Studies on the breakdown of hormone herbicides and allied compounds by soil organisms

by

Kathleen Vera Symonds

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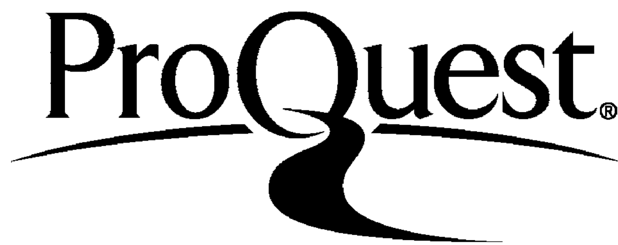

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ABSTRACT OF THESIS

Experiments were carried out using pure cultures of a soil bacterium (B. globiforme) to determine optimum conditions of growth for this organism in 2:4-D culture. The effect of aeration of liquid cultures and the addition of various other substances to the media was recorded. The ability of this organism to grow on other substrates was studied. Assays of phytotoxicity in the culture media were undertaken and the effect of concentration of 2:4-D on its decomposition studied.

Isolations of fresh organisms from garden soil capable of the detoxication of 2:4-D were attempted and these organisms subsequently grown, singly and in mixtures, in 2:4-D culture in an attempt to produce a breakdown in culture similar to that found in soil. Experiments using a soil perfusion technique and also mixtures of organisms obtained from perfusate enriched to 2:4-D were also carried out.

The original isolates became ineffective in 2:4-D decomposition after culture of approximately twelve months and fresh isolations had to be made. These resulted in the eventual isolation from fresh garden soil of a strain of the Actinomycete Nocardia which proved capable of utilising 2:4-D as a carbon source in agar and in aerated liquid culture. Subsequent experiments were carried out using sub-cultures of this organism.

The effects of concentration of 2:4-D and of the presence of a soil extract and agar dialysate on detoxication of the culture fluids were recorded. The growth of this organism in unaerated liquid culture on various carbon substrates was studied and the subsequent ability of organisms so cultured to decompose 2:4-D in agar recorded.

The decomposition of 2:4-D in liquid culture was followed using radioactive samples of 2:4-D labelled in either the methylene or carboxyl groups of the side chain. The changes in radioactivity and phytotoxicity were followed chromatographically and measurements of chloride and phenolic material present in the culture media made throughout the detoxication period. Measurements of radioactivity and phenolic materials absorbed in an alkali trap through which air leaving the cultures was passed were also recorded.

A possible route for the early stages of breakdown of the 2:4-D molecule by this strain of Nocardia is suggested involving the formation of 2:4-dichlorophenol, o-chlorophenol and phenol.

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STUDIES ON THE BREAKDOWN OF HORMONE HERBICIDES AND ALLIED COMPOUNDS BY SOIL ORGANISMS

I. Introduction

When agricultural soils are treated with a solution of 2:4-dichlorophenoxyacetic acid (2:4-D) in the form of the sodium, potassium or ammonium salt, for the purpose of selective weed killing it is found that the herbicidal properties disappear within five to six weeks and the treated soil is then able to support normal growth of susceptible plants. (Newman, Thomas and Walker, 1952). These authors also report that during the detoxification of 2:4-D a substance is produced which stimulates root elongation in cucumber. This substance can be destroyed by steaming. The presence of this stimulatory substance may mask the presence of small quantities of 2:4-D.

The disappearance of 2:4-D from normal soils can be accounted for, as suggested by Audus 1949, by various factors including leaching by rain. Detoxification is not entirely due to leaching since herbicides disappear from normal moist soils in its absence. The main detoxification is therefore assumed to be either a chemical or a biological process. Audus suggested that a biological detoxification occurred and confirmed the hypothesis, 1950, by the isolation of an effective organism subsequently identified as belonging to the Bacterium globiforme group of Bergey. This isolation was made onto a medium containing 0.1% 2:4-D as the only

added carbon source, and shewn to be the organism responsible for 2:4-D breakdown in enriched soils by adding a suspension of the organism to a non-enriched perfusate and obtaining a breakdown rate comparable to that of an enriched soil, and by perfusing a suspension of the organism through a plug of sterile glass wool, in place of soil and obtaining a similar rapid breakdown. The isolate shewed a steady loss of vigour with each successive sub-culture onto 2:4-D agar.

For the work to be described in this thesis it was thought that the breakdown of 2:4-D by this organism could best be studied using liquid cultures containing the essential mineral salts and appropriate concentrations of 2:4-D as the only added carbon source. In view of the instability of the organism in the absence of soil and the general paucity of growth, preliminary experiments were carried out to study the growth and attempt to obtain an improved growth of this organism in liquid culture.

In this work the effect of aeration of the culture fluid and the addition to the medium of various substances in small quantities was studied. The substances used include: low concentrations of agar, insufficient to cause gelling of the liquid, soil extract, yeast extract, calcium chloride, vitamin B_{12}, menaphthane, biotin, casein hydrolysate, and extra phosphate. In addition, the effect of adding various inert materials such as sterile brickdust, glass wool, cotton wool and soil to the medium was noted. The effect on the cultures of aeration by carbon dioxide free air was also studied.

The ability of the organism to utilise additional or alternative carbon sources was also investigated. Carbon sources used in these experiments include:- glucose, sodium benzoate, phenoxyacetic, phenylacetic, 4-chlorophenoxyacetic, 3:4 dichlorophenoxyacetic, 3-chlorophenoxyacetic, 2:5 dichlorophenoxyacetic, 2-chlorophenoxyacetic and 2:4-dimethyphenoxyacetic acids; o-chlorobenzoic, 4-iodophenoxyacetic, α-4-chlorophenoxypropionic, 2:4:5-trichlorophenoxyacetic, α-2:4-dichlorophenoxypropionic and 2:3:5-tri-iodobenzoic acids. The effect of size of inoculum on growth and of additional doses of 2:4-D given both before and after detoxification of the original application was noted.

During these experiments growth of the organism was measured by direct counting using a haemacytometer, phytotoxicity of the solutions by the inhibition produced by a sample on the growth of cress seedling roots, and a preliminary investigation made of the culture solution by paper chromatography, for intermediate substances possibly produced during the detoxification process.

The subcultures of the original Bacterium globiforme isolate proved incapable of detoxificating 2:4-D in liquid culture after a period of twelve months storage on 2:4-D agar with subcultures onto fresh slopes made every four to six weeks.

Fresh isolations of organisms were made from 2:4-D enriched soil in perfuser and aerated flask suspensions. Subsequent experiments were carried out with one of these isolates which detoxificated 2:4-D in pure culture and shewed morphological similarities in 2:4-D liquid culture, to the original organism.

This second organism was subsequently identified as belonging to the genus Nocardia.

Experiments carried out with the Nocardia organism include studies to determine the effect of 2:4-D concentration on detoxification, the effect of soil extract, and of dialysate of agar on detoxification. Investigations on the breakdown of radioactive 2:4-D labelled with C^{14} in the methylene or carboxyl group of the sidechain were carried out with this organism. Growth studies of the Nocardia in unaerated liquid culture on various carbon substrates were made.

From the findings recorded in the results of these experiments a possible route for the destruction of the 2:4-D molecule by this organism is suggested, involving the production of 2:4-dichlorophenol as a first step and the subsequent neutralisation of the toxic effect of this substance on the organisms concerned, in pure culture.

II. Experiments carried out with 2:4-D decomposing organism of the Bacterium globiforme group

1. Introduction

This organism was isolated by Audus, 1950, from a soil perfusate enriched to 2:4-D and plated onto agar containing 100 p.p.m. 2:4-D as the only added carbon source. Experiments were carried out to study the growth characteristics of this organism in liquid culture containing an appropriate concentration of 2:4-D and in the presence of other carbon substrates. An attempt was made to correlate growth with the rate of breakdown of 2:4-D in the cultures. The possible presence of phytotoxic intermediate products in the culture medium was investigated chromatographically.

2. Experimental methods

a) Maintenance of stock cultures of B.globiforme

Stock cultures were as far as possible kept in liquid media containing:-

Magnesium sulphate	0.2 grm.
Potassium chloride	0.2 grm.
Ammonium phosphate	1.0 grm.
Ammonium salt of 2:4-D	0.1 grm.
Glass distilled water	1000 mls.

The solutions were made up into 25ml. aliquots in boiling tubes plugged with cotton wool and having a narrow glass tube passing through the centre of the plug to the base of the culture tube. This tube was also plugged at its outer end. The solutions were sterilised with steam pressure at 15 lbs. for 15 minutes, inoculated, when cool, with equal inocula from previous sub-cultures and sterile, moist air bubbled continuously through them. The tubes were incubated in the dark at a temperature of 25°C. The air was purified by passage through carbon towers, humidified by bubbling through distilled water and rendered sterile by passing it through a tower of sterile non-absorbent cotton wool. Sub-cultures were made every 12 to 14 days depending on the rate of detoxication - usually 7 to 10 days. In this manner a constant supply of bacteria of a similar age was maintained for experimental purposes.

b) Measurement of bacterial growth

Growth in liquid culture was measured by direct counting under a haemacytometer. A small sample, approximately 0.5 ml., was removed from the culture aseptically after vigorous shaking and put into a small clean sample tube 0.25" x 1.0", together with a crystal of water soluble methylene blue. The tube was tightly corked to prevent evaporation, shaken to dissolve the dye and left at least thirty minutes for the bacteria to take up the stain. At the end of this period each tube was well shaken and sufficient of the fluid placed on the haemacytometer slide to fill the cell. A preliminary count of organisms was made in

volumes taken at random over the area covered by the grating. If the numbers of organisms in each count were approximately the same a count of ten consecutive volumes of 0.1 x 1/400 cmm. was made and recorded. If the numbers of cells in the random counts differed widely the sample was discarded the suspension reshaken and the process repeated until a sample containing evenly distributed organisms was obtained on the haemacytometer slide. The mean of the final ten counts was multiplied by 4×10^6 to give the number of cells per ml. of the original culture solution. When the random counts shewed large numbers of organisms per volume, above 20-30 per count, a dilution was made with sterile distilled water to reduce the numbers to a level easily counted accurately, less than 20 per volume.

c) Estimation of the concentration of 2:4D in culture solutions

The concentration of 2:4-D in solutions was estimated by the inhibitory effect of the herbicide on the growth of cress seedling roots. The variety used was Carter's Plain Cress No.4015 and the method adopted a modification of that described by Audus, 1951.

Measured samples, 0.1 - 0.2 ml. of the culture fluids under test were removed from the culture vessels aseptically, boiled in a water bath for 30 minutes to kill the organisms and diluted with glass distilled water to bring the original concentration of 2:4-D in the medium to 1.0, 0.1 and 0.01 p.p.m. 5 mls. of solution of each of these dilutions was added to each

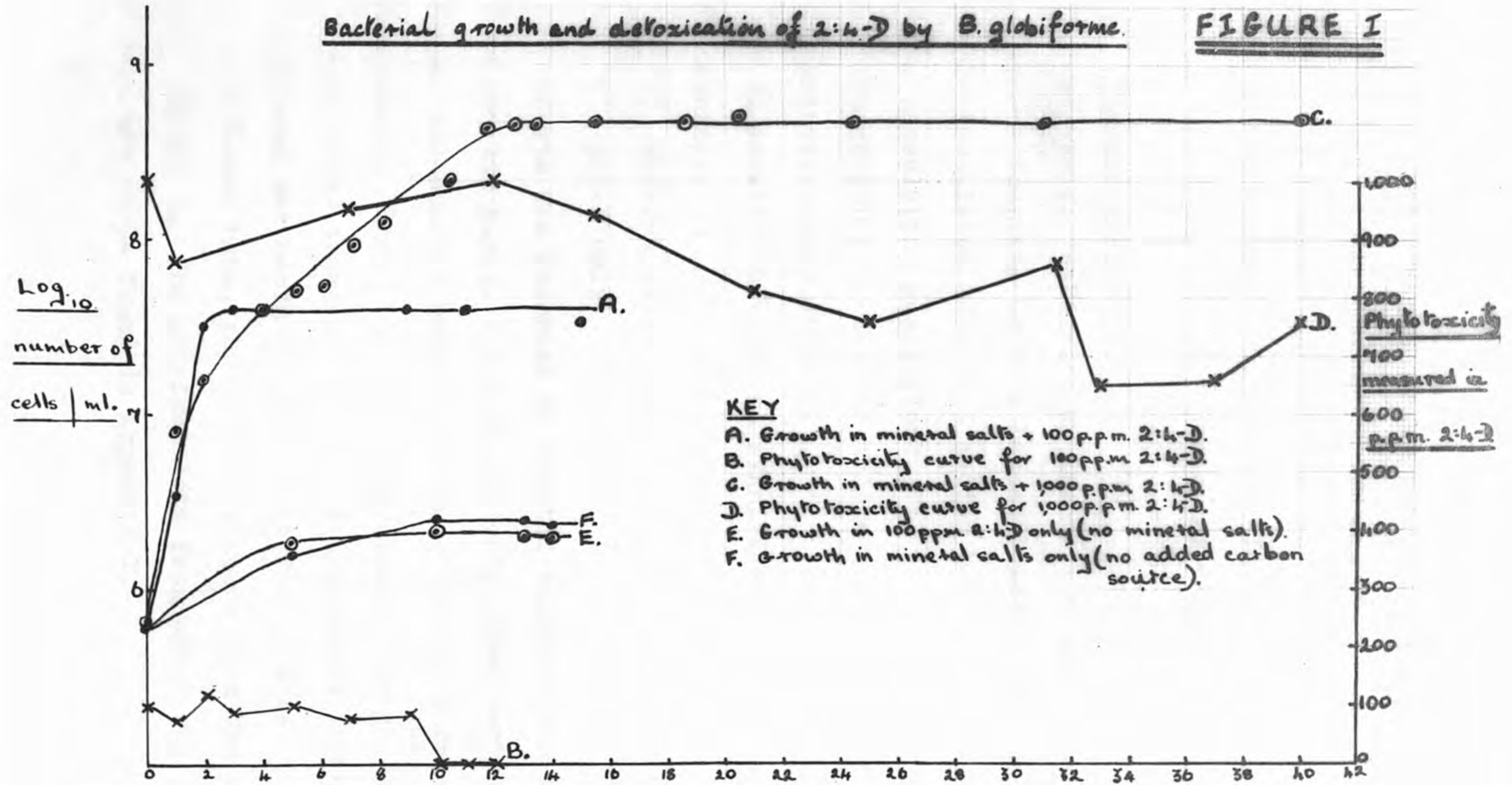
of two tubes containing approximately 20 cress seeds supported on filter paper three inches from the base of the tube. Control tubes received 5 ml. glass distilled water only. These tubes were incubated in the dark at 25°C for four days and then the four longest roots in each tube measured in mms. The measurements were compared with a standard curve obtained with known concentrations of 2:4-D and the quantity of 2:4-D present in the culture solution at the time of assay estimated.

3. Growth experiments

a) Culture technique

Growth experiments were carried out in boiling tubes containing 25 mls. of the culture solution plugged with cotton wool and including an air inlet tube as for the stock culture vessels.

Liquid cultures contained a mineral salt medium composed of: magnesium sulphate, potassium chloride and ammonium phosphate, in the same proportions as the stock cultures, together with the stated concentration of the appropriate carbon source, plus any additional substances for the particular experiments. Stable materials were added to the media before sterilisation unstable or volatile compounds were made up aseptically in sterile glass distilled water and added to the main solution after autoclaving.


Bacterial growth and detoxication of 2:4-D by B. globiforme.
FIGURE I
Log.10 number of cells / ml.
9
8
7
6
A.
B.
C.
D.
E.
F.
KEY
A. Growth in mineral salts + 100 p.p.m. 2:4-D.
B. Phytotoxicity curve for 100 p.p.m. 2:4-D.
C. Growth in mineral salts + 1,000 p.p.m. 2:4-D.
D. Phytotoxicity curve for 1,000 p.p.m. 2:4-D.
E. Growth in 100 p.p.m. 2:4-D only (no mineral salts).
F. Growth in mineral salts only (no added carbon source).
Phytotoxicity measured in p.p.m. 2:4-D
1,000
900
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Time from inoculation in days.

Equal inocula of organisms from stock cultures of the same age were used for all experiments. Stock cultures inoculated at the same time and incubated together with the experimental cultures were used as controls in each experiment. Incubation was carried out in the dark at 25°C unless otherwise indicated.

b) Results obtained

Growth was estimated by increase in cell numbers measured by direct counting under a haemacytometer; the initial number of cells inoculated into each medium being calculated from a count of the inoculating suspension.

No growth was recorded unless the cultures were continuously and vigorously aerated.

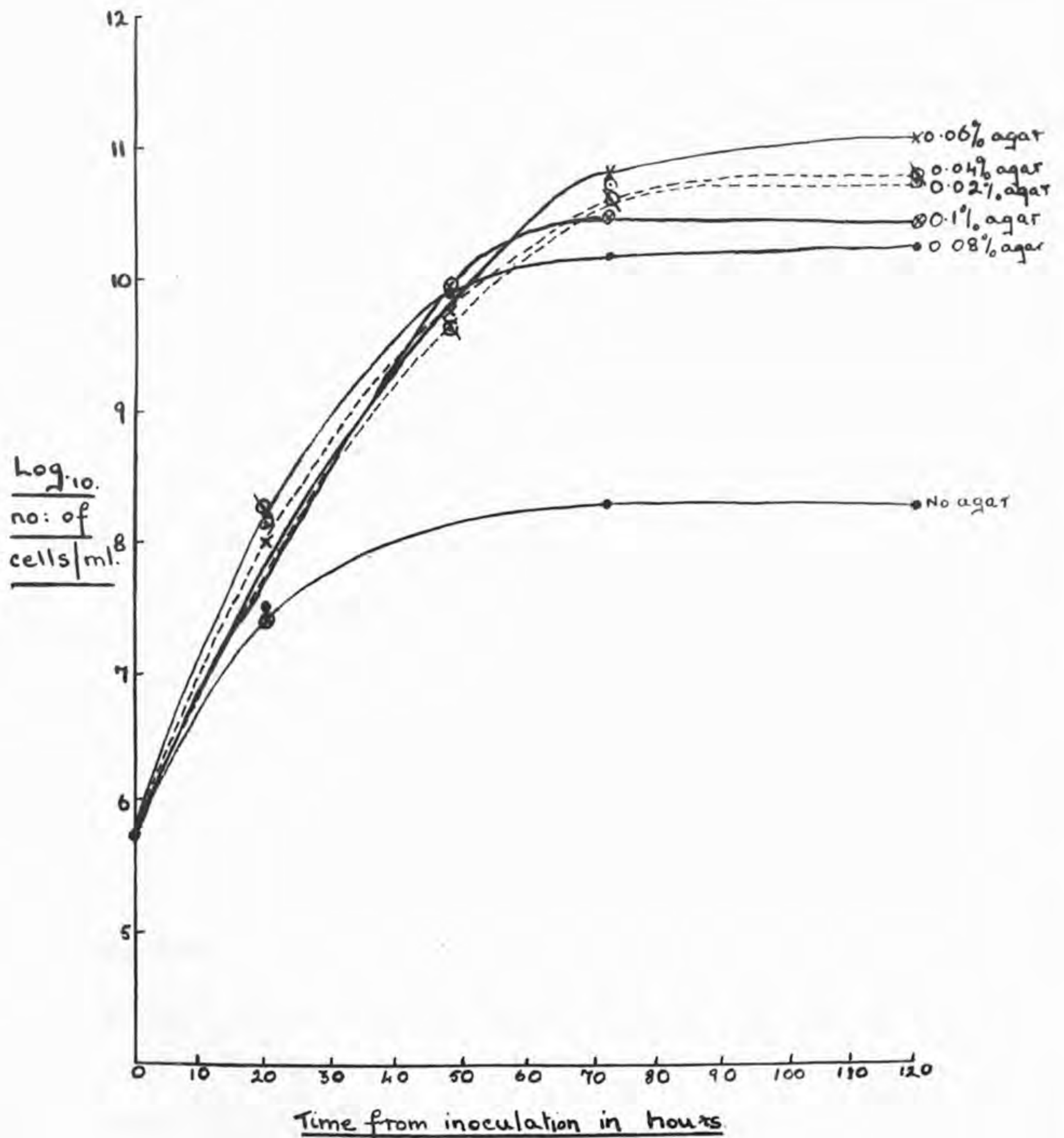
In aerated cultures no significant growth occurred in media containing:-

1) Mineral salts only
2) 2:4-D only.

Growth was recorded in aerated cultures containing mineral salts and 100 p.p.m. 2:4-D as the only added carbon source. No increase in cell numbers occurred after 48-72 hours from the time of inoculation. Growth in media containing mineral salts and 1,000 p.p.m. 2:4-D commenced at a rate comparable to that in cultures containing one tenth the concentration of 2:4-D but continued, at a slower rate, for up to 384 hours from the time of inoculation. Figure I. The original data from which this figure was constructed are to be found in Appendix I.

FIGURE II

The effect of small quantities of agar on bacterial growth in aerated liquid culture containing 100ppm 2:4:D



Growth on this medium was never prolific. Attempts to improve it were made by the addition of various accessory growth factors.

4. The effect on growth of adding various substances to the media.

a) Agar

Reasonable growth was supported by solid agar media containing 100 to 1,000 p.p.m. 2:4-D as the only added carbon source. The effect of the addition of small quantities of agar insufficient to cause gelling of the medium was studied.

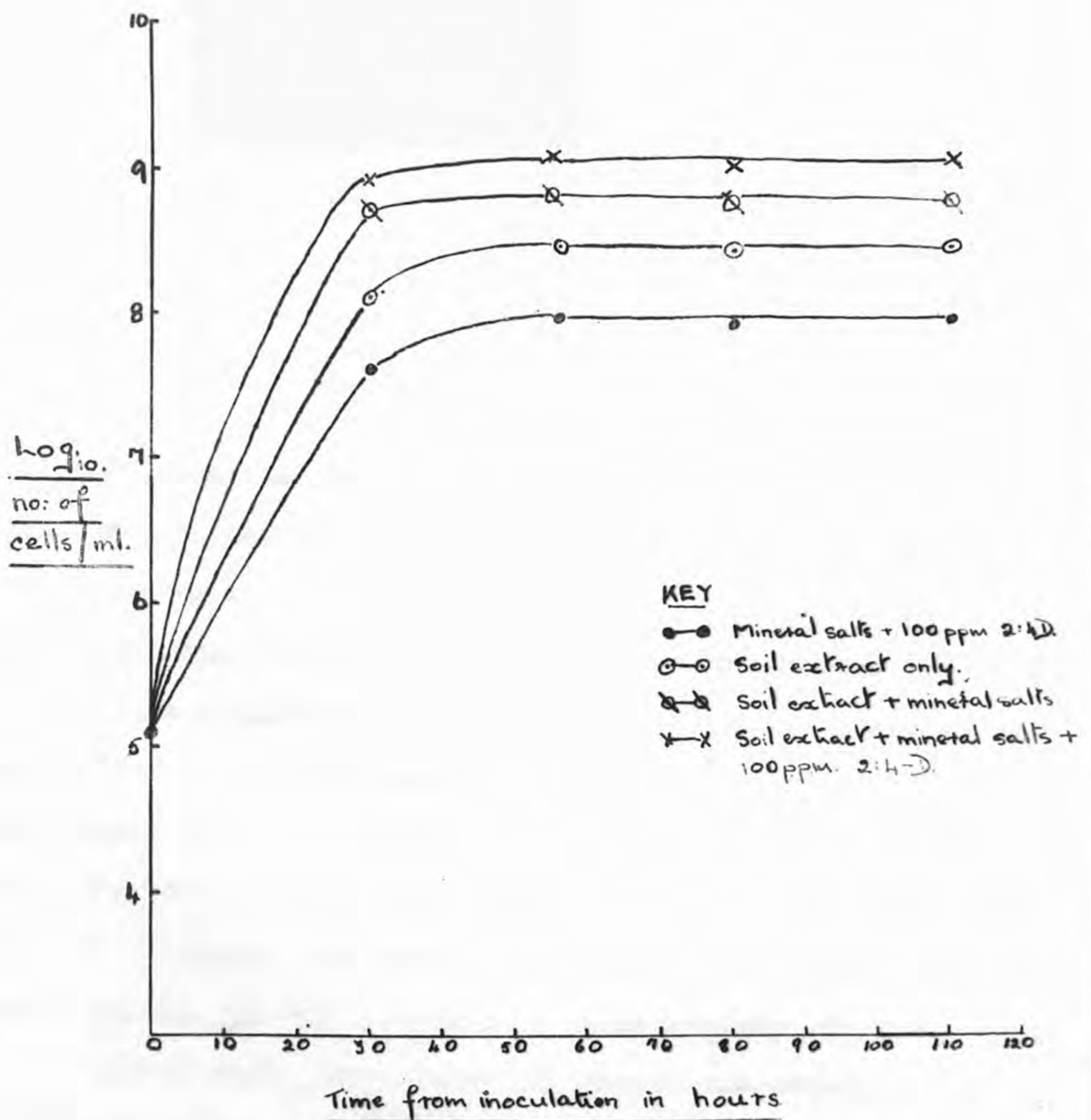
Growth was improved by the presence of agar in the medium down to concentrations of 0.02% agar - the lowest concentration used. There was no significant increase of growth with increasing concentrations of agar up to 0.1% the highest concentration used. The addition of agar in these quantitities to the medium did not affect the detoxication process in cultures containing originally 100 p.p.m. 2:4-D. Figure II. The original data being given in Appendix II.

b) Soil extract

An extract of soil was prepared by autoclaving together equal quantities of fresh garden soil and glass distilled water at 15 pounds pressure for 30 minutes. The solution was allowed to cool, filtered and neutralised with a solution of sodium hydroxide. This solution was added to the media prior to sterilisation in the proportion of 9 parts medium to 1 part soil extract.

FIGURE III

The effect of soil extract on bacterial growth in aerated liquid culture containing 100 p.p.m. 2:4-D.



Greater growth took place on soil extract enriched media than on the 2:4-D mineral salt medium. In fact more growth was recorded on the soil extract diluted with distilled water only than on the full medium. Detoxication of solutions containing 100 p.p.m. 2:4-D by this organism was delayed by the addition of soil extract to the normal culture medium. This suggests that more easily available carbon sources are present in the soil extract and that the 2:4-D molecule is not attacked until these have been exhausted. Figure III. Original data recorded in Appendix III.

c) Yeast extract

Liquid cultures containing 0.1%, 0.05% and 0.01% "Yeastrel" were used. During the course of these experiments it was found that the main growth on yeast extract media was composed of rod shaped organisms at least five times as long as the diameter. On all other media the organism appeared as small ovoid cells or cocci. At the time it was thought that the cultures containing the rod shaped organisms had become infected, by an organisms capable of existing on the yeast extract in the presence of 2:4-D, but in view of later work it is now felt that this was merely another form of the same organism. No trouble with this "infection" was experienced with any other media even with the use of sugars and amino acid media which would have been capable of supporting the growth of a wide variety of organisms.

Again some improvement of growth was obtained but no significant effect on the detoxication process was noted.

FIGURE IV

The effect of yeast extract on bacterial growth in aerated liquid culture containing 100 p.p.m. 2:4-D

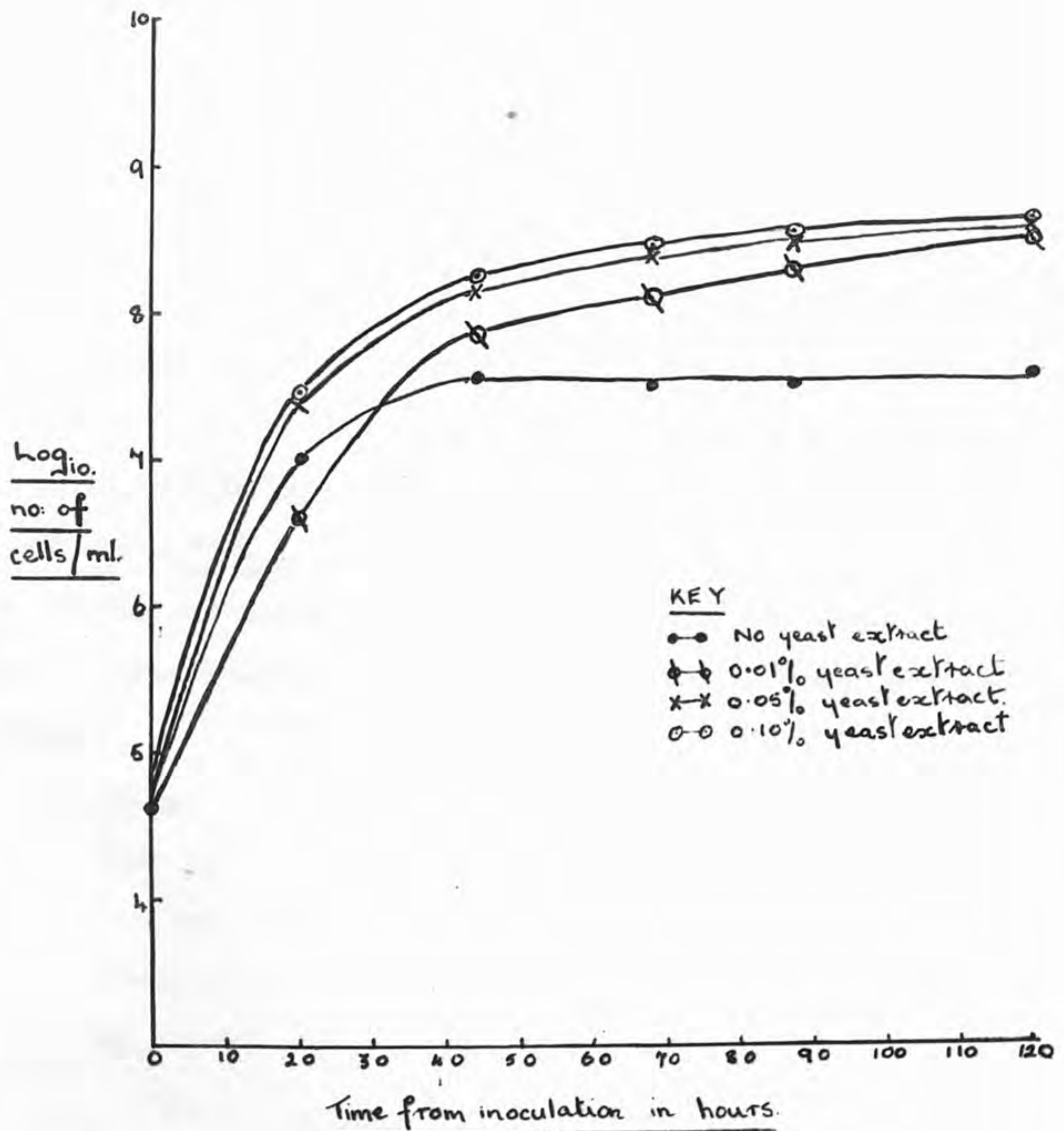


Figure IV, the data being recorded in Appendix IV a.
The numerical results recorded in the following experiments (d-j) are to be found in Appendix IVb.(d-j).

d). Calcium chloride and calcium carbonate

The presence of small quantities of calcium is essential for the growth of many micro-organisms. Taylor 1951. The effect of adding M/1000 calcium chloride and 0.1% calcium carbonate was studied.

No effect at all was observed in the cultures containing calcium chloride. Counting was impossible in the cultures containing the carbonate due to the small particles which obscured the bacteria and also to the fact that the bacteria attached themselves to the particles of chalk and formed clusters around them. The addition of either of these compounds had no effect on the detoxication process.

e) Vitamin B_{12}

The presence of 1.0, 0.10, or 0.01 and 0.005 p.p.m. vitamin B_{12} in the media had no effect on the growth of these organisms.

f) Menaphthone

This accessory growth factor also appeared unnecessary to these organisms in 2:4-D culture.

g) Biotin

No effect

h) Casein hydrolysate containing vitamins and vitamin free

No effect unless present in sufficient quantity to be used as substrate in place of 2:4-D. The presence or absence of vitamins had no noticeable effect.

i) Additional phosphate

Quantities up to twice that normally used for culture had no effect.

j) The effect of adding inert materials to the medium

Materials used:- Sterile brick dust
Sterile cotton wool strands
Sterile glass wool
Sterile soil.

The organisms became attached to the particles present in the liquid media and in no case was it possible to obtain counts of numbers of organisms. No effect on detoxication was however noted and it was assumed therefore that the presence of solid material in the culture medium could not affect the organisms to any considerable extent.

5. The effect of aerating liquid cultures with CO_2 free air

a. Experimental method

The technique was kept as similar as possible to that used in the previous experiments. Rubber bungs containing inlet and outlet tubes replaced the cotton wool plugs at the mouth of the tubes and a column of small granules of soda lime was

inserted into the air line leading to each of the appropriate tubes. A small ignition tube containing lime water was suspended inside the culture tube to ensure that the air was in fact free of carbon dioxide.

b. Results

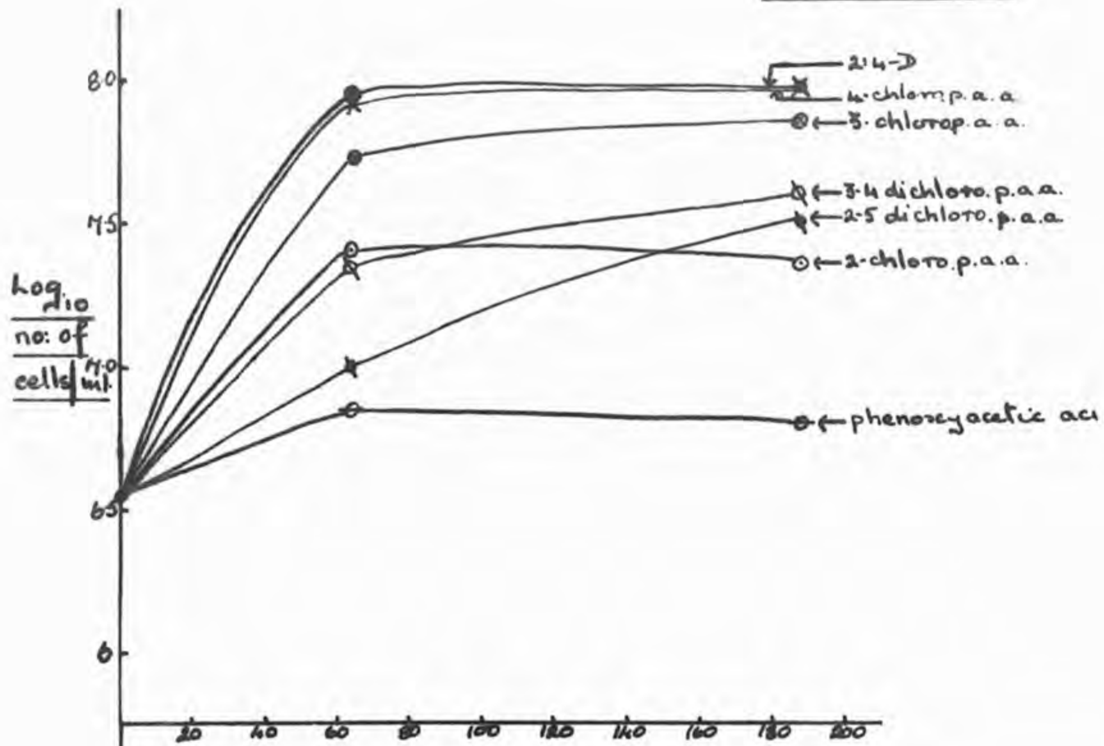
The removal of carbondioxide from the aerating stream of air had no effect on the growth of these organisms in 2:4-D culture but no drop in phytotoxicity of the culture solutions was recorded during the time for which the experiment was run. A similar result was obtained using a mixture of organisms from an enriched soil perfusate solution as will be described later (p.47)

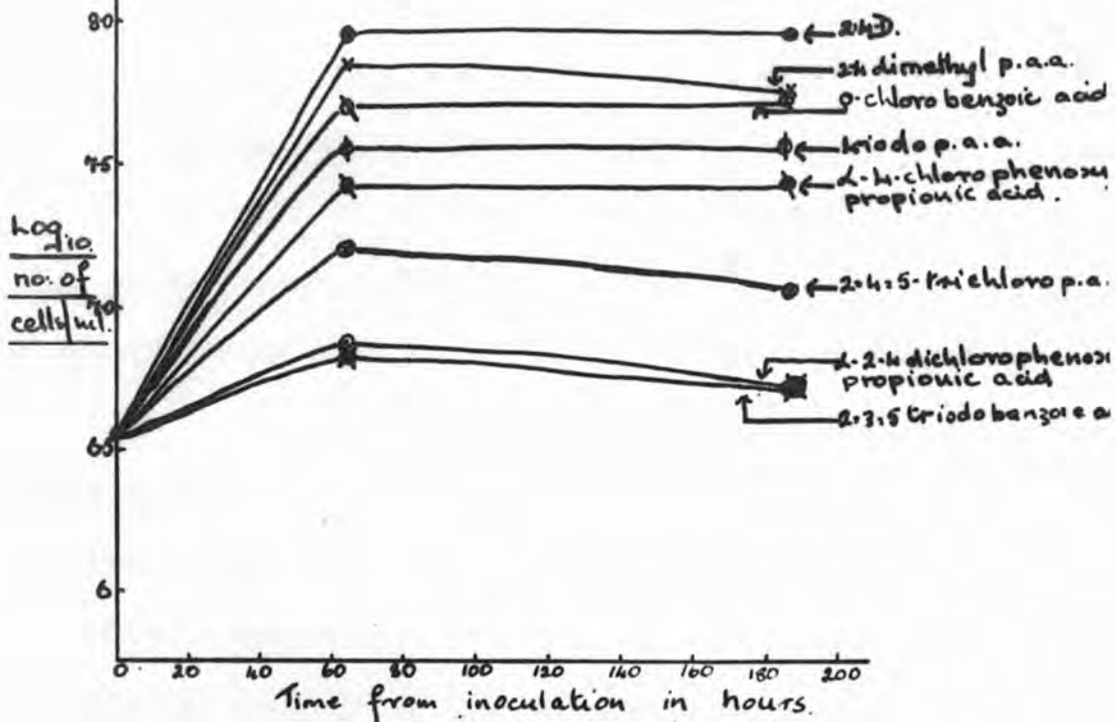
The enrichment of the aerating stream with additional quantities of carbondioxide up to 5% of the total air had no effect on growth or detoxication of 2:4-D. The extra carbon dioxide was obtained from a cylinder of the gas and fed into the main air stream at the required rates.

