measurements of carbon

as indicated earlier, there was no effect of low concentrations of

The respiratory quotient

-47-
Table 6. Aerobic, endogenous oxygen uptake, carbon dioxide evolution, and respiratory quotients of yeast treated with 2,4-D.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Oxygen uptake (mm/20 mg wet wt.hour)</th>
<th>Carbon dioxide evolution (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.0</td>
<td>51.9</td>
</tr>
<tr>
<td>2x10^{-2}</td>
<td>74.8</td>
<td>69.0</td>
</tr>
<tr>
<td>3x10^{-2}</td>
<td>86.4</td>
<td>80.0</td>
</tr>
<tr>
<td>4x10^{-2}</td>
<td>121.6</td>
<td>111.4</td>
</tr>
<tr>
<td>5x10^{-2}</td>
<td>16.0</td>
<td>42.8</td>
</tr>
<tr>
<td>6x10^{-2}</td>
<td>4.2</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table 7. Aerobic, exogenous oxygen uptake, carbon dioxide evolution, and respiratory quotients of yeast treated with 2,4-D, in 0.5 per cent glucose.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Oxygen uptake (mm/5 mg wet wt.hour)</th>
<th>Carbon dioxide evolution (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>122</td>
<td>185</td>
</tr>
<tr>
<td>3x10^{-2}</td>
<td>124</td>
<td>228</td>
</tr>
<tr>
<td>4x10^{-2}</td>
<td>122</td>
<td>223</td>
</tr>
<tr>
<td>5x10^{-2}</td>
<td>77</td>
<td>131</td>
</tr>
<tr>
<td>6x10^{-2}</td>
<td>32</td>
<td>46</td>
</tr>
</tbody>
</table>
Untreated yeast had an RQ of 1.51; there was an increase to near 1.8 at 3, 4, and 5\times10^{-2} \text{ M} 2,4-D. When the highly inhibitory concentration of 6\times10^{-2} \text{ M} was reached, the RQ decreased to 1.44.

**Endogenous and exogenous respiration**

The type of respiratory response of yeast to 2,4-D is dependent upon the presence and amount of exogenous carbohydrate in the suspending medium. In these experiments glucose was used as the carbohydrate source. Concentrated glucose solutions were made up fresh for each experiment, and added to the yeast suspensions in the Warburg flasks to produce the desired concentration.

The effect of 2,4-D on oxygen uptake and carbon dioxide evolution in the presence of 0.5 per cent glucose is presented in Table 7. There was progressive inhibition of oxygen uptake with increasing concentration of 2,4-D from 3 to 6\times10^{-2} \text{ M}. However, carbon dioxide evolution was stimulated slightly at the lower rates, as reflected in the higher RQ values at 3 and 4\times10^{-2} \text{ M}. At 5 and 6\times10^{-2} \text{ M} 2,4-D, carbon dioxide evolution was progressively inhibited with a resultant lowering of the RQ to near control levels.

Results typical of the effect of 2,4-D on aerobic, exogenous oxygen uptake are presented in Figure 5. The 2,4-D was tipped into the flasks at zero time and the respiratory response in 0.5 per cent glucose was determined over a period of two hours. Oxygen uptake was progressively inhibited from 2 to 5\times10^{-2} \text{ M} 2,4-D.
Fig. 5 The aerobic, exogenous oxygen uptake of yeast at several concentrations of 2,4-D. The external carbohydrate used was 0.5 per cent glucose.
In Table 8 is presented a comparison of a range of concentrations of 2,4-D on the endogenous and exogenous oxygen uptake of yeast. Oxygen uptake increased to a maximum of 238.6 per cent of the untreated control at 4x10⁻² M 2,4-D in the endogenous treatments. Further increase in concentration caused a drop to 8.5 per cent of the control at 6x10⁻² M. However, in the presence of 0.5 per cent glucose there was a steady decrease in oxygen uptake from the lowest to the highest concentration of 2,4-D. At 5x10⁻² M 2,4-D oxygen uptake was only 11.7 per cent of the control. The 2,4-D was inhibitory both exogenously and endogenously at a concentration of 5x10⁻² M, but the degree of inhibition was markedly greater in the presence of 0.5 per cent glucose.

The results of Table 9 show the effect of a range of glucose concentrations on the inhibitory effect of 2,4-D on aerobic oxygen uptake. Glucose increased respiration of the controls by a factor of 10, with no pronounced difference between the several concentrations employed. As has been noted previously, certain levels of 2,4-D increased the endogenous respiration of yeast. In contrast, oxygen uptake was progressively inhibited with increasing concentration of 2,4-D at all levels of glucose. There was a tendency, at 3 and 4x10⁻² M 2,4-D toward lower respiration at the highest concentration of glucose. However, at 5x10⁻² M 2,4-D there was no clear relationship between inhibition and concentration of glucose.
Table 8. The effect of 2,4-D on the aerobic, endogenous and exogenous oxygen uptake of yeast. (The exogenous source of carbohydrate was 0.5 per cent glucose.)

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Endogenous</th>
<th>Exogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$O_2$ uptake (cm³/10 mg x hr)</td>
<td>percentage of control (%)</td>
</tr>
<tr>
<td>Control</td>
<td>24.6</td>
<td>100.0</td>
</tr>
<tr>
<td>$2 \times 10^{-2}$</td>
<td>30.2</td>
<td>122.7</td>
</tr>
<tr>
<td>$3 \times 10^{-2}$</td>
<td>42.7</td>
<td>173.6</td>
</tr>
<tr>
<td>$4 \times 10^{-2}$</td>
<td>58.7</td>
<td>238.6</td>
</tr>
<tr>
<td>$5 \times 10^{-2}$</td>
<td>15.1</td>
<td>61.3</td>
</tr>
<tr>
<td>$6 \times 10^{-2}$</td>
<td>2.1</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Table 9. Respiratory response of yeast treated with 2,4-D to a range of concentrations of glucose.

<table>
<thead>
<tr>
<th>Glucose concentration (%)</th>
<th>Oxygen uptake 2,4-D concentration (moles/liter)</th>
<th>Control (cm³/10 mg wt. x hour)</th>
<th>$3 \times 10^{-2}$</th>
<th>$4 \times 10^{-2}$</th>
<th>$5 \times 10^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td></td>
<td>28</td>
<td>36</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>298</td>
<td>263</td>
<td>254</td>
<td>144</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>282</td>
<td>263</td>
<td>254</td>
<td>120</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>282</td>
<td>263</td>
<td>242</td>
<td>192</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>282</td>
<td>254</td>
<td>188</td>
<td>148</td>
</tr>
</tbody>
</table>
Further evidence for the reversal of the effect of 2,4-D in the presence of glucose is presented in Table 10. In this case $5 \times 10^{-3} \text{M}$ 2,4-D acid was used at pH 5.0 in $\text{M/40}$ phthalate buffer. The 2,4-D was tipped into the yeast after 60 minutes. The initial rates of oxygen uptake were uniform, and permitted close comparison of subsequent treatments. Untreated, endogenous oxygen uptake decreased markedly after the first hour and then remained fairly steady for the duration of the experiment. Untreated, exogenous respiration was maintained at a high level throughout. Treatment with 2,4-D without glucose produced an initial stimulation, followed by a decrease in the fourth hour. However, the final rate in 2,4-D alone was higher than that of the control. In 2,4-D with glucose there was a reduction from the initial untreated rate of 283 ccm per hour to a treated rate of 49 ccm per hour. This inhibited rate of oxygen uptake was maintained for the remainder of the experiment.

Experiments with low concentrations of glucose showed that addition of an exogenous carbohydrate did not cause permanent inhibition of oxygen uptake by 2,4-D. The results of Table 11 are typical of the effect of $5 \times 10^{-3} \text{M}$ 2,4-D acid at pH 5.0 on aerobic oxygen uptake in several dilute solutions of glucose. The 2,4-D was tipped in after the 60 minute reading. Therefore, the first two readings in each column are for untreated yeast respiring in the different concentrations of glucose.

Untreated, endogenous oxygen uptake declined for the first hour and then maintained a steady rate for the duration of the experiment. Treated, endogenous respiration declined similarly before addition of the 2,4-D and then rose to a high value which decreased slightly toward the
Table 10. Effect of $5 \times 10^{-3}$ M 2,4-D acid on aerobic, endogenous and exogenous oxygen uptake. (The 2,4-D was added after the 60 minute reading.)

<table>
<thead>
<tr>
<th>Time interval (minutes)</th>
<th>Oxygen uptake</th>
<th>Control</th>
<th>Control + 0.5% glucose</th>
<th>2,4-D</th>
<th>2,4-D + 0.5% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-60</td>
<td></td>
<td>23</td>
<td>283</td>
<td>23</td>
<td>283</td>
</tr>
<tr>
<td>60-120</td>
<td></td>
<td>7</td>
<td>260</td>
<td>35</td>
<td>49</td>
</tr>
<tr>
<td>120-180</td>
<td></td>
<td>8</td>
<td>264</td>
<td>34</td>
<td>49</td>
</tr>
<tr>
<td>240-300</td>
<td></td>
<td>5</td>
<td>260</td>
<td>9</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 11. Regulating effect of glucose on the stimulation or inhibition of yeast respiration by 2,4-D acid. (The 2,4-D was tipped into yeast in the several glucose concentrations after the 60 minute reading.)

<table>
<thead>
<tr>
<th>Time interval (minutes)</th>
<th>Oxygen uptake</th>
<th>Control</th>
<th>Glucose concentration (%)</th>
<th>5$x10^{-3}$ M 2,4-D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>0-30</td>
<td></td>
<td>20</td>
<td>140</td>
<td>310</td>
</tr>
<tr>
<td>30-60</td>
<td></td>
<td>16</td>
<td>74</td>
<td>266</td>
</tr>
<tr>
<td>60-90</td>
<td></td>
<td>8</td>
<td>26</td>
<td>158</td>
</tr>
<tr>
<td>90-120</td>
<td></td>
<td>8</td>
<td>30</td>
<td>98</td>
</tr>
<tr>
<td>120-150</td>
<td></td>
<td>8</td>
<td>30</td>
<td>88</td>
</tr>
</tbody>
</table>
The results of Table 11 demonstrate that the type of action pro-

the control.

Just half hour the respiration increased but did not reach the level of the respiration of yeast in 0.1 per cent glucose for an hour. During the respiration of yeast throughout the experiment, the Z.4-x-4 depressed respiration of yeast in 0.1 per cent glucose remained nearly constant throughout the experiment. The Z.4-x-4 depressed respiration of yeast in 0.05 per cent glucose was depressed in the presence of 0.05 per cent glucose for 4 hours. In the presence of 0.025 per cent glucose showed a decrease of 0.025 percent respiration by 2.4-x-4 of the usual rate throughout the experiment. The rate of respiration increased in the initial minutes and of the experiment. At 0.025 percent glucose the initial rate of the respiration carboxydrase in the medium to show the usual initial rate. The apparatus was the standard reached 4 minutes in 0.025 per cent glucose, there was a gradual elevation of respiration in 0.025 percent glucose. Upon addition of Z.4-x-4 at 0.025 to yeast respiration in the respiration of the carboxydrase. The uninhibited rate then increased dropped from 740 to 74 per 60 minutes, indicating a rapid end of the experiment.
progressive stimulation to a final level well above that of the control which contained no 2,4-D. Not only the concentration of glucose added, but also the rate at which the carbohydrate was utilized by the yeast influenced the type and degree of 2,4-D effect on respiration.

**Aerobic and anaerobic respiration**

Anaerobic conditions in the Warburg flasks were produced by flushing with nitrogen gas previously freed of oxygen. The data presented in Table 12 are summarized from a series of experiments on the effect of 2,4-D on carbon dioxide evolution of yeast respiring endogenously and exogenously, and aerobically and anaerobically. In these experiments, the 2,4-D was tipped into the yeast suspension at zero time. Glucose solution was added to the yeast suspension in the Warburg flasks during preparation of the flasks. The experiments were run at pH 6.0.

**Table 12.** Aerobic and anaerobic carbon dioxide evolution of yeast respiring endogenously and exogenously in several concentrations of sodium 2,4-D.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (mole)</th>
<th>Carbon dioxide evolved</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endogenous 0.5% Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>574</td>
<td>1</td>
<td>745</td>
</tr>
<tr>
<td>2×10⁻²</td>
<td>14</td>
<td>502</td>
<td>10</td>
<td>688</td>
</tr>
<tr>
<td>3×10⁻²</td>
<td>44</td>
<td>474</td>
<td>23</td>
<td>710</td>
</tr>
<tr>
<td>4×10⁻²</td>
<td>45</td>
<td>556</td>
<td>22</td>
<td>690</td>
</tr>
<tr>
<td>5×10⁻²</td>
<td>21</td>
<td>503</td>
<td>13</td>
<td>724</td>
</tr>
<tr>
<td>6×10⁻²</td>
<td>4</td>
<td>91</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>
Yeast respiring aerobically and endogenously showed a stimulation of carbon dioxide evolution when treated with 3 and $4 \times 10^{-2}$ M 2,4-D, and marked inhibition at $6 \times 10^{-2}$ M. There was no effect of 2,4-D on aerobic, exogenous (0.5 per cent glucose) carbon dioxide evolution except at $6 \times 10^{-2}$ M 2,4-D, where respiration was greatly inhibited. Under anaerobic conditions, endogenous carbon dioxide evolution was stimulated markedly at all concentrations of 2,4-D used. Greatest stimulation occurred at 3 and $4 \times 10^{-2}$ M, the same concentrations which produced maximum stimulation under aerobic conditions. Anaerobic, exogenous respiration was unaffected except at $6 \times 10^{-2}$ M 2,4-D.

No stimulation of respiration by 2,4-D was noted under either aerobic or anaerobic conditions in the presence of 0.5 per cent glucose. In both cases respiration was severely inhibited by $6 \times 10^{-2}$ M 2,4-D. Aerobic, endogenous respiration was stimulated at the intermediate concentrations and inhibited strongly at $6 \times 10^{-2}$ M. Endogenous carbon dioxide evolution in nitrogen was stimulated at all concentrations of 2,4-D used, but maximum stimulatory concentrations coincided with those active in aerobic, endogenous respiration. Unlike the aerobic, endogenous effects, however, there was no inhibition of anaerobic, endogenous respiration.
Effects of pH and 2,4-D Compounds

The experiments on the effect of pH on the respiratory response of yeast to 2,4-D were carried out at pH's 3.3, 4.0, 5.0, 6.0, and 8.0. M/40 phthalate buffer was used at pH's 3.3, 4.0, and 5.0. M/40 phosphate buffer was employed at pH's 6.0 and 8.0. Exploratory studies with these buffers showed that there was no effect of either buffer on yeast respiration at the concentrations used.