From these observations it appears that a small quantity of CO_2 is necessary for the complete detoxication of 2:4-D by this organism in pure culture but that the quantity normally present in the air passed through the culture in the course of aeration is sufficient. Many bacteria will not grow in the complete absence of CO_2 but this organism does not appear to be one of these since normal growth took place in CO_2 free cultures. This growth could not have been supported by CO_2 already present in the culture fluid since CO_2 free air was bubbled through the solutions for several hours prior to inoculation. Small quantities of CO_2

Growth of B. globiforme on various carbon substrates. Concentration of each:- 100 p.p.m.

FIGURE V





may leak through the rubber connections but it would not seem that this is responsible for the growth recorded since no difference in growth on standard media in CO_2 free and normal air has been noted.

6. The effect of size of inoculum on growth

The rate of growth did not appear to be affected initially by the size of inoculum but it was found that growth continued for a slightly longer period with the smaller inocula so that the final counts gave a similar number of organisms in parallel cultures whatever the size of the original inocula - within reasonable limits.

7. Utilisation of alternative or additional carbon sources by B. globiforme

i. Growth recorded on substrates having similar molecules to that of 2:4-D

a) Growth of the organism in media containing 100 p.p.m. of the stated carbon source was recorded. The results of these experiments are shewn in Figure V and Appendix V.

None of the carbon sources used in these experiments proved to be more efficient substrates than 2:4-D for this organism. This was shewn by the fact that less growth took place on these compounds than on 2:4-D and also no measurable reduction in the concentration of the substrate was recorded for any of the cultures. It will be seen that very little if any growth occurs on phenoxyacetic acid.

FIGURE VI

Growth of B. globiforme on various concentrations of phenylacetic acid

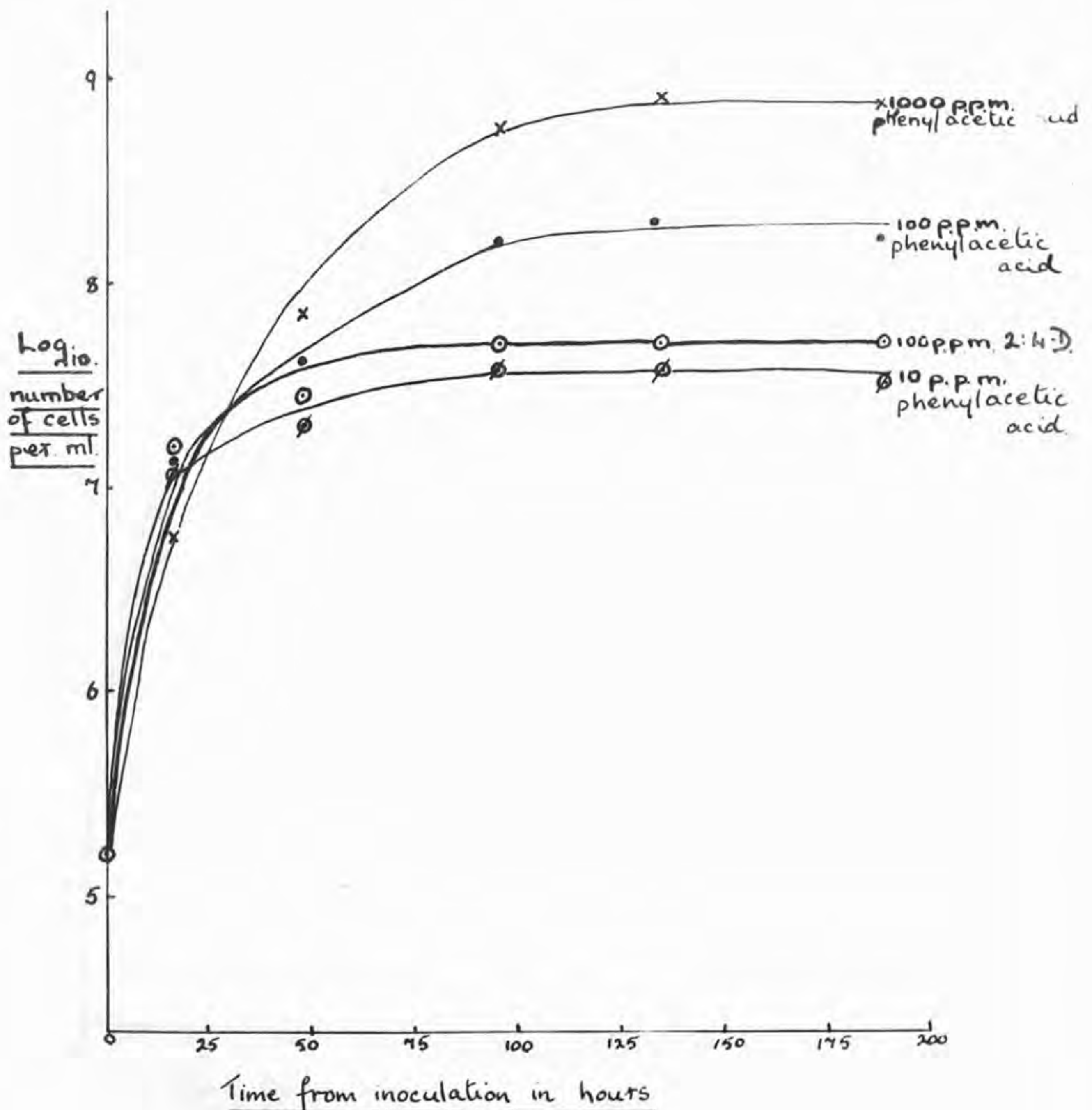
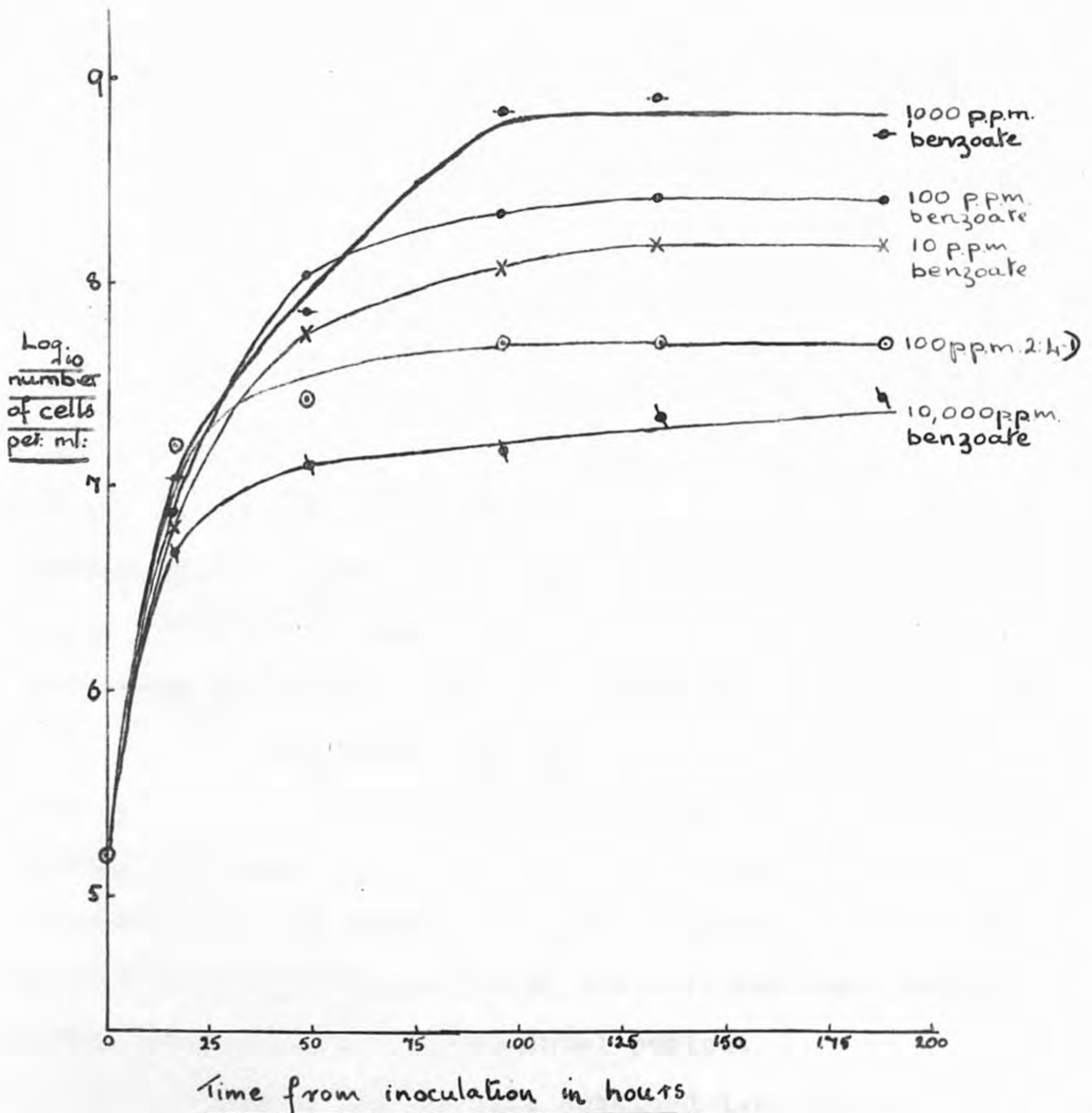


FIGURE VII

Growth of B. globiforme on various concentrations of sodium benzoate



b) Preliminary experiments shewed that two carbon substrates having a similar molecule to that of 2:4-D, namely, phenylacetic acid and benzoic acid, produced greater growth than the 2:4-D medium. Benzoic acid was used in the form of its sodium salt.

The growth recorded for these two compounds at concentrations from 10 to 1,000 p.p.m. phenylacetic acid and 10 to 10,000 p.p.m. sodium benzoate compared with the growth obtained on 100 p.p.m. 2:4-D can be seen in Figures VI and VII and Appendices VI and VII.

ii. The effect on growth of carbon substrates unlike 2:4-D

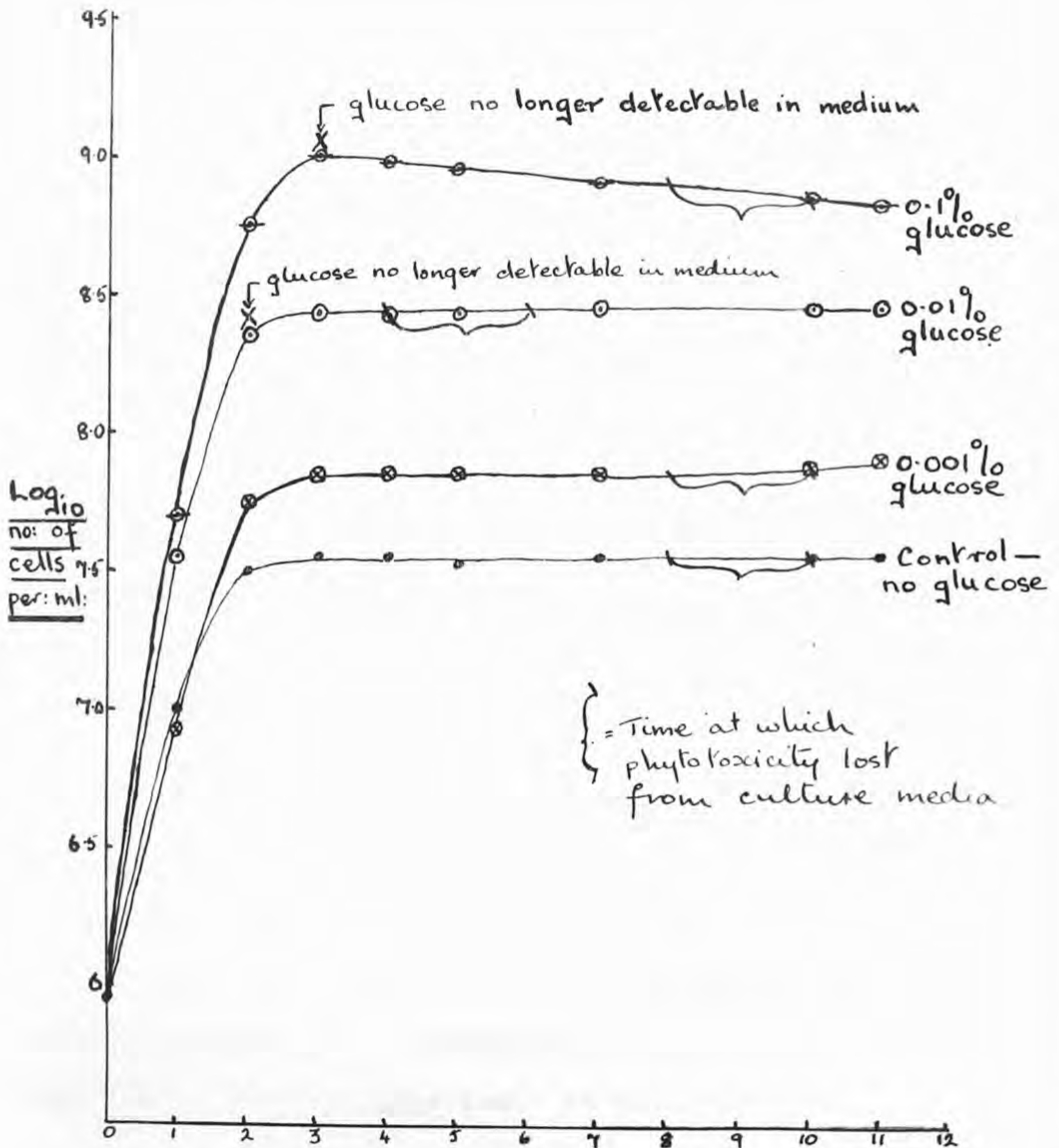
It has previously been reported by Audus that this strain of Bacterium globiforme whilst growing vigorously on such carbon substrates as glucose and sucrose completely lost all adaptation to 2:4-D and could not then be re-adapted. Large quantities of the organism could not therefore be grown on such a substrate and subsequently inoculated into 2:4-D solutions in order to facilitate the study of the decomposition of the herbicide.

It was decided to study the effect of adding small quantities 0.1%, 0.01% and 0.001% of glucose to the media already containing 100 pp.m. 2:4-D. Experiments using 1% glucose were also carried out. In these experiments numbers of bacteria, sugar concentration and phytotoxicity of the cultures were recorded at intervals throughout the experimental period.

Some of the results obtained i.e. those for cultures containing 0.1% glucose or less are shewn in Fig.VIII and Appendix

FIGURE VIII

The effect of glucose on the growth and detoxication of 2:4·D, by B. globiforme



VIII. It was impossible to count the numbers of organisms in cultures containing 1% glucose initially since the bacteria formed flakes in the liquid which coalesced producing a skin like growth over and near to the surface of the media in spite of vigorous aeration. No loss in phytotoxicity was recorded for these cultures i.e. 1% glucose growth curve not shewn on graph. For the lower concentrations of glucose some counts were obtained although there was still the tendency of the organisms, when rapidly dividing, to form flakes in the medium. The counts should therefore perhaps be regarded as the minimum number of organisms likely to be present at the time of counting, rather than an accurate assessment of the number of bacterial cells actually present in the culture at that time. As will be seen from the figure, actual growth is dependent upon the initial concentration of glucose in the medium - but the time of detoxication of 2:4-D is not so easily related. In all cases no glucose was detectable by the time detoxication was recorded.

The presence of 0.10% glucose apparently has no effect on the detoxication time in spite of the increase in numbers of organisms. With initially a concentration of 0.01% glucose however there appears to be a decrease in the detoxication time - from a maximum of 10-11 days to a maximum of 5-6 days from inoculation.

It was hoped that the addition of small quantities of glucose to the 2:4-D medium might result in being able to culture this organism effectively on a large scale in order to study the bio-

chemistry of 2:4-D decomposition by the bacteria. Subsequent experiments proved however that although the presence of 0.01% glucose did in fact increase the numbers of organisms over those produced in the control cultures and the rate of loss of toxicity from it, see Fig.VIII, subcultures from the parent culture were unable to decompose 2:4-D either in the presence or absence of glucose and that any further applications of 2:4-D to the parent cultures remained toxic for several weeks - until the experiments had to be stopped due to evaporation and reduction of volume by sampling for assay.

One possible explanation of the behaviour of this organism when growing in cultures containing both 2:4-D and glucose is that the culture in fact contains a mixture of organisms. The culture may then contain organisms of varying efficiency in the destruction of the 2:4-D molecule. Growth on 2:4-D only would encourage development of organisms capable of carrying out all stages of the detoxication process. If however glucose, or any other carbon source which is easily available to the organisms, is added to the media, growth of 2:4-D non-efficient organisms is encouraged and where this growth is very rapid - as it is when 1% glucose is employed - may cause staling of the medium adversely affecting the 2:4-D detoxicating cells before breakdown has occurred. With only one tenth of the concentration of glucose initially this effect would not be so noticeable and detoxication could occur. The use of an even lower concentration of glucose should on this explanation have little if any effect on the detoxication of 2:4-D.

This is not the case. 0.01% glucose actually increased the rate of detoxication. Increase in growth in these cultures is not so marked as that in solutions containing higher concentrations of glucose, and it is possible that the increase in numbers is due partly to increase in 2:4-D efficient cells and partly to increase in non-efficient cells. The increase in 2:4-D breakdown efficient cells being sufficient to cause the noticeable decrease in detoxication time and the increase in non-efficient cells not sufficient to cause exhaustion or staling of the medium before detoxication is complete.

If the culture of organisms used is in fact a pure strain of 2:4-D destroying cells it would seem that rapid growth on a carbon substrate other than 2:4-D causes deadaptation of the organisms. The repeated divisions taking place in the presence of 1% glucose producing complete deadaptation whether or not 2:4-D is added to the medium as well as the glucose. 0.1% glucose reduces the efficiency oftthe organisms whilst not completely deadapting them since detoxication takes the same length of time as in the control in spite of the increase in numbers of organisms. The effect of adding 0.01% glucose to the culture media is more difficult to explain on this hypothesis but it is possible that this low concentration of sugar whilst sufficient to cause an increase in cell numbers is not great enough to produce a noticeable reduction in efficiency of the organisms so that an apparent increase in rate of detoxication is observed. 0.001% glucose is insufficient to cause any recordable effect in the parent cultures.

The vitality of the organisms is affected adversely at least as far as the breakdown of 2:4-D is concerned since no further charges of 2:4-D can be dealt with either in the same culture or after subculture of the organisms into fresh media either in the presence or absence of glucose.

8. Phytotoxicity of cultures

Phytotoxicity in liquid cultures was estimated by the inhibitory effect of the herbicide on the growth of cress seedling roots as previously described (on page 7.).

Culture media containing 100 p.p.m. 2:4-D as the only added carbon source

There was little change in phytotoxicity for approximately nine days from the time of inoculation and then a sudden drop so that all toxicity disappeared within the next twenty four hours. Thus detoxication does not shew any close correlation with bacterial proliferation. These results are shewn graphically in Figure I and the original data recorded in Appendix I.

Culture media containing 1,000 p.p.m. 2:4-D as the only added carbon source

At this higher concentration of 2:4-D no experimental culture using an inoculum of Bacterium globiforme has completely lost its toxicity. During the phase of bacterial proliferation no significant change in toxicity was observed; but when growth had ceased a slow drop in toxicity became apparent and continued

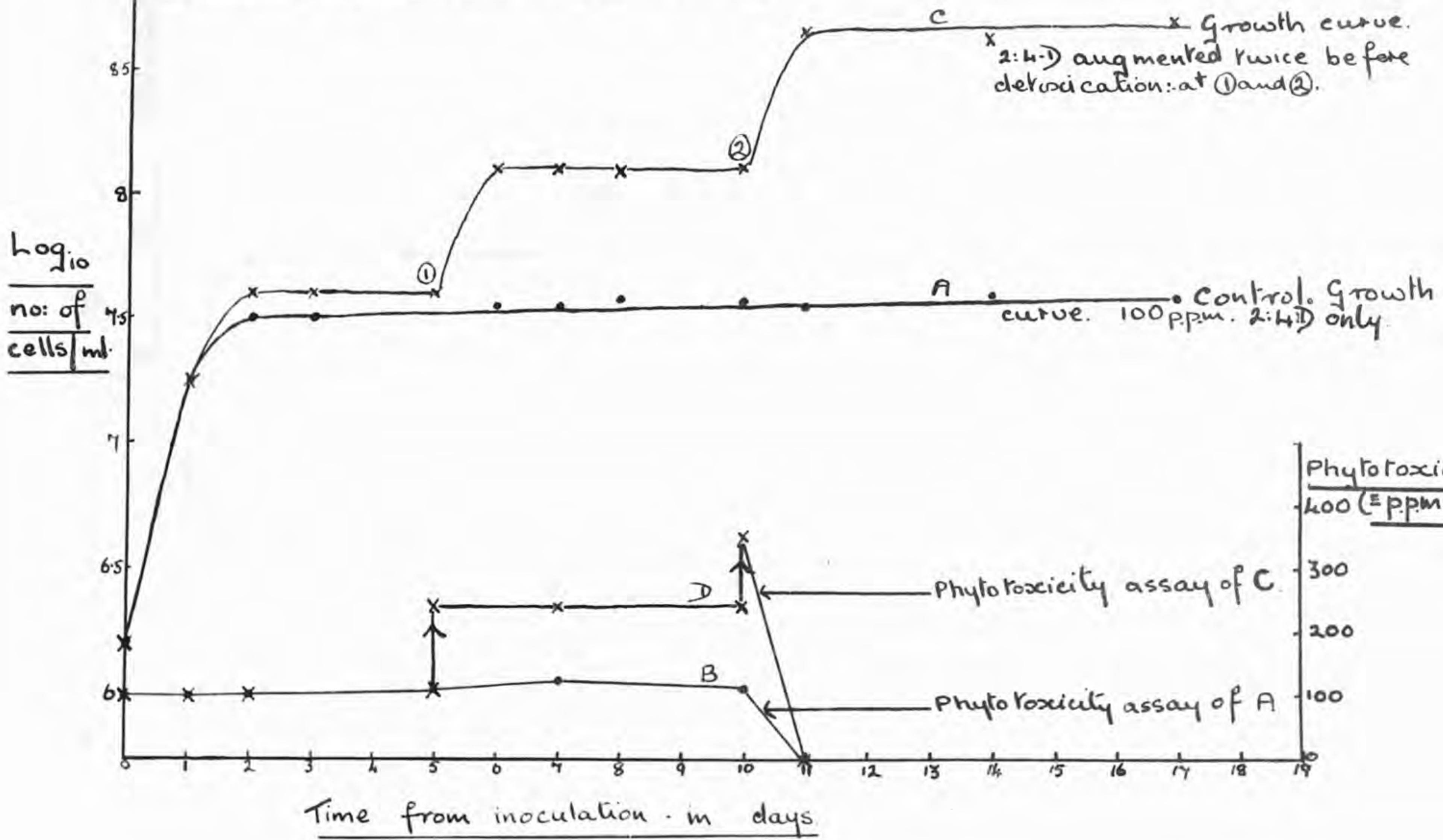


FIGURE IX
Bacterial growth and 2:4·D decomposition.
The effect of additional charges of 2:4·D added before detoxication sets in.
Log10 no: of cells/ml.
Growth curve. 2:4·D augmented twice before detoxication: at ① and ②.
Control. Growth curve. 100 p.p.m. 2:4·D only
Phytotoxicity assay of C
Phytotoxicity assay of A
Phytotoxicity 400 (≡ p.p.m. 2:4·D)
A
B
C
D
①
②
Time from inoculation · in days
9
8.5
8
7.5
7
6.5
6
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
12
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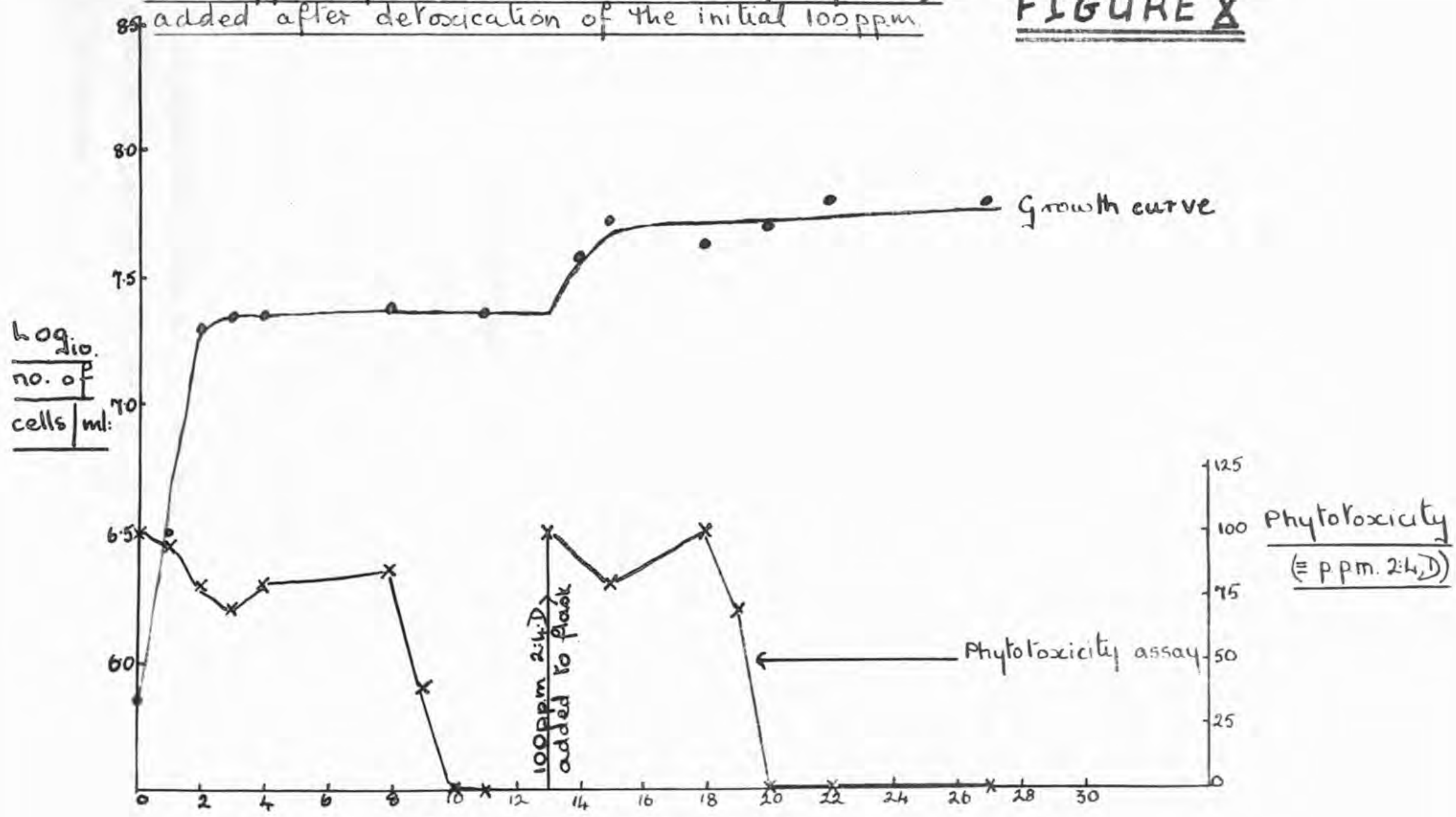

Bacterial growth and 2:4-D decomposition.
The effect of an additional charge of 2:4-D
added after detoxication of the initial 100 ppm
FIGURE X
Growth curve
Log10 no. of cells/ml.
8.5
8.0
7.5
7.0
6.5
6.0
100 ppm 2:4-D added to flask
Phytotoxicity assay
Phytotoxicity (≡ p.p.m. 2:4-D)
125
100
75
50
25
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time from inoculation - in days.

until the experiment was terminated 40 days after inoculation. These results are also illustrated on Figure I.

9. The effect of additional dosage of 2:4-D on growth of the organisms and toxicity of the culture solutions:

a. before and

b. after the detoxication of the initial charge

a. Additional charges of 100 p.p.m. 2:4-D were added to the culture media when growth of the organisms ceased and before detoxication set in. A small increase in cell numbers occurred with each addition of 2:4-D and detoxication of the final concentration of 300 p.p.m. 2:4-D took place at the same time as that of the control experiment which received no additional charge of 2:4-D after the initial 100 p.p.m. If a final concentration of greater than 300 p.p.m. 2:4-D was reached no subsequent detoxication took place during the experimental time. Figure IX and Appendix IX.

b. Additional charges of 2:4-D were added to the culture media after detoxication of the previous dose was complete. There was a slight reduction in time for the second detoxication but no further on the third application of 2:4-D and if further charges were added they were not detoxicated. Figure X and Appendix X.

c. A possible explanation of the results obtained from these two sets of experiments is that the 2:4-D molecule is immediately attacked by the bacteria utilising it as a source of

carbon for increase in cell material and as a respiratory substrate but that the initial rate of breakdown is retarded by the formation of a substance toxic to the bacteria themselves. This intermediate may or may not be phytotoxic also. An adaptation to this material would have to occur before complete breakdown of the 2:4-D and therefore detoxication of the culture solutions could take place. The "lag"phase between cessation of growth and completion of detoxication would be the period of adaptation to this toxic intermediate product.

It is also possible that the overall toxicity of the solutions remains high due to the formation of a phytotoxic intermediate substance. The apparent level of 2:4-D concentration could be maintained not by pure 2:4-D but by a changing mixture of 2:4-D and a phytotoxic intermediate having approximately the same degree of toxicity as 2:4-D. In this case no drop in the level of phytotoxicity in the culture solutions would be recorded until almost all the original 2:4-D had been converted into this intermediate and that itself started to disappear.

10. Experiments planned to test these hypotheses

In order to discover whether the toxicity present in the culture solutions after the cessation of growth of the bacteria was in fact due to 2:4-D or a phytotoxic intermediate or a mixture of these substances some attempt at analysis of the solutions was made.

a. Experimental methods

Cultures of Bacterium globiforme were grown as in the previous experiments with 100 or 1000 p.p.m. 2:4-D as the only added carbon source, but in addition to growth measurements of the organisms and phytotoxicity assays of the solutions samples were also taken to be analysed chromatographically.

Chromatographic method

Whatman No.2 paper cut into 2" wide strips was used. The strips were washed in the developing solvent by allowing it to run through the paper until it reached and dripped off the front of the strip. The paper was then dried and a pencil line drawn across it 10 cms. from the top. The sample taken from the culture was streaked in a narrow band along this line. The chromatograms were developed overnight in a solvent containing iso-propyl alcohol, ·880 ammonia solution and distilled water in the proportions 10;1:1. After development for approximately 14-16 hours, allowing the solution to run about 25 cms. from the origin, the chromatogram was taken from the tank, the solvent front marked and the solvent dried off in a gentle stream of air.

Identification of spots on the chromatograms

Chromatropic acid will detect 0.05γ 2:4-D per ml. (Freed V.H. 1945). This test is however not specific for 2:4-D; a positive reaction being given by a wide range of other organic compounds of similar constitution. It was hoped that by eluting sections of the developed chromatograms and treating the eluate with chromatropic acid as described by Freed some indication

would be obtained as to whether the positively reacting substance or substances ran to the same Rf as 2:4-D or if one or more spots in other positions might be recorded during the detoxication process. The developed chromatograms were divided into 10 sections corresponding to Rf 0.1 - 1.0 and eluted in various solvents. For complete list see Appendix Xa.

The only process by which sufficient material was eluted, to give a positive reaction from the 2:4-D spot area, was the treatment with hot concentrated HCl. The colour however was so faint as to make the use of this process impracticable for studying the gradual disappearance of the substance from the solutions.

The identification of the 2:4-D and any other possibly phytotoxic spots on the chromatograms was therefore undertaken by the method of Audus and Thresh 1953, using the growth of pea root sections as an indication of the position and intensity of phytotoxicity on the developed chromatograms.

b. Experiments using culture solutions containing 100 p.p.m. 2:4-D

The culture vessels were set up and inoculated in the same way as for all previous experiments and samples taken at intervals, usually every twenty four hours, until detoxication was complete. 0.1 ml. samples were placed on the starting line of the chromatograms. It was found that the use of large samples on the chromatograms caused tailing of the 2:4-D spot and

FIGURE XI

Phytotoxicity recorded on chromatograms. Measured as inhibition of growth of pea root sections.
Culture solutions containing initially 100 p.p.m. 2:4-D

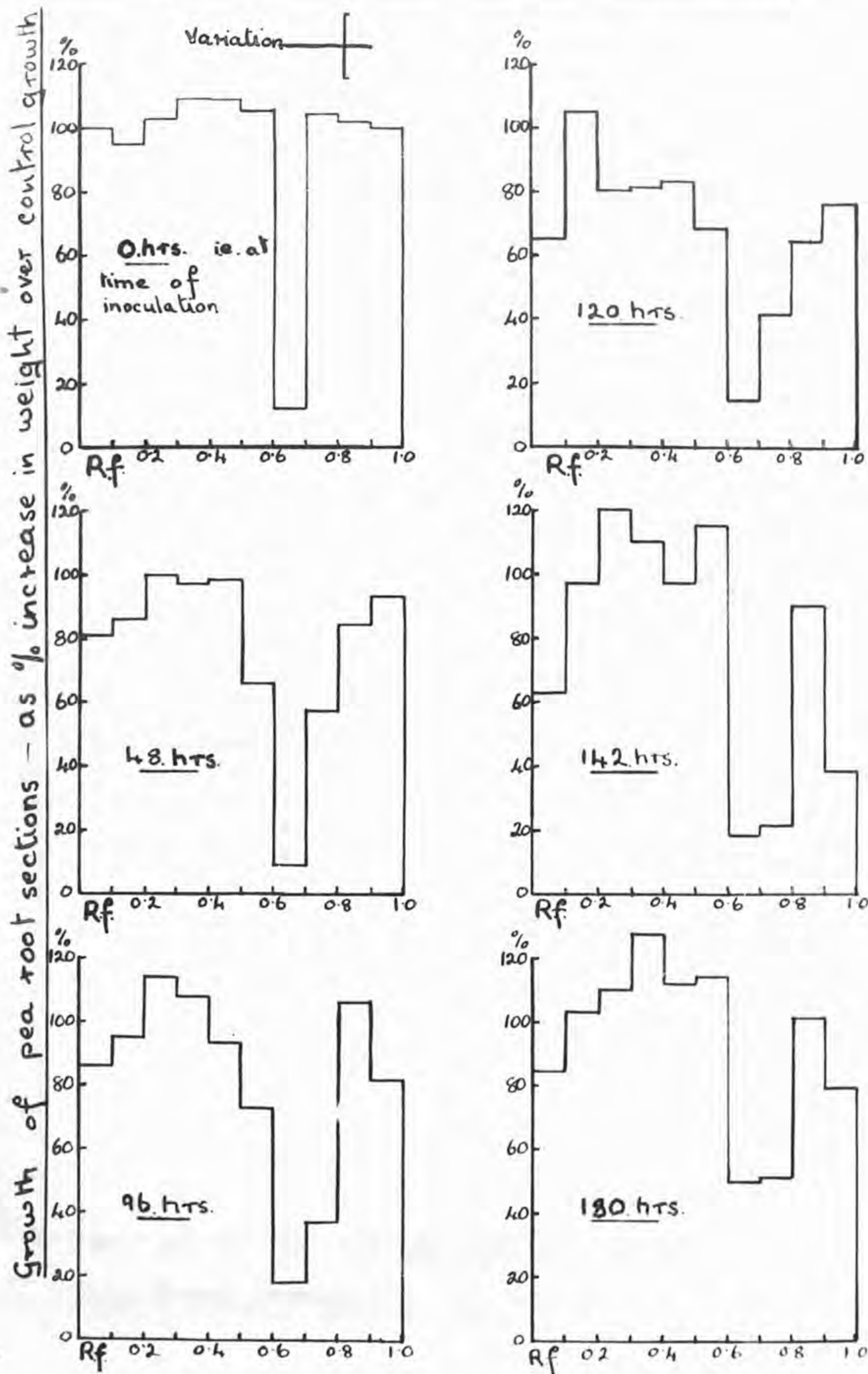


FIGURE XII

Total inhibition of growth recorded on chromatograms. Culture solutions containing 100 ppm 2:4-D

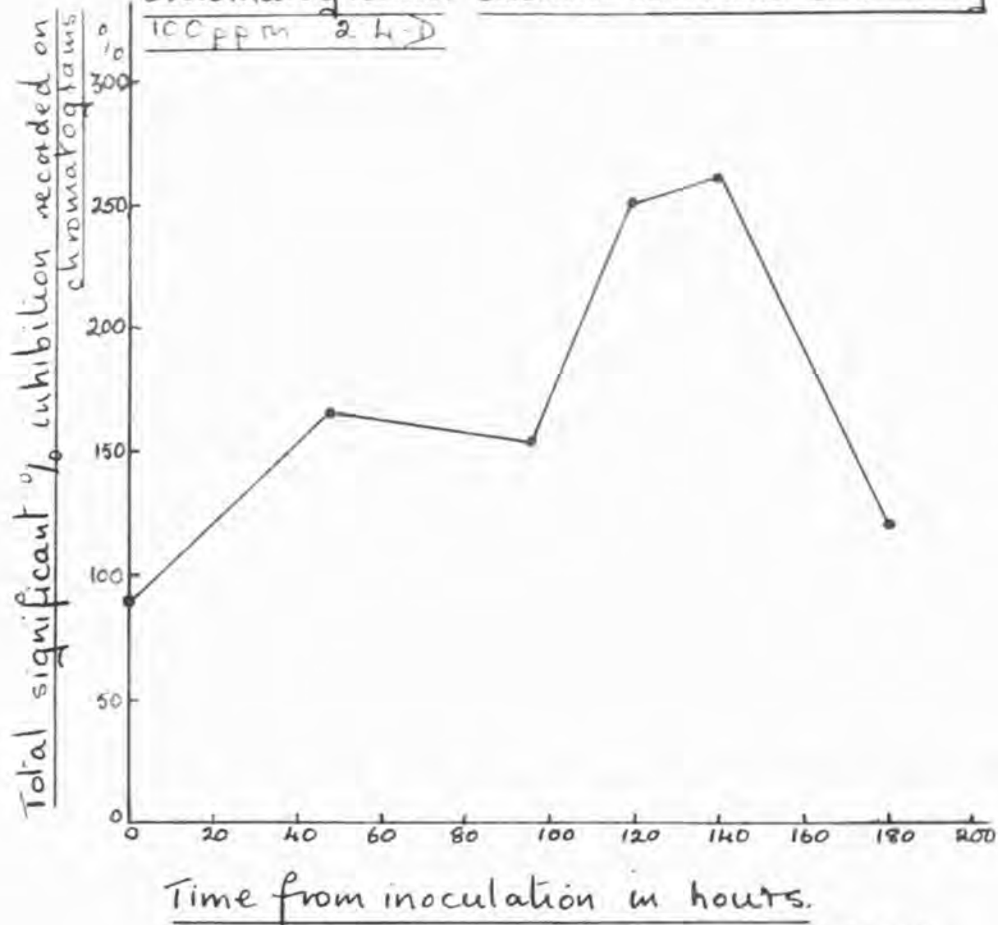
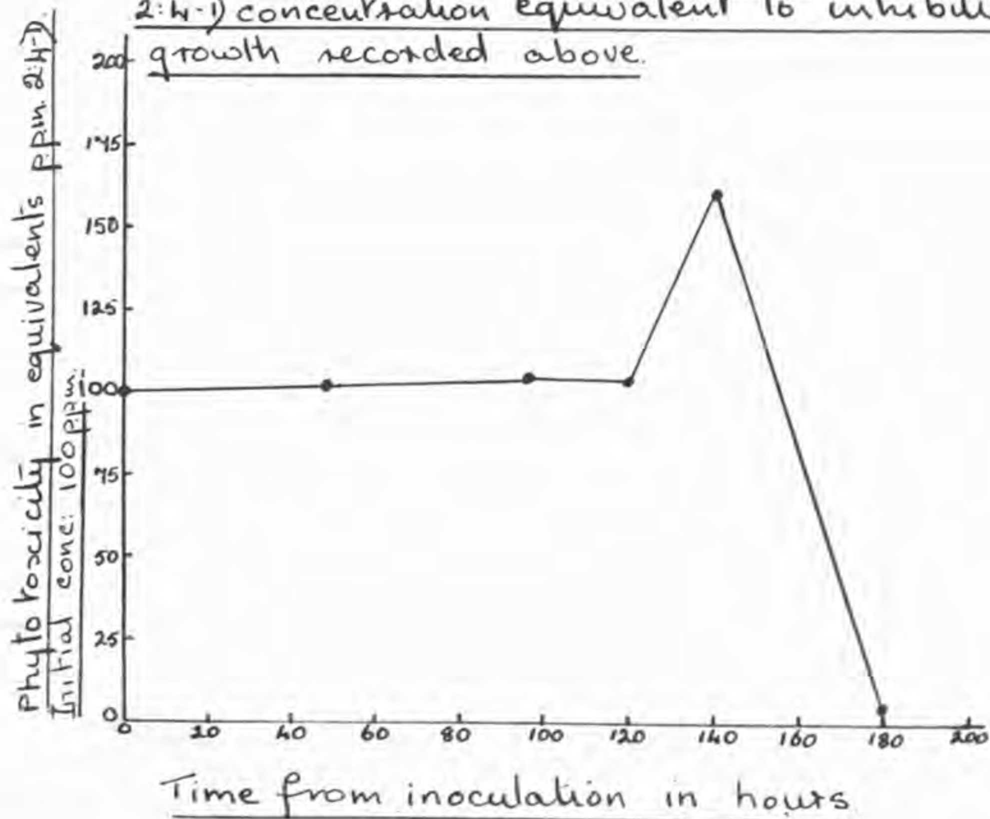


FIGURE XIII

2:4-D concentration equivalent to inhibition of growth recorded above.



therefore marked inhibition in the region behind the main spot which in some cases extended to over two or three sections. Larger samples were used when wider chromatograms were run.