The effect of 2,4-D on aerobic, endogenous oxygen uptake at each pH level was determined by running a concentration curve of the chemical. The results of Table 13 show that there was no effect of 2,4-D at any concentration between $10^{-4}$ and $10^{-2}$ M when run at pH 6.0. However, at pH 4.0, there was marked stimulation of oxygen uptake at $10^{-3}$ M 2,4-D, and at concentrations of $5x10^{-3}$ M and higher, the chemical was severely inhibitory.

Over the concentration range of 3 to $5x10^{-2}$ M 2,4-D, the chemical was shown to be strongly inhibitory at pH 4.0 for all concentrations used (Table 14). However, at pH 6.0, oxygen uptake was stimulated at all concentrations, with a maximum stimulation at $4x10^{-2}$ M 2,4-D.

The relationship between pH and response to 2,4-D is summarized in Figure 6. The percentage oxygen uptake is plotted against concentration of 2,4-D for each pH level. As the pH of the 2,4-D solution was raised, a greater concentration of the chemical was required to produce stimulation or inhibition of respiration. There was an increased stimulation with increase in the pH, with some anomaly.
Table 13. Effect of pH on the aerobic, endogenous oxygen uptake of yeast treated with several concentrations of sodium 2,4-D.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Oxygen uptake</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0</td>
<td>pH 6.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55.6</td>
<td>55.6</td>
<td></td>
</tr>
<tr>
<td>1x10^-4</td>
<td>54.2</td>
<td>52.6</td>
<td></td>
</tr>
<tr>
<td>1x10^-3</td>
<td>108.0</td>
<td>54.2</td>
<td></td>
</tr>
<tr>
<td>5x10^-3</td>
<td>2.8</td>
<td>56.9</td>
<td></td>
</tr>
<tr>
<td>1x10^-2</td>
<td>0.7</td>
<td>56.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 14. Aerobic, endogenous oxygen uptake of yeast treated with sodium 2,4-D at two levels of pH.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (moles)</th>
<th>Oxygen uptake</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.0</td>
<td>pH 6.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>49.5</td>
<td>58.1</td>
<td></td>
</tr>
<tr>
<td>3x10^-2</td>
<td>12.0</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td>4x10^-2</td>
<td>15.8</td>
<td>121.0</td>
<td></td>
</tr>
<tr>
<td>5x10^-2</td>
<td>16.5</td>
<td>76.2</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 6 Aerobic, endogenous respiration of yeast at several pH levels and a range of 2,4-D concentrations.
showing in either a high value at pH 4 or a low value at pH 5. Possibly the peak concentration was missed at pH 5. It was found also that as the pH was raised, the difference in concentration between maximum stimulation and inhibition decreased. The data for pH 8.0 are incomplete because of limitations in the solubility of 2,4-D. Table 15 presents the critical concentrations of 2,4-D required to produce a standard response at the several pH values.

The compounds of 2,4-D of greatest agricultural importance at present are the various amine salts and the alkyl esters. The sodium salt was used in earlier practice. Little practical use has been found for the acid form of 2,4-D because of its low solubility. In the present investigations the effects of the several 2,4-D compounds on respiration have been studied in an attempt to understand more clearly the differences in their herbicidal activity.

The results presented in Table 16 are typical of six experiments comparing the activity of several 2,4-D compounds. The experiment was carried out at pH 5.0 and the chemicals were tipped in from the sidearm at zero time. The sodium and amine salts, and the n-butyl ester of 2,4-D gave an immediate stimulation of oxygen uptake. There was an initial depression of respiration by the 2,4-D acid. The depressing effect of the acid was not always apparent, but in all the experiments the initial stimulation caused by the 2,4-D acid was markedly less than that of the other compounds. The stimulation of respiration caused by 2,4-D increased gradually with time for the salts and the ester. There was a rapid stimulation in the 2,4-D acid treatments to levels well
Table 15. The effect of pH on maximum stimulation and 50 per cent inhibition of aerobic, endogenous oxygen uptake by 2,4-D-treated yeast.

<table>
<thead>
<tr>
<th>Respiratory response</th>
<th>Concentration of 2,4-D required to produce response (moles per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>Maximum stimulation</td>
<td>(1 \times 10^{-4})</td>
</tr>
<tr>
<td>50 per cent inhibition</td>
<td>(5 \times 10^{-4})</td>
</tr>
</tbody>
</table>

Table 16. The differential effect of several compounds of 2,4-D on the aerobic, endogenous respiration of yeast.

<table>
<thead>
<tr>
<th>Time interval (minutes)</th>
<th>Sodium salt monohydrate (percentage of control)</th>
<th>Monoisopropenol amine salt</th>
<th>Acid</th>
<th>n-Butyl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30</td>
<td>142.3</td>
<td>127.9</td>
<td>89.4</td>
<td>128.7</td>
</tr>
<tr>
<td>30-60</td>
<td>127.0</td>
<td>128.5</td>
<td>136.3</td>
<td>128.5</td>
</tr>
<tr>
<td>60-90</td>
<td>148.2</td>
<td>143.5</td>
<td>191.2</td>
<td>143.0</td>
</tr>
<tr>
<td>90-120</td>
<td>147.7</td>
<td>147.7</td>
<td>227.3</td>
<td>143.2</td>
</tr>
<tr>
<td>120-150</td>
<td>125.2</td>
<td>147.2</td>
<td>229.8</td>
<td>132.5</td>
</tr>
<tr>
<td>150-210 *</td>
<td>160.4</td>
<td>170.5</td>
<td>183.7</td>
<td>154.2</td>
</tr>
<tr>
<td>250-310 **</td>
<td>187.3</td>
<td>180.0</td>
<td>143.7</td>
<td>170.0</td>
</tr>
<tr>
<td>1250-1430</td>
<td>189.3</td>
<td>221.4</td>
<td>126.2</td>
<td>305.0</td>
</tr>
</tbody>
</table>

*Flasks opened for 40 minutes after the 210 minute reading.

**Flasks opened overnight.
above those of the other compounds for a short while. This was followed by a decrease in the stimulatory action of the acid. The measurements taken after long exposure to the 2,4-D compounds showed that the salts continued to increase stimulation slightly. Stimulation due to the acid continued to decrease, and the stimulatory effect of the ester increased greatly.

Figure 7 shows the effect of the several 2,4-D compounds on the rate of oxygen uptake. At 5x10^{-3} M the salts and ester were found to stimulate oxygen uptake throughout the experiment. Within the experimental period, the stimulation due to the 2,4-D acid treatment did not exceed that of the salts, but the rate of inception of stimulation was slower and the rate of oxygen uptake was steady after the first hour, while that of the control and the other treatments decreased.

The data of Table 17 represent the results obtained from three experiments on the effect of concentration of the several 2,4-D compounds on respiration. It was found that at 10^{-3} M, the salts and the acid produced little stimulation. At 5x10^{-3} M, the salts and the acid were highly stimulatory, and at 7.5x10^{-3} M these 2,4-D compounds severely inhibited oxygen uptake. However, the ester proved to be stimulatory at all three concentrations.
Fig. 7 Aerobic, endogenous oxygen uptake of yeast treated with four 2,4-D compounds.
Table 17. Effect of three concentrations of 2,4-D on the differential effects of several 2,4-D compounds on yeast respiration.

<table>
<thead>
<tr>
<th>2,4-D compounds</th>
<th>Oxygen uptake</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of 2,4-D</td>
<td>1x10^-3 M</td>
<td>5x10^-3 M</td>
<td>7.5x10^-3 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monohydrate sodium salt</td>
<td></td>
<td>103.0</td>
<td>166.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Monoisopropanol amine salt</td>
<td></td>
<td>103.0</td>
<td>160.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td>104.6</td>
<td>185.5</td>
<td>2.1</td>
</tr>
<tr>
<td>γ-Dutyl ester</td>
<td></td>
<td>129.2</td>
<td>117.6</td>
<td>144.2</td>
</tr>
</tbody>
</table>

*0.5 Per cent Tween 20 was added to all treatments.
Effects of 2,4-D on Glucose Utilization and Polysaccharide Content

The pronounced alteration in level of respiration, as well as the change in the RQ of yeast treated with 2,4-D, indicated that there was an effect of the chemical on carbohydrate metabolism. The utilization of added glucose was determined for yeast treated with 2, 4, and 6x10^{-2} M 2,4-D. These concentrations were selected because of their marked effects on growth and respiration at pH 6.0.

The data of Table 18 show that 2,4-D inhibited the utilization of glucose by yeast, as measured by the residual glucose in the medium. There was only slight inhibition at 2x10^{-2} M, but increase in concentration decreased glucose utilization. The 2,4-D did not interfere with reducing sugar determinations. A very low value was obtained for 2,4-D alone and there was complete recovery of added glucose in a glucose and 2,4-D mixture.

Where no glucose was added to the yeast suspension in 2,4-D, there was a slight increase in the reducing sugar content of the medium. In order to investigate this effect more fully, determinations of glucose utilization were carried out with 4x10^{-2} M 2,4-D. A nutrient solution, identical to that used in the growth experiments, was included.

The data of Table 19 show the results of one of the three experiments run with 4x10^{-2} M 2,4-D. In the presence of added glucose, there was inhibited glucose utilization by 2,4-D in both the nutrient and distilled water media. Glucose utilization was nearly complete within one hour in distilled water when no 2,4-D was added. In nutrient medium
Table 18. Effect of 2,4-D on the aerobic glucose utilization by yeast. (Two hour incubation period.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose in medium (mg/gm yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Yeast + $2 \times 10^{-2}$ M 2,4-D</td>
<td>2.2</td>
</tr>
<tr>
<td>Yeast + $4 \times 10^{-2}$ M 2,4-D</td>
<td>2.2</td>
</tr>
<tr>
<td>Yeast + $6 \times 10^{-2}$ M 2,4-D</td>
<td>2.8</td>
</tr>
<tr>
<td>Yeast + glucose + $2 \times 10^{-2}$ M 2,4-D</td>
<td>4.4</td>
</tr>
<tr>
<td>Yeast + glucose + $4 \times 10^{-2}$ M 2,4-D</td>
<td>10.2</td>
</tr>
<tr>
<td>Yeast + glucose + $6 \times 10^{-2}$ M 2,4-D</td>
<td>18.0</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.8</td>
</tr>
<tr>
<td>Yeast + glucose*</td>
<td>3.4</td>
</tr>
<tr>
<td>$6 \times 10^{-2}$ M 2,4-D</td>
<td>1.6</td>
</tr>
<tr>
<td>Glucose + $6 \times 10^{-2}$ M 2,4-D</td>
<td>51.9</td>
</tr>
<tr>
<td>Glucose*</td>
<td>53.5</td>
</tr>
</tbody>
</table>

*50 mg glucose added at the beginning of the experiment.
Table 19. Effect of $4 \times 10^{-2}$ M 2,4-D on aerobic glucose utilization by yeast. (One hour incubation period.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose in medium (mg/gm yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.31</td>
</tr>
<tr>
<td>Yeast + 2,4-D</td>
<td>1.88</td>
</tr>
<tr>
<td>Yeast + glucose *</td>
<td>2.05</td>
</tr>
<tr>
<td>Yeast + glucose * + 2,4-D</td>
<td>14.76</td>
</tr>
<tr>
<td>Yeast + nutrient</td>
<td>11.40</td>
</tr>
<tr>
<td>Yeast + nutrient + 2,4-D</td>
<td>14.13</td>
</tr>
<tr>
<td>Yeast + nutrient + glucose *</td>
<td>13.56</td>
</tr>
<tr>
<td>Yeast + nutrient + glucose * + 2,4-D</td>
<td>47.64</td>
</tr>
</tbody>
</table>

*50 mg glucose added at the beginning of the experiment.
the reducing sugar concentration was 11.4 mg. This was largely due to
the yeast extract component of the medium. Upon addition of glucose
to the medium the reducing sugar concentration was 13.6 mg, indicating
nearly complete utilization of the glucose. However, when 2,4-D was
added to the nutrient-glucose medium, the reducing sugar value was 47.5
mg, indicating that 2,4-D markedly inhibited glucose utilization in
the nutrient medium.

There was a small but consistent increase in reducing sugar content
of the medium in both the distilled water and nutrient media when
treated with 2,4-D in the absence of added glucose.

The data of Table 20 are a summary of the results of three experi-
ments on the effect of 2,4-D on glucose utilization. The inhibitory
effects of 4x10^-2 M 2,4-D on utilization of added glucose were highly
significant. The increase in reducing sugar content of the yeast sus-
pensions treated with 2,4-D but no glucose was highly significant for
the non-nutrient medium and significant at the 5 per cent level for the
nutrient medium.

Polysaccharide content of treated and untreated yeast was measured
as the reducing substances in the medium after one hour incubation in
the treatments, followed by hydrolysis with HCl. The results of one
experiment presented in Table 21 are typical of the effect of 2,4-D on
the polysaccharide content of yeast. The polysaccharide content of
yeast in distilled water and in nutrient medium was equal. In all cases
where 2,4-D was added to the medium, the polysaccharide content was less
than that of the controls. The dissimilation or inhibited synthesis
Table 2. Effect of 4x10<sup>-5</sup> M 2,4-D on the proflavine content of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reducing substances in hydrolysed yeast (mg/g yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Effect of 4x10<sup>-5</sup> M 2,4-D on the proflavine content of yeast. (One hour incubation period)

Mean ± S.E. Mean

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reducing substances in hydrolysed yeast (mg/g yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Effect of 4x10<sup>-5</sup> M 2,4-D on the proflavine content of yeast. (One hour incubation period)
of polysaccharide was greater due to treatment in the distilled water medium than in the nutrient medium.