Result

The results obtained are shewn on Figures XI and XII and Appendix XI.

It will be seen that at the time of inoculation (0 hours) only one growth active spot is present on the chromatogram that of 2:4-D with an Rf of 0.65. The sample for the 0-hour chromatogram is taken after inoculation of the flask so as to include any materials carried over in the inoculum which may possibly shew growth activity. From the results obtained from chromatographing samples taken at this time and subsequent assay of growth activity on the developed chromatograms it was concluded that no growth active substances were present in the culture solutions, apart from 2:4-D at the commencement of the experiment. During incubation however at least two other phytotoxic spots appear; one at the origin of the chromatogram and the other in advance of the 2:4-D spot at an Rf of 0.75 - 0.80. The spot at the origin is unlikely to be 2:4-D prevented from moving on the paper since early samples do not shew it and the later ones are taken in the same manner. The spot in advance of the 2:4-D spot is unlikely to be due to tailing of the 2:4-D since this occurs behind the normal 2:4-D Rf and although it may cover several sections of the chromatogram if unduly large quantities of 2:4-D are placed on the starting line the growth activity does not

advance to this Rf value.

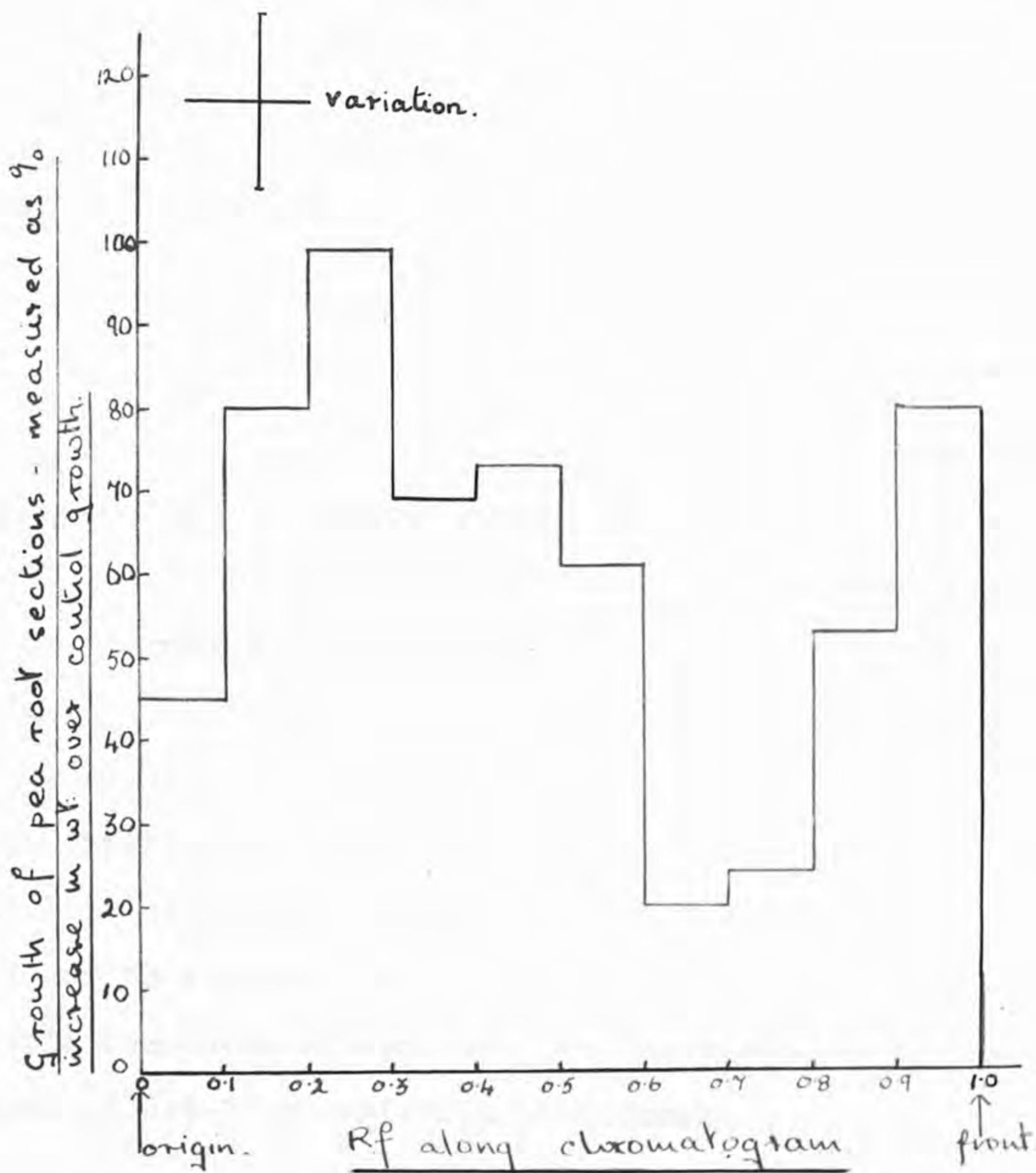
These results suggest therefore that during the detoxication of 2:4-D by Bacterium globiforme at least two phytotoxic intermediate compounds are produced. It is not possible by these means to determine which is produced first since both appear on the chromatogram at similar times and as detoxication proceeds decrease together. That phytotoxic intermediate compounds are indeed produced is confirmed by summing the total inhibition recorded on each chromatogram and plotting this figure against time. This is illustrated in Figure XII ~~Appendix XII~~ where it will be seen that the total inhibition of growth increases during incubation up to 260% at 142 hours from inoculation - compared with 88% recorded at inoculation time. The high level of phytotoxicity recorded during the "lag-phase" in the decomposition process could well be accounted for by a combination of 2:4-D and the phytotoxic intermediate compounds and not, as has been previously assumed, by 2:4-D alone.

c. A similar experiment was carried out using a culture medium containing mineral salts and 1,000 p.p.m. 2:4-D. A similar increase in phytotoxic areas on the chromatograms was recorded but no significant decrease in overall toxicity took place over the experimental period. After 40 days incubation the culture solution was acidified by the addition of 10 ml. concentrated HCl. It was then extracted by successive shakings with ether. The combined ether extracts were then evaporated to dryness until a brown crystalline residue remained. This residue consisted of

FIGURE XIV

Chromatogram of unknown substance from 40 day culture of B. globiforme grown on 1,000 p.p.m. 2:4-D.

(Chromatogram developed in propanol, ammonia, water, solvent)



at least two components, one readily soluble in warm 30% ethanol (presumably unchanged 2:4-D) while a very small fraction, a brown crystalline material, was insoluble. Separation was carried out by allowing the hot solution in 30% ethanol to cool. The brown material first separating out was filtered off and air dried. It weighed 0.3 mgs., equivalent to 0.04% of the total quantity of 2:4-D present initially in the culture. This residue was dissolved in a small quantity of concentrated ammonia solution, the excess ammonia boiled off and a standard solution of 0.01% by weight made up. 0.5 ml. of this solution was placed as a spot on the starting line of a chromatogram. A similar spot of 2:4-D was placed on another chromatogram and the two chromatograms developed together in the same tank with the propanol-ammonia-water solvent. Phytotoxicity on the chromatograms was assayed as previously using pea-root sections.

The result obtained from the assay of the chromatogram on which the unknown substance was run is shewn in Figure XIV and Appendix XIV. This shews phytotoxic spots in the same positions as those obtained when running samples of culture fluids from the previous experiments using an initial concentration of 100 p.p.m. 2:4-D. This experiment therefore tends to confirm the previous results and to endorse the hypothesis that at least two phytotoxic compounds are produced as intermediates during the breakdown of 2:4-D by Bacterium globiforme.

Figure XIII shews the concentration of 2:4-D equivalent to the total inhibition of growth recorded on the chromatograms during the decomposition of 2:4-D in an initial concentration of 100 p.p.m. in the culture solutions. It will be seen from this that although there is marked spreading of the phytotoxic areas on the chromatograms there is little significant increase in overall toxicity, as measured in equivalents of 2:4-D, until, in this case 142 hours from inoculation, just prior to the main detoxication.

From the results of all of these experiments using cultures of the organism Bacterium globiforme of a strain effective in the process of detoxication of 2:4-D the following facts emerge concerning the process.

Detoxication is only effected if the cultures are continuously and vigorously aerated.

Concentrations of up to 300 p.p.m. 2:4-D only, are detoxicated - however the application is made - either by an initial dose of 300 p.p.m. or three successive doses of 100 p.p.m. applied either before or after detoxication of the previous applications.

The time for complete detoxication varies but is generally 7-10 days from the time of inoculation. There is a lag period prior to detoxication and the phytotoxicity present in the culture solutions is lost, generally within twenty/four hours, at the end of this lag phase.

Bacterial growth, as measured by increase in cell numbers, commences immediately upon inoculation and is complete within 48-72 hours; well before detoxication of the culture solution sets in.

Although no detoxication of high concentrations (1,000 p.p.m. 2:4-D) takes place bacterial growth occurs at approximately the same rate initially, as in culture media containing the lower concentration of 2:4-D. Growth may continue at a reduced rate for up to 14 days from the time of inoculation but eventually ceases and no further change has then been recorded.

When successive applications of 2:4-D are made after the detoxication of previous doses the lag phase is retained but if the applications are made prior to the detoxication of the first dose the entire concentration is decomposed at the same time and no increase in the length of the initial lag period is recorded.

Adaptation to the breakdown of 2:4-D is very quickly lost if sub-culture of the organisms is made onto other carbon substrates. This deadaptation takes place whether or not 2:4-D is also present in the medium. Organisms so deadapted have not so far been found susceptible to re-adaptation.

During the detoxication of 2:4-D by this organism at least two phytotoxic compounds distinct from 2:4-D are produced. At present there is no evidence which suggests any particular structure for these substances.

These results shew certain differences from those reported by Audus (1952) for the process of detoxication of 2:4-D in soil. Here a lag phase occurs on the first application of 2:4-D to the soil followed by a rapid detoxication. Further applications however are immediately attacked and rapid detoxication takes place, the process being complete within 2 or 3 days. There is no lag phase once the soil is adapted or enriched to 2:4-D and no apparent limit to the number of charges which can be decomposed.

These results are explained as either one of two processes. Firstly, that a few organisms presumably arising by mutation from a parent strain, may be capable of attacking the herbicide molecule. The lag phase before detectable detoxication occurs, is the period required for the build up of a sufficiently large population of these organisms in the presence of the substrate for their activities in the destruction of the herbicide to be measurable. The second possibility considered is that a considerable proportion of the total population of the species concerned may be capable of responding to the herbicide and that as these organisms grow and divide enzyme systems in the daughter cells slowly become modified to allow the destruction of the 2:4-D molecule. In this case the lag phase would represent the period of enzyme adaptation.

The results obtained with the culture of B. globiforme isolated from an enriched soil cannot be easily explained along similar lines. Growth in the cultures ceases long before

detoxication is detectable. It would be expected that the greatest rate of detoxication would occur during the fastest rate of division of the organisms, that is during the first two days of culture. Also, once an adapted population had been built up, that any further charge of 2:4-D would be dealt with immediately. This is not the case. Adaptation in the soil is retained for considerable periods in the absence of the herbicide - this does not happen with the pure culture experiments. Comparatively small quantities of the herbicide can be detoxicated in pure culture.

The fact that growth in the 2:4-D containing cultures ceases so early is suggestive of the production of a substance toxic to the organisms producing it during the decomposition of the 2:4-D molecule. This substance may or may not also be phytotoxic. The limiting factor in the rate of breakdown of 2:4-D would then be the rate at which enzyme systems in the organisms could further break down or else "neutralise" this toxic compound. In this case it must be postulated that the enzyme system produced to deal with this hypothetical substance is unstable in the absence of its substrate, rapidly disappears after detoxication is complete, and must be reformed when further applications of the substrate are made. The rapid loss of adaptation in the presence of other substrates would thus be explained and the retention of the lag phase for successive applications of 2:4-D to the same culture. The toxic effect of large quantities of 2:4-D would be explained by the production

of too large a concentration of the toxic intermediate before the neutralisation mechanism was fully established. No increase in the initial lag phase would occur when successive applications of 2:4-D were made prior to the detoxication of the initial charge since there would be no stage - before detoxication set in - where the substrate for this particular system was absent and so no disappearance of the adaptation would take place.

The difference in the situation in soil could be due to the breakdown in soil being carried out in various stages with different organisms, or strains of organisms, responsible for each. B. globiforme may be only one componant of this complex, responsible for the initial stages of the breakdown and therefore most easily isolated on 2:4-D agar plates. No accumulation of any toxic intermediate would occur in soil, and adaptation to the molecule as a whole would be retained by the soil in so far as each component organism in the complex remained adapted to its particular substrate produced in the overall detoxication process.

Experiments were set up to isolate organisms from soil throughout the detoxication process of 2:4-D and to use these isolates singly and in various combinations in an attempt to produce a similar picture of detoxication in cultures containing a brown organism or series of organisms to that presented by the study of the process in soil.

III. Isolations of other organisms from soil

1. Experimental method

500 ml. conical flasks plugged with cotton wool and containing a narrow glass tube sufficiently long to pass through the centre of the plug to the base of the flask were sterilised each containing 200 ml. glass distilled water and 20 mgs. 2:4-D. After cooling, fifty grms. of fresh garden soil were added to each flask. A steady stream of moist sterile air was bubbled through the vessels and samples taken every twentyfour hours to assay phytotoxicity and, after vigorous shaking, to plate onto 2:4-D (100 p.p.m.) and nutrient agars. Plating of samples of the liquid was continued until it was completely detoxicated. The first plates were made before the 2:4-D was added to the soil suspension.

2. Result

Large numbers of different organisms appeared on the nutrient agar plates at various times. These sometimes included a very poor fungal mycelial growth. No significant increase in the numbers of any one type or change in the overall density of theccolonies during the detoxication process was noted. Throughout the detoxication process the following types of bacterial colony were constantly apparent on the nutrient agar plates:

1. White colonies, matt surface, rhizoidal growth at fringes.
2. White round glistening colonies.
3. Yellow round glistening colonies. Pigment developing gradually and reaching a maximum depth after 3-5 days incubation.

Colonies on 2:4-D agar

The growth supported by 2:4-D agar looked to be ~~apparently~~ a pure culture. The colonies all appeared whitish opalescent small (1-2mm. diameter) and round. But the organisms 1-3 above when sub-cultured from nutrient agar onto 2:4-D agar shew:

1. No rhizoidal fringes to the colonies and the surface of the colonies moist.
2. As on nutrient agar.
3. No development of yellow pigment for at least 4 weeks, if at all.

It is therefore impossible to distinguish between these organisms by morphological characteristics on 2:4-D agar and also impossible to determine by this means whether other organisms apart from these may not also be present on the plates. The differences become visible if the colonies are re-transferred to nutrient agar.

Cultures of these organisms were isolated on nutrient agar and then sub-cultured onto 2:4-D agar and subsequently into liquid cultures containing 100 p.p.m. 2:4-D and aerated.

Transferences into liquid culture were made singly and with mixtures of the organisms and in combination with the original strain of Bacterium globiforme. Growth and the detoxication process was followed for each culture. See Appendix XIVa.

1. Organism 1. Very little growth and no detoxication within the experimental period (up to 40 days).

2. Organism 2. Indistinguishable in growth and detoxicating characteristics from the original culture of B.globiforme.

3. Organism 3. Grows in liquid culture with 2:4-D as the only added carbon source but no detoxication occurred. This organism also grew on agar plates with no added carbon source if kept in the dark and normal air. No growth occurred on agar plates kept in light or in a CO_2 free atmosphere. Although organism 3 in pure culture was unable to detoxicate solutions of 2:4-D containing 100 p.p.m. it was found that the time for detoxication of solutions of this concentration by organism 2, using subcultures which had not before been grown in the presence of 2:4-D could be reduced from 28 to a maximum of 16 or 17 days from inoculation by adding an inoculum of organism 3 to the culture at the beginning of the experiment.

3. The results of these preliminary experiments with these isolations suggest that in fact more than one strain of organism is concerned with the decomposition of 2:4-D in the soil and that most efficient decomposition in liquid culture

could best be obtained using a mixed inoculum of all the bacterial components if they can be isolated. The organism B. globiforme is capable of completely detoxicating 2:4-D but there are considerable differences between the results of experiments obtained using this organism and the results of experiments using soil.

These organisms were kept on 2:4-D agar for a period of twelve months with sub-cultures approximately every 4-6 weeks. All continued to grow satisfactorily on the agar but no detoxication occurred when any of the sub-cultures was inoculated into liquid culture containing 2:4-D as the sole carbon source.

Fresh isolation experiments to obtain effective organisms had therefore to be set up.

IV. Experiments carried out with soil using perfusion technique of Lees and Quastel modified by Audus

1. Introduction

Whilst is was considered that the breakdown of 2:4-D by soil organisms could best be investigated using pure cultures of an effective organism in the simplest possible medium, in the absence of such an isolated organism it was decided to carry out experiments with soil using the perfusion technique and at the same time to use the 2:4-D enriched perfusate as a source of bacteria for isolation of a 2:4-D utilising organism. The technique employed in the isolations is described later under "Isolation of a fresh effective organism". No effective organism was in fact isolated from the 2:4-D enriched perfusate but the experiments produced some interesting facts concerning the breakdown of 2:4-D in soil.

2. Experimental methods

50 grms. of air-dried sieved soil (2-4 mm. particles) were used together with 200 mls. of the perfusing fluid. The soil was obtained from the Botany Garden of Bedford College, London. Perfusion of the soil with sterile glass distilled water was continued for 24 hrs. to allow the soil population to stabilise and become fully active in the moistened soil. 20 mgs. of 2:4-D were then added to the perfusate. Evaporation losses were made up with sterile glass distilled water.

20 ml. samples of perfusate were taken aseptically at intervals throughout the detoxication process and placed, together with the appropriate quantity of mineral salts, in sterile boiling tubes fitted with cotton wool plugs and air inlet tubes. These tubes were then aerated by bubbling a continuous stream of moist sterile air through the solutions. The cultures so obtained were assayed for phytotoxicity and the time taken for complete detoxication recorded and compared with the time taken for the solution in the perfusion to become non-toxic. The samples taken from the perfuser were replaced by 20 mls. of a sterile solution of 2:4-D containing 100 p.p.m. 2:4-D. Growth curves for the organisms in these perfusate cultures are not included since the organisms tend to form visible clumps in the perfusate making accurate estimations of numbers impossible. It is usually possible to separate the organisms in counting samples by the addition of small quantities of alcohol and formalin but since the original clumps are very large it is difficult to obtain an accurate sample in the first place - even with vigorous shaking.

The first samples were taken from the perfusers at the time of the addition of the 2:4-D.

Other experiments were carried out in which the entire perfusate was drained and replaced by a fresh sterile solution daily. The drained perfusates were then aerated and sampled for 2:4-D assay as in previous experiments.

Finally experiments were carried out in which samples of perfusate taken during the first forty-eight hours of perfusion were aerated in normal and CO_2 free air, with, and without the addition of mineral salts.

3. Results from these experiments

(a) 20 ml. samples taken throughout the detoxication process, mineral salts added and the solutions aerated.

1. The removal of 20 ml. aliquots daily from the perfusate and their replacement by 20 mls. fresh sterile 2:4-D made no difference to the length of the lag phase occurring before detoxication in the perfuser.

2. The fact that continual fresh applications of 2:4-D were being made to the perfusate had no effect on detoxication time. This is in accordance with experiments carried out with the original isolation of B. globiforme where additional applications of 2:4-D to the culture medium before the completion of the lag phase did not prolong this stage.

3. The removal of 10% of the perfusate and therefore 10% of the total microbial population of the perfusate daily had no effect on the rate of 2:4-D breakdown in the perfuser. This suggests, as would be expected that considerable numbers of the organisms remain closely attached to the soil particles and are not washed out into the perfusate.

4. When perfusion was carried out with water only-the initial sample - detoxication occurred when 2:4-D was added to this sample. The lag phase though shewing some variability being approximately equal to that normally found under the perfusion conditions. At least a small number of organisms capable of metabolising the 2:4-D molecule must therefore be normally present in the soil and perfusate prior to the application of the substrate.

This first sample shewed a tendency to slightly increased lag in some cases - 12 to 13 days. This could be due to the slower build up of an adapted population or enzyme systems in an already present population owing to the fewer organisms of the effective strain or strains normally present in the soil in the prior absence of this particular substrate. In other experiments a considerably reduced lag phase, 5 to 6 days, was recorded for this first sample (as compared with later samples, the "parent" perfuser and a similar non-sampled perfuser). It would appear that the activity of this first sample, which has not been exposed to 2:4-D in the presence of the whole soil, varies quite considerably but that in any case effective organisms are consistently present in perfusate, though probably in very small numbers, before 2:4-D is added. The lag phase then represents the time taken for the production of an effective population capable of attacking the herbicide molecule at measurable rates.

The fact that detoxication in this initial sample consistently occurred suggests that the process of adaptation is

FIGURE XV a.

The effect of removal of samples of perfusate from the perfuser on the duration of the lag phase before detoxication in the removed perfusate.

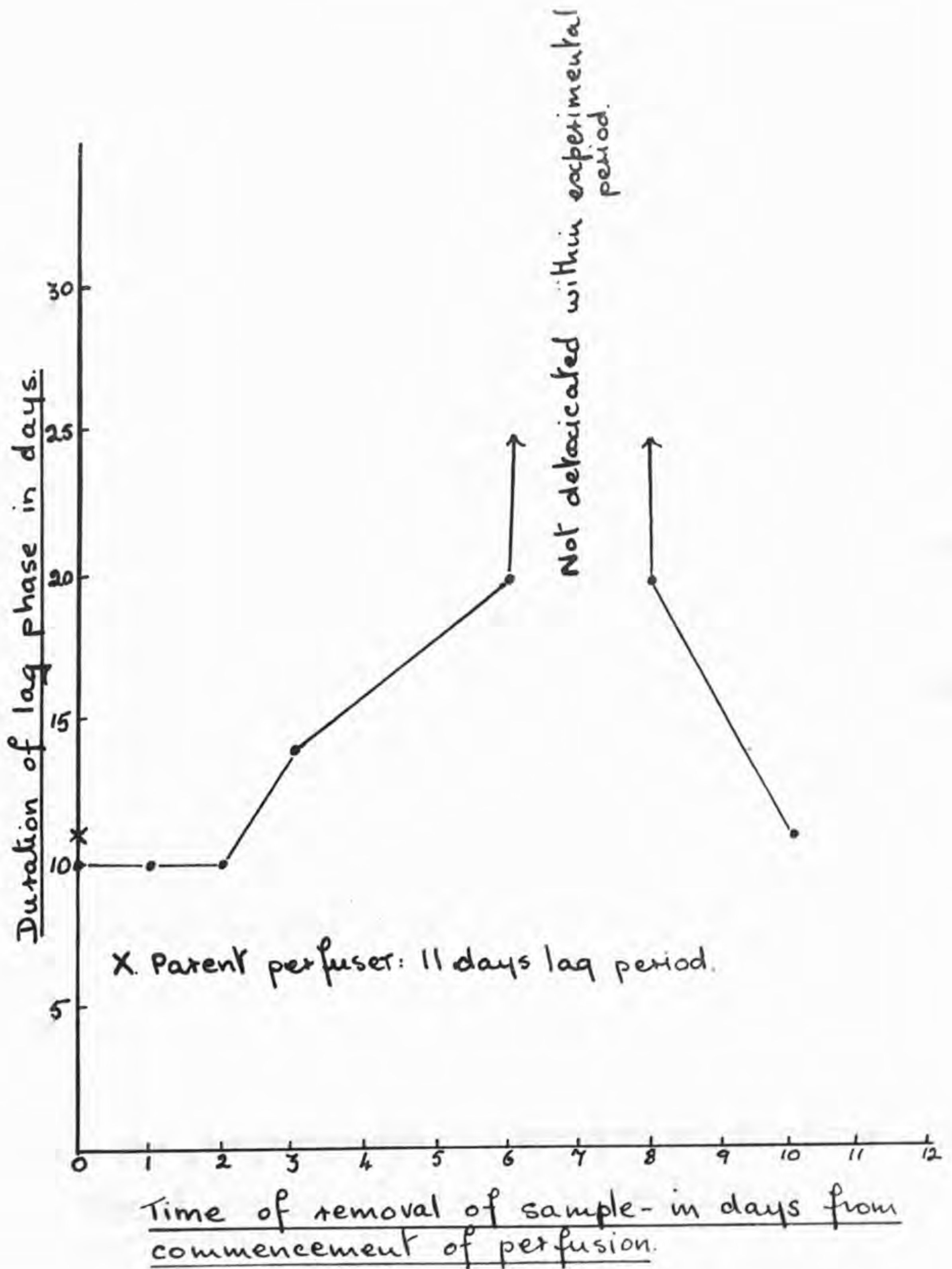
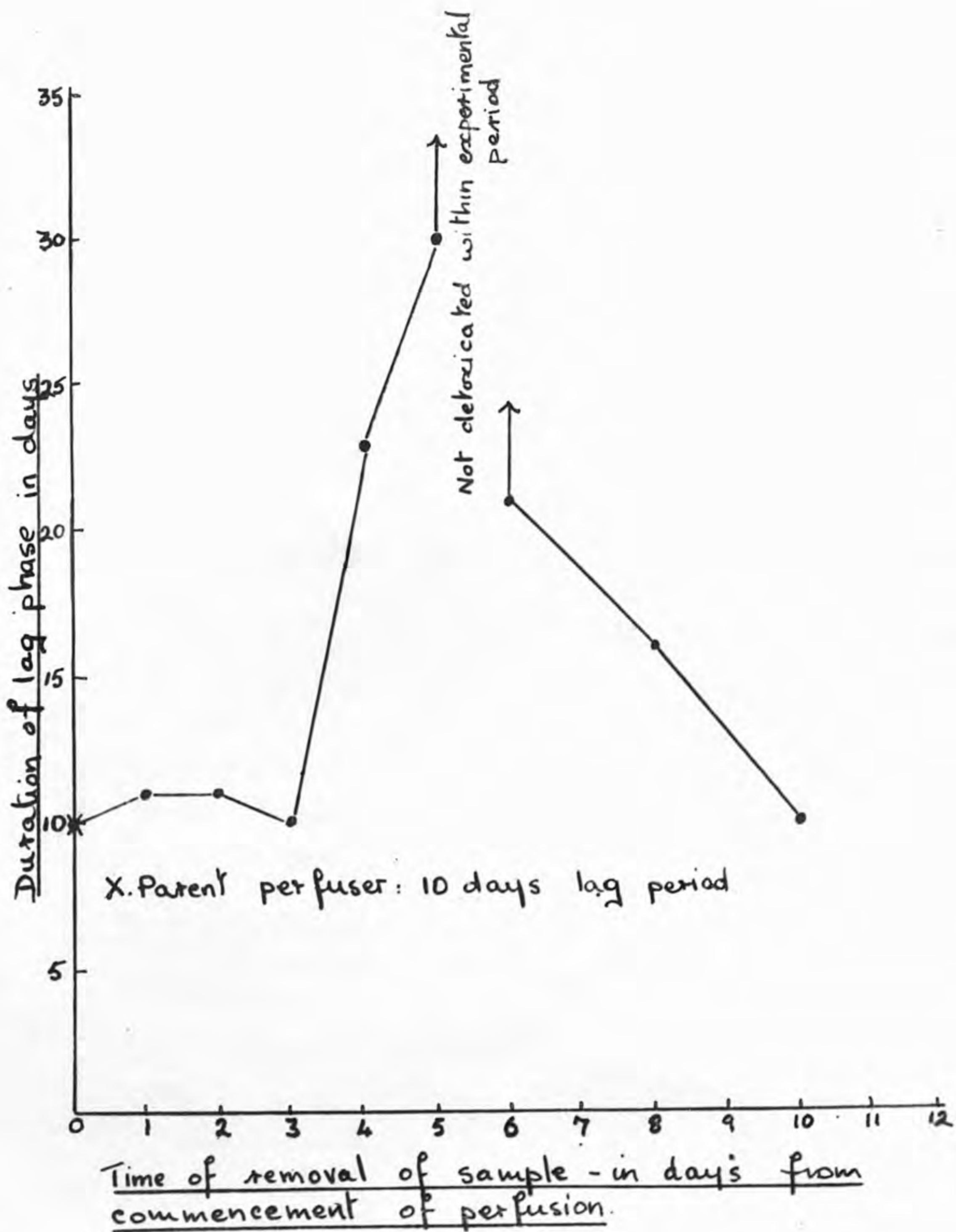


FIGURE XV b.

The effect of removal of samples of perfusate from the perfuser on the duration of the lag phase before detoxication in the removed perfusate.



is one of enzyme modification of already existing organism types rather than dependent upon the production of a few 2:4-D destroying mutants and the growth of an effective population from them, since the chances of always sufficient of these mutants in any sample, which has never been in contact with 2:4-D, to maintain a rate of herbicide destruction comparable with that recorded for the whole soil must be remote.

Later samples taken during the detoxication process - after at least 24 hours perfusion with 2:4-D

Early samples taken during the first two or three days of perfusion detoxicate at approximately the same rate as the perfuser. Later samples taken during the third to ninth day from commencement of perfusion shew an increased lag phase continuing indefinitely in the case of samples taken at about the middle of the normal lag phase period, approximately 5 to 7 days from commencement of perfusion. Later samples taken 9-10 days after the start of the experiment detoxicated as the "parent" perfuser. These results are illustrated in Figures XV a), and b), and Appendix XV. Figure XV a) represents results obtained using soil from the Botany Garden of Bedford College, Regent's Park and b) results obtained when these experiments were repeated using soil from King's College Botany Department at Herne Hill. Both experiments were duplicated.

The increase in lag phase duration of the middle samples cannot be due to exhaustion of an essential factor from the soil by continual removal of samples since later aliquots detoxicate. The early samples detoxicate without any increase in the lag phase therefore the perfusate population then present is capable of detoxicating the given quantity of 2:4-D in the absence of the whole soil. Detoxication is delayed or prevented by removal of the soil during the middle part of the lag phase. The mechanism of adaptation may therefore be impaired at this stage by the separation from soil. Alternatively if the lag phase represents the period of build up of an adapted population in the soil it would seem that this must be complete at about this time, i.e. approximately 5 days from commencement of perfusion, and that little further growth normally takes place after this in the soil so that many fewer, if any, adapted, or partially adapted organisms become washed out into the perfusate at this stage. The population has re-stabilised by the end of the lag phase. Absolute numbers of organisms would not affect materially the breakdown rate in soil - a very small proportion of the total present being "lost" in the removed perfusate sample - but a reduced number in the relatively small volume of perfusate sampled would affect considerably the rate of breakdown in that sample.

Alternatively the behaviour of the perfusate samples could be explained as: since adaptation takes place in the absence of the whole soil it is possible that there exists more

than one possible adaptation route. One route, the more efficient, occurring in the presence of soil but <u>not</u> of perfusate only and the other in the absence of soil. The latter route would be taken by the initial samples of perfusate and encouraged in the experiments involving isolation of organisms onto agar and subsequently into liquid culture. The former route is normally followed in soil and may also be encountered on agar plates since - as will be discussed later - agar dialysate is partially effective in producing a more efficient detoxication, comparable with that induced by the presence of soil or of soil extract. Soil extract prepared by the method described on page 10 is effective in place of soil but not just perfusate, that is water which has been merely in contact with the soil. The regularity with which detoxication occurred on agar plates by organisms incapable of destroying 2:4-D in liquid culture would thereby be explained. Samples of perfusate to which 1,000 p.p.m. 2:4-D was added shewed no loss of phytotoxicity during experimental periods of up to 6 weeks from the application of 2:4-D whereas the addition of soil extract was effective in producing detoxication of this concentration in similar cultures within 10 days.

The less efficient detoxication of 2:4-D in the absence of soil particles or of soil extract may be due, as suggested, to an alternative route being developed by the organisms, or the same set of intermediate products being formed, one or more stages in the process being carried out at less than maximum

efficiency in the absence of the active factor from soil. The organisms are limited in dealing with the herbicide by the available concentration of this substance or any of the components required for its metabolism. It would appear most likely that this factor is required for the "neutralisation" of the toxic effect and further metabolism of the bacteriotoxic intermediate postulated as limiting the growth of the organisms. The concentration of 2:4-D dealt with in pure culture in the absence of soil extract or in isolated perfusate depends on the quantity of this substance which can be produced. In its absence lethal concentrations of the intermediate may be built up and the organisms perish before the substrate is exhausted.

The detoxication of 2:4-D in soil shews various features differing from the kinetics of the herbicide breakdown in pure culture away from soil. One very possible explanation of this is that in soil, as has already been suggested, more than one strain of organism is responsible for the complete destruction of the molecule, at least one of these microbial components closely adhering to the soil particles and not washed into the perfusate, or else obligately dependent upon a factor not present in the perfusate. The perfusate population then adapting to the breakdown of 2:4-D along its own route and the whole soil along another. The intermediates produced in these two processes may or may not be the same but the enzyme adaptations of particular organisms would be different. That the biochemical routes of

detoxication may not be the same in all cases when different organisms attack the same compounds is suggested by Steenson and Walker, 1956, for three different organisms concerned with the oxidation of chlorophenoxyacetic acids.

b) <u>Experiments carried out in which the entire perfusate was drained daily and replaced by a fresh sterile solution of 2:4-D</u>.

The first draining, 24 hours from the commencement of perfusion was carried out before 2:4-D was added to the perfusing fluid and the appropriate quantity of the herbicide added to the drained perfusate.

The total lag phase varied in duration from 9 to 12 days for all perfusates drained between 1 and 12 days from the commencement of perfusion, that is the detoxication of the later ones commenced during the first 24 hours i.e. during perfusion and the organisms remaining in the soil became adapted to the herbicide breakdown in the same time as was usual under these conditions in spite of being exposed to different samples of 2:4-D each day.

Draining and replacement of the perfusate was continued for some time after the initial detoxications were complete. The duration of the lag phase then gradually increased and by the seventeenth sampling no loss in toxicity was recorded after a further 30 days incubation. This was assumed to be due to the

exhaustion of the soil by continual leaching of soluble materials and effective organisms by the draining of perfusates and addition of fresh liquid daily. Appendix XVb

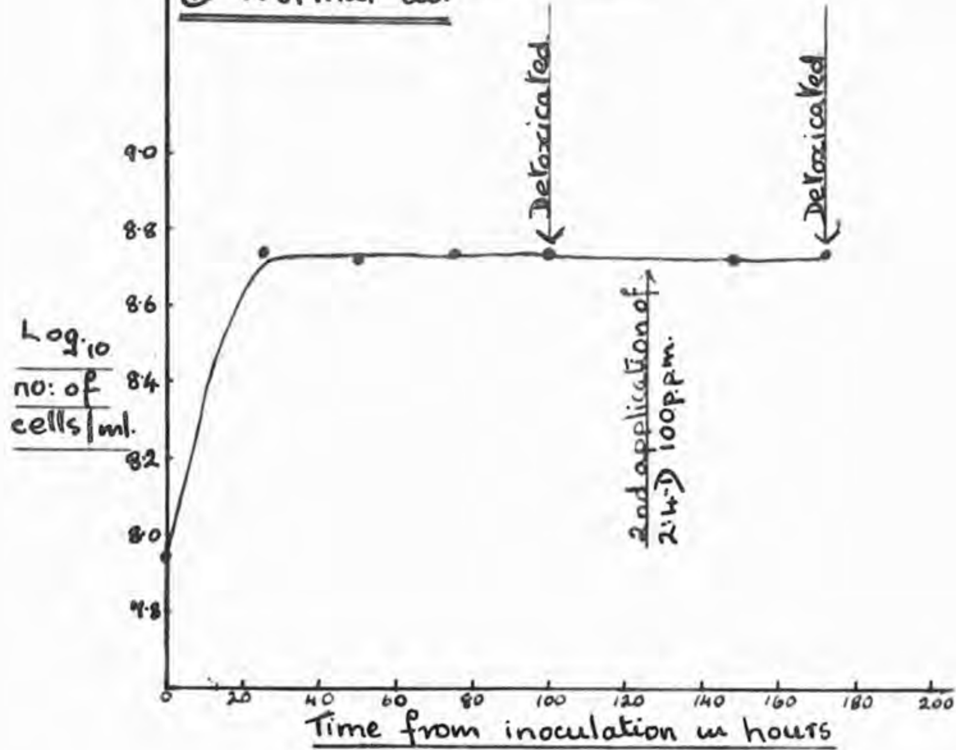
The organisms in the soil became adapted to the 2:4-D molecule in approximately the same length of time as when the initial perfusing fluid was retained throughout the period of adaptation. No increase in lag phase duration was recorded for samples removed during the adaptation period as in the previous experiments. This result was unexpected in the light of previous results and tends to negative the suggestion that an alternative route for detoxication is present in the soil since in this case the same phenomenon of increase in duration of the lag phase would be expected to appear. The draining technique in these last experiments may have contributed to this unexpected result. Perfusers of a modified design were used. The perfusate draining to a flask at the base of the apparatus. This flask was removed daily and replaced by a fresh flask containing a new sterile solution. Small quantities of soil collect in these flasks and without filtering, it has not been possible to separate perfusate completely from soil. The 20 mls. samples taken in the first series of experiments were removed from near to the top of the flasks, using sterile pipettes, thus avoiding the inclusion of soil particles as far as possible.

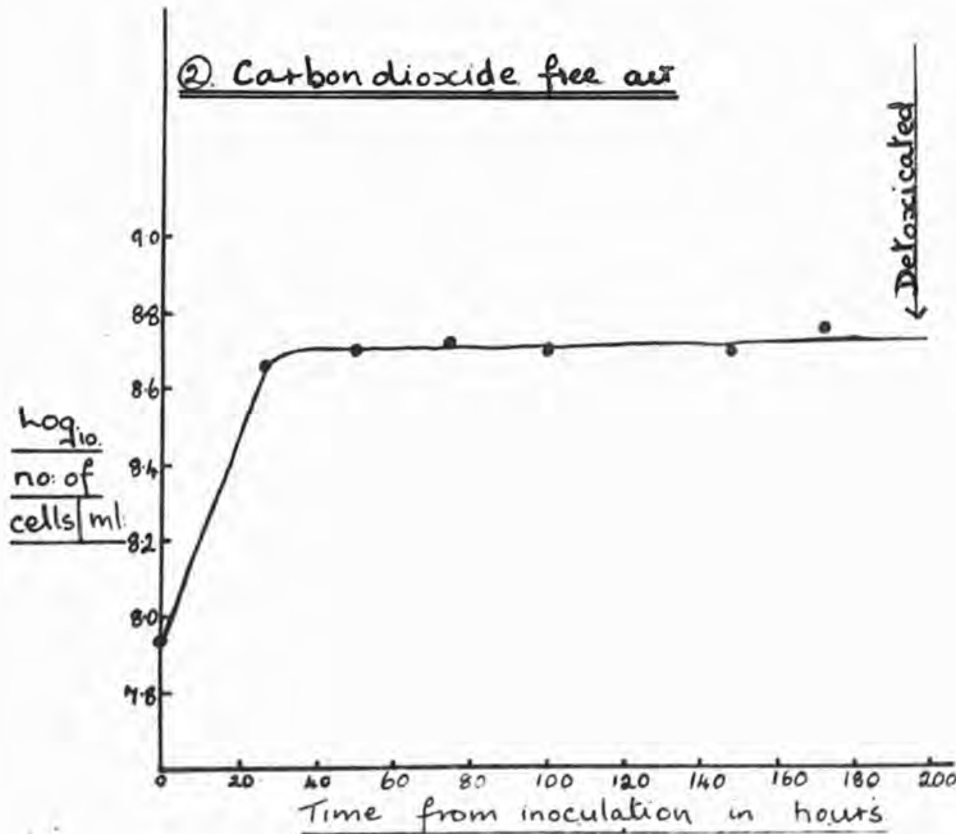
4. The aeration of perfusate with a) Normal and b) CO_2 free air with and without the addition of mineral salts to the solutions.

a) Experimental methods

Perfusers were set up in the usual manner using sterile water as the perfusing fluid. After 45 hours 25 mls. aliquots were removed aseptically, placed in sterile boiling tubes containing air inlet tubes, together with 2.5 mgs. 2:4-D and the appropriate quantities of mineral salts. Other samples were similarly treated but no mineral salts were added to the medium. Further samples were placed in sealed boiling tubes containing air inlet and outlet tubes, together with a vessel of soda lime inserted into the air line leading to them in order to remove the CO_2 from the air stream passing through them. All the cultures were incubated in the dark at 25°C and aerated continuously. Samples were taken at intervals throughout the detoxication process and an attempt made to measure growth in the cultures and phytotoxicity assayed using the cress technique. Samples from the cultures were also run on one way paper chromatograms developed in isopropyl alcohol, ammonia and water solvent and assayed for growth activity using root sections of pea seedlings as previously described.

FIGURE XVI





b) Results obtained from these experiments - See Appendices XVI, XVII and XVIII

The addition or omission of mineral salts to the perfusate had no effect on the detoxication. No quantity of 2:4-D greater than that giving a final concentration of 300 p.p.m. was detoxicated however applied. The inability of the organisms to deal with more than three successive applications of 100 p.p.m. 2:4-D is not due to exhaustion of the mineral medium.

As has been stated previously it is impossible to achieve an accurate absolute measurement of the numbers of organisms in the perfusate owing to the tendency of the bacteria to form large flake like clumps. An attempt to count organisms after dissolution of the clumps by alcohol and formalin was made for comparison of growth in normal and CO_2 free air. The curves so obtained are illustrated in Figure XVI. As will be seen there is no obvious difference between the growth characteristics of cultures aerated with normal air and those aerated with CO_2 free air.

There is a considerable difference between the duration of the lag phase when normal and CO_2 free are used. The lag phase being almost doubled by the lack of CO_2. This result is in accordance with results obtained from experiments in which pure cultures of B. globiforme were aerated by streams of CO_2 free air and growth of the organisms but no detoxication of the herbicide was recorded.

FIGURE XVII

25 mls water perfusate. Mineral salts and 100ppm. 2:4:D added and the solutions aerated with Normal Air

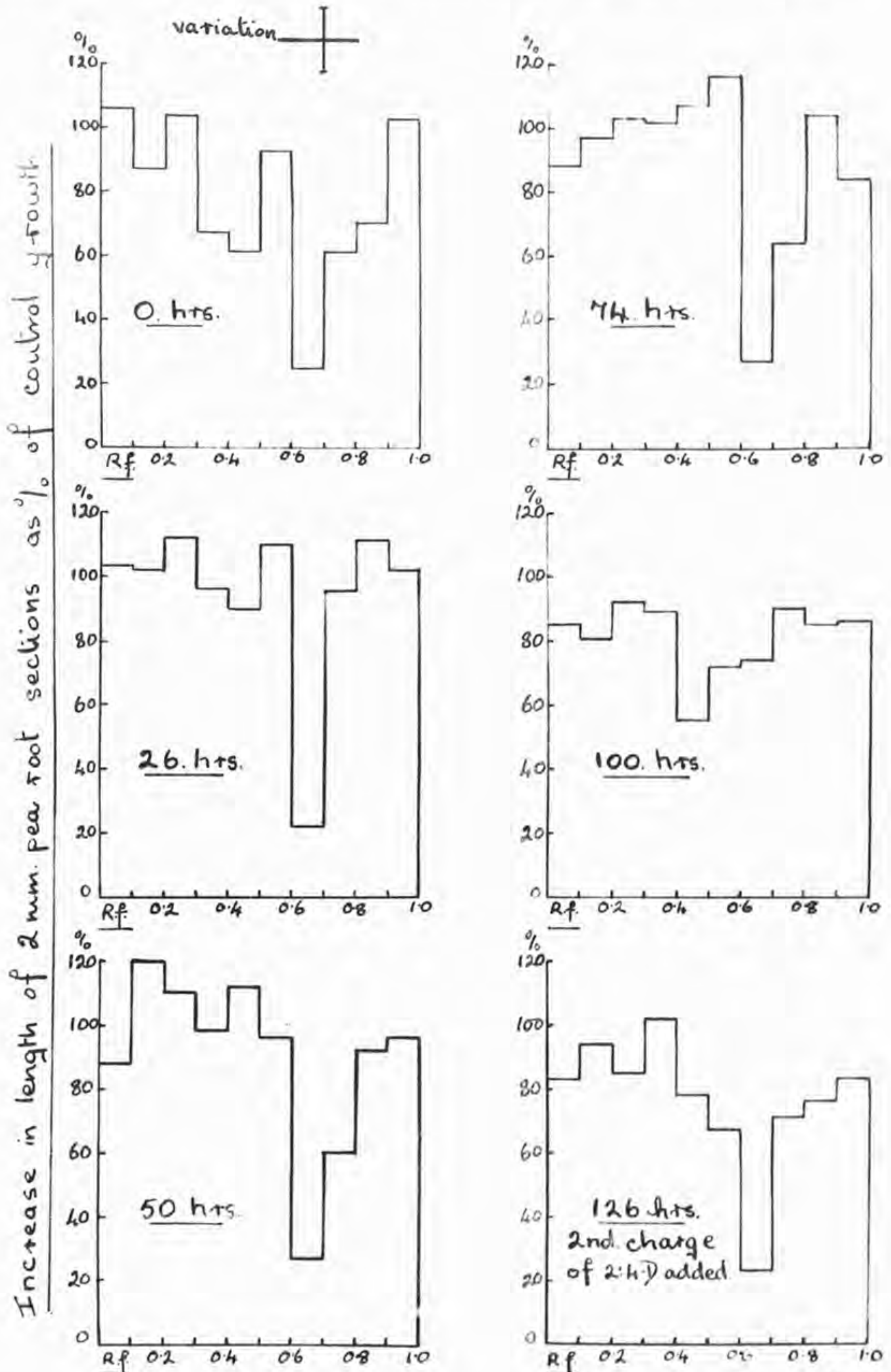


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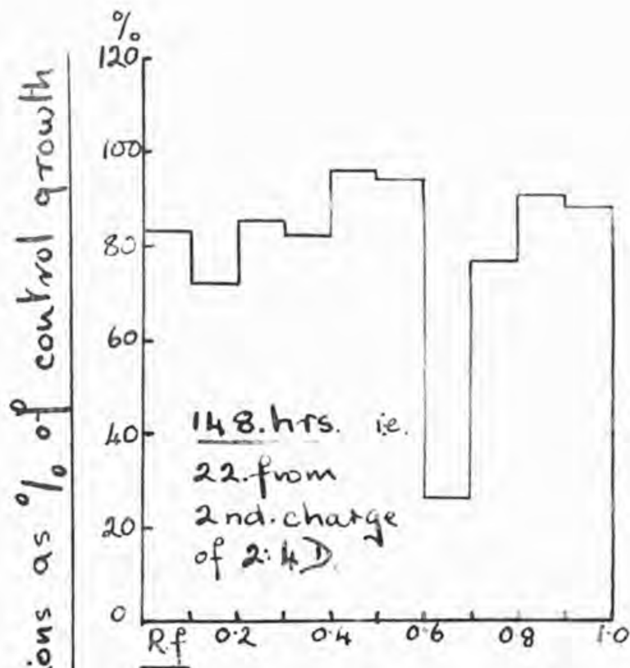
FIGURE XVII continued.



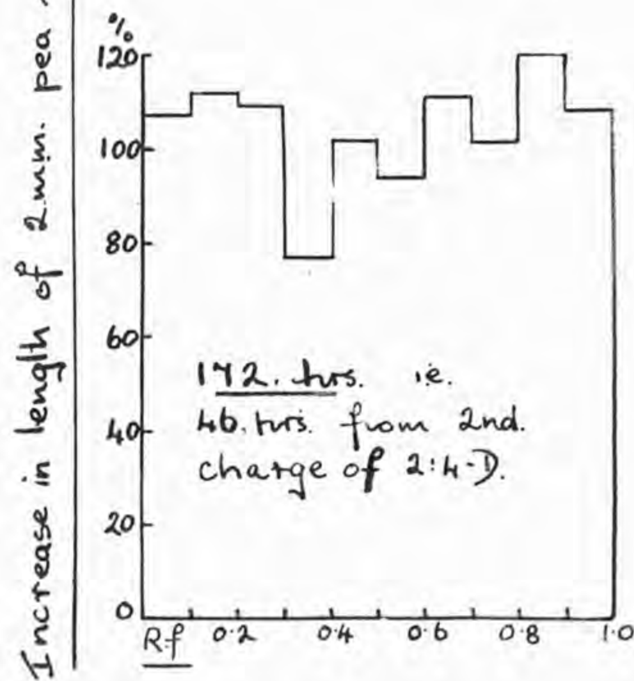


FIGURE XVIII

25 mls. water perfusate. Mineral salts and 100 p.p.m. 2:4-D added and the solutions aerated with CO_2 free air

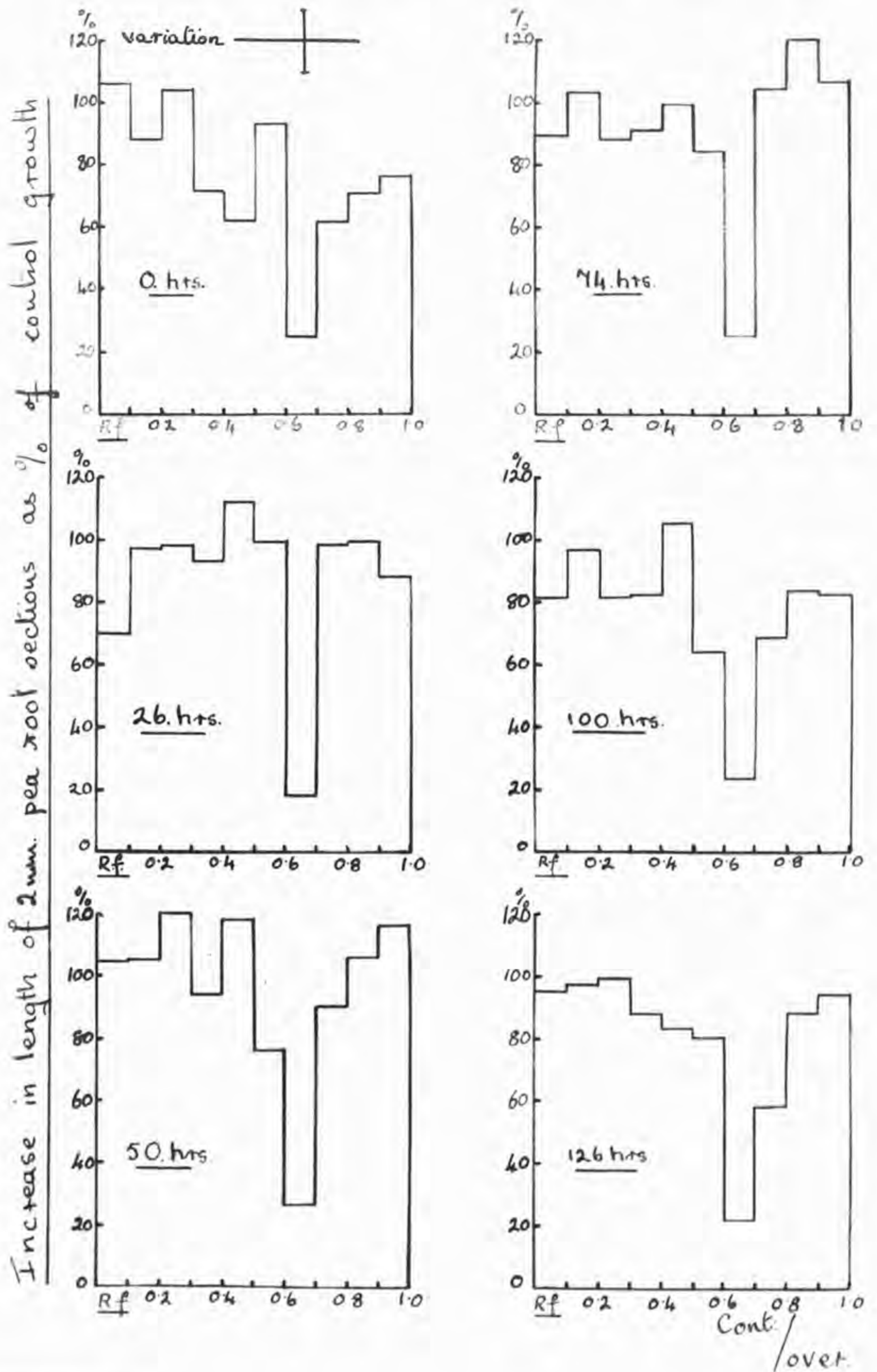


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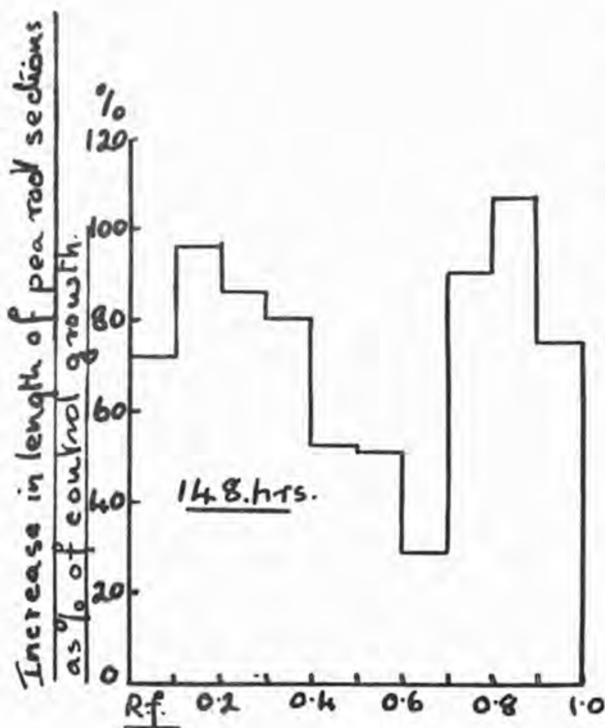
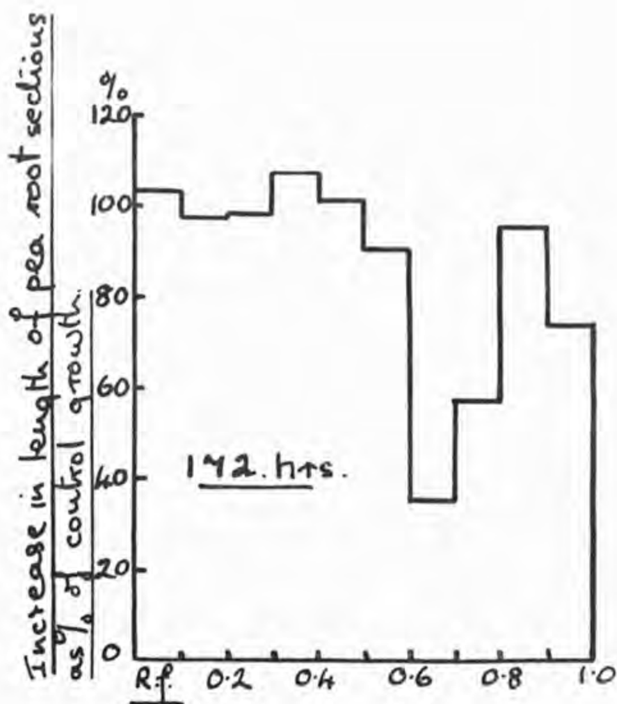
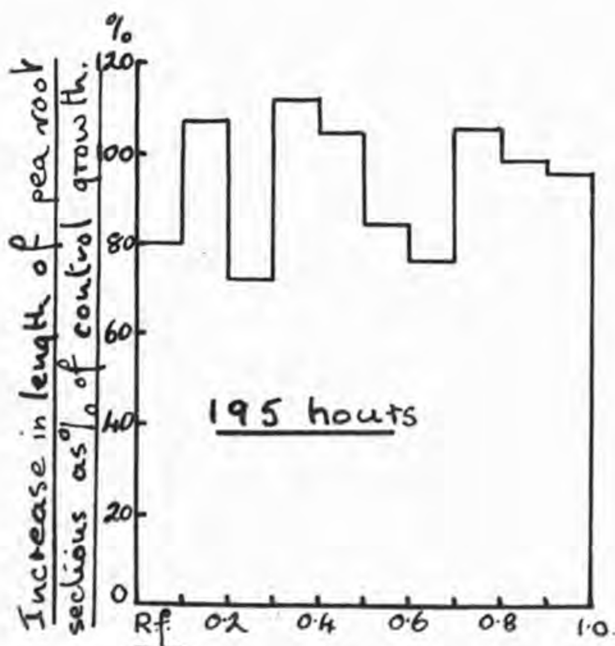
FIGURE XVIII continued.







Figures XVII and XVIII shew the results obtained by chromatographic analysis of the perfusates. They confirm that growth inhibition is present in the 2:4-D spot area up to 195 hrs. from incubation of the CO_2 free air aerated cultures and disappears from the normally aerated at approximately 100 hrs. The growth activity of the chromatograms as a whole is impossible to interpret owing to the large amount of growth active material carried over from the soil, which can be seen to be present at 0 hrs. i.e. the start of incubation. Soil extract presents the same problem and it has not therefore been added, in order to increase the efficiency of breakdown, to experimental cultures intended for chromatographic analysis.

V. Isolation of a fresh effective organism

1. Method of culture

Flasks containing 200 mls. of glass distilled water and 2 mgs. 2:4-D were sterilised, cooled, 50 grms. of fresh garden soil added, and the suspensions aerated. Samples were taken daily after vigorous shaking of the flasks. 0.1 ml. of the suspension was plated onto nutrient agar, 0.1 ml. onto 2:4-D agar - containing 100 p.p.m. and 1,000 p.p.m. 2:4-D. Each set of plates was duplicated. The flasks and plates were incubated in the dark at 25°C. Sampling and the plating out of samples was continued throughout the detoxication of the initial and two further applications of 2:4-D. Phytotoxicity of the soil suspension was assayed throughout the experiment using the cress seedling root test.

After 14 days incubation nutrient agar plates were checked for numbers and types of colonies. 2:4-D agar plates were assayed for phytotoxicity using cress seedlings as the sensitive bio-assay method. The assays were repeated after a further 7 and 14 days incubation if toxicity was present in the first samples.

2. Method of assay of phytotoxicity in agar plates

A small volume of agar, approximately 0.2 ml., was cut from the centre of each plate with a sterile needle and

transferred into 5 mls. glass distilled water in a 10 ml. graduated cylinder and the total volume immediately noted. The agar and water were tipped into a test tube, boiled in a water bath for thirty minutes to thoroughly melt the agar and to destroy the organisms present. The volume of the solution was then made up with glass distilled water to that giving a concentration equivalent to 1 p.p.m. 2:4-D of the original in the agar medium. This diluted sample was then used in the cress assay for phytotoxicity as previously described.

An uninoculated plate was incubated and assayed with the inoculated ones in order that any inaccuracies caused by drying out of the agar during incubation could be accounted for. As no discrepancies in the assays were noted this practice was discontinued after the first few experiments. A plain agar plate, that is containing no 2:4-D, was also assayed. No growth effedt of agar, in this low concentration, on cress seedlings was recorded.

It was thought that a simpler technique for the assay of phytotoxicity in agar might be developed by transferring the appropriate volume of agar containing the herbicide directly to the assay tube. This however was not possible since diffusion of 2:4-D out of the agar at the incubation temperature was so slow that only 10% of the usual inhibition of growth was recorded for the control plates i.e. containing the full concentration of 2:4-D. This method was therefore abandoned.

The method of Jensen 1952 of remelting the agar and growing the cress seeds directly on it was also considered but rejected since this involved the destruction of bacterial growth over the surface of the plate and the object of these experiments was to obtain a pure culture of an organism, able to utilise 2:4-D as a source of carbon, for use in liquid culture experiments. It was therefore of primary importance to retain alive the colonies on detoxicated plates. It would have been possible to overcome this difficulty by making sub-cultures of all distinct colonies on the plates before assay but this would be a very laborious process involving extremely large numbers of sub-cultures the majority of which would not need to have been made if the first plate could be assayed without the destruction of its colonies.

3. Isolation of organisms from primary growth plates and their subsequent culture

Agar plates originally containing 100 p.p.m. 2:4-D and shewing no reduction of phytotoxicity within 28 days were rejected.

Plates in which there was a loss of phytotoxicity during 28 days incubation

All discrete colonies were picked off from one of each pair of duplicate plates and streaked onto both 2:4-D (100 and 1,000 p.p.m. 2:4-D) and nutrient agar slopes.

All the organisms from the other plate of the pair were washed into a liquid culture medium consisting of the three mineral salts, previously used in culture experiments, in glass distilled water together with 100 p.p.m. 2:4-D, aerated and tested at intervals for phytotoxicity.

The streak cultures obtained from the first of each pair of plates were incubated in the dark at 25°C for 14 days. The 2:4-D slopes were then assayed for phytotoxicity and the assays repeated after a further 7 and 14 days if necessary. The parallel nutrient agar slopes were used to check the morphological characteristics of the colonies and to ensure, as far as possible by this method, that the cultures were in fact pure.

All cultures growing on 2:4-D agar slopes from which toxicity was lost were washed into 2:4-D liquid media, aerated and assayed as before.

4. Results of isolation experiments

Regular detoxication of 2:4-D on agar containing 100 p.p.m. 2:4-D occurred during periods of 14-21 days incubation. Detoxication of agar plates containing 1,000 p.p.m. 2:4-D was erratic and in cultures in which the level of toxicity dropped complete removal was seldom recorded.

No detoxication took place in liquid culture even with the mixed cultures washed directly from the detoxicated agar plates into flasks. Mixtures of organisms were also made from the isolated sub-cultures on agar slopes and these inoculated into

liquid media without success.

Eventually an organism was isolated by the methods described above from a flask containing 2:4-D enriched soil and 2:4-D in a concentration of 100 p.p.m. which detoxicated 2:4-D in pure culture in an aerated liquid medium containing 100 p.p.m. 2:4-D. Sub-cultures of this organism were used for subsequent experiments on the breakdown of 2:4-D.

5. Characteristics of this effective organism

The composition of media used and the tests applied are given in Appendix XIXa.

10 tubes of each culture medium were inoculated using organisms from five separate sub-cultures.

Nutrient broth

Dense growth especially at the surface. Whitish skin over surface of the liquid continuing for a short distance along the glass over the liquid.

Nutrient agar

Continuous streak approximately 2 m.m. wide. Raised. Discrete colonies 1-2 mm. diameter. Smooth and glistening. Slight indication of rhizoidal extension at edges after 7-14 days.

Inorganic broth

No visible turbidity but slight growth could be masked by the presence of calcium carbonate.

Inorganic agar

Definite growth. Colonies translucent, whitish growth similar to that on 2:4-D agar but even poorer. On washing the colonies off the slopes the agar was found to be pitted beneath them.

Nutrient gelatin-slopes

Luxuriant growth confluent - similar to nutrient agar in early stages. Surface convoluted. Edges rhizoidal after 3 days. Centre coccoid cells. Very slow liquifaction - not noticeable for at least 14 days and then proceeds very slowly.

Gelatin stab

Luxuriant crateriform surface growth. Very little down stab. Stab growth is rhizoidal. Very slow crateriform liquifaction - not noticeable for at least 14 days and then proceeds very slowly.

Starch broth

Turbidity concentrated at and just under the surface. Whitish skin forms over the surface of the liquid and glass.

Potato slopes

Very luxuriant growth. Raised, lobed colonies. Confluent. Dry white chalky at first then becoming moist and greyish. Later (after 2 months) whole growth and plug becomes brown and slimy. Isolated colonies at first have raised edge with low centre.

Glucose

Skin forms over surface. Very little acid, no gas.

Sucrose

Growth at surface. No acid or gas.

Lactose

Growth at surface. No acid or gas.

Maltose

Growth at surface. No acid or gas.

Mannitol

Good growth at surface. No acid or gas.

Glycerol

Good growth, skin forms at surface. No acid or gas.

Nitrate reduction medium

Surface growth with skin formation. No gas. Nitrite test very positive, using Griese reagent. Negative reaction for ammonia, using Nessler's solution. Uninoculated tubes of medium incubated with tests used as controls - negative.

Amino acid medium

Heavy growth near surface. Whitish skin over surface of the liquid.

Dorset's Egg Medium

Whitish confluent raised moist streak at first. Pigment - dull orange develops very slowly; first visible 7-14 days from inoculation.

Litmus milk

Clot formed. Liquid acidified very slowly.

Phenol

Good growth in media containing phenol as the only carbon source.

Catalase production

Tremendous activity in all cultures tested with H_2O_2 whether on nutrient agar, nutrient gelatine or inorganic agar. No reaction on uninoculated plates.

Acid fast stain

Variable. Some acid fast organisms in mainly non-acid fast cultures.

Gram stain

Positive.

One point to be noted in connexion with the reactions involving the use of Durham tubes for collection of gas which may be formed in the medium is that since all growth occurs at the surface of the media gas even if formed is unlikely to accumulate in the inverted Durham tube which is well below the surface.

6. General morphology of the organism in 2:4-D culture

Organisms from vigorously aerated liquid cultures always appeared as small ovoid cocci one diameter being slightly greater than the other. Films made from 2:4-D agar plate

cultures shew the same type of organism the longer diameter being slightly further extended in some cases. Short rods if produced never exceeded twice their diameter in length. No mycelial forms or long rods seen, even in very young liquid culture.

Growth on 2:4-D agar was relatively slow. Small whitish translucent smooth colonies formed 1-2 mms. in diameter. Maximum size reached after approximately 14 days incubation. No mycelial or rhizoidal growth noted.

Growth on 2:4-D media is so different from that recorded on the standard media that it is doubtful if it can be of use in identifying the organism.

7. Identity of organism

From the results of the characterisation tests the organism isolated from soil and capable of detoxicating 2:4-D in aerated liquid culture containing no organic materials apart from 2:4-D, the carbon source, was identified, by the use of Bergey Manual of Determinative Bacteriology VI Edition as belonging to the genus Nocardia in the family Actinomycetaceae of the order Actinomycetales.

The determination of the species proved less satisfactory since the characteristics of the isolated organism are not exactly similar to those of any of the recognised strains

described in this Manual. However it is possible that in the cultivation of this organism on such a highly selective medium as 2:4-D the strain now being cultured has lost or altered some of its original characters in addition to forming an adaptation to this carbon substrate.

The species most closely resembling this organism appear to be:-

Nocardia pulmonalis

Nocardia minima

Nocardia coeliaca

There are however several important differences in each case:-

Nocardia pulmonalis

Initial mycelium of this organism is well developed and it produces a lemon yellow pigment on potato. The habitat is considered to be bovine infections - the original source being the lungs of a cow.

Nocardia minima

This species does not liquify gelatin. A pink pigment is developed on agar and only grows slowly on potato and also develops a pink colouration on this medium also.

Nocardia coeliaca

This species produces an alkaline reaction in litmus milk medium, forms hollow lobes in deep gelatine culture and does not reduce nitrates to nitrites.

VI. Experiments using pure cultures of the effective strain of Nocardia

1. Introduction

This strain of Nocardia was found to detoxicate 2:4-D in liquid cultures, containing not more than 300 p.p.m. 2:4-D as the only added carbon source, if the culture solutions were vigorously and continuously aerated.

The effects of 2:4-D concentration, soil extract and agar dialysate on detoxication were studied. Studies on the breakdown of radioactive 2:4-D were carried out with this organism. Growth studies of the organism in unaerated liquid cultures containing various carbon substrates were made and the effect of growth on these media of subsequent ability to detoxicate 2:4-D in agar media recorded.

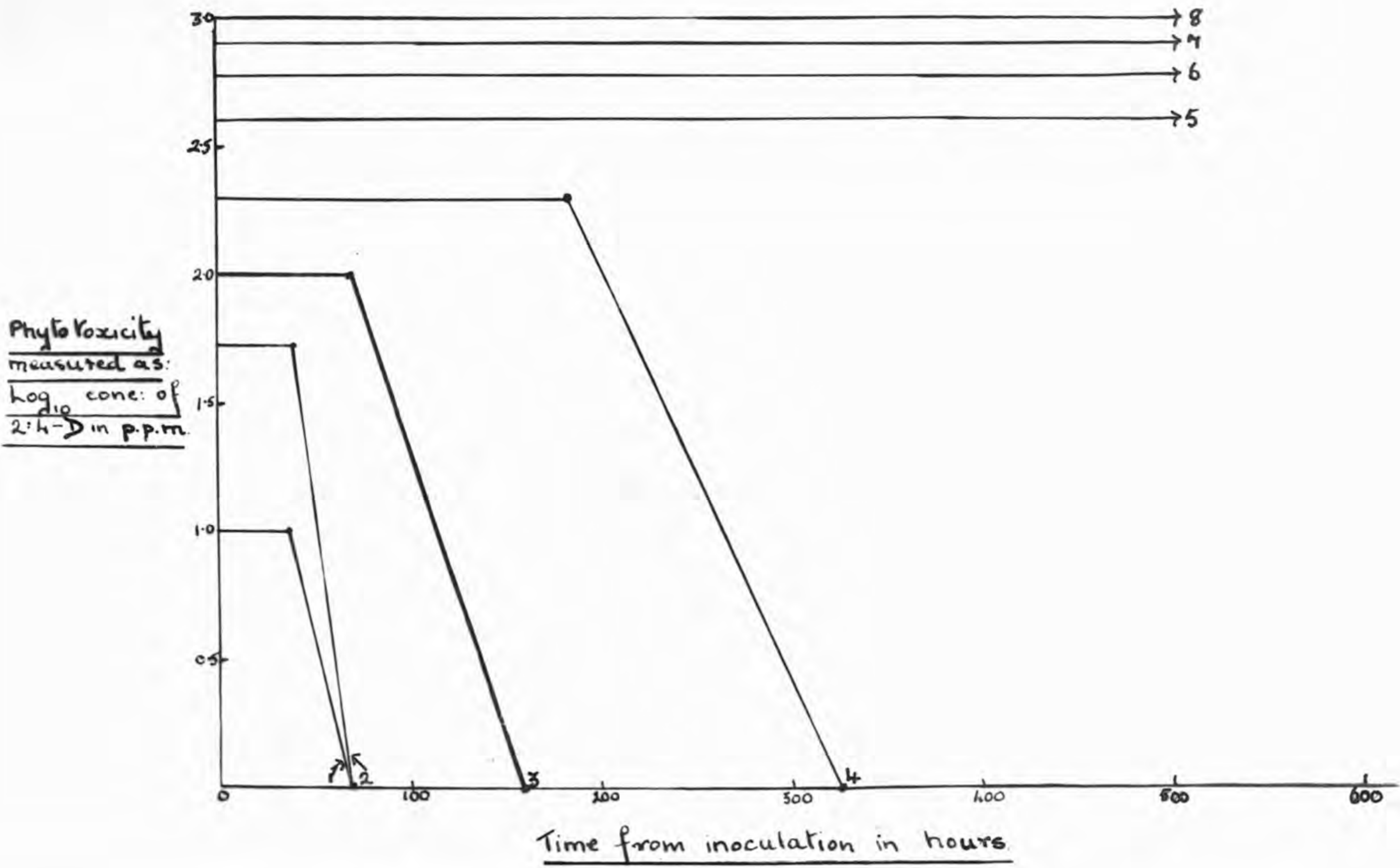
The effect of 2:4-D concentration on detoxication

Experimental Method

Series of culture tubes were set up containing concentrations of 2:4-D ranging from 10 p.p.m. to 1,000 p.p.m. Equal inocula of the same suspensions of bacteria were transferred into each, the tubes aerated and tested at intervals, for phytotoxicity.

FIGURE XIX

The effect of initial concentration of 2:4-D on its detoxication



Result

The main results are given in Appendix XIXb and are shewn in diagrammatic form on Figure XIX.

Detoxication of 2:4-D occurs in liquid cultures containing 300 p.p.m. 2:4-D or less. In cultures containing sufficiently low quantities of 2:4-D for the organisms to decompose, the initial concentration affects the time taken for complete loss of toxicity - the higher the concentration the longer the time taken to detoxicate. This result would be expected if detoxication commenced at the time of inoculation and continued steadily until all the 2:4-D had been consumed. As can be seen from the figure such is not the case but there is no drop in toxicity, within the limits of accuracy of the assay method, for a considerable period and then a rapid drop. At concentrations of 400 p.p.m. 2:4-D and above no detectable drop in phytotoxicity occurred; the lag phase was extended indefinitely.

As will be seen later experiments with 2:4-D labelled C^{14} in the side chain produce evidence that this portion of the 2:4-D molecule is attacked straight away. These results suggest that the level of phytotoxicity is maintained not by 2:4-D alone but partly by another toxic substance produced by the bacteria during the destruction of 2:4-D under these conditions. There is at this stage no evidence to indicate whether or not this hypothetical substance acts as a bacteriocidal or bacteriostatic

agent for this organism under these conditions.