A summary of the effect of 2,4-D on the polysaccharide content of yeast is presented in Table 22. The loss of polysaccharides in yeast

Table 22. The effect of $4 \times 10^{-2}$ M 2,4-D on synthesis and degradation of polysaccharides in yeast. (The data are means of two experiments replicated three times.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reducing substances in hydrolysed yeast</th>
<th>Significance of difference between means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (mg/gm yeast)</td>
<td>2,4-D (mg/gm yeast)</td>
</tr>
<tr>
<td>Yeast</td>
<td>97.15</td>
<td>90.90</td>
</tr>
<tr>
<td>Yeast + 50 mg glucose</td>
<td>104.40</td>
<td>94.25</td>
</tr>
<tr>
<td>Yeast + nutrient</td>
<td>97.77</td>
<td>38.66</td>
</tr>
<tr>
<td>Yeast + nutrient + 50 mg glucose</td>
<td>100.09</td>
<td>98.82</td>
</tr>
</tbody>
</table>

*Significant at the 5% level.  **Significant at the 1% level.

treated with 2,4-D in distilled water was highly significant. Greater variability was noted in nutrient solution, but the lower polysaccharide content in the 2,4-D treatment was significant at the 5 per cent level. Synthesis of polysaccharide from added glucose by untreated yeast was highly significant in the distilled water medium and significant at the 5 per cent level in nutrient medium. In the presence of glucose in the distilled water medium, 2,4-D not only inhibited synthesis
but also caused a loss of polysaccharide. The difference in poly-
saccharide content between treated and untreated yeast was highly
significant. Synthesis of polysaccharide from added glucose was inhib-
ited significantly by 2,4-D in nutrient medium.
A variety of hypotheses have been advanced for the action of 2,4-D on plants. It has been suggested (101) that 2,4-D combines with certain proteins and stimulates the liberation of organic phosphate from phosphorylated compounds. A generation of energy-rich phosphate bonds in the metabolism of phenoxyacetic acids in plants has also been postulated (74). A common mode of action of 2,4-D and tetraethyl pyrophosphate through their effects on aerobic metabolism has been suggested (37). In seeds (43) and microorganisms (116) it has been postulated that 2,4-D acts by rendering oxygen unavailable to the organisms, indicating that the aerobic phase of respiration is the most sensitive to 2,4-D. The hypothesis that 2,4-D acts through its effect on respiratory metabolism (86) or directly to inhibit certain respiratory enzymes (57) has much support.

It was suggested (103) that 2,4-D may act by inducing accumulation of toxic intermediates, such as coumarin derivatives, but further work (107) showed that the coumarin compounds do not produce typical 2,4-D responses when applied to plants. Another hypothesis was that 2,4-D may compete with endogenous auxin (107). The deficiency of normal auxin at the sites of attachments produced by 2,4-D competition would then lead to malformation through nonpolarized cell division, alteration of physiological responses, and finally to death.
According to one suggestion, 2,4-D may be oxidized in the plant to 2,6-dichloro-3-glycolic acid quinone which could then be reduced to the corresponding quinol (49). The toxicity of these compounds might be the determining factor in producing injury or death of the plant. However, no direct evidence of the toxic effect of these compounds has been reported. The destruction of the 2,4-D molecule in the presence of light and riboflavin has been demonstrated (39), and it was suggested that the reaction between 2,4-D and riboflavin may have physiological significance.

It has been shown in these investigations that 2,4-D does not stimulate the growth of yeast in nutrient medium containing exogenous carbohydrate. The 2,4-D was only slightly inhibitory over a wide range of lower concentrations. When the critical level of $10^{-3}$ M was exceeded, growth was severely inhibited. Complete inhibition was apparently obtained with $3x10^{-2}$ M. However, complete inhibition of growth is not a good indication of the effectiveness of an inhibitor. The concentration at which complete inhibition is obtained is difficult to determine directly, because near the zero growth level there is often a range over which growth is gradually suppressed. Furthermore, with microorganisms the inhibition may be transient and reversible. Although growth of most of the cells may be completely inhibited, a few remaining viable cells may, upon removal of the inhibitor, produce a population density equaling that of the control.

A more easily obtained and more readily reproducible measure of the effect of 2,4-D and other inhibitors, is the ED$_{50}$, the 50 per cent
effective dose. Interpolation of the data obtained for growth of yeast in 2,4-D showed an ED$_{50}$ of 6.5x10$^{-3}$ M at pH 6.0. The concentrations found to be completely inhibitory to yeast growth are considerably lower than the 10$^{-1}$ M reported by Currier (21) to be required to kill Allium epidermis, Anacharis leaf tissue, and Beta root parenchyma tissue.

Under normal conditions of herbicide application, a plant cell or group of cells is not continually exposed to lethal concentrations of 2,4-D. It is of importance, therefore, to understand not only the growth inhibition produced, but also the capability of affected cells to recover after treatment. Studies of this nature in higher plants are complicated by environmental factors within the plant. Alteration of translocation, water uptake, and normal auxin effects mask the response at the cellular level. With yeast it was possible to evaluate cellular reaction independently of the many variables encountered in work with tissues or whole plants.

It must be assumed initially that the physiology of plants or plant tissues is basically that of the component cells, and that gross alterations in morphology, chemical composition, and other responses of the plant are simply manifestations of a complex of stimulatory or inhibitory reactions occurring at the cell level. It must be realized, however, that the internal economy of an intact plant is governed by the interaction and interdependence of the component cells and tissues, and projection of effects of growth regulators from single-celled organisms to higher plants must be done with care. However, the basic principles of cell physiology are applicable for a wide variety of
simple and complex organisms. Blackman et al. (10) have noted the similar effects of the dinitrophenols on the physiological responses of diverse organisms. The cellular approach to the physiology of 2,4-D action may be expected to yield information of useful application on higher plants.

It has been demonstrated in the present studies that the inhibitory effect of 2,4-D on yeast growth is reversible. At pH 6.0, concentrations above $2 \times 10^{-2}$ M were severely inhibitory. With concentrations up to $3 \times 10^{-2}$ M, growth was resumed rapidly after removal of the 2,4-D; with 3,5 and $4 \times 10^{-2}$ M, growth recovered slowly, and with $4.5 \times 10^{-2}$ M and above, growth was permanently inhibited. Only at the highest concentrations can 2,4-D be considered to have been lethal under the conditions of these experiments.

Brian and Rideal (13) found that adsorption of 2-methyl,4-chlorophenoxyacetic acid to monolayers of wheat protein was 4 to 5 times as great as to tomato monolayers. They suggested that the foundations of species susceptibility may be based upon the extent of adsorption of the chemical to sites not concerned in the physiological response. The results of the present studies support the hypothesis that the activity of 2,4-D is correlated with the concentration reaching the sites directly concerned in physiological responses. Up to a critical concentration of $4.5 \times 10^{-2}$ M there was apparently a direct relationship between growth and degree of saturation of the growth sites with 2,4-D. A loose bonding between the 2,4-D molecule and its substrate is postulated because growth was resumed readily when 2,4-D solutions were removed and
the cells washed. Crafts (19) suggests a flexible mechanism of chemical and physical attachment in which a balance of forces produces the combination of growth regulator and substrate.

At and above the critical concentration of $4.5 \times 10^{-2}$ M, the growth sites were probably saturated completely with 2,4-D, and removal of the chemical did not permit resumption of growth. This result seems to be best explained by postulating an alteration in structure of the protein or protoplasm upon complete saturation of the points of attachment by 2,4-D. This disruption or destruction of the normal protoplasmic structure may be the cause of the cessation of growth. Virgin (104) noted that herbicides such as 2,4-D produce a decrease in firmness of the protoplasmic membrane and a decrease in the time for plasmolysis. He concluded that herbicides have an extremely pronounced effect on the state of the protoplasm.