The effect of soil extract and agar dialysate on detoxication

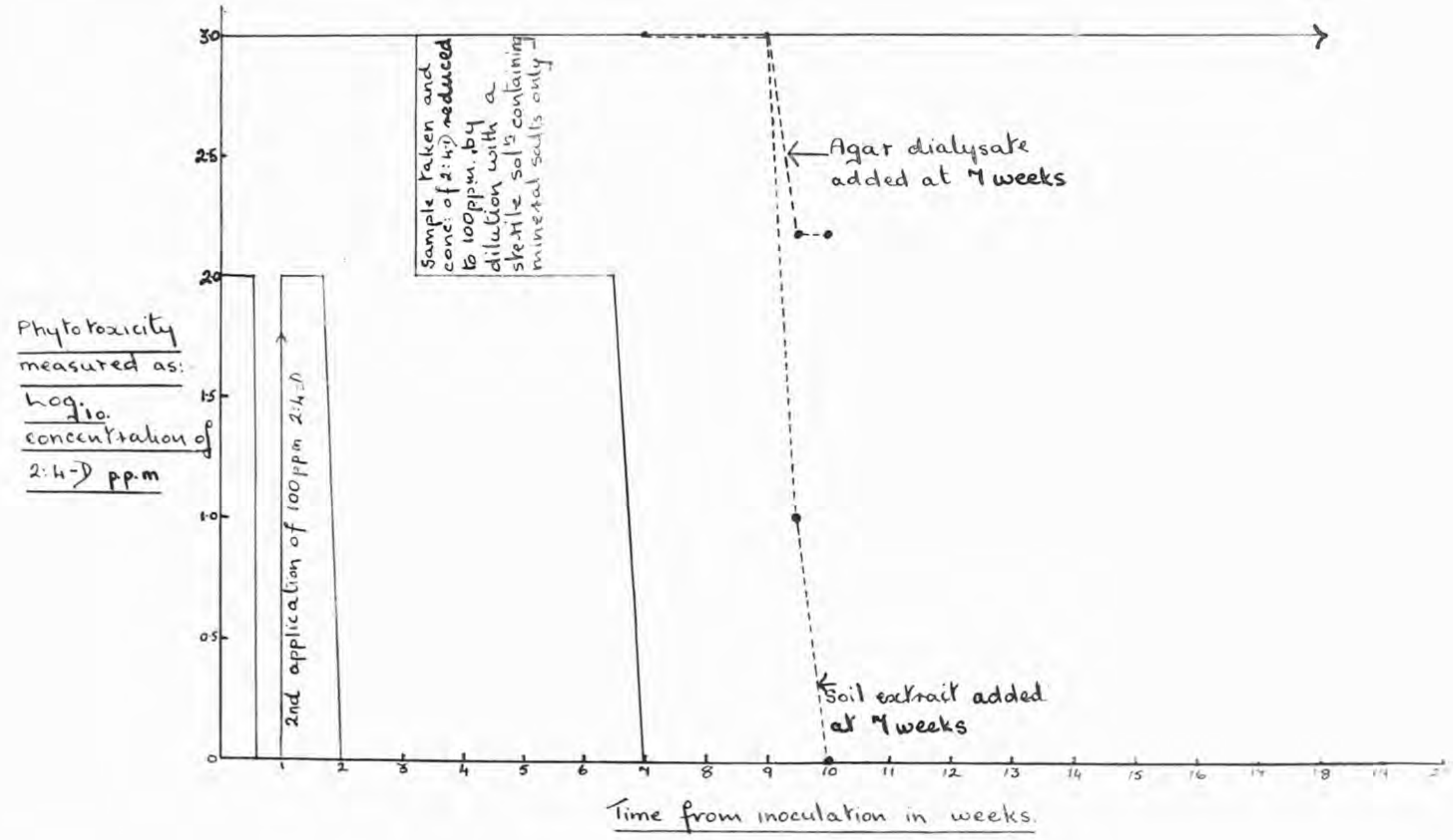
a) Preparation of soil extract and agar dialysate

Soil extract was prepared as previously described in the section dealing with growth studies of Bacterium globiforme on page 10.

Agar dialysate was prepared from fine powdered agar. The agar powder was poured into a length of "sausage skin" dialysing membrane one end of which was sealed. The agar was moistened, all air bubbles excluded and the other end then sealed. The complete tube was immersed in glass distilled water, in proportion: 10 mls. water to 1 grm. of agar, and left for one week at a temperature of 1°C. The agar was then removed and the dialysate sterilised and used in the preparation of culture media as stated.

b) Cultures containing initially 1,000 p.p.m. 2:4-D

Paired flasks of 500 mls. capacity one containing 250 mls. culture medium with 1000 p.p.m. 2:4-D and the other of the pair 250 mls. culture medium with 100 p.p.m. 2:4-D were sterilised and inoculated together with equal inocula from the same stock culture. Both were aerated and incubated in the dark at 25°C. Samples were taken at intervals for phytotoxicity



The effect of soil extract and agar dialysate on detoxication of 2:4-D FIGURE XX

assay. A further application of 100 p.p.m. 2:4-D was made to the second flask after detoxication of the initial charge was complete.

A sample from the flask containing the high concentration of 2:4-D was removed after 3 weeks from inoculation, diluted 10 times with sterile salts solution, aerated and assayed at intervals for phytotoxicity.

Further 25 ml. samples were removed 7 weeks from the time of inoculation and to one 1 ml. of soil extract and to the other 1 ml. of agar dialysate was added. These cultures were aerated and phytotoxicity followed. The results are shewn in Figure XX and Appendix XX.

Although no significant fall in the level of phytotoxicity occurred during the experimental period in the flask originally charged with 1000 p.p.m. 2:4-D, the organisms it contained were effective in detoxicating lower concentrations as shewn by the fact that repeated detoxication occurred in the other flask of the pair initially charged with 100 p.p.m. 2:4-D Growth of the organisms in the high concentration of 2:4-D was complete by the end of the second week, but the organisms were still alive and effective in detoxicating 2:4-D since the sample taken after 3 weeks and diluted ten times detoxicated although with an extended lag period of a further three weeks. This suggests that the breakdown mechanism of the organisms was impaired by contact with this high concentration or that a

considerable proportion of the organisms were in fact dead. Small quantities of organisms removed from the flasks and inoculated into 100p.p.m. 2:4-D flasks failed to grow.

The addition of small quantities of soil extract and agar dialysate to further samples taken from the main flask 7 weeks after inoculation caused a rapid detoxication with a further lag of only two weeks. This detoxication was complete in the case of the sample to which soil extract was added but only partial - reducing the concentration to approximately 120 p.p.m. or 12% of the original in the flask to which agar dialysate was added. This only partial detoxication effect was probably due to the limited amount of agar dialysate added and to the fact that the "active component" of the dialysate was present in only very minute quantities.

c) Further experiments with these two materials

Experiments were set up in which concentrations of 100 and 1000 p.p.m. 2:4-D were used in association with:-

Soil extract

Agar dialysate

Soil extract and agar dialysate

Neither substance

Results (See Appendix XXb)

It was again found that concentrations of 1000 p.p.m. 2:4-D would be detoxicated by these organisms in the presence of soil extract and/or agar dialysate. At the lower concentration of 2:4-D (100 p.p.m.) no significant effect of either substance on the rate of detoxication of the herbicide was observed.

The addition of soil extract to culture media containing 100 p.p.m. 2:4-D increased the number of subsequent applications of the same quantity of 2:4-D which could be dealt with by the cultures from a maximum of 2 (without soil extract) to a maximum of 10 or 12. Fresh soil extract must be added with each dose of 2:4-D for this effect to occur.

Various quantities of soil extract and agar dialysate were added to the 25 ml. medium used. All concentrations used for soil extract, down to 0.25%, were effective in producing complete detoxication of 1,000 p.p.m. 2:4-D. It was found that at 10% agar dialysate was needed to ensure complete detoxication of 2:4-D at the same concentration.

Growth on various carbon substrates in unaerated liquid culture both in the presence and absence of 2:4-D and the effect of such culture on subsequent ability to detoxicate 2:4-D in agar

a) Introduction

No growth, as measured by increase in cell numbers, of this organism takes place in still cultures with 2:4-D as the only added carbon source and no decrease in toxicity occurs. Growth of the organism and detoxication of the herbicide however occurs regularly in cultures aerated vigorously and continuously with moist sterile air. This difference in behaviour of the organism in aerated and unaerated culture could be due to a variety of factors.

That the organism is strictly aerobic became abundantly clear in the characterisation tests but it proved able to grow at the surface of still liquid cultures on a variety of substrates. It does not appear that a continuous air current through the cultures is necessary for growth to occur on all media. The possibility therefore arises that vigorous aeration of the cultures is necessary only for the destruction of 2:4-D. This suggests, if true, that this substance itself may be toxic to the bacteria, especially in view of the small quantities which can be tackled, or that during the decomposition of the herbicide an intermediate compound is produced which is

toxic and which may be "neutralised" by oxidation - either actively by the bacteria under conditions of high oxygen concentration or spontaneously under these specialised culture conditions. Another possibility is that this toxic substance is volatile and the passage of a continuous stream of air through the cultures reduces its concentration below the lethal level for the organisms in question.

It was therefore decided to investigate the behaviour of this organism in still culture on other carbon substrates both with and without 2:4-D in the culture medium.

b) Experimental method

Boiling tubes of 1" diameter containing 10 mls. of the culture solutions and plugged with cotton wool were used in order that the liquid should be sufficiently shallow to provide reasonably aerobic conditions for growth. The tubes were autoclaved at 15 lbs. pressure for fifteen minutes to sterilise. Volatile or unstable carbon substrates were made up aseptically in sterile glass distilled water and added to the culture fluids after autoclaving.

Series of tubes for each medium were prepared and inoculated with equal aliquots from a suspension of organisms which was actively decomposing 2:4-D in aerated culture. The cultures were incubated at 25°C in the dark for seven days and growth estimated by eye on an arbitrary scale of turbidity 0 - 5.

Further observations were made after 14, 21 and 28 days but in no case was any increase in turbidity recorded after the 7 days reading.

Scale

0 - perfectly clear - no turbidity

1 - turbidity just visible, faint milkiness

2 - fairly turbid

3 - turbid

4 - very turbid

5 - extremely thick growth usually associated with the formation of flakes in the medium and very dense surface growth.

All carbon substrates were used in a concentration of 100 p.p.m., with and without 100 p.p.m. 2:4-D.

Subcultures of 0.1 ml. of each fluid from all tubes were made onto 2:4-D agar containing 100 p.p.m. 2:4-D and the plates incubated in the dark at 25°C for 14 days. Phytotoxicity in the agar was then assayed using the cress seedling technique previously described. Plates shewing a high level of phytotoxicity were re-assayed after a further 7 and 14 days.

The results of these experiments are set out in Table I.

TABLE I

Growth of 2:4-D decomposing strain of Nocardia on various carbon substrates in the presence and absence of 2:4-D in unaerated liquid culture and the effect of such cultures on subsequent ability to decompose 2:4-D on agar plates.

Carbon substrate	Growth No 2:4-D	Subsequent detoxication	Growth + 2:4-D	Subsequent detoxication of 2:4-D in agar
No additional	0	✓	0	erratic
Acetic acid	4	0	4	0
Glycollic acid	1	0	1	0
Glyoxylic acid	1	✓	1	✓
Succinic acid	3	✓	3	✓
Glucose	2	0	2	0
Sucrose	2	0	2	0
Phenol	3	✓	3	✓
o-chlorophenol	2	✓	2	✓
p-chlorophenol	2	0	2	0
2-4-dichlorophenol	0	0	0	0
2-4-dichloranisole	2	0	2	0
4-chlororesorcinol	4	erratic	0	0
4-chlorocatechol	2	0	2	0
3-5-dichlorocatechol	0	erratic	0	0
chlorohydroquinone(BDH)	0	0	0	0

Cont/over

TABLE I - continued

Carbon substrate	Growth No 2:4-D	Subsequent detoxication of 2:4-D in agar	Growth + 2:4D	Subsequent detoxication of 2:4-D in agar
Hydroquinone	2	0	2	0
Quinhydrone	0	0	0	0
chlorohydroquinone (M & B)	4	0	4	0
chloro-p-benzoquinone (M & B)	0	0	0	0
4-chloro-2-hydrophenoxyacetic acid (M & B)	5	0	5	0
2-chloro-4-hydrophenoxyacetic acid (M & B)	5	0	5	0
4-chlorophenoxyacetic acid (M & B)	5	0	5	0
2-chlorophenoxyacetic acid (M & B)	2	✓	2	✓
2-4-dichloro-6-hydroxyphenosyacetic acid (M & B)	2	✓	2	✓
2-4-dihydroxyphenoxyacetic acid	0	0	0	0
1-2-4 trihydroxybenzene	0	0	0	0

c. Result

It will be seen that whilst 2:4-D itself does not support growth of this organism in unaerated liquid culture its presence does not affect the growth produced on other carbon substrates. 2:4-D itself in this concentration is not actively toxic to this particular organism. Growth in still cultures is supported in varying degrees by a large proportion of the substrates tested - some with molecules very similar to that of 2:4-D. No growth is recorded in solutions of 2:4-dichlorophenol, 2,4-dihydroxyphenoxyacetic acid, 1,2,4-trihydroxybenzene, 3,5-dichlorocatechol, chloro-p-benzoquinone, quinhydrone, chlorohydroquinone (B.D.H.). This last substance is not guaranteed pure. A specially pure sample of chlorohydroquinone and of chloro-p-benzoquinone was obtained (from May and Baker) with the result shewn on the Table namely that chlorohydroquinone supports good growth of the organism but that chloro-p-benzoquinone does not. It was therefore concluded that pure chlorohydroquinone could be utilised as a carbon source by the strain of Nocardia in still culture but that chloro-p-benzoquinone could not and that this or some similar substance having the same action was most probably present in the first sample of chlorohydroquinone used.

Growth in aerated liquid cultures containing 100 p.p.m. 2:4-D is equal approximately to "1" on this arbitrary scale. Very little growth takes place in 2:4-D compared with that

produced by similar concentrations of other carbon sources the most noticeable being the extremely heavy growth on the substances having very similar molecules to that of 2:4-D namely:-

4-chlorophenoxyacetic acid

2-chloro-4-hydroxyphenoxyacetic acid

4-chloro-2-hydroxyphenoxyacetic acid.

This heavy growth coupled with the fact that organisms from these cultures subsequently transferred to 2:4-D agar plates failed to detoxicate the 2:4-D would seem to exclude these substances from being considered as intermediates in 2:4-D breakdown.

Very few of the compounds used left the bacteria capable of detoxicating 2:4-D in agar. Exceptions to this general rule being glyoxylic and succinic acids from the side chain possibilities and 2-4-dichloro-6-hydroxyphenoxyacetic acid, 2-chlorophenoxyacetic acid, phenol and o-chlorophenol of the ring compounds. It should also be noted that whereas no growth took place in media containing no carbon source i.e. mineral salts only the organisms inoculated into these tubes remained viable and adapted to 2:4-D breakdown but organisms inoculated into media containing 2:4-D shewed only erratic retention of the adaptation. The absence of a carbon substrate does not cause death or deadaptation and the mere presence of 2:4-D in company with another carbon substrate does not either

but the inoculation of organisms into solutions containing 2:4-D only as a source of carbon without aeration impairs their adaptation to 2:4-D breakdown. These observations again suggest that the intact 2:4-D molecule is not toxic to the organisms but that an intermediate produced very early on in the detoxication process is, and that this can only be dealt with in aerated cultures.

d) The effect of soil extract and tryptone on growth in unaerated liquid culture

i. Introduction

The results of previous experiments had shewn that large quantities of 2:4-D, up to 1000 p.p.m., could be detoxicated in the presence of soil extract in aerated culture. It was therefore decided to find the effect of soil extract on the retention of 2:4-D adaptation by these organisms during culture in still liquids containing 2:4-D in concentrations of 100 and 1000 p.p.m.

ii. Experimental method

An exactly similar method to that used in the experiments just described was employed. Concentrations of 100 p.p.m. 2:4-D and 1000 p.p.m. were used. Concentrations of 1% and 10% soil extract and also 1 p.p.m. and 10 p.p.m. tryptone were employed.

The results of this experiment are shewn in Table II below.

TABLE II

Substrate	Growth no 2:4-D.	Subsequent detoxication of 2:4-D on agar	Growth + 2:4-D	Subsequent detoxication of 2:4-D on agar
1 ppm.Tryptone	0-1 very little if any	✓	0-1	✓
10 ppm.Tryptone	1	✓	1	✓
1% soil extract	1	✓	1	✓
10%soil extract	2	✓	2	✓
10 ppm. 2:4-D	0	0	-	-
1000 ppm.2:4-D	0	0	-	-
1 ppm.Tryptone 1000 ppm.2:4-D	2	✓	-	-
10ppm.Tryptone 1000ppm.2:4-D	3	✓	-	-
1% soil extract 1000ppm.2:4-D	2	✓	-	-
10%soil extract 1000ppm.2:4-D	3	✓	-	-

iii. Two interesting features emerge from this experiment. The first remarkable fact is that no loss in adaptation to 2:4-D breakdown occurs with the organisms inoculated into either 100 ppm. or 1000 ppm. 2:4-D in the presence of soil extract or of very small quantities of the pancreatic digest of casein known as tryptone. These substances on their own in these

small quantities cause no de-adaptation in these concentrations. The second fact is that whilst no growth occurs in cultures containing only mineral salts and 2:4-D, some growth occurs on soil extract and tryptone in the absence of 2:4-D, no increase in this growth is measurable on the addition of 2:4-D (100 p.p.m.) to tryptone or soil extract in the cultures, yet cultures containing soil extract or tryptone plus 1000 p.p.m. 2:4-D shew some increase in turbidity over that recorded for the soil extract or tryptone alone. This suggests that a minute, undetectable growth occurs in cultures containing the lower concentration of 2:4-D. No measurable detoxication occurred in any of these cultures.

These facts again suggest the partial neutralisation of the toxic effect of an intermediate substance produced early on in the decomposition of the 2:4-D. The neutralisation cannot be complete since then it would be expected that detoxication of the medium would take place. This however seems only possible in aerated media. The neutralisation is sufficient, in unaerated culture, to prevent loss of adaptation; no reduction in the vitality of the organisms as a whole, or of the particular enzyme systems involved in the breakdown of 2:4-D being detectable under these conditions.

Tryptone is a pancreatic digest of casein and its effectiveness in replacing soil extract in the above experiments, in very low concentrations, suggests that the active factor in

soil extract may well be an amino acid. Rogoff and Reid 1956, have obtained decomposition of up to 3,000 p.p.m. 2:4-D by cultures of a Corynebacterium in a medium containing glutamic acid, but this particular acid is not effective in producing more efficient breakdown of 2:4-D by Nocardia. Appendix XXII

e). The compounds of interest in the further study of the mode of breakdown of 2:4-D by this organism emerging from this series of experiments are: firstly those which whilst being utilised as carbon substrates do not cause any subsequent loss of adaptation to the metabolism of the 2:4-D molecule by the organisms so using them. These compounds are possible intermediates in the breakdown process but with one important reservation, they are metabolised in the absence of aeration; 2:4-D is not, therefore the metabolism of any of these substances cannot be the first step in the process - one or more stages requiring aeration must occur prior to the destruction of any of these compounds. The compounds considered under this category are:-

Glyoxylic acid
succinnic acid
2:4-dichloro-6-hydroxyphenoxyacetic acid
2-chlorophenoxyacetic acid
ortho-chlorophenol
phenol

There is also the possibility that any or all of these compounds follow a completely different metabolic pathway from that of 2:4-D and the latter mechanism is left unimpaired. This possibility is rendered less likely by the fact that attempts to grow 2:4-D destroying organisms on any media containing carbon substrates other than 2:4-D and at the same time retain their adaptation to 2:4-D has met with failure.

The second group of compounds of interest are those upon which this organism is unable to grow in the absence of aeration. These substances are either materials which cannot be utilised by this organism as a carbon substrate or they may be actively toxic to the bacteria in this concentration.

The compounds to be considered under this category are:-

2:4-D itself
2-4 dichlorophenol
3-5 dichlorocatechol
chlorohydroquinone (B.D.H.)
Quinhydrone
chloro-benzoquinone
2-4 dihydroxyphenoxyacetic acid
1-2-4 trihydroxybenzene

Considering this last group of compounds

Chlorohydroquinone has already been discussed.

2:4-D. As far as it is possible to ascertain the intact 2:4-D molecule is not toxic to these organisms. No reduction in growth or viability on any other substrate has been recorded for this organism in the presence of 2:4-D as compared with exactly similar cultures incubated in the absence of 2:4-D.

The remaining six compounds

No detectable growth took place on these materials (100 p.p.m.) No subsequent detoxication of 2:4-D occurred when organisms from these cultures were plated out onto 2:4-D agar. Organisms remain viable and adapted to 2:4-D breakdown in the mere absence of a carbon substrate therefore it appears that not only are these substances unable to support growth of this organism under these circumstances but that at this concentration, 100 p.p.m., they are actively toxic to it.

Concentrations of up to 100 p.p.m. of any of these materials would be most unlikely to occur in any experimental culture containing initially only 100 p.p.m. 2:4-D.

Experiments to find the effect of much smaller quantities of these materials on the organism were set up.

f) <u>The effect of these compounds on growth of the organism in liquid culture containing 0.1% glucose as a carbon substrate</u>

The methods used were exactly similar to those used in the previous still culture experiments. The requisite quantities of the additional organic materials were added aseptically after sterilisation of the main medium. Concentrations of 10, 1.0 and 0.1 p.p.m. of the substances were used - that is 10%, 1% and 0.1% of the initial concentration of 2:4-D normally used.

TABLE III

Growth of 2:4-D effective organism on 0.1% glucose in the presence of toxic substances

	Concentration - p.p.m.			
Compound	10	1.0	0.1	0
	Growth measured on arbitrary scale as before			
Quinhydrone	2	2	2	2
2-4 dichlorophenol	0	0	1	2
3-5 dichlorocatechol	2	2	2	2
chloro-p-benzoquinone	0	0-1	1-2	2
2-4 dihydroxyphenoxy-acetic acid	1	1-2	2	2
1-2-4 trihydroxybenzene	2	2	2	2

These results shew that at concentrations of 10 p.p.m. or less (i.e. equivalent to 10% or less of the original 2:4-D concentration in aerated cultures) quinhydrone, 3-5 dichloro-catechol, and 1:2:4 trihydroxybenzene do not affect growth on glucose in still culture. It appears that these substances are not themselves metabolised since a denser growth than that recorded for the control tubes containing glucose only would then be expected, but that a concentration greater than 10 p.p.m. is necessary for toxicity to be noticeable. No growth was recorded in cultures containing these substances in the previous set of experiments. These substances do not then support growth in unaerated culture but at concentrations of 10 p.p.m. or less do not inhibit growth on other carbon substrates.

<u>2:4 dichlorophenol</u> entirely prevents growth of the organism in concentrations as low as 1 p.p.m. and less growth is recorded at 0.1 p.p.m. than for the control tubes. This substance is toxic to this organism in extremely low concentrations and a concentration equivalent to only 1% of the original 2:4-D added to aerated cultures is sufficient to completely inhibit growth in still cultures.

<u>Chloro-p-benzoquinone</u>. Here a similar picture is presented but slight growth in this case took place in some of the cultures containing 1 p.p.m. of this substance. The growth at this level was erratic - shewing toxicity was still present.

Toxicity had almost disappeared at the 0.1 p.p.m. level but some inhibition of growth as compared with the controls was still recorded.

<u>2:4 dihydroxyphenoxyacetic acid</u>. This substance shewed some interference with growth of this organism at the 10 p.p.m. level but very little at concentrations lower than this. Concentrations greater than 10% of the original 2:4-D would therefore be needed to completely prevent growth.

From these observations two substances emerge as being highly toxic to this particular organism in low concentrations and also having a molecular structure which could derive from the breakdown of the 2:4-D molecule. These two substances are 2,4 dichlorophenol and chloro-p-benzoquinone. 2:4 dichlorophenol could theoretically be derived from 2:4-D by the removal by hydrolysis of the acetic acid side-chain as put forward as a possible first step by Audus 1952. The formation of chloro-p-benzoquinone involves the removal of the side chain and of the para position chloride ion and its replacement by OH. The formation of the quinone from hydroquinone would then presumably be dependent upon cultural conditions - neutral to alkaline pH and rapid aeration. If this is so it would seem that unaerated cultures should be more effective in breaking down 2:4-D since chlorohydroquinone is easily and rapidly metabolised in unaerated culture.

Both of these substances - 2:4-dichlorophenol and chloro-p-benzoquinone are volatile in so far as they are present in the air stream leaving flasks containing a watery solution of them and they can be trapped by passing the air stream through an alkaline solution such as NaOH. Both substances react with Folin and Ciocalteu's phenol reagent and may thus be detected in such an alkaline trap.

5. <u>The effect of these toxic compounds on detoxication of 2:4-D in aerated liquid culture</u>

a. <u>Experimental methods</u>

Culture tubes were set up as previously described for aerated liquid culture experiments. The requisite quantities of the appropriate compounds were added after autoclaving and just prior to inoculation of the culture solutions. Samples were taken at intervals throughout the experimental period.

The culture solutions contained the usual mineral salts together with 100 p.p.m. 2:4-D and 1.0 or 10 p.p.m. of one of the compounds under investigation. Control tubes contained mineral salts and 100 p.p.m. 2:4-D only. The experiments were discontinued after 23 days owing to evaporation of the culture solutions and reduction in volume by sampling.

b. The results of these experiments are set out in Table IV Page 83 and Appendix XXI. It will be seen that all of the compounds delay the detoxication of 2:4-D in spite of the fact that the 2:4-dichlorophenol, for example, is bubbled out of the solution within 48 hours. At the concentrations used these compounds are all toxic to the organism concerned in so far as they act as inhibitors to the breakdown process of 2:4-D the most striking effect is seen with 1-2-4-trihydroxybenzene and 2-4-dihydroxyphenoxyacetic acid. In neither of these cases was any drop in the phytotoxicity of the solutions detected even at

the lower concentration. 3-5 dichlorocatechol behaves in the same manner for the higher concentration but detoxication of the lower was recorded after 20 days incubation and aeration. If any of these substances appear as intermediates in the breakdown process of 2:4-D, concentrations of less than 1% of the initial (100 p.p.m.) 2:4-D must be ensured in the culture medium if detoxication is to occur in the usual (7-10 days) time for this type of experiment.

Since no accumulation of intermediate has been found in the culture solutions, and the process of detoxication of the herbicide molecule appears to be continuous, it is thought more likely that a toxic substance is produced early in the process and that the overall rate of the decomposition depends on the rate of neutralisation of the toxicity of this initial material. Therefore the formation of 2-4:dichlorophenol is considered most likely as a first step in the breakdown. Chloro-p-benzoquinone is a possibility but in that case its precursor would probably be 2-4 dichlorophenol giving chloro-hydroquinone oxidising to chloro-p-benzoquinone in the culture solutions.

Continued on p. 84.

TABLE IV

Additional Substrate	Time taken to detox.100 p.p.m. 2:4-D in days in presence of additional substrate	
	Concentration	
	10 p.p.m.	1 p.p.m.
2:4 dichlorophenol	16	15
chloro-p-benzoquinone	21	15
1-2-4 trihydroxybenzene	22+	22+
2-4 dihydroxyphenoxy-acetic acid	22+	22+
3-5 dichlorocatechol	22+	20
Quinhydrone	21	21
No additions	10	–

The effect of adding small quantities of toxic substances to aerated solutions containing 100 p.p.m. 2:4-D

If chlorohydroquinone is produced as an intermediate, presumably from 2:4-dichlorophenol then it should be decomposed rapidly to the next stage in the breakdown process since it is easily metabolised even in unaerated solutions. Small quantities of chloro-p-benzoquinone may form in the culture flasks due to the experimental conditions. This would cause a slowing down of the overall process of 2:4-D decomposition owing to the toxic effect of this substance on the organisms concerned. 2:4-Dihydroxyphenoxyacetic acid is a possible intermediate, if in the breakdown of the 2:4-D molecule, the first stages are the replacement of the 2 and 4 position chloride ions by hydroxyl groups. 2:4-Dihydroxyphenoxyacetic acid is highly toxic, to the Nocardia strain used in this work, in unaerated culture and has not been found to be volatile under the usual experimental conditions. If 2:4-Dihydroxyphenoxyacetic acid is formed the next step would logically appear to be the formation of 1:2:4-trihydroxybenzene. This latter substance is as toxic to these organisms as 2:4-dihydroxyphenoxyacetic acid and is unlikely to be produced in any quantity as a second stage from it. Neither of these two substances support growth of the organism when supplied as the only added carbon source and they do not disappear, in measurable quantities, from such culture solutions. It is therefore reasonable to suppose that firstly:- if 2:4-dihydroxyphenoxyacetic acid is formed it must accumulate in the culture medium and secondly (but this is considered unlikely) if it is further metabolised to form

1:2:4-trihydroxybenzene this latter substance will accumulate in the culture medium and in an alkali trap attached to the air supply outlet tube from the flask. Both 2:4-dihydroxyphenoxyacetic acid and 1:2:4-trihydroxybenzene give a positive reaction for phenolic material with Folin and Ciocalten's phenol reagent. Either, therefore, if present in the culture solutions in sufficient quantity (i.e. 1 p.p.m.) will give a positive reaction with this test. As will be seen later (page 102) no continuous accumulation of phenolic substances has been detected.

3:5-dichlorocatechol again could not be the first step in the breakdown process and it is difficult to see what compound not giving a phenolic reaction could be formed as the next step, yet retaining sufficient of its structure to be further metabolised into a phenolic substance later on in the process. This point is further discussed on page 119.

6. Carbon substrates utilised by the 2:4-D destroying strain of Nocardia in the absence of aeration of the liquid cultures and without producing subsequent deadaptation to the breakdown of 2:4-D.

Of the compounds tested only 6 fulfilled the above requirements namely:-

2:4-dichloro-6-hydroxyphenoxyacetic acid

2-chlorophenoxyacetic acid

o-chlorophenol

phenol

succinnic acid

glyoxylic acid.

These substances are all metabolised in the presence or absence of 2:4-D in aerated or still cultures. Little growth is recorded in aerated culture for phenol and o-chlorophenol. This it is felt is due to loss of the substrate by volatilisation rather than to any inability of the organism to utilise it. Both of these substrates are very volatile. Since these substrates are utilised in preference to 2:4-D, even though not causing deadaptation to the herbicide molecule, it is thought likely that either their metabolism in fact does not follow the same pathway as 2:4-D - that is these substances are not intermediate in the breakdown of 2:4-D - or that if they are intermediate products in the metabolism of the herbicide they are produced at a stage _after_ the formation of the postulated toxic intermediate. Their metabolism therefore produces no substance or substances deleterious to the organism and at the same time leaves the enzyme systems involved in the metabolism of 2:4-D unimpaired. Considering each of these substances in turn:-

2:4-dichloro-6-hydroxyphenoxyacetic acid

The only possible stage in the breakdown of 2:4-D at which this substance could be produced is the initial one, by the addition of a hydroxyl group into the ring at position 6. If this is the initial stage then, and this substance is not poisonous to the organisms there is no reason for the cessation of growth before the exhaustion of the substrate unless a later intermediate proves toxic. In this case 2:4-dichloro-6-hydroxyphenoxyacetic acid would be no more easily utilised as a source of carbon than the 2:4-D molecule. The heavier growth recorded on this substance both in the presence and absence of 2:4-D and in still cultures suggests that such a toxic intermediate is not formed andthe metabolic pathway followed by 2:4-dichloro-6-hydroxyphenoxyacetic acid is in fact different from that followed by 2:4-D. This substance is further considered on page 108.

2-chlorophenoxyacetic acid

This substance could theoretically be produced as a first stage in decomposition by the removal of the 4 chloride ion and its replacement by H (see page 116). This again if it occurs must be the first step, with the next - either the replacement of the 2-position chloride by H with the consequent formation of phenoxyacetic acid or, the removal of the acetic acid sidechain and the formation of o-chlorophenol by hydrolysis.

This organism has not been demonstrated to be able to grow on phenoxyacetic acid at all. This substance if formed must accumulate. That it does not accumulate is shewn by the loss of the sidechain as carbon dioxide in experiments to be described in section VII using radioactive labelled carbon C^{14}, in both methylene and carboxyl positions. It is logical to assume that phenoxyacetic acid is not formed at any stage. If 2-chlorophenoxyacetic acid is the initial stage in decomposition then o-chlorophenol must logically be supposed to be the next.

o-chlorophenol

This substance is very volatile and is evolved in measurable quantities within a few hours of its addition to aerated cultures. Any o-chlorophenol produced during decomposition of 2:4-D must therefore be further metabolised immediately or a large proportion would be lost and the chloride ion released into the medium consequently not reach a value approximating to 100% of the chlorine initially present in the 2:4-D. As is shewn later (page 102) chloride recovery is complete. Again, if o-chlorophenol is produced from 2-chlorophenoxyacetic acid there appears to be no stage during which a toxic compound, causing cessation of growth in the organisms, is formed and no necessity for continuous aeration. The formation of o-chlorophenol from 2:4-dichlorophenol is discussed later. Page 117.

Phenol

This is again a volatile substrate. It could not be formed as the first intermediate substance in the decomposition of 2:4-D by the Nocardia strain. Its formation at a later stage is discussed at the end of this work (page 118).

VII. Experiments using radioactive 2:4-D labelled with C^{14} in the methylene and carboxyl groups of the side chain

1. Introduction

Samples of 2:4-D containing C^{14} in either the methylene or carboxyl group of the side chain were used. Experiments were carried out using a mixture of organisms from a sample of 2:4-D decomposing Nocardia isolated from soil. Growth of the organisms was measured by direct counting as in the previous experiments.

Phytotoxicity was assayed by the inhibition of growth of pea root sections. Changes in phytotoxicity and radioactivity were followed on chromatographed samples of the culture fluid throughout the detoxication process.

Carbon dioxide evolved from the cultures was collected in an alkali trap and radioactivity estimated by counting samples of the trap liquid dried onto planchettes.

The experiments with samples of radioactive 2:4-D fall into two sections. Firstly experiments in which small

quantities of culture fluid were used (25 mls) and in which the entire quantity of 2:4-D added (2.5 mgs.) was from the labelled sample. Secondly experiments in which much larger volumes of medium, 500 mls., were employed in order that more measurements could be taken without causing the total volume of the culture medium to be reduced by more than one half. Small quantities (2.5 mgs.) only of labelled 2:4-D were added together with 47.5 mgs. of unlabelled 2:4-D to make the concentration in the medium up to 100 p.p.m. 2:4-D.

2. Experimental methods

First series of experiments - using small volumes of culture fluid.

a) 25 mls. of the medium containing 2.5 mgs. C^{14} labelled 2:4-D were sterilised in a ground glass stoppered boiling tube, the stopper being fitted with inlet and outlet tubes. The inlet tube goes to the bottom of the vessel and ends in a sintered glass filter. The apparatus was assembled in series:

1. Sterile cotton wool filter
2. Humidifying bottle containing sterile distilled water
3. Experimental vessel
4. Alkali trap for carbon dioxide
5. Second similar trap. Vessels of the same type as those used for culturing the organism under these conditions were used for the traps

6. Soda lime tube as a final precaution to ensure that no active carbon dioxide escaped into the atmosphere.

Air was drawn through the apparatus at a steady rate, this rate being maintained throughout the experiment by the the insertion of a resistance manometer into the series. Samples of the culture fluid and of the alkali were taken at intervals throughout the experimental period. The experimental vessel was kept in the dark and experiments were carried out at room temperature.

Growth of the organism was measured by direct counting of organisms as in previous work.

Total carbon dioxide output was estimated by absorption in baryta water and titration against standard HCl and the evolution of labelled CO_2 by counting the activity in samples of uniform size dried onto planchettes.

b) Chromatographic procedure

This was similar to that used in previous wrk with the original organism except that care was taken to place the sample on the starting line of the chromatogram in a spot not exceeding 1 cm. in diameter. This was achieved by making repeated small applications of the material and allowing each to dry before the next was made. 0.1 ml. samples of the culture fluid removed in sterile micropipettes were used. The chromatograms were developed overnight in a solvent containing isopropyl alcohol: ·880 ammonia: water in the proportions 10:1:1 the solvent

Diagrammatic section through lead castle to show relative positions of G-M Counter and slide carrying radioactive chromatogram

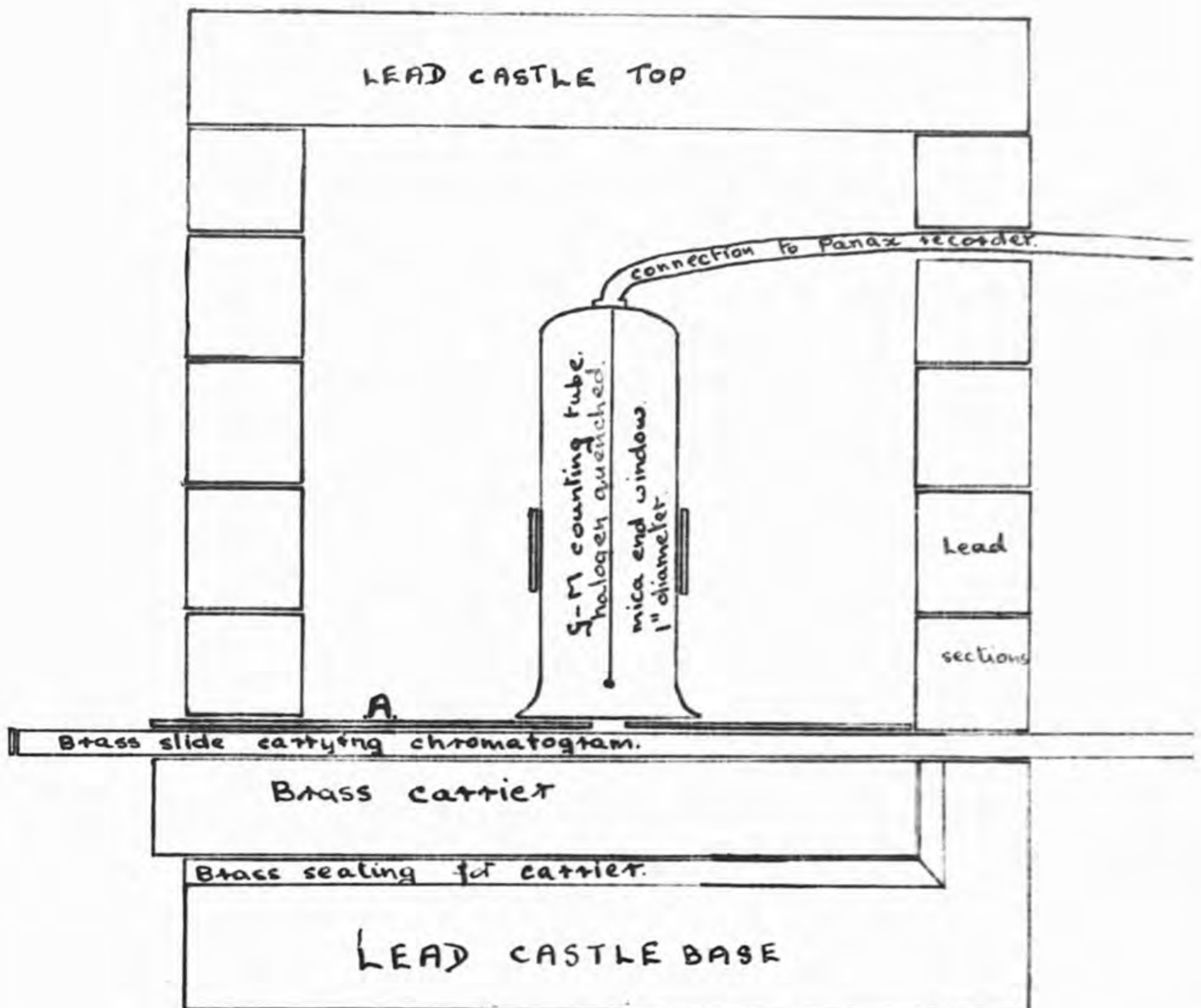


FIGURE XX

Brass carrier and slide for chromatogram.