Rate of recovery from treatment is related not only to the concentration, but also to the length of time the yeast cells are in contact with 2,4-D. Progressive inhibition with increase in length of treatment may be associated with the time factor in attachment to active growth sites. That this attachment is reversible is shown by the fact that growth may be resumed slowly (Table 4). With a concentration of $6 \times 10^{-2}$ M, 8 hours pretreatment delayed but did not prevent growth. Pretreatment for 16 to 32 hours produced an irreversible static effect on growth, independent of duration of exposure to 2,4-D. This stasis may be the inception of irreversible protoplasmic disruption.
Two possible explanations for the mechanism involved in the alteration of inhibition with duration of exposure to 2,4-D are worthy of consideration. Either penetration is slow and inhibition of growth is a function of the rate of penetration, or attachment or chemical combination at the active growth sites is limited by some factor other than internal concentration of 2,4-D. The hypothesis that penetration is slow seems unlikely because respiratory reactions are altered immediately upon addition of 2,4-D. The complex of aerobic and anaerobic respiratory changes produced by 2,4-D makes the suggestion (77) that reaction occurs at the cell surface in yeast treated with uranium seem unlikely in the case of 2,4-D. Therefore it is assumed that rate of penetration is not limiting. Attachment at the growth sites may be limited by competition for the substrate. A stoichiometric relationship between 2,4-D and growth does not seem likely on the basis of the present results.

Giese and Swanson (33) state that oxygen consumption may still occur in disorganized cells, but the coupling of respiratory energy to the synthetic processes may no longer exist. That this may hold true for the action of 2,4-D on yeast is shown by the relationship between growth and respiration. Although growth was irreversibly inhibited at 4.5x10^{-2} \text{M}, oxygen uptake continued at or above control levels.

Although growth and respiration were both affected by 2,4-D, the growth response was more sensitive. Growth was severely inhibited at 2x10^{-2} \text{M}, while respiration was stimulated slightly. Maximum stimulation of oxygen uptake occurred at 4x10^{-2} \text{M}, a concentration above which
growth was irreversibly inhibited. The relationship of growth and respiration of yeast treated with 2,4-D is very similar to that produced by 2,4-dinitrophenol (DNP). It has been shown (11,12,56) that DNP inhibits growth at concentrations which have no inhibitory effect on respiration. When the concentration of the nitrophenols is raised, both respiration and growth are inhibited (10). The work of Loomis and Lipmann (51) suggests that DNP acts to uncouple phosphorylation from oxidation, thus increasing respiration in tissues in which the phosphorylating system limits the respiratory rate. It has been shown by Brody (15) that 2,4-D uncoupled oxidative phosphorylation in phosphate-deficient rat liver mitochondria. The parallel effects of inhibitors and growth regulators such as 2,4-D have been noted by Smith (86). The data presented on the relationship between growth and respiration of yeast treated with 2,4-D suggest, but do not prove, the operation of a mechanism similar to that of DNP.

Aerobic, endogenous oxygen uptake and carbon dioxide evolution were uniformly stimulated by 2,4-D at 2, 3, and $4 \times 10^{-2}$ M, but at 5 and $6 \times 10^{-2}$ M, carbon dioxide formation was inhibited less than oxygen use. It is significant to note that the high RQ values occurred at the concentrations of 2,4-D which caused irreversible inhibition of growth. Concentrations of 2,4-D just below the critical which caused irreversible inhibition of growth, stimulated respiration but did not affect the RQ. This result indicates that there was no change in the type of substrate oxidized in respiration at 2,4-D levels below $4.5 \times 10^{-2}$ M.
Normally respiring yeast cells dissimilate their carbohydrate reserves aerobically (91,94,95,96). As shown in the present studies, the endogenous, anaerobic carbon dioxide evolution was negligibly low in untreated yeast. However, addition of 2,4-D induced anaerobic dissimulation of reserve carbohydrates, as indicated by the high values for carbon dioxide evolution in nitrogen. The ratio of anaerobic to aerobic, endogenous carbon dioxide evolution increased from approximately 0.5 at 4x10^{-2} M to 2 at 6x10^{-2} M. Aerobic metabolism is in most instances a mixture of the aerobic and anaerobic processes in yeast (90). Therefore, the high RQ values obtained with 5 and 6x10^{-2} M 2,4-D were probably caused by the resultant of inhibited aerobic oxygen uptake and carbon dioxide evolution, and the induction of anaerobic fermentation. McElroy (55) has attributed the action of inhibitors to a greatly stimulated glycolysis which competes with oxidative synthesis for the available hydrogen acceptors, thus inhibiting synthesis.

The data do not clearly indicate whether the high RQ in certain treatments was caused solely by induction of anaerobic fermentation or whether aerobic fermentation was also induced. However, it can be stated with certainty that 2,4-D markedly stimulated endogenous fermentation. It has been found (91,94,96) that any physical or chemical disturbance of the normal structure of the yeast cell leads to induction of endogenous fermentation. Field et al. (27) noted that DNP stimulated fermentation as well as respiration in yeast, and an accelerated endogenous fermentation has been reported in yeast irradiated with ultraviolet light by Giese and Swanson (33). Newcomb (64) found that DNP
As intimated, fermentation in the presence of 2,4-D was recorded action real of rapidity with time. In the presence of 2,4-D a concentration of ethylene occurred at a concentration below 0.1 per cent, rapid—

reached the maximum when a concentration of 22.0 per cent was reached (approximately 0.1)

Acorns et al. (1) noted that the rate of oxygen uptake in yeast

*General description of metabolism functions*

the ethylene fermentation were several hundred-fold higher.

22.0 per cent was reached (approximately 0.1)

and the hydrogen gas values, those of an aerobic container.

increased to the lower levels of 2,4-D and inhibited at higher

reached the maximum at the lower levels of 2,4-D and inhibited at higher

proceeded at higher concentration, carbon dioxide evolution was stimulated.

In the presence of 0.5 per cent ethylene, 2,4-D did not stimulate

metabolites of the compounds of oxidation and synthesis.

metabolites of the compounds of oxidation and synthesis.

were dropped in explaining the induction of fermentation in tobacco

that DMP reversibly undergoes phosphorylation from oxidation has

the suggestion by Locors and Lipman

evolution of carbon dioxide (28). The stimulation by locors and lipman

and berke (26). In yeast, dehydrogenases which in yeast have also been demonstrated by robstein

tobacco cells, tissue. The stimulatory effect of DMP on endogenous

stimulated aerobic fermentation and induced aerobic fermentation in
the 2,4-D. Pickett and Clifton (69) and Reiner and Spiegelman (73)
have demonstrated that DNP and sodium azide block synthesis from glucose
by yeast. Brockmann and Stier (14) found that the rate of glucose
metabolism by yeast is controlled primarily by the rate at which high
energy phosphate bonds are dissipated. Fales (25) concluded from his
studies on the effect of azide on carbon dioxide and ethanol production,
and glucose utilization in yeast, that alteration of these functions may
be explained on the basis of an inhibition of conversion of inorganic
to organic phosphate. He stated that this hypothesis is valid only if
a single locus of action is concerned, and suggested that the complex
changes induced by azide may indicate that there are several loci of
action.

Inhibition of respiration by 2,4-D in the presence of glucose
depended on the concentration of glucose in the medium. At the lower
concentrations of glucose, 2,4-D caused an immediate inhibition of
respiration followed by an increase above control levels with time.
Commoner (17) found that KCN inhibited respiration only above a certain
critical concentration of glucose. He attributed this effect to an
inhibition of the increase in respiration that normally follows the
addition of glucose.