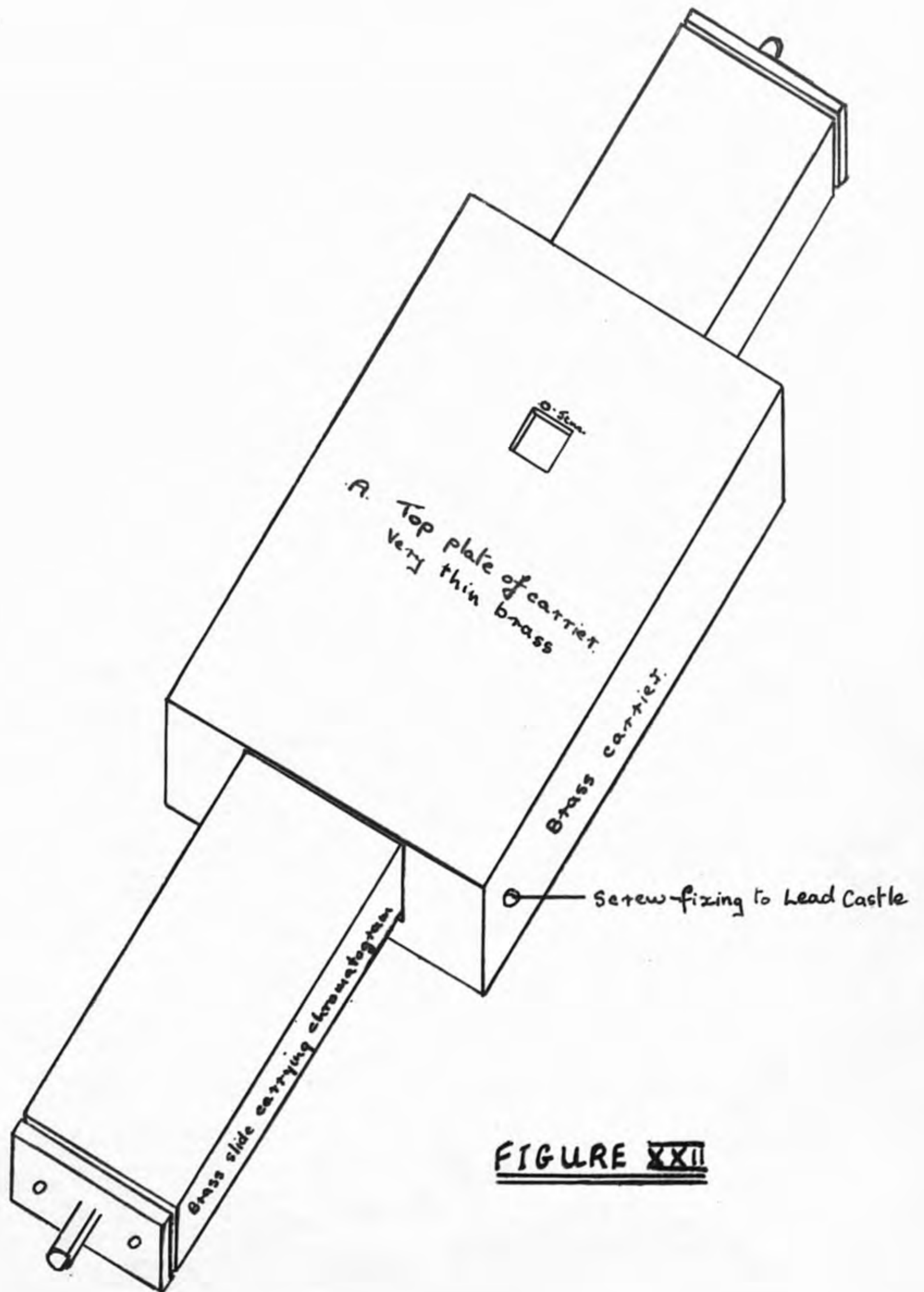


FIGURE XXII

front travelling approximately 25 cms. from the origin.

After development the chromatograms were removed from the tanks, dried in a gentle stream of air and examined for fluorescence in ultra violet light. This process proved useful in establishing accurately the exact path spots travelled during development. A line was drawn down the chromatogram passing through the centre of the placing spot at the origin and through the centres of any fluorescent spots. The chromatogram was subsequently fixed to the slide for estimation of radioactivity in such a position that this line passed through the centre of the counting square.

c) Estimation of radioactivity on chromatograms

A diagrammatic representation of the apparatus used is shewn in Figures XXI and XXII. Radioactivity counts were made from a distance of 0.5 cms. above the origin by 0.5 cm. squares to the front of the chromatogram.

Initially each section was counted for five minutes with twenty minutes over active and doubtful regions. This proved too time consuming a process with chromatograms running distances of approximately 25 cms., and counting was therefore limited to one minute over sections shewing only background activity (less than 18 counts per minute) and five minutes over doubtful or active regions.

The chromatograms were then sectioned and pea root sections grown on each for assay of phytotoxicity.

FIGURE XXIII

Phytotoxicity and radioactivity recorded on chromatograms after development in ammonia/propanol/water solvent. Samples of culture fluid taken at intervals throughout the detoxication process after inoculation of cultures with a mixture of organisms taken from a soil perfusate enriched to 2:4-D. 2:4-D labelled with C^{14} in the methylene group of the side chain.

FIGURE XXIII

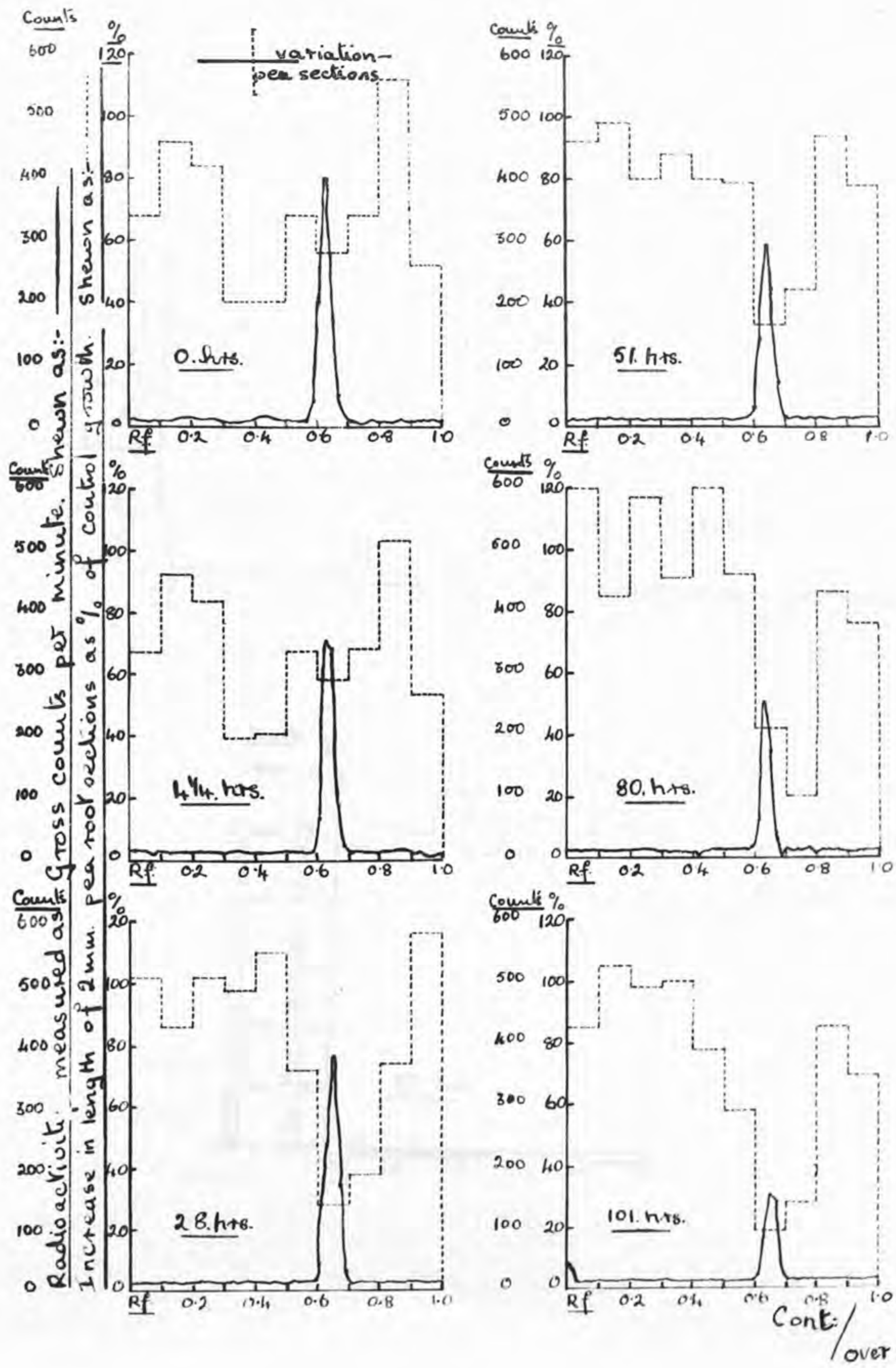


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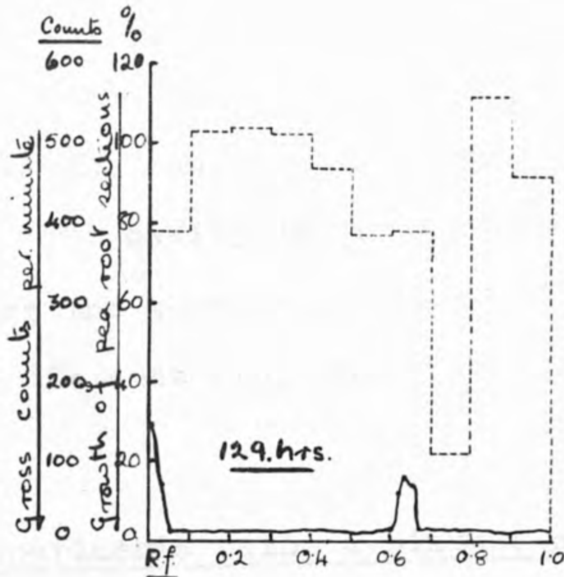
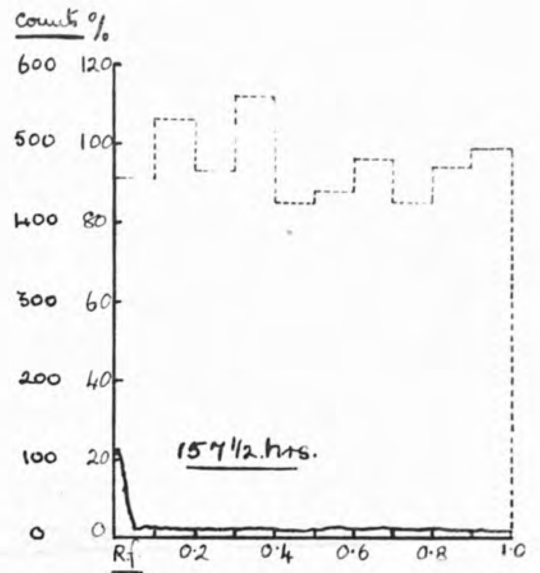
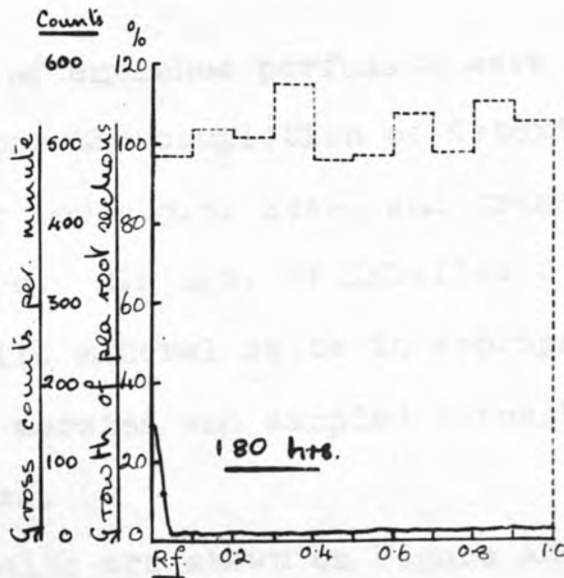
FIGURE XXIII continued.







d) Assay of radioactivity in carbondioxide trap

An exactly similar carrier to that used to take the movable slide for assay of radioactivity on chromatograms was used except that the small square hole was replaced by a circular cavity exactly fitting a 2 cm. diameter planchette with its centre in exactly the same position relative to the counter as the centre of the square window in the chromatogram carrier. The cavity in the planchette carrier was of such a depth that the surface of the planchette when in position was the same distance from the counter window as the chromatogram paper.

3. Experiments using a mixture of organisms in 2:4-D enriched perfusate with 2:4-D labelled in the methylene group of the side chain

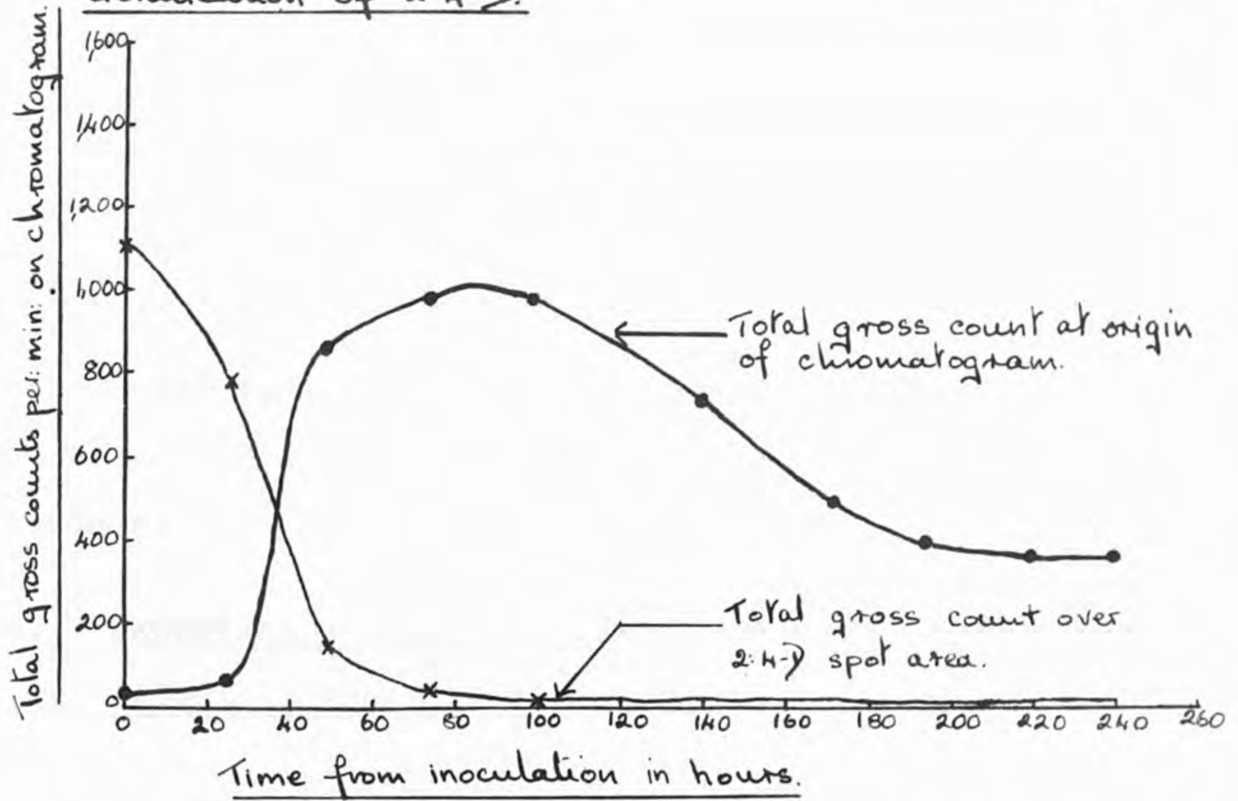
25 mls. of enriched perfusate were taken aseptically from a perfuser after the completion of detoxication of a solution containing 100 p.p.m. 2:4-D and transferred to a sterile culture tube. 2.5 mgs. of labelled 2:4-D were added together with sterile mineral salts in appropriate quantity. The solutions were aerated and sampled throughout the detoxication process.

The results are shewn on Figure XXIII and Appendix XXIII. These results are given for comparison with those obtained using a pure culture of the effective Nocardia strain.

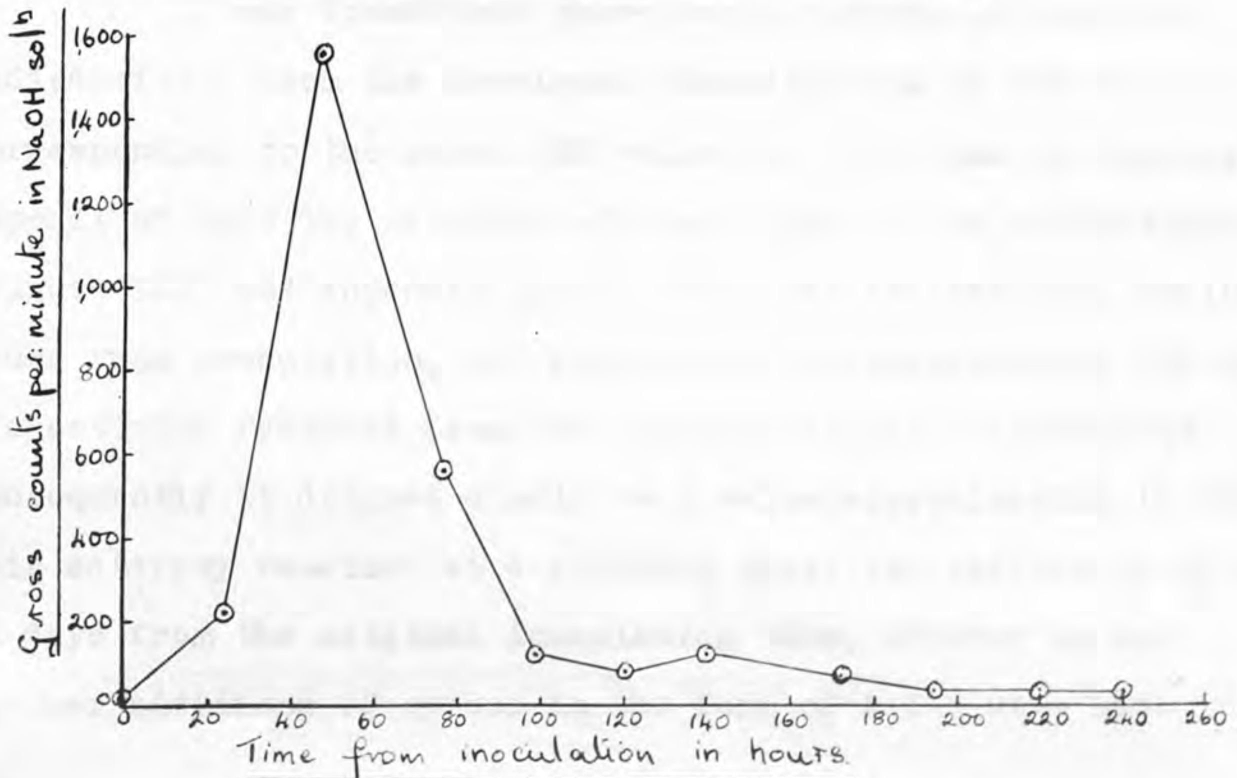
FIGURE XXIV

Methylene labelled 2:4-D.

Radioactivity measured on chromatograms during detoxication of 2:4-D.



Evolution of $C^{14}O_2$ during detoxication of 2:4-D.



The use of the mixture of organisms and materials obtained in a perfusate was not persisted with since it was found that results of phytotoxicity on chromatograms were not consistent and that "streaking" of phytotoxicity back from the main 2:4-D spot frequently occurred. This made any assessment of changes in phytotoxicity due to decomposition of 2:4-D impossible since greater differences were produced by materials present in the perfusate than those to be expected from the experimental materials.

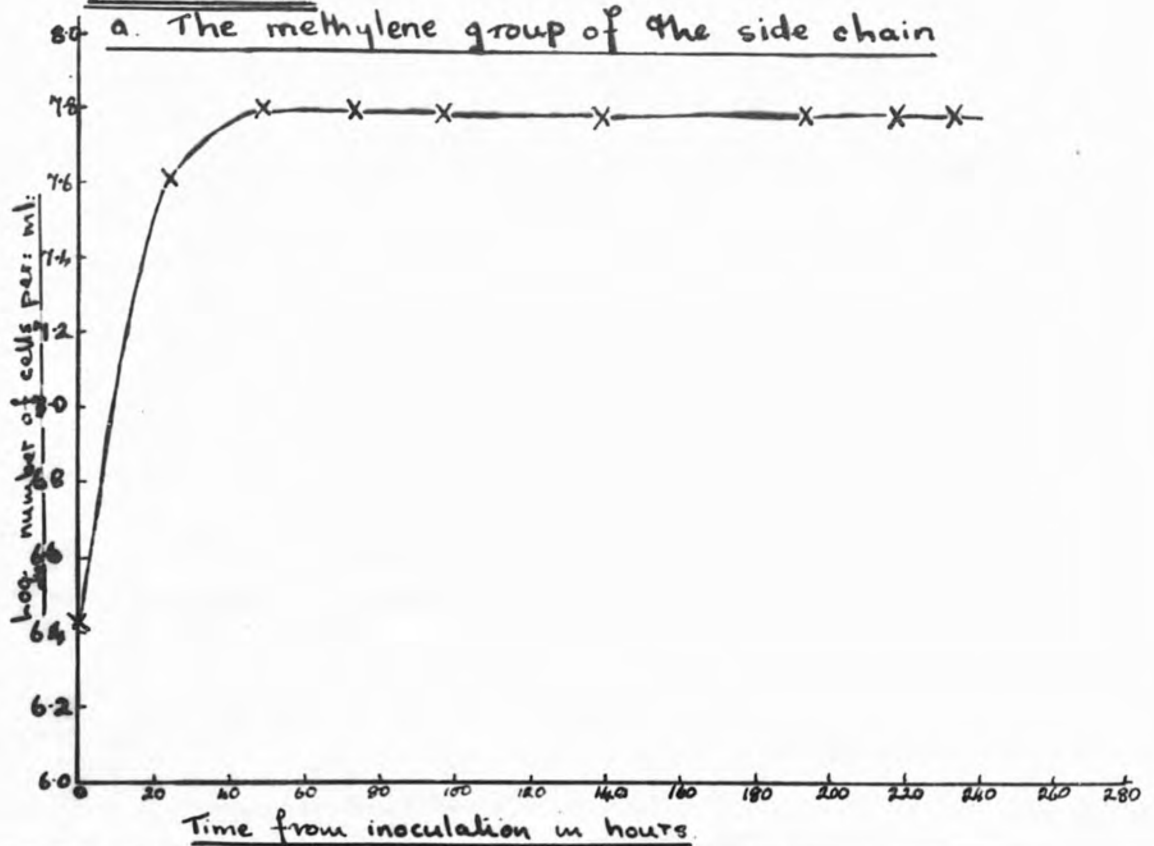
4. Experiments using sub-cultures of organisms of the effective Nocardia strain.

a) Preliminary experiments were carried out along the lines described above using samples of 2:4-D labelled in the methylene group of the side chain.

It was found that there was a continuous loss of radioactivity from the developed chromatograms in the region corresponding to the normal Rf value for 2:4-D and an increasing deposit of labelled material at the origin of the chromatogram. (Figure XXIV and Appendix XXIV). This, at its maximum, 80-100 hours from inoculation, was equivalent to approximately 90% of the activity recorded from the original sample of 2:4-D but subsequently it dropped slowly to a value approximating to 35%. This activity remained at a constant level for periods of up to 21 days from the original inoculation time, whether or not further additions of carbon in the form of 2:4-D were made to

FIGURE XXV

Growth of bacteria during detoxication of 100 p.p.m. 2:4D labelled with C^{14} in:-



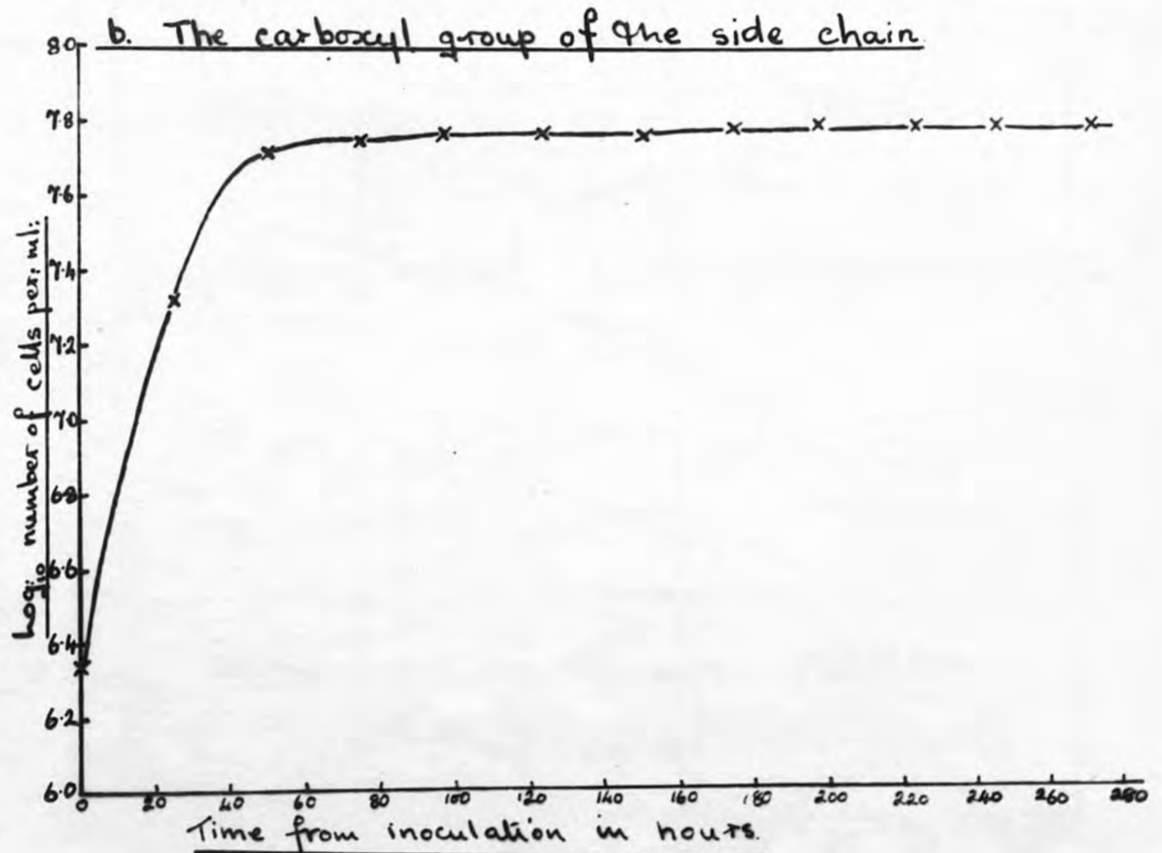


FIGURE XXVI

Phytotoxicity and radioactivity recorded on chromatograms after development in ammonia / propanol / water solvent.
Samples of culture fluid taken at intervals throughout the detoxication process after inoculation of cultures with the 2:4-D decomposing strain of Nocardia isolated from garden soil.
2:4-D labelled with C^{14} in the methylene group of the side-chain.

FIGURE XXVI

Methylene labelled 2:4-D

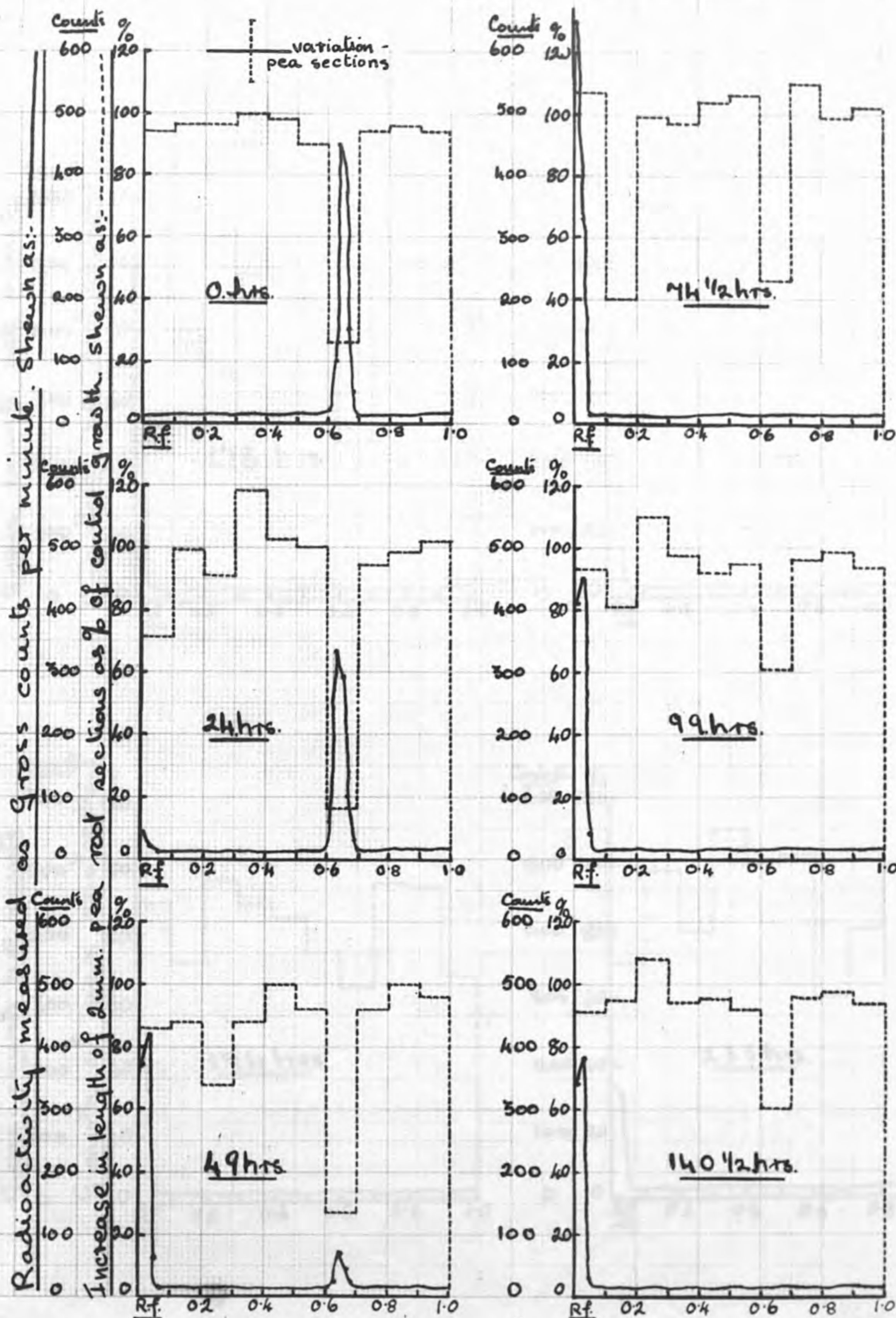


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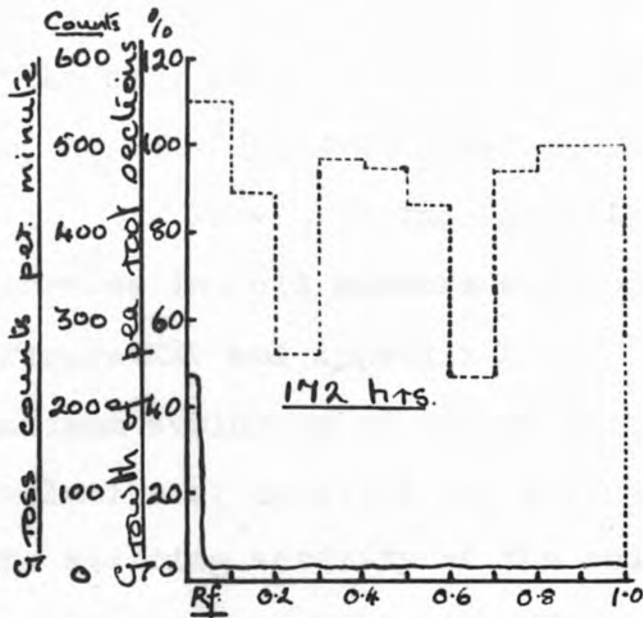
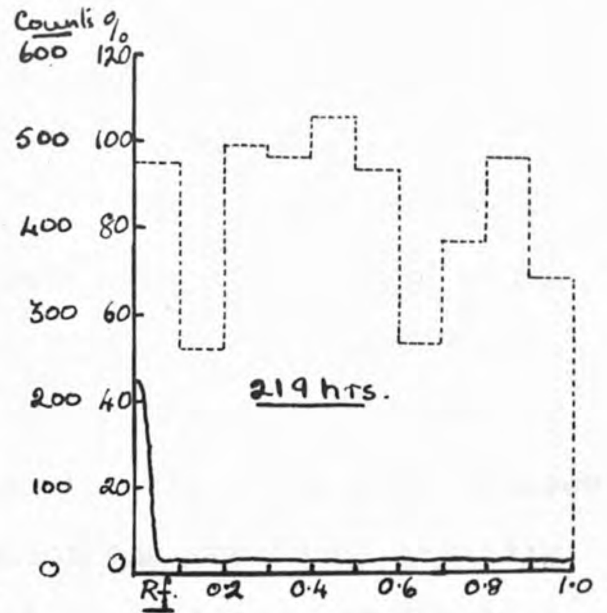
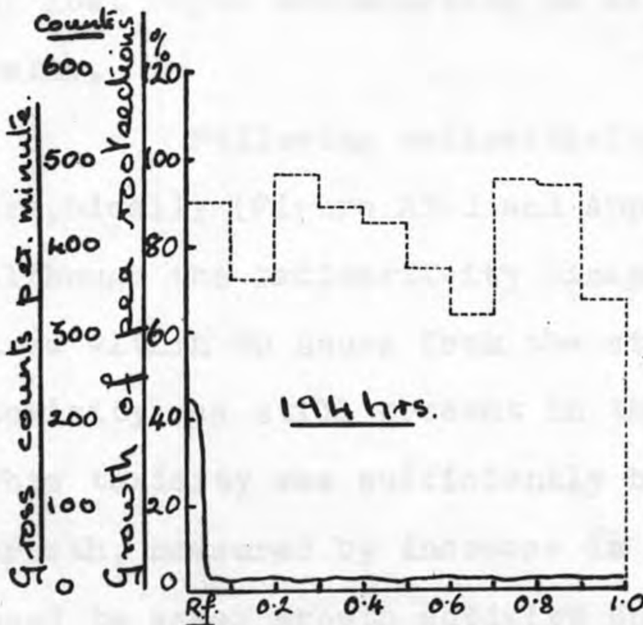
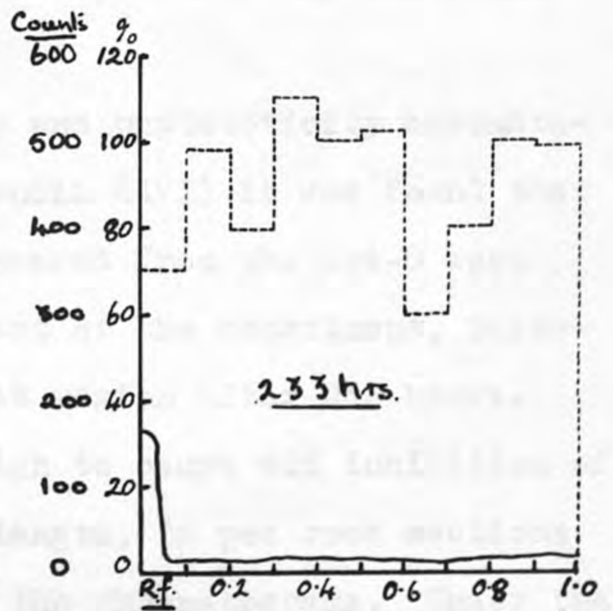
FIGURE XXVI continued









the medium. This activity is retained by the cells even under conditions of carbon starvation. This labelled carbon was found to be within the bacterial cells and not in a substance free in the culture medium. Centrifuged and washed cells retained their activity whereas the supernatant fluid remaining after centrifugation lost it. No measurable activity remained on the 2:4-D spot area by 80 hours after inoculation.

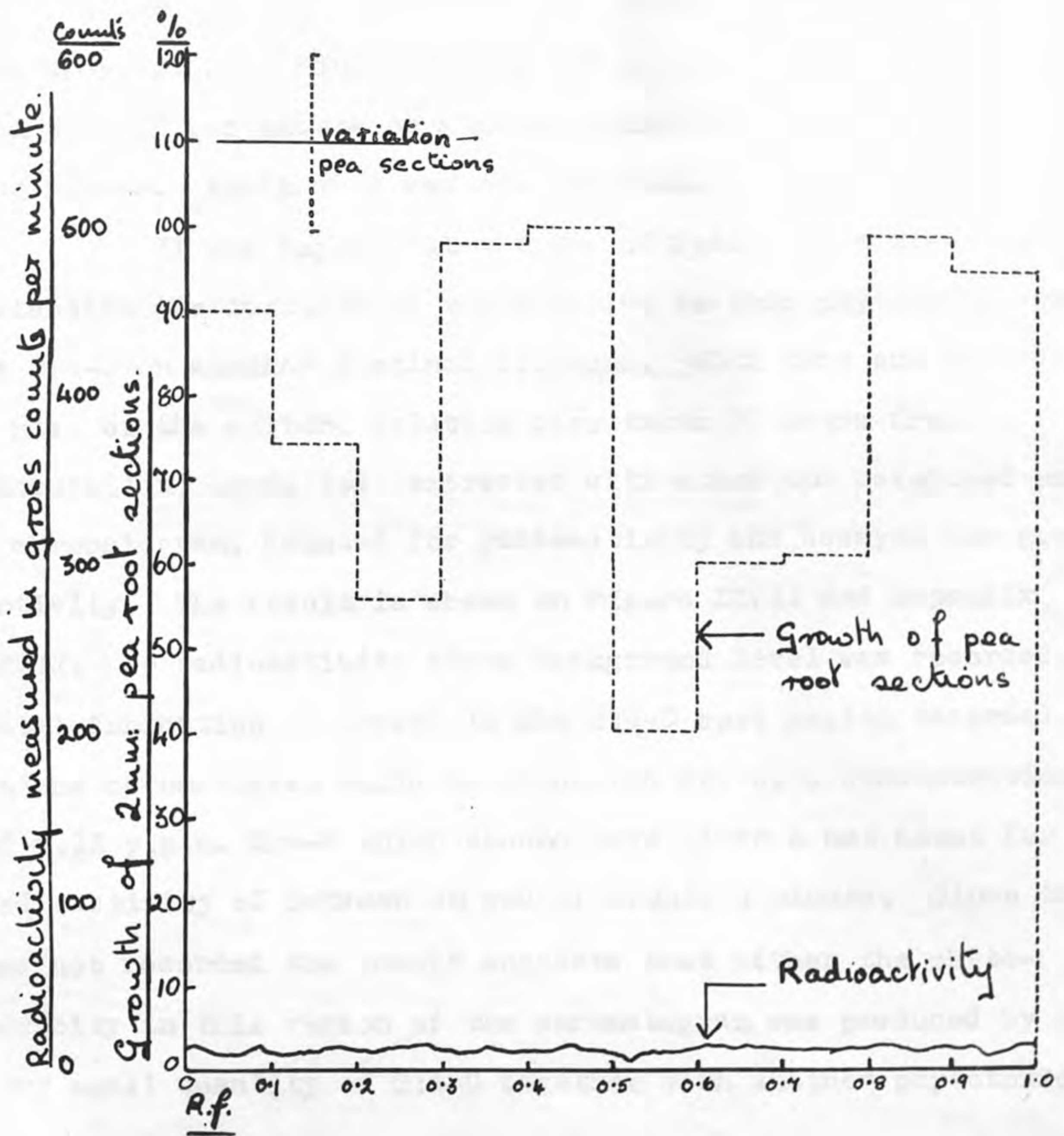
Growth in the bacterial cultures as measured by increase in cell numbers was complete by the end of 48 hours. (Figure XXV and Appendix XXV). This same period gave the maximum evolution of carbon dioxide and also of labelled CO_2. Evolution of labelled CO_2 accounted for the difference between the starting activity of the solution and the final activity retained in the bacteria. The maximum evolution of labelled CO_2 came towards the end of the growing period and at the time of most rapid accumulation of activity within the bacterial cells.

Following radioactivity and phytotoxicity chromatographically (Figure XXVI and Appendix XXVI) it was found that although the radioactivity disappeared from the 2:4-D spot area within 80 hours from the start of the experiment, phytotoxicity was still present in that region after 233 hours. This toxicity was sufficiently high to cause 40% inhibition of growth, measured by increase in length, in pea root sections used to assay growth activity on the chromatograms. Under the

FIGURE XXVII

Methylene labelled 2:4-D.

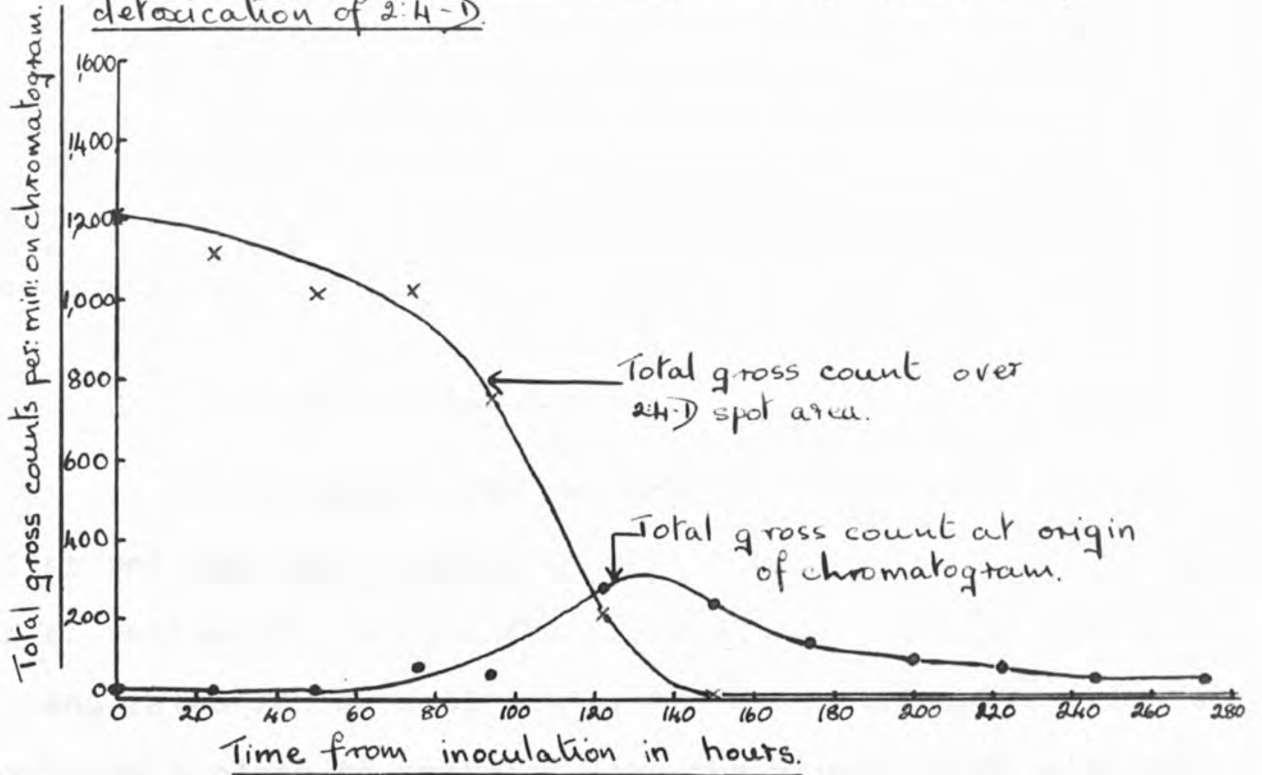
Radioactivity and phytotoxicity recorded on chromatogram. Acid ether extract of 99 hr. culture solution.

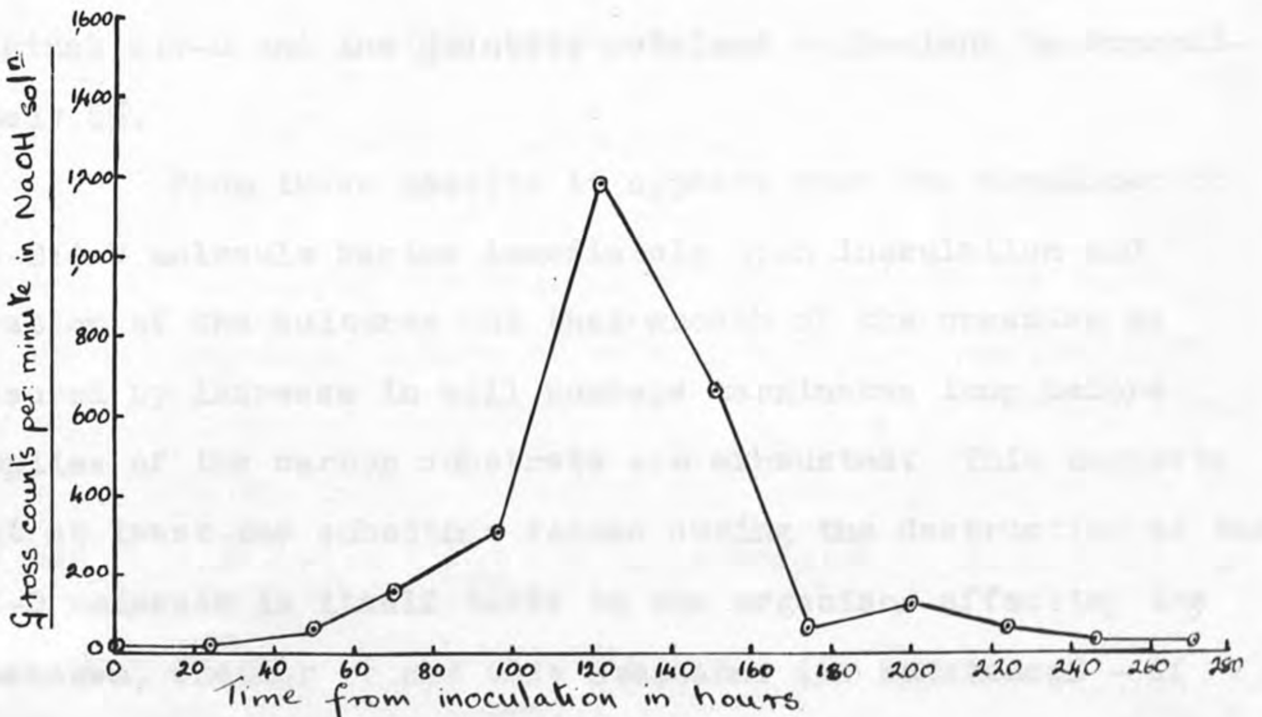


conditions of the experiment 40% inhibition was associated with an original concentration of 0.46 p.p.m. 2:4-D. See Appendix XXXV for calibration curves. This concentration of 2:4-D should have produced a net radioactive count of 16 per minute above the background level, or a total gross count of between 26 and 36 counts per minute. No count above background was recorded in this area. At 73.5 hours the inhibition was of the order of 54%, equivalent to 0.7 p.p.m. 2:4-D and a net count of 19 per minute or a gross count of between 29 and 39 per minute. Again this was not recorded.

It was hoped that the use of radioactive 2:4-D would establish the identity of the material in this phytotoxic spot, as 2:4-D on another distinct compound. With this end in view 2 mls. of the culture solution were taken 99 hours from inoculation, acidified extracted with ether and developed on a chromatogram, counted for radioactivity and assayed for growth activity. The result is shewn on Figure XXVII and Appendix XXVII. No radioactivity above background level was recorded. Total inhibition of growth in the 2:4-D spot region recorded on the chromatogram could be accounted for by a concentration of 4.12 p.p.m. 2:4-D which should have given a net count for radioactivity of between 50 and 60 counts a minute. Since this was not recorded the result suggests that either the phytotoxicity in this region of the chromatogram was produced by a very small quantity of 2:4-D together with another phytotoxic

FIGURE XXVIII





compound which was unlabelled or that some measure of exchange takes place between the labelled side chain of the 2:4-D molecule and acetic acid or other radicals already present in the bacterial cells, so that either unlabelled 2:4-D is produced or else another very similar compound - with the same ring structure possibly but another side chain arrangement.

b) Carboxyl labelled 2:4-D

Experiments with carboxyl labelled 2:4-D gave similar results for phytotoxicity assayed on chromatograms and loss of radioactivity from the 2:4-D spot. Entry of activity into and retention of activity by the bacterial cells did not however take place to anything like the extent found with the methylene labelled material (Figure XXVIII and Appendix XXVIII). The maximum counts recorded at the origin of the chromatogram were equivalent to less than 25% of the total activity in the original 2:4-D and the quantity retained equivalent to approximately 5%.

From these results it appears that the breakdown of the 2:4-D molecule begins immediately upon inoculation and aeration of the cultures but that growth of the organism as measured by increase in cell numbers terminates long before supplies of the carbon substrate are exhausted. This suggests that at least one substance formed during the destruction of the 2:4-D molecule is itself toxic to the organisms effecting the breakdown, whether or not this substance (or substances - if

there are more than one -) is also phytotoxic is another matter. Since the side chain of the 2:4-D molecule is attacked at the commencement of the experiments - as shewn by the evolution of radioactive CO_2 - it would appear that this toxic agent must be formed from the ring portion of the molecule with possibly a different side chain attached.

In order to study the reactions occurring during the breakdown of 2:4-D more fully the experimental methods were modified to give a much larger bulk of culture fluid without increasing the consumption of radioactive 2:4-D.

5. Modified Experimental Methods.

Second series of experiments using large volumes of culture fluid

a) A similar arrangement of apparatus to that used for the first series of experiments was employed but:-

1. The experimental tube was replaced by a litre flask having similar inlet and outlet tubes. 500 mls. of medium were used containing the requisite quantities of the three mineral salts, 47.5 mgs. of unlabelled 2:4-D together with 2.5 mgs. of radioactive 2:4-D.

2. The carbon dioxide traps were replaced by gas washing bottles containing 250 mls.concentrated NaOH solution.

Samples were taken daily throughout the detoxication process as follows:-

1. <u>From the culture solution</u>

a. 5 mls. for chromatograms. This sample was acidified with concentrated HCl shaken with two 5 ml. samples of ether, the two extracts mixed and two 4 ml. samples of the extract in ether drawn off. The ether was evaporated to a small bulk and each sample placed as a spot of diameter less than 1 cm. on the starting line of a paper chromatogram. One chromatogram of each pair was developed in the isopropanol, ammonia, water solvent as used in the previous experiments and the other in a n-butanol acetic acid water solvent. After development both chromatograms were treated as previously.

b. 5 mls. for estimation of free chloride ions in the culture solution. Saturated potassium chromate solution was added to the sample as an indicator and titrated against a N/500 solution of silver nitrate.

c. 1 ml. tested for the presence of substances giving a positive reaction with Folin and Ciocaltin's phenol reagent. This reagent will detect 1 p.p.m. phenol in 1 ml. if 0.5 ml. of the reagent are added to the sample followed by 1.5ml of 20% sodium carbonate solution and the blue colour developed by placing the sample in a water bath at 30°C for 30 minutes.

The depth of colour was estimated on a Unicam colorimeter and some estimation of relative quantity made by comparison with a standard curve obtained by the depth of colour produced by reactions using known concentrations of 2-4 dichlorophenol.

d. 0.2 ml. for bacterial count.

e. 0.1 ml. for overall phytotoxicity assay.

2. <u>From the alkali traps</u>

a. 0.1 ml. dried onto planchettes and assayed for radioactivity. It was found in every case that the first trap was efficient in collecting all the radioactive material which was evolved from the culture flasks.

b. 1 ml. was tested for the presence of phenolic substances in the same way as the sample taken from the culture flask for the same purpose. This estimation of phenolic substances appearing in the alkali trap set to absorb radio CO_2 was carried out since it had been previously found that, under the conditions in which these experiments were carried out, 2:4 dichlorophenol added to the culture media all evaporated out of the solutions within 48 hours and accumulated in traps containing either concentrated alkali or alcohol. Alkali traps were used in preference to alcohol since the former also served to absorb carbon dioxide and evaporation losses from them were considerably smaller, and therefore more easily replaced over

considerable periods of time, than when using alcohol. Again it was found that the first trap was efficient in collecting all the phenolic material passing out of the culture medium and no positive reactions were obtained from the second trap.

Using this modified method it was hoped to check the phytotoxic spots appearing on the chromatograms for identity with 2:4-D or otherwise and to separate if possible, by running identical samples in two separate solvents, any toxic substance which may have been running in close association with 2:4-D in the original isopropanol, ammonia, water solvent used. To estimate the free chloride present in the growth medium at the beginning of the experiment and to follow any changes throughout the detoxication process which might produce evidence of an attack on the ring portion of the 2:4-D molecule. To test for the presence of any substance giving a positive reaction with Folin and Ciocaltin's phenol reagent both in the culture media and the alkali traps. Since the evidence from previous experiments shewed that the side chain of the molecule was immediately attacked the possibility of the presence of a compound having a phenolic structure cannot be overlooked.

The larger quantity of material placed on the chromatograms - 20 times that originally used - to bring the radioactive counts given by the sample to rates comparable with those obtained in the previous experiments and also to increase

FIGURE XXIX

The production of phenolic substances during the detoxication of 2:4-D. (100 p.p.m.)

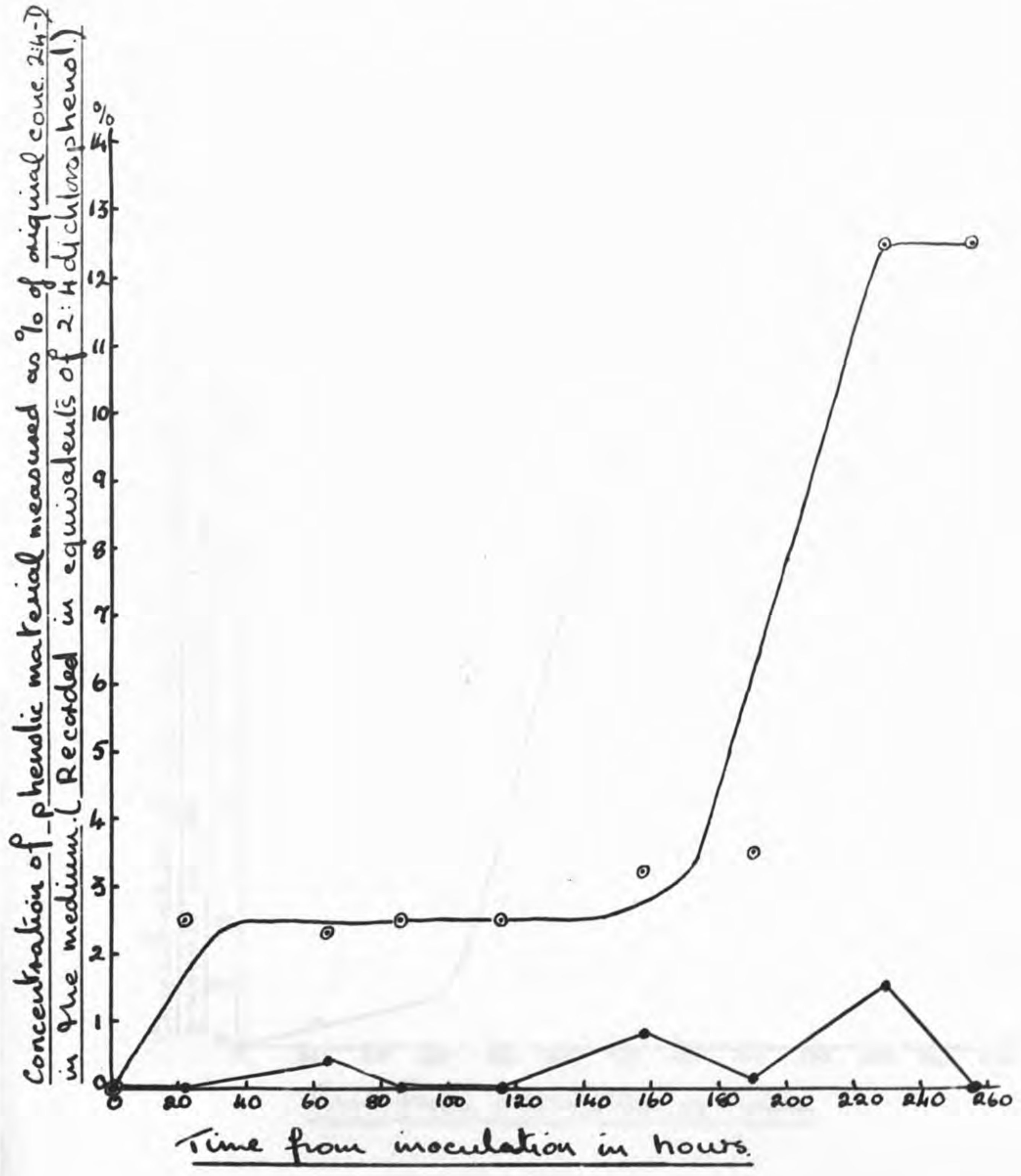
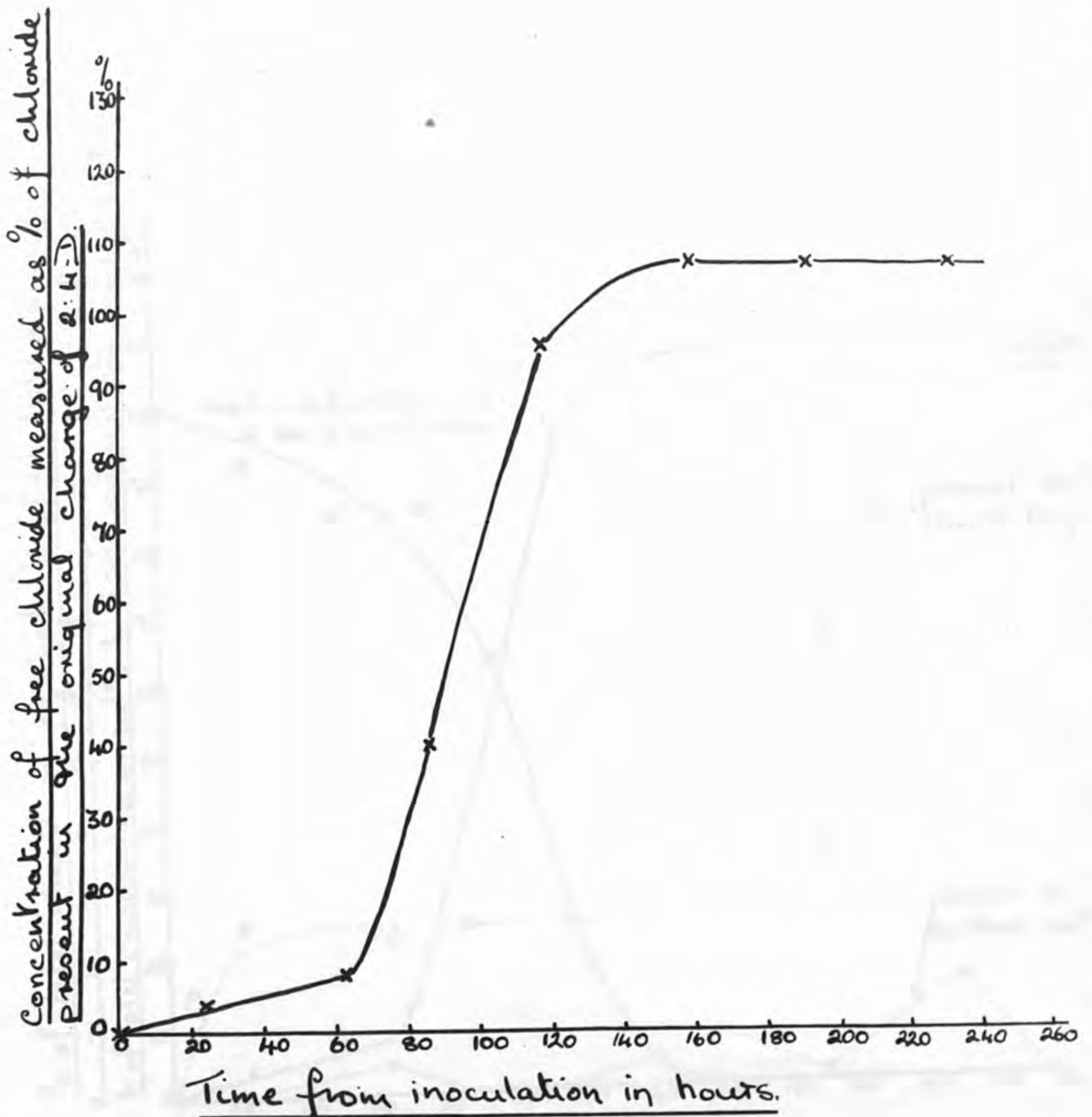


FIGURE XXX

The accumulation of free chloride in the culture medium during the detoxication of 2·4-D.



the quantity on the chromatogram of any acidified ether soluble intermediate present in the culture fluid meant that the 2:4-D could no longer be expected to run as a very sharp spot as when small quantities were developed. This however it was decided would not seriously interfere with the interpretation of results obtained even if the 2:4-D spot should spread or produce "tailing" behind the main spot.

b) Results obtained

The results of these experiments are shewn in Figures and Appendices XXIX, XXX and Figure XXXI.

Growth curves are exactly similar to those obtained in all previous experiments. Similar observations are recorded for phytotoxicity* Chloride accumulates in the medium up to a total of up to 108% of that estimated as present in the original 2:4-D it is assumed that the total chloride is recovered and that the excess is probably due to small evaporation losses not being accurately made good. Chloride accumulates most rapidly during the period of most rapid loss of radioactivity by 2:4-D spot region on the developed chromatograms. Phenolic substances are detectable within 24 hours in the alkali trap, no further increase in concentration is then noted until after the completion of chloride accumulation and loss of measurable radioactivity. After this period there is a rapid accumulation of phenolic material in the alkali trap. Traces of

[*Spreading of spots and tailing occurred - due to the large quantities of material placed on the starting line. Phytotoxicity results are not therefore considered from this set of experiments.]

phenolic materials are recorded at intervals in the culture medium throughout the detoxication process but no accumulation is recorded. The general picture of the appearance of phenolic substances in both the trap and culture fluids suggests that there is a rapid production of a phenolic compound at the beginning of the incubation period, during the initial growth period of the organisms, that this substance is volatile and only small quantities - less than 1% of the original 2:4-D measured in equivalents of 2:4 dichlorophenol - remain unchanged in the culture solution. There is then no further production of this material in measurable quantities. A second volatile substance giving a phenolic reaction is rapidly formed towards the end of the detoxication process.

The chloride graph suggests that the loss of chloride ions from the organic molecule is a continuous process starting slowly and reaching a maximum rate during the period of most rapid evolution of radioactive carbon dioxide.

The loss of radioactivity from the 2:4-D spot region of the developed chromatograms is also a continuous process and follows the chloride production curve quite closely in time.

VIII. Conclusions from and Discussion of Preceding experiments

From the results obtained during this work it would appear that, in the decomposition of 2:4-D by the strain of Nocardia isolated from flask cultures of soil enriched and 2:4-D :-

Attack on the molecule begins immediately upon inoculation and aeration of cultures containing 2:4-D. This is shewn by the fact that growth of the organisms begins immediately and radioactivity is lost from solutions containing C^{14} labelled 2:4-D. This first stage in the breakdown of the herbicide molecule would seem to be the removal of the acetic acid side chain as a whole leaving a ring structure of probably 2:4-dichlorophenol. The side chain is utilised as a carbon source, partly respired, as the evolution of radioactive CO_2 from both methylene and carboxyl groups indicates: and partly, especially that originally comprising the methylene grouping, incorporated into the substance of the bacterial cells, and not respired even under conditions of starvation.

The ring compound which is left is volatile and evaporates from the culture medium and may be collected in an alkali trap. Loss of radioactivity is continuous but the production of "phenol" is not.

The next step does not then seem to be the straightforward decomposition of this phenol.

Growth of the bacterial cells during detoxication is slight compared with growth on the same concentration of other similar substrates and ceases within 48 - 72 hours of inoculation that is, during the time of production of this phenolic compound. This first phenolic compound appears to be the one which is produced in quantities sufficiently great to cause cessation of growth in the cultures and perhaps to prevent the detoxication of concentrations of 2:4-D greater than 300 p.p.m. Of the compounds investigated 2:4 dichlorophenol and chloroparabenzoquinone completely inhibit growth of this organism at very low concentrations of the order of 0.1 p.p.m. These observations were made in still cultures containing 0.1% glucose as a carbon source. These two substances are also volatile under the conditions of the experiments and may be collected in an alkali trap. Higher original concentrations, up to 10 p.p.m. are needed to inhibit growth in vigorously aerated cultures since evaporation takes place, but a very low maintained concentration is sufficient. A very small quantity only, continuously produced, would be necessary to prevent cell division in the culture medium. The production of chloroparabenzoquinone if it occurs would come by oxidation of chlorohydroquinone. This oxidation would occur under the experimental conditions employed if chlorohydroquinone was produced by the organisms. Pure chlorohydroquinone is not toxic in concentrations of up to 100 p.p.m. to this organism. In the event of chlorohydroquinone

being the first phenolic substance formed the toxic effect on the bacteria would be caused by the conditions of the experiment and not a direct effect of an intermediate produced during the breakdown of 2:4-D.

After this short period of "phenol" production no more evidence of phenol is found although side chain breakdown and chloride accumulation continue.

In the absence of evidence for the production of a second phenolic compound it would be assumed that the 2:4-D molecule, side chain and ring structures were attacked simultaneously and that destruction was complete when chloride accumulation reached a maximum and radioactivity disappeared. This view is taken by Rogoff and Reid, 1956.

The production of the second phenolic compound and the presence of phytotoxic spots on the chromatograms after the completion of chloride and radioactive reactions does not allow this simple explanation to be considered as the complete explanation for the breakdown of 2:4-D by this organism.

During the period of maximum rate of accumulation of chloride and loss of radioactivity there is no evidence of volatile phenol being carried over into the trap or of non-volatile material remaining in the culture solution. If, as it is logical to suppose, the first reaction is continuing, the first phenolic substance must be further changed into a substance not reacting with Folin and Ciocalten's reagent, at approximately the rate at which it is produced.

The first phenolic material formed during the first 48 hours of culture could theoretically be produced in one of several different ways:-

1. The introduction of a hydroxyl group into the ring forming most probably 2:4 dichloro-6-hydroxyphenoxyacetic acid in a manner comparable with the formation of 2 hydroxy-4-chlorophenoxyacetic acid from p-chlorophenoxyacetic acid as reported by W.C.Evans and P.Moss (1957).

2. Cleavage of the 2:4-D molecule separating the acetic acid side chain from the ring with the formation of 2:4-dichlorophenol by hydrolysis as postulated by Audus 1952.

3. The removal of one (or both) chloride ions and their replacement by hydroxyl groups forming:-

2 chloro-4-hydroxyphenoxyacetic acid

2 hydroxy-4-chlorophenoxyacetic acid or

2-4 dihydroxyphenoxyacetic acid.

4. The simultaneous removal of the side chain and one or both of the chloride ions and their replacement by hydroxyl groups giving

chlorohydroquinone

4-chlorocatechol or

1-2-4 trihydroxybenzene.

5. The removal of one or both chloride ions and their replacement by H giving2-chloro or 4-chlorophenoxyacetic acid or phenoxyacetic acid. The next stage being the formation of the corresponding phenol.

From the results already obtained it has not been possible to decide whether chloride ions and radioactive CO_2 are released simultaneously from the 2:4-D molecule or if the removal of one precedes that of the other.

Considering each of these possible compounds in turn:-

2,4 dichloro-6-hydroxyphenoxyacetic acid

The organisms grow with this substance as the only organic carbon source in unaerated liquid media. No deadaptation to 2:4-D is then subsequently recorded whether or not 2:4-D is included in the liquid medium. This suggests that this substrate is more easily attacked than the 2:4-D molecule and producing no deadaptation might well be considered as an intermediate in the breakdown of 2:4-D. This compound has not however been found to be volatile under the experimental conditions used. It cannot therefore be considered as the "phenolic" material detected in the alkali trap. No accumulation of "phenolic" material occurs in the detoxication process and it has therefore been concluded that either 2:4 dichloro-6-hydroxyphenoxyacetic acid is not an intermediate in the destruction process of the 2:4-D molecule or if it is produced as the first step then it is rapidly broken down - at the rate of production, the former suggestion being the more likely since much heavier growth than is obtained would be expected if 2:4-D-6-OH is an intermediate since heavier growth is obtained on it alone than on 2:4-D.

2-4 dichlorophenol

This substance limits the growth of this 2:4-D destroying Nocardia strain in very small quantities. It is volatile under the experimental conditions and may be accumulated in an alkali trap. If this substance were produced it could account for the very limited amount of growth recorded on 2:4-D media and for the need for vigorous aeration of the cultures throughout the detoxication process. It would also account for the apparent toxicity of 2:4-D in unaerated cultures when no other carbon substrate is present. If this substance is formed during the detoxication process then the quantity of 2:4-D dealt with by any culture will be limited by the efficiency of the organisms in dealing with this toxic substance. The neutralisation mechanism appears to be associated with or dependent upon a factor present in soil extract and, in limited quantities, in agar. That the 2:4 dichlorophenol if produced, is further metabolised is shewn by the fact that chloride is recovered from the culture solutions.

2 chloro-4-hydroxyphenoxyacetic acid
and 2 hydroxy-4-chlorophenoxyacetic acid

These two substances were found not to be volatile under the experimental conditions. Also they support very heavy growth of the organism even in unaerated culture. This growth is not recorded during 2:4-D breakdown. They also cause deadaptation of the organism to 2:4-D.

2:4 dihydroxyphenoxyacetic acid

This substance has not been found to be volatile under the conditions of the experiments. It does not support growth of the organism but does not prevent growth on glucose up to concentrations of 10 p.p.m. but growth is reduced at this concentration (10 p.p.m.). If formed, any of these three substances should be detectable in the medium.

Chlorohydroquinone

This substrate must be considered in conjunction with the related quinone, chloro-p-benzoquinone.

Chlorohydroquinone is not toxic to the organisms. They can utilise it as a carbon substrate and grow in both aerated and unaerated media containing it as the only added carbon source. No growth takes place on chloro-p-benzoquinone in aerated or unaerated cultures in concentrations of the order of 10-100 p.p.m. Chloro-p-benzoquinone reduces growth on glucose when present in concentrations down to 0.1 p.p.m. and completely inhibits growth at 10 p.p.m. These substances under the experimental conditions are volatile and may be collected in an alkali trap and estimated by the use of Folin and Ciocalten's phenol reagent. The presence of either of these substances produces subsequent deadaptation of the organisms to 2:4-D. This would not be surprising in the case of chloro-p-benzoquinone if, being a substance toxic to the

organisms, it appears in the culture medium merely as a product of the cultural conditions and not as a true intermediate in the breakdown process of 2:4-D. If chlorohydroquinone is an intermediate it is more difficult to see why growth on the pure substance should produce deadaptation to the 2:4-D molecule unless the enzyme systems involved in the earlier stages of the breakdown, that is in the production of the chlorohydroquinone, are unstable in the absence of their particular substrate, the intact 2:4-D molecule. In this case the utilisation of chlorohydroquinone as a carbon substrate or of any other carbon source not involving this particular unstable enzyme system would result in deadaptation. 2:4dichlorophenol also produces deadaptation. This suggests that, in the event of either of these substances being intermediate compounds produced in the destruction of the 2:4-D molecule, the unstable reaction precedes their formation. The splitting off of the acetic acid side chain must precede the formation of either of these two compounds from 2:4-D. On this hypothesis any compound composed of only ring or side chain elements should produce deadaptation and compounds including the ether linkage present between the two should not..

Results of experiments set out in Table I (following page 68) shew that:-

Acetic acid)
) produce deadaptation
Glycollic)

Glyoxylic acid)
) cause no deadaptation
Succinic acid)

4 chloro-2-hydroxyphenoxyacetic acid)
)
2 chloro-4-hydroxyphenoxyacetic acid) produce
)
4 chlorophenoxyacetic acid)

deadaptation and extremely heavy growth in still culture suggesting that the toxic intermediate postulated as being formed from 2:4-D is not produced here. The ether linkage is present here - both side chain and ring formation are materially unaltered. The breaking of the link between ring structure and side chain in each of these three compounds would produce respectively

4 chlorocatechol)
)
chlorohydroquinone)
)
parachlorophenol) These three substances under the same conditions also produce deadaptation and support growth of the organism. This suggests very strongly that if any of these first three substances is formed then it is in _their_ formation that is again the very first stage in detoxication that the unstable enzyme system exists. There are also compounds in this table which, being utilised as a carbon substrate, do not cause subsequent deadaptation to 2:4-D:-

2:4-dichloro-6-hydrozyphenoxyacetic acid

2 chlorophenoxyacetic acid.

These when subjected to separation of ring and side chain components would yield respectively:-

3-5 dichlorocatechol

and orthochlorophenol.

This latter also retains adaptation and so does phenol itself. 3-5 dichlorocatechol causes deadaptation again suggesting that it is not produced as an intermediate in the breakdown of the 2:4-D molecule.

4-chlorocatechol

This material is volatile under the conditions of the experiments. It is utilised by the 2:4-D organism in still cultures both in the presence and in the absence of 2:4-D. Growth on 4-chlorocatechol causes deadaptation of this organism to 2:4-D breakdown. This compound could be produced from for example 2-4 dichlorophenol as a second stage in the breakdown process by the removal of the 2-position chloride ion and its replacement by OH as of course could chlorohydroquinone if the 4-position chloride was removed instead in a similar way. The production of 4-chlorocatechol is suggested as the second stage in the breakdown of 2:4-D, the first being the production of 2-4 dichlorophenol, by Steenson and Walker (1957). There are however several points of difference between the behaviour of their organisms and that of this strain of Nocardia. Both 4-chlorophenoxyacetic acid and 2-chlorophenoxyacetic acid are utilised more readily than 2:4-D by the Nocardia but not by the organisms used by Steenson and Walker. Again 2-chlorophenol and phenol are not oxidised by these workers' organisms but they

are readily utilised by the 2:4-D effective strain of Nocardia, and this organism also gives an extremely heavy growth on 2-hydroxy-4-chlorophenoxyacetic acid. Whilst it is not impossible that breakdown of 2:4-D should follow the same pathway with both these organisms it also seems quite possible that this is not so. 4-chlorocatechol is not thought to be the first "phenolic" substance appearing during the initial 24 hours incubation period of this Nocardia strain of organisms with 2:4-D media since there appears to be no difficulty in its utilisation by the organisms and no reason therefore why its production should not be continuous - and the production of the "phenol" is not. Production of ionic chlorine in the medium is a continuous process and goes on steadily after the evolution of the "phenol" has ceased. If the chloride was being released by the formation of 4-chlorocatechol a break in the chloride curve shewing either an increased or a decreased rate of production would be expected at or about the 24 hour stage. An increased rate as the efficiency of further breakdown of the 4-chlorocatechol occurred preventing the evolution of 4-chlorocatechol from the solution. A decreased rate if the evolution stopped due to a slowing down or stoppage in the production of the catechol at this stage. The first possibility is thought to be the more feasible but in fact this does not happen and it is another 40 hours approximately before an increase in chloride production is detectable.

1-2-4 trihydroxybenzene

This substance seems highly toxic to the organisms and produces deeply pigmented solutions even in high dilution. This pigmentation has not been noticed in any cultures growing on 2:4-D. 1-2-4 trihydroxybenzene has not been considered as the first volatile substance released from the culture media though theoretically it is a possibility for the later phenolic substance evolved. It seems unlikely that both chloride ions and the acetic acid side chain would be simultaneously released as a first step in breakdown, particularly as there is no close time connection between the "phenol" and chloride release.

4-chlorophenoxyacetic acid

The production of 4-chlorophenoxyacetic acid is considered unlikely since its further decomposition should produce the extremely heavy growth recorded for this substrate on Table I. Its utilisation as a carbon source also causes deadaptation to the 2:4-D molecule as does p-chlorophenol a possible derivative from it by loss of the sidechain. Para-chlorophenol also produces greater growth than a similar quantity of 2:4-D even in unaerated culture and does not appear to be toxic to the organisms in concentrations of up to 100 p.p.m.