Untreated yeast respiring aerobically in glucose, oxidized 18.4 mg
more glucose per gm of yeast than that treated with \(4 \times 10^{-2} \text{ M} \) 2,4-D,
when calculated on the basis of oxygen uptake. Direct measurement of
glucose uptake showed that 16.2 mg more glucose per gm of yeast was
taken up in one hour by untreated yeast than by treated. The close
agreement between these measurements may indicate that the inhibition of respiration produced by 2,4-D in the presence of glucose is caused by an inhibition of glucose uptake, thus depressing the normal increase produced by added glucose, although a direct depression of respiratory processes is equally possible.

It was shown also that the reserve carbohydrate content of yeast was lowered by treatment with 2,4-D. The fact that there was a net loss of reserve carbohydrates, probably glycogen (93,94), even in the presence of glucose, indicates that 2,4-D can have an important effect in accelerating this dissimilation process, and suggests some interference with phosphorylations. Rainer and Spiegelman (73) postulate that the inhibition of carbohydrate uptake by yeast in the presence of DNP can be explained by the fact that the DNP accelerates dissimilation of stored carbohydrate reserves and thus prevents synthesis from added carbohydrate.

On the basis of respiratory carbon dioxide evolved, 4.0 mg more glucose per gm of treated yeast respiring endogenously was oxidized per hour in the treated samples. Determination of reducing sugars in the acid-hydrolyzed samples showed that in the absence of glucose, treated yeast contained 6.3 mg per gm less glycogen (as glucose) than the untreated. Therefore, approximately two thirds of the dissimilated carbohydrate reserves may be accounted for by the increased respiration. From these calculations it becomes apparent that the stimulatory effect of 2,4-D on yeast respiring aerobically and endogenously is caused by
the increase in respirable substrate produced in the dissimilation of reserve carbohydrates in the presence of 2,4-D.

The data lead to the conclusion that 2,4-D probably acts through stimulation of anaerobic, or aerobic and anaerobic, fermentation, with a simultaneous inhibition of the oxidative synthetic processes involved in growth, possibly through an uncoupling of phosphorylation from oxidation. Stimulated endogenous respiration may be attributed to the accelerating effect of 2,4-D on the dissimilation of reserve carbohydrates, resulting in an increase in respirable substrate. The reduction of exogenous respiration by 2,4-D may be due to an interference with glucose absorption, or may indicate a direct interference with respiratory processes which is not evident when the substrate is limiting in endogenous respiration. Anker (2) studied the effect of indoleacetic acid on yeast respiration and found that stimulation was obtained only at glucose concentrations below the optimum. He showed also that indoleacetic acid accelerated the mobilization of carbohydrate reserves, and concluded that the stimulated respiration resulted from an increased glycogen mobilization which provided a substrate for the endogenous respiration.

The biological activity of weak acids and bases is markedly affected by the pH of the medium in which the compound is dissolved. Recent investigations by Simon and coworkers (81,82,83,84) have shown that the concentration of weak acids necessary to produce a standard response increases as the pH of the medium is raised above the pK. It was noted (82) that the concentrations of hydrogen fluoride, hydrazoic acid,
iodoacetic acid, and 3,5-dinitro-2-cresol required to produce a 50 per cent inhibition of bakers' yeast increased with increasing pH. Below the pK, pH changes did not affect the concentrations required to produce a standard response.

In the present studies the concentration of 2,4-D required to produce maximum stimulation and 50 per cent inhibition of aerobic, endogenous oxygen uptake in bakers' yeast was determined. With a unit increase in pH between 3.3 and 8.0, a 5- to 10-fold increase in total concentration of the weakly acidic 2,4-D was required to produce a standard response. The total concentration of a weak acid is composed of undissociated molecules and ions in varying proportions, depending on the dissociation constant of the compound and the pH of the medium. The concentration of undissociated 2,4-D molecules may be calculated with the equation

$$pH = pK + \log \frac{[\text{ion}]}{[\text{undissociated molecule}]}.$$ 

The pK of 2,4-D has been reported by Audus (4) to be 3.28 at 5x10^{-4} M. Using the value 3.28 for the pK of 2,4-D, the percentages of undissociated molecules at pH 3.3, 4.0, 5.0, 6.0, and 8.0 are 48.85, 16.00, 1.87, 0.19, and 0.002 respectively. The product of the total concentration of 2,4-D and the percentage of undissociated molecules gives the molar concentration of undissociated 2,4-D required for the standard response at each pH. Maximum stimulation at pH 3.3, 4.0, 5.0, 6.0, and 8.0 was produced by 5x10^{-5}, 1.6x10^{-4}, 10^{-4}, 8x10^{-5}, and >2x10^{-5} M undissociated 2,4-D respectively. Similarly, 50 per cent inhibition of
oxygen uptake at the same pH values was obtained with 2.5x10^{-4}, 9x10^{-4},
2x10^{-4}, 10^{-4}, and >2x10^{-5} M undissociated 2,4-D. The solubility of
2,4-D was too low to permit complete determinations at pH 8.0. The
other values in both series are uniform within one unit of magnitude.

Barron et al. (6) have shown that only the undissociated form of
pyruvic acid will penetrate the cell membrane. In the present experi-
m ents the maximum stimulation of oxygen uptake was attained with
approximately 10^{-4} M undissociated 2,4-D throughout the range of pH
values used. Calculation of the molar concentration of undissociated
2,4-D shows a comparable narrow range of concentrations for 50 per cent
inhibition of respiration. One interpretation of these observations
would be that activity is confined to the undissociated molecules. It
is more likely, however, that penetration is limited to undissociated
2,4-D, and that the responses are governed by the pH effects in the
cell.

There was an indication that some factor other than the degree of
dissociation in the external medium influenced the activity of 2,4-D.
The data showed that the concentration of undissociated molecules
required to produce the standard responses increased slightly as the
pH increased from the pK to pH 4.0. Further increase in pH resulted in
a decrease in the concentration of undissociated molecules required to
produce the standard responses. The effect of 2,4-D at pH 4.0 was
further characterized by a higher maximum stimulation than at pH 3.3 or
5.0 (Fig. 6). The non-linear relationship between maximum stimulation
or 50 per cent inhibition of oxygen uptake at several pH values, and
the concentration of undissociated 2,4-D indicates that the ions also contribute to 2,4-D activity. This suggestion is in agreement with the postulates of Simon and Beevers (62), based on investigations which included the effects of several weak acids on yeast respiration and fermentation.

The decrease in the level of maximum stimulation of oxygen uptake with decreasing pH of the 2,4-D solution requires special consideration. It was found that there was marked stimulation at pH 6.0, and the stimulation decreased as the pH was lowered. At the higher pH values, only a small amount of undissociated 2,4-D is present. Lowering the external pH will decrease dissociation, and is assumed to permit increased entry of 2,4-D. In the buffered plant cell, the 2,4-D tends to dissociate equally at the internal pH. Therefore the internal concentration of 2,4-D ions should increase as the pH is lowered. As the concentration of ions increases, the physiological response of stimulated respiration may be suppressed and the more generalized inhibitory reactions become dominant. Thus the degree of stimulation may be dependent upon the internal concentration of 2,4-D ions.

An apparent discrepancy with the postulate that the level of stimulation and the ionic concentration of 2,4-D are related, was the lesser stimulation at pH 5.0 than 4.0 (Fig. 6). The concentration range over which 2,4-D stimulated oxygen uptake was narrow. Because fewer points were used in determining the response at pH 5.0 than at pH 4.0 and 6.0, it is possible that the lower level of stimulation at pH
5.0 was a result of having missed the exact concentration which would cause maximum stimulation.

Experimentally, it has been possible to investigate cellular reaction to 2,4-D only on the basis of external concentration and pH of the medium. However, Simon and coworkers (82,84) and James (44) have pointed out that entry and internal activity may be influenced by both the external pH of the inhibitor medium and the internal pH of the cell. Respiratory response of yeast to 2,4-D in relation to pH is very similar to that of such compounds as hydrogen fluoride and iodoacetic acid (82). Therefore, it is reasonable to assume that a similar mechanism of penetration and pH-activity effects applies for both 2,4-D and these inhibitors.

Crafts (19) postulated that 2,4-D moves through the cuticle of higher plants as nonpolar molecules which dissociate in the cell and react as ions. According to this view (19, p. 264):

Finally, interaction by multipoint attachment at the site of maximum physiological response (meristems) must take place from the ionic form for the protein-regulator complexes postulated involve linkages or bonding through ionic structures.

Brian and Rideal (13) studied the interaction of 2-methyl,4-chloro-phenoxycetic acid with protein monolayers in a Langmuir trough. They noted that alteration of pH changed the ionization of protein carboxyl groups and consequently the interactions of the protein and growth regulator ion were affected.

Before it will be possible to understand completely the activity of 2,4-D and other compounds having growth and respiratory activity, more
knowledge of the internal pH of the cell and its variation within the cell structure will be required. The varied physiological responses of a cell or tissue to an inhibitor or growth regulator such as 2,4-D show that the major effects are produced within the cell rather than at the cell surface. Thus, internal pH of the cell may be a controlling factor in the response of an organism. Whether this controlling influence is exerted through alteration of the internal concentration of the active form of 2,4-D, or whether it is related to ionization of protein and subsequent complexing with the growth regulator ion, must await further experimentation. It seems likely that both mechanisms may be concerned.

The important effects of pH on the activity of 2,4-D in vitro are, to a large extent, nullified when small quantities of the chemical are applied to massive tissues, as in normal herbicidal applications (81). However, the pH of the cells or tissues through which the 2,4-D moves may have profound effects on activity.

The sodium and amine salt compounds of 2,4-D produced nearly equal effects on yeast respiration. The stimulatory effect of the butyl ester was slightly less than that of the salts at $5 \times 10^{-3}$ M and pH 5.0. The 2,4-D acid was initially less stimulatory, then more stimulatory, and finally less stimulatory than the salt compounds with time after treatment. Stimulation of respiration by the ester after extended contact with the yeast was considerably greater than that of the other 2,4-D compounds. Further results showed that the ester was stimulatory both at concentrations which produced no effect and at those which produced severe inhibition of respiration with the 2,4-D salts and acid.
The difference in activity of the ester of 2,4-D may be attributed to the fact that it does not dissociate. Therefore, penetration of the ester of 2,4-D at pH 5.0 would be considerably greater than that of the salts or acid. However, since the ester was added to the yeast in the form of an emulsion, it is expected that less intimate contact with the yeast cells would be produced than with the solutions. Within the cell the ester would have to be hydrolyzed to the active ion form. Therefore, the constant stimulatory effect of the ester over a range of concentrations at which the other compounds have markedly different effects may be explained by the greater penetration of the ester and a slower production of ions within the cell. Beever et al. (8) have shown that the diethyl ester of malonic acid inhibited corn root respiration while the acid had no effect.

The variation in activity of the acid from that of the salt compounds of 2,4-D is difficult to interpret. Simon et al. (84) found that 2,4-D acid was slightly more toxic than its sodium salt. Hamner et al. (38) reported a greater effect of 2,4-D on bean plants when applied as the acid. Van Overbeek (102) suggests that the greater activity of the acid may be explained by the fact that acids move in the lipoid phases of plant cells as the undissociated molecules. Therefore, penetration of the cuticle by the acid would be enhanced.

The work presented here may serve as a foundation for further studies on the effects of 2,4-D in cellular metabolism. Further investigations are needed to determine the role of phosphorylation in the 2,4-D response. The strikingly similar effects of 2,4-D to those of DNP and indoleacetic acid suggest that additional work to determine whether there exists a basic cellular reaction common to many inhibitors would be profitable.
SUMMARY

The effect of 2,4-D on cellular growth, respiration, and carbohydrate metabolism was investigated, using bakers' yeast as the plant material. The use of yeast permitted study of the effect of 2,4-D on cellular metabolism without the complicating factors of translocation and tissue interaction encountered in higher plants.

(1) Yeast growth was not stimulated by any concentration of 2,4-D. It was found that up to a critical concentration and duration of treatment, the inhibitory effect of 2,4-D was reversible. Inhibition of growth was presumably related to the degree of saturation of the active growth sites by 2,4-D. Complete cessation of growth occurred at and above the critical concentration. It is suggested that saturation of the growth sites caused disruption or disorganization of the normal protoplasmic structure.

(2) There was less effect of 2,4-D on aerobic, endogenous respiration than on growth. At the ED_{50} for growth there was no measurable respiratory response, and maximum stimulation of respiration occurred at a concentration which inhibited growth completely.

(3) Aerobic oxygen uptake and carbon dioxide evolution, and anaerobic carbon dioxide evolution in endogenous respiration were stimulated by 2,4-D. The high RQ produced at high concentrations was attributed to induction of fermentation of reserve polysaccharides.
(4) With added carbohydrate, aerobic oxygen uptake and carbon dioxide evolution were inhibited at concentrations of 2,4-D which markedly stimulated endogenous respiration. Anaerobic, exogenous carbon dioxide evolution was unaffected except at the highest concentration used. At this concentration aerobic and anaerobic, exogenous respiration as well as aerobic, endogenous respiration were strongly inhibited, indicating a general disruption of metabolic functions.

(5) Dissimilation of stored polysaccharides was induced by 2,4-D, and glucose uptake was inhibited. It was shown that the stimulatory effect of 2,4-D on aerobic, endogenous respiration could be attributed largely to the production of a respirable substrate during the dissimilation of polysaccharides induced by 2,4-D. The inhibitory effect of 2,4-D on respiration in the presence of glucose may be caused by an inhibited glucose uptake, thereby depressing the normal increase in respiration produced by added glucose, although a direct depression of respiratory processes is also possible.

(6) Lowering the pH of the medium increased the effect of 2,4-D on respiration. The results indicated that penetration was limited to the undissociated molecules, but the respiratory response was also affected by the concentration of 2,4-D ions within the cell.

(7) The 2,4-D ester was initially less stimulatory than the salts, presumably because of less intimate contact of the yeast cells with the 2,4-D in an emulsion. Greater final stimulation of respiration by the ester was attributed to increased penetration of the undissociated compound. The wider range of concentrations over which the ester stimulated
respiration was considered to be caused by the slower production of 2,4-D ions from the nonelectrolytic ester compound.

(8) The marked similarity of reaction of yeast to 2,4-D, DNP, and indoleacetic acid is noted and discussed.
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