2 chlorophenoxyacetic acid

This substrate supports growth of the 2:4-D effective Nocardia strain without causing deadaptation in unaerated culture as does orthochlorophenol its nearest phenolic derivative. Less growth is recorded for 2-chloro than for organisms growing on 4-chlorophenoxyacetic acid. The fact that both 2-chlorophenoxyacetic acid and 2-chlorophenol are utilised as carbon substrates by this 2:4-D organism without causing it to lose its ability to deal with 2:4-D would suggest that the production of first 2-chlorophenoxyacetic acid and secondly 2-chlorophenol from it as a probable route for 2:4-D disintegration. It still has to be considered that both these substrates can be utilised in the absence of aeration; 2:4-D cannot. If these two substances are intermediates in 2:4-D breakdown this last fact leaves as the only possible reaction requiring aeration the removal of the 4-position chloride ion and its replacement by hydrogen but, parachlorophenoxyacetic acid and parachlorophenol are utilised in the absence of aeration. Also since neither the acid nor the phenol appears toxic to the organism concerned, in concentrations up to the level of the initial substrate strength of the 2:4-D, there seems to be no reason why, once it has started, production of the phenol should not be continuous. The results illustrated on Figure XXXI shew that this is not so. There is a lag of 100 to 120 hours from the time of cessation of evolution of the first phenolic material until any further measurable quantity

is evolved and none is detectable in the culture fluid. Meanwhile all radioactivity has disappeared from the 2:4-D spot area and accumulation continues during the intermediate "phenol" lag period.

Since it is apparent that at least one stage in the decomposition of 2:4-D by this organism produces a substance which limits the growth of the organism before the substrate is exhausted, and from the experimental work already carried out it seems possible that 2:4-dichlorophenol may be this substance, the formation of o-chlorophenol from 2:4 dichlorophenol must be considered. o-chlorophenol could be produced from 2:4 dichlorophenol by the removal of the 4 position chloride ion and its replacement by a hydrogen ion. Chlorohydroquinone could be formed in a similar manner if the 4 position chloride is replaced by a hydroxyl group. In either case, since neither of these monochloride compounds is toxic to the organism the overall rate of decomposition would be limited by the ability of the organism to effect this conversion. If o-chlorophenol is produced in this manner then it would explain the retention of 2:4-D adaptation by organisms grown on o-chlorophenol as the sole carbon source. The retention of adaptation to 2:4-D by organisms grown on 2-chlorophenoxyacetic acid would be similarly explained since in its breakdown only intermediates produced during the decomposition of 2:4-D would be formed. Aeration is unnecessary since the volatile toxic substance

2:4 dichlorophenol would not in this case be produced. The removal of the second chloride ion and its replacement by a hydrogen ion would in both cases produce phenol which is metabolised by this organism as a sole carbon source and its metabolism does not produce subsequent deadaptation to the 2:4-D molecule. The route:- 1. 2:4-D

2. 2:4dichlorophenol + [glycollic acid]

3. o-chlorophenol + chloride ion

4. phenol + chloride ion

can thus account for some of the phenomena encountered in these experiments <u>but</u> it does not explain the absence of "phenol" production during the period of chloride accumulation in the medium. Also there would seem to be no reason why once the decomposition process has started it should not be continuous irrespective of the initial concentration of 2:4-D. This is not, so but such a system can be produced by the addition of soil extract and in some measure by the addition of dialysate of agar to the medium. The effective substance present in these extracts may be the material which neutralises the toxic effect of the first formed 2:4-dichlorophenol on the organisms and at the same time renders it non-volatile and its reaction to the Folin and Ciocaltin's phenol reagent negative. The acetic acid side chain not being replaced merely by OH but by this unknown substance X. The phenol escaping into the trap is that which does

not react to form this compound with X and aeration is necessary to keep the concentration of free phenol below the lethal level.

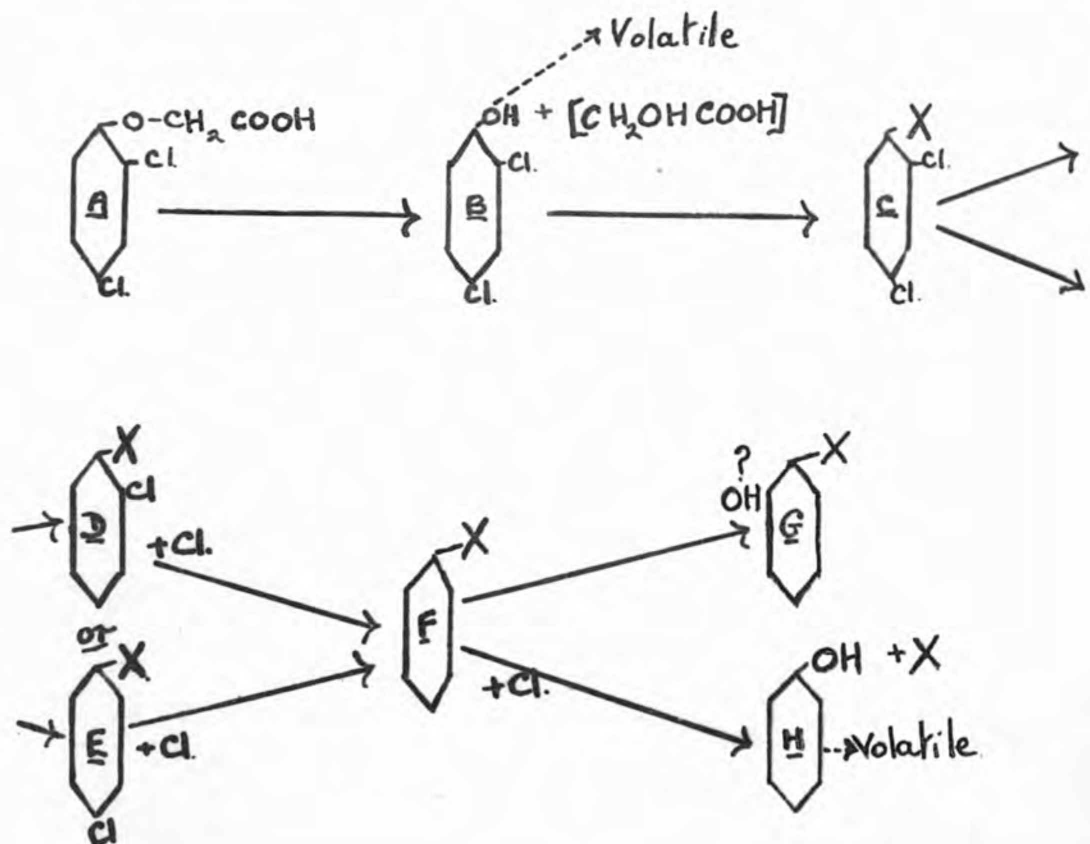
The overall picture presented by all of these facts suggests that, in the breakdown of 2:4-D by this particular organism the disintegration of the molecule commences immediately upon inoculation and aeration of the cultures. The initial reaction releases a phenolic material which is volatile under the experimental conditions and the production of this phenol involves the separation of the side chain from the ring portion of the molecule. The further metabolism of the side chain portion releases radioactive CO_2 from both carboxyl and methylene carbon atoms in radioactivity labelled samples. The phenolic material is toxic to the organisms in very small quantities and is responsible for the early cessation of growth in cultures. This toxic phenol bubbles out of the solution initially - at the commencement of incubation but cannot be found after 24-48 hours. If as it is logical to suppose its production, once started, is continuous since the next (or later) stage, the removal of chloride is, then a neutralisation of this toxic material must be effected by the organism keeping the concentration below the lethal value. This neutralisation of the toxic effect also removes the phenolic reaction of the substance and renders it non-volatile. The next step in this series of reactions would then be the removal of chloride ions - 4 position first and then 2 position but not then replacement by OH but by H. ion, since this would again give a phenolic

reaction and this is not recorded. Finally a second volatile phenolic substance is produced possibly by the release of the added neutralising sidechain. This latter substance is at present unknown but if it is involved is most likely to be the "active principle" found in soil extract and agar dialysate which enables the organisms to deal with much larger quantities of 2:4-D.

A second possibility for the formation of the later phenolic material is by the introduction of an OH group or groups into the remaining ring compound preparatory to its being split as is frequently the case in the oxidation of aromatic ring structures by micro-organisms.

The following Figure XXXII sets out this scheme diagrammatically.

FIGURE XXXII



The compounds C, D and E not being volatile else chloride recovery in culture media would not be complete.
Compound B is volatile. A very small quantity only is lost before conversion to C.
Any or all of compounds F, G and H may be volatile. F must give a negative reaction for "phenol," G and/or H positive. The production of D is more probable than that of E.

A possible route for the early stages of 2:4-D decomposition by an effective strain of Nocardia

IX. Experiments using C^{14} methylene labelled 2:4-D in high concentration.

1. Introduction

From the preceding work a hypothetical route for the early stages of 2:4-D breakdown by the effective Nocardia strain isolated from soil flask culture can be worked out. None of the postulated substances has up to this time been isolated from culture media and identified. No accumulation of intermediate substances in the culture vessels has been detected, and,owing to the very small quantities of the herbicide the organisms are able to tackle,analysis of the fluid from the culture flasks or alkali traps by ordinary chemical means has not been possible.

Chromatographic analysis of these solutions whilst suggesting the presence of substances other than 2:4-D have produced no really conclusive evidence. The disappointing results obtained from chromatographic studies of the culture solutions are it is felt, due to the volatility of the compounds involved and the transient nature of their appearance in solutions.

In the following experiments an attempt to separate the first stages in the breakdown has been made. Carbon dioxide including carbon atoms from groups of the side chain is evolved from aerated culture solutions, containing initially 100 p.p.m. 2:4-D, during the first 24 hours of incubation.

This fact has been established by the use of C^{14} side chain labelled 2:4-D. (See Figures XXIV and XXVII). Free chloride ions are detectable in the culture fluids during this same period and a positive reaction for phenolic substances is given by the alkali trap. (Figure XXXI). The postulated scheme of reactions set out in Figure XXXII shews 2:4-dichlorophenol as the first intermediate product. If this in fact is the case it should be possible, if the reaction can be stopped at an appropriate time, to separate the formation of the phenol and carbon dioxide on the one hand from the subsequent release of chloride ions on the other.

Growth, as measured by numbers of organisms, occurs in cultures containing initially 1,000 p.p.m. 2:4-D but little if any loss in phytotoxicity takes place. Since no growth takes place in the absence of an added carbon substrate it is reasonable to assume that this initial growth is due to the metabolism of a very small quantity of the herbicide or an impurity present in it. This latter possibility is unlikely since the 2:4-D used is a very pure sample. If growth in cultures containing this high concentration of 2:4-D takes place at the expense of the herbicide, and 2:4-dichlorophenol is produced as a first step in the breakdown, in the absence of complete metabolism of the substrate at this initial concentration it may be possible to detect either or both of the following:-

1. The evolution of C^{14} labelled carbon dioxide before the liberation of free chloride into the culture solution.

2. The presence of phenolic material in the culture solution or in the alkali trap prior to or in the absence of the production of ionic chloride in the medium.

2. Experimental methods

An experimental set up similar to that designed for the previous experiments in which samples of radioactive 2:4-D were involved was used. This is described on page 90.

A volume of 60 mls. of culture fluid was used with an alkali trap containing 20 mls. of 2Normal NaOH. The critical time in the experiments envisaged appeared to be during the first few days only. It was therefore decided that the use of a small volume of fluid containing 1,000 p.p.m. 2:4-D made up of 20 mgs. of radioactive sample and 40 mgs. of non-labelled 2:4-D would be best for this work. Methylene labelled 2:4-D was employed.

The use of 20 mgs. of active sample in a total of 60 mls. culture fluid meant that a high count could be expected in the alkali trap as soon as radioactive CO_2 was evolved from the solution. The use of 60 mls. instead of employing 20 mls. with all the 2:4-D taken from the active sample enabled 5 ml. samples to be taken from the culture flask, and titrated against N/500 $AgNO_3$ for chloride estimation, at intervals throughout the experimental period. The first

FIGURE XXXIII

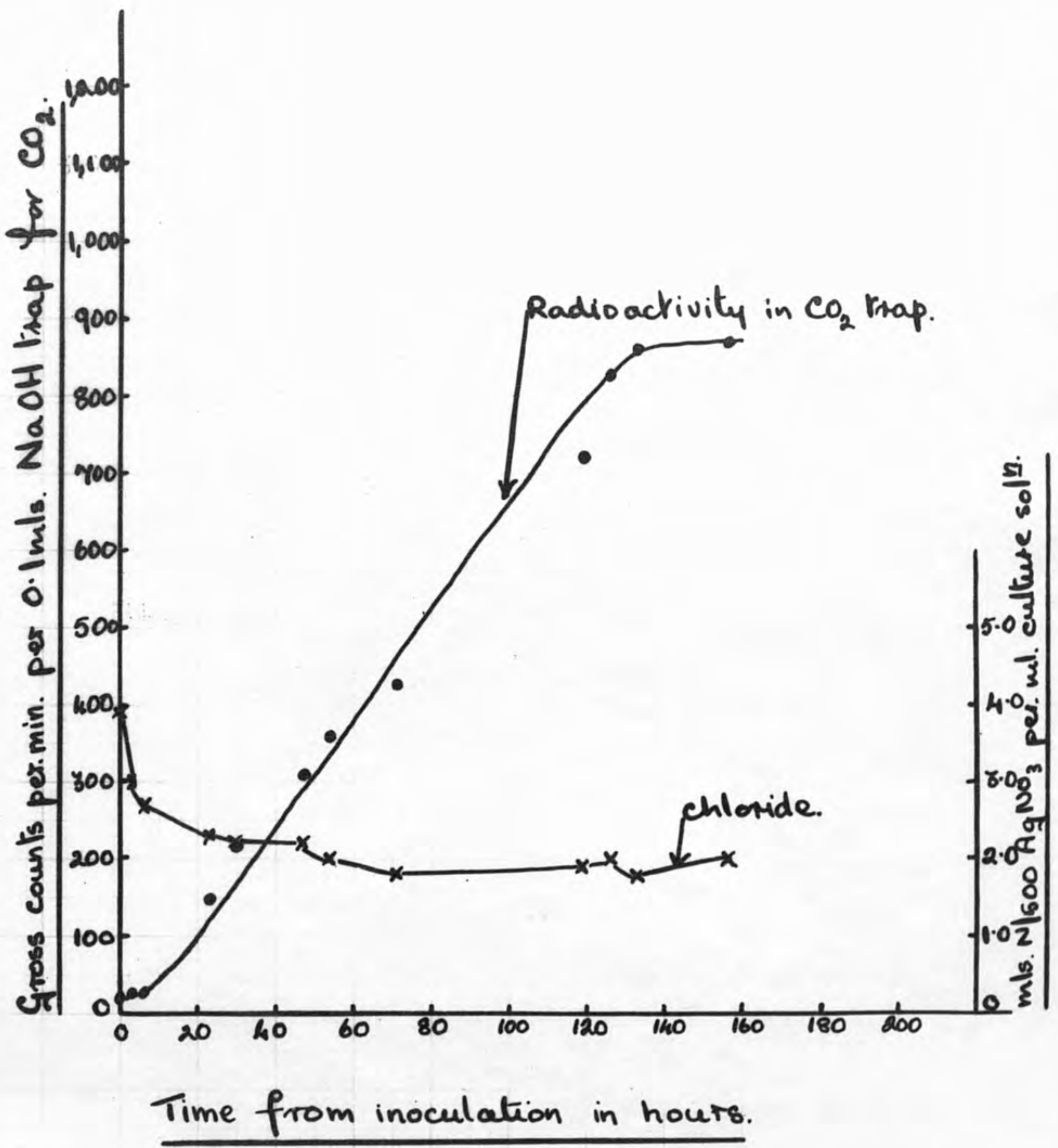
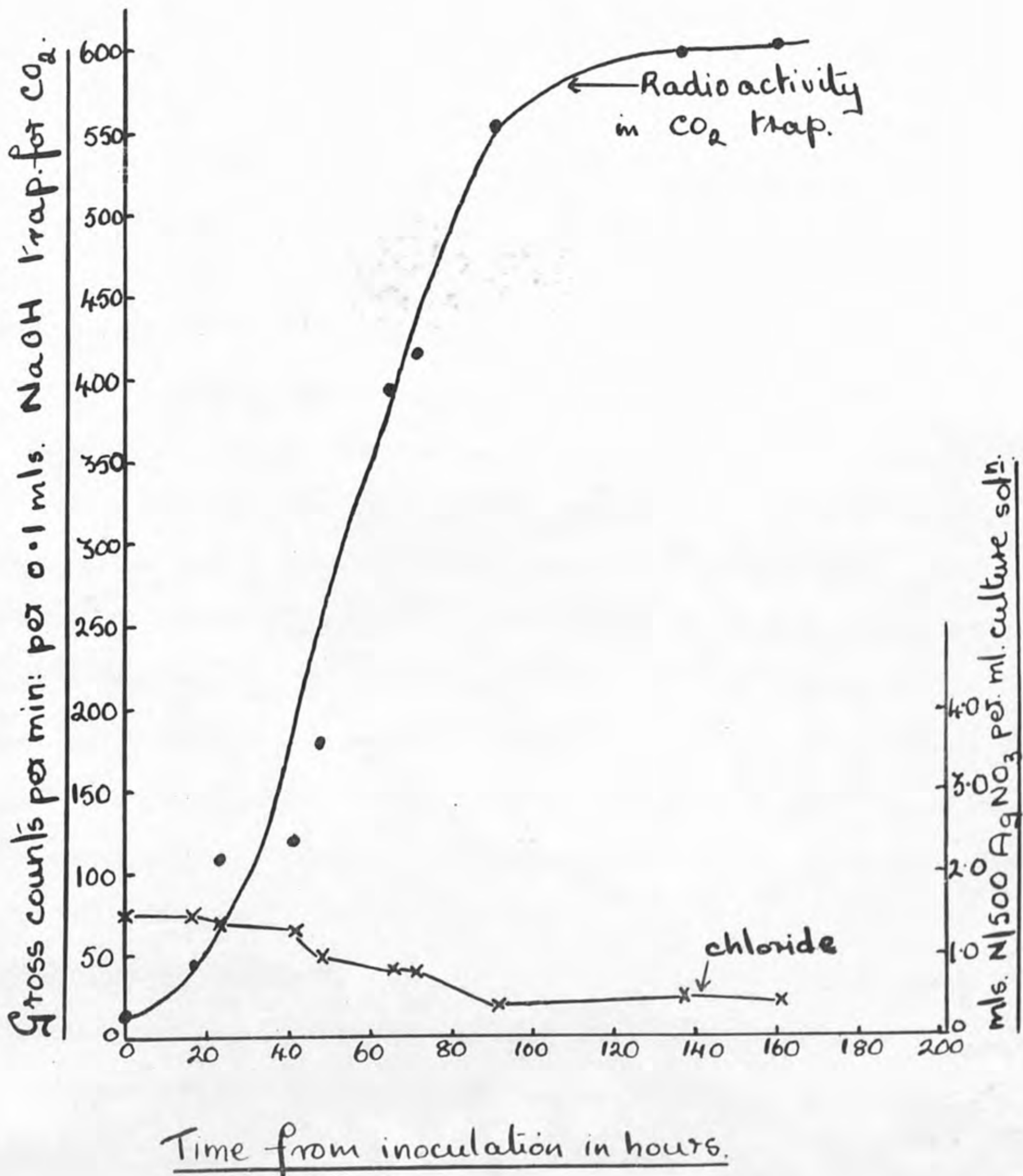


FIGURE XXXIV



experiment was carried out using the normal growth medium of the organism in 2:4-D culture - that is containing potassium chloride. In the second experiment this was replaced by potassium dihydrogen phosphate.

Results.

The measurements of radioactivity recorded in the alkali trap and the results of the chloride titrations are shewn in Figures XXXIII and XXXIV and given in Appendices XXXII and XXXIV.

Figure XXXIII shews results obtained using media containing potassium chloride and Figure XXXIV those obtained when potassium chloride was replaced by potassium dihydrogen phosphate. It will be seen that in this latter case chloride is still present in the culture fluid. This is due to the inevitable transference of chloride in the inoculum. The chloride results have plotted directly as titration records and not calculated as percentages of the added chloride (i.e. in the 2:4-D charge) since it is apparent that no measurable increase in chloride content of the media has been noted. In fact there is a decrease. It appears unlikely therefore that any chloride has in fact come from the 2:4-D.

Radioactive carbon dioxide has undoubtedly been evolved from the cultures so that it may confidently be stated that the initial growth on media containing 1,000 p.p.m. 2:4-D

as the only added carbon source is due to the metabolism of this substrate.

The total final radioactivity count obtained in the alkali traps after six days incubation (600-900 counts per minute) is equivalent to that recorded from a concentration of 7.5 to 12.6 p.p.m. 2:4-D under the same conditions. One third only of the 2:4-D in the culture solution was added from the radioactive sample, this result therefore represents a total of carbon dioxide released from metabolised 2:4-D equivalent to 22.5 to 37.8 p.p.m. 2:4-D. The initial concentration of 2:4-D in the culture medium was 1,000 p.p.m. therefore something like 2-4% of the CO_2 from the side chain has been evolved. 22.5 p.p.m. 2:4-D releases into 5 mls. of solution chloride equivalent to 0.478 mls. of N/500 $AgNO_3$. 37.8 p.p.m. 2:4-D releases into 5 mls. of solution chloride equivalent to 0.802 mls. of N/500 $AgNO_3$. In neither of the experiments carried out was this increase recorded at the time of CO_2 evolution. This required increase is however very small and in view of the fact that the inoculum, and in one case the medium, was not free of chloride, it cannot be assumed that no chloride is in fact produced.

In these two experiments the reaction of the trap fluid diluted 1:3 for phenolic material was tested after 6 days incubation only. The percentage trahsmission of light after reaction with

Folin and Ciocaltens Phenol reagent - the control tube containing NaOH solution and the reagent was:

	at inoculation	after 6 days
Control	100	100% transmission
First experiment (Figure XXXIII)	100	55.3% "
Second experiment (Figure XXXIV)	100	68% "

These readings are associated with a concentration of phenolic material equivalent to 50.12 and 26.61 p.p.m. 2:4-dichlorophenol respectively. These concentrations are approximately equivalent to 5% and 2.66% respectively of the initial charge of 2:4-D.

This formation of phenol in the absence of complete metabolism of the 2:4-D at this high concentration further evidence in support of the hypothesis that 2:4-dichlorophenol is formed at the first intermediate product in the decomposition of 2:4-D and that it is the substance which being toxic to the organism concerned inhibits further breakdown at high concentrations.

X. Analysis of the alkali trap fluid by a method of gas chromatography.

Dr.B.Heywood of May & Baker Ltd. Dagenham, Essex, who kindly supplied 13 chemical compounds of guaranteed purity for use in the work described in this thesis, also offered to undertake the analysis of the materials present in the alkali trap, if the volume of the trap could be reduced to 25 mls. or less without decreasing the volume of the culture fluid below 500 mls. It proved difficult to fulfil these requirements, to ensure that bubbling was slow enough to ensure the trapping of all volatile materials evolved from the cultures, and at the same time maintain a sufficiently vigorous rate of aeration to obtain decomposition of the 2:4-D.

A sample of trap fluid was eventually obtained and sent to Dr.Heywood.

A preliminary report on its contents gave :-

2:4-dichlorophenol
chlorohydroquinone
a monochlorophenol
phenol
and three as yet unidentified materials as present in this fluid.

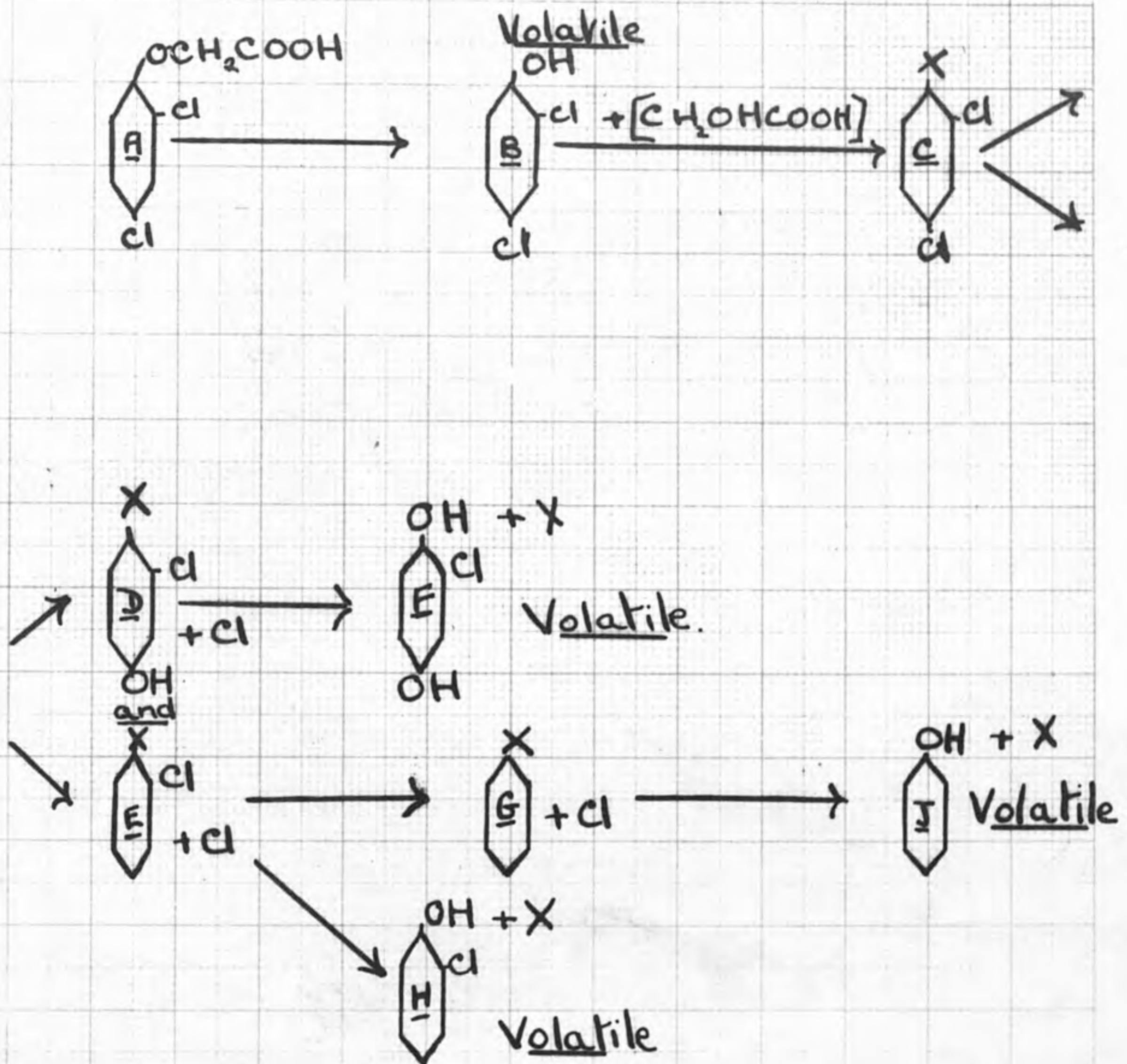
In view of this some modification of Figure XXXII can now be made.

The monochlorophenol is assumed to be o-chlorophenol having regard to the evidence from previous experiments already

discussed and to the presence of chlorohydroquinone in the trap. Also o-chlorophenol could be produced from 2:4-dichlorophenol by replacement of the 4-position chloride ion with a hydrogen ion. Similarly chlorohydroquinone can be formed by the replacement of the <u>same</u> position chloride with a hydroxyl group. Thus attack on the same position produces both substances. If 4-chlorophenol were assumed to be formed, two separate attacks on the molecule must take place. Evidence from growth experiments already discussed is also against the formation of 4-chlorophenol.

Figure XXXV sets out the scheme for the early stages in 2:4-D decomposition as now suggested.

FIGURE XXXV



Very small quantities only of B, F and H liberated and found to be present in air bubbling from the media. Small quantities of J also present, the remainder used as a carbon source by this organism.

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APPENDICES

Data relating to the text.

One representative set of figures is given for each experiment.

Cultures other than those involving use of samples of radioactive 2:4-D were carried out in triplicate and the experiments repeated three times i.e. using nine tubes in all.

Experiments involving the use of radioactive material were carried out three times, only using one culture tube or flask in each experiment.

APPENDICES

1. Data relating to Figures in the text.

In all cases time measured from inoculation.

Haemacytometer cell factor = 4×10^6

Appendix I. Figure I.

A. Growth of B. globiforme in mineral salts 100p.p.m. 2:4-D

Time in hrs.	Cell counts	Av.	Cells/ ml.	Log_{10} cells/ml.
0	Calculated from inoculating suspension		6.06×10^5	5.78
24	0,2,1,1,1,0,1,0,1,2	0.9	3.6×10^6	6.56
48	8,6,6,9,10,7,8,7,11,9	8.1	3.24×10^7	7.51
72	16,15,15,16,18,17,15,16,16,17	16.1	6.44×10^7	7.81
100	16,17,17,14,16,16,14,13,17,18	15.8	6.32×10^7	7.80
216	16,14,14,16,17,16,14,19,18,15	15.9	6.36×10^7	7.80
264	15,15,16,16,18,15,17,15,16,18	16.1	6.44×10^7	7.81
360	11,13,12,15,10,13,16,11,14,11	12.6	6.04×10^7	7.70

APPENDIX I

B. Phytotoxicity of culture A. Cress seedling root test.

Culture solution diluted to give initial conc. of:-

Length of roots in mms.

Time in days	1 ppm. 2:4-D	Av.	0.1	Av.	0.01	Av.	Control	Av.
0	2,1,1,1	1.23	13,11,10,10	11	60,60,56,45	50.2	85.78,78,72	78.3
1	4,1,2,3	2.5	17,17,14,10	14.5	72,70,62,60	66	96,81,79,70	81.5
2	1,1,0,0	0.5	10,6,5,5	6.4	54,51,42,36	45.7	83,80,78,76	79.3
3	3,2,2,1	2.0	15,12,10,8	11.3	65,59,52,42	56.5	89,87,81,75	83
5	3,2,1,1	1.8	16,10,8,7	10.3	61,60,53,43	54.3	86,82,78,74	80
7	4,3,2,2	2.8	17,15,10,10	13	69.63.54.51	59.3	91,83,76,72	80.4
9	5,2,1,1	2.3	18,14,11,11	13.5	62,60,58,57	59.3	87,84,76,75	80.4
10	87,80,75,75	78	94,92,80,77	83.3	98,95,80,80	88.3	87,86,79,76	82.5
11	80,90,88,86	88.5	95,93,89,76	88.3	90,86,86,80	85.5	85,81,78,77	80.3
12	97,90,88,87	90.5	93,90,80,79	85.5	88,78,75,75	79	92,88,79,78	84.3

APPENDIX I

C. Growth of B. globiforme in mineral salts 1,000p.p.m.2:4-D

Time in hrs.	Cell counts	Av:	cells/ml	Log_{10} cells/ml
0	Calculated from inoculating susp.		4.56×10^5	5.66
24	4,0,4,4,2,1,1,0,1,3	2.0	8.0×10^6	6.90
48	3,2,6,4,3,6,4,4,3,4	3.9	1.56×10^7	7.19
96	11,10,10,10,8,11,12,7,8,9	9.6	3.84×10^7	7.59
124	13,12,12,13,13,12,12,14,12,12	12.5	5.00×10^7	7.70
148	11,12,14,13,14,14,13,13,15,14	13.3	5.32×10^7	7.73
172	22,21,20,25,26,23,20,21,25,23	22.6	9.04×10^7	7.96
	Diluted 1:10			
196	4,3,2,2,2,3,5,1,5,4	3.1	1.24×10^8	8.10
252	4,7,6,4,3,6,2,5,3,3	4.3	2.12×10^8	8.33
284	11,13,12,10,10,11,13,10,10,9	10.9	4.36×10^8	8.64
308	13,12,10,12,9,11,10,9,14,15	11.5	4.60×10^8	8.66
332	10,13,9,14,10,15,8,11,14,12	11.6	4.64×10^8	8.67
372	12,13,11,11,9,13,13,12,11,12	11.7	4.68×10^8	8.67
448	13,12,11,12,11,12,12,14,10,10	11.7	4.68×10^8	8.67
492	13,13,14,13,11,13,13,14,10,14	12.8	5.12×10^8	8.71
584	13,10,11,9,10,12,13,14,13,11	11.6	4.64×10^8	8.67
744	11,15,10,11,11,10,13,10,14,12	11.7	4.68×10^8	8.67
960	10,12,16,13,14,12,13,9,11,12	12.2	4.88×10^8	8.69

APPENDIX I

D. Phytotoxicity of culture D. Cress seedling root test.

Length of roots in mms.

Time in Days.	1 p.p.m. 2:4-D	Av.	0.1 p.p.m.	Av.	0.01p.p.m.	Av.	Control	Av.
0	3,3,2,2	2.5	16,12,8,6	10.5	63,54,53,50	55	85,80,80,79	81.0
1	5,5,4,3	4.3	20,20,19,16	18.8	69,63,55,50	59.2	83,82,82,80	81.3
7	3,2,2,1	2.0	19,17,14,10	15	62,60,53,41	54.0	80,80,79,79	79.5
12	3,2,1,1	1.8	17,16,13,10	14	62,58,50,42	53.0	80,79,79,75	78.3
15	4,2,1,0	1.8	22,20,15,11	17.5	67,62,51,38	54.5	86,84,80,80	82.5
21	5,4,2,2	3.2	28,17,17,12	18.5	69,67,52,40	57	82,80,79,77	79.5
25	5,5,2,2	3.5	27,25,16,10	19.5	59,58,58,56	57.8	81,80,79,79	79.8
31	3,3,3,1	2.5	25,23,8,11	19.3	67,65,54,49	58.8	91,82,81,80	83.5
33	4,4,4,3	3.7	30,23,19,18	22.5	72,64,57,53	61.5	90,83,80,75	82.0
37	5,4,4,3	4.0	29,25,20,17	22.7	71,70,59,56	64.0	88,86,82,76	83.0
40	4,4,3,2	3.3	24,24,15,14	19.2	69,65,55,52	60.3		

APPENDIX I

E. Growth of B. globiforme in 2:4-D only (no mineral salts)

Time in hrs.	Cell counts	Mean	Cells/ ml.	Log_{10} cells/ml.
0	Calculated from inoculating susp.		$6.06x10^5$	5.78
120	1,0,0,0,1,0,0,2,0,0	0.4	$1.6\ x10^6$	6.1
240	1,0,2,0,0,1,0,0,0,1	0.5	$2.0\ x10^6$	6.3
312	0,0,0,2,0,0,1,0,1,0	0.4	$1.6\ x10^6$	6.1
336	0,0,1,0,0,1,1,1,0,0	0.4	$1.6\ x10^6$	6.1

F. Growth of B. globiforme in mineral salts only (no.2:4-D)

Time in hrs.	Cell counts	Mean	Cells/ml.	Log_{10} cells/ml
0	Calculated from inoculating susp.		$6.06x10^5$	5.78
120	0,1,0,0,1,0,0,2,0,0,	0.4	$1.6x10^6$	6.20
240	1,0,0,1,0,2,0,2,0,0	0.6	$2.4x10^6$	6.40
312	0,1,2,1,0,1,0,1,0,0	0.6	$2.4x10^6$	6.40
336	1,0,0,0,2,0,0,2,0,1	0.6	$2.4x10^6$	6.40

APPENDIX II Data relating to Figure II.

<u>The effect of small quantities of agar on bacterial growth in cultures containing 100 p.p.m. 2:4-D.</u>

Conc. of Agar		Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
All cultures	0	Inoculating suspension gives		5.60×10^{5}	5.75
No agar	20	7,6,9,5,8,10,7,9,10,8	7.9	3.16×10^{7}	7.5
	48	4,3,1,1,2,3,5,2,2,2,	2.5	1.0×10^{8}	8.0
	72	6,2,9,3,4,4,8,7,4,3	5.0	2.0×10^{8}	8.3
	120	5,2,3,3,4,5,5,8,7,8	5.0	2.0×10^{8}	8.3
		Diluted 1:10			
0.02% agar	20	5,2,3,3,2,6,5,4,2,3	3.5	1.40×10^{8}	8.15
		Diluted 1:100			
	48	7,3,3,6,7,4,5,4,6,5,	5.0	2.0×10^{9}	9.3
		Diluted 1:1000			
	72	11,10,13,19,18,8,21,13,13,14	14.0	5.6×10^{10}	10.75
	120	15,13,17,12,14,10,16,14,18,11	14.0	5.6×10^{10}	10.75
		Diluted 1:10			
0.04% agar	20	4,5,6,2,6,4,4,5,4,4	4.4	1.76×10^{8}	8.25
	48	Diluted 1:100			
		11,10,11,15,18,12,13,8,11,12	11.1	4.44×10^{9}	9.65
		Diluted 1:1000			
	72	14,11,11,13,11,12,10,12,8,9	11.1	4.44×10^{10}	10.65
	120	16,17,17,14,18,18,20,19,20,18	17.7	7.08×10^{10}	10.85
0.06% agar		Diluted 1:10			
	20	2,4,2,2,2,5,1,3,2,2	2.5	1.00×10^{8}	8.0
		Diluted 1:100			
	48	15,12,13,14,16,15,13,15,14,14	14.1	5.64×10^{9}	9.75
		Diluted 1:1000			
	72	15,25,17,13,14,18,21,17,20,18	17.7	7.08×10^{10}	10.85
	120	35,36,32,38,39,35,33,31,36,37	3.50	1.40×10^{11}	11.15
		Diluted 1:10			
0.08% agar	20	4,3,4,4,5,3,4,3,4,6	4.0	1.60×10^{8}	8.2
		Diluted 1:100			
	48	20,18,17,19,20,17,22,23,25,18	19.9	7.96×10^{9}	9.9
	72	Diluted 1:1000			
		5,3,4,3,3,5,6,2,9,4	4.4	1.76×10^{10}	10.25
	120	7,5,4,6,2,3,4,8,5,6	5.0	2.0×10^{10}	10.3
0.10% agar	20	3,4,3,6,5,6,4,6,7,6	5.0	2.0×10^{7}	7.3
		Diluted 1:100			
	48	23,25,20,22,21,20,24,25,22,21	22.3	8.92×10^{9}	9.95
		Diluted 1:1000			
	72	9,7,9,10,8,7,7,5,8,9	7.9	8.16×10^{10}	10.5
	120	7,8,9,6,8,8,10,9,6,8	7.9	3.16×10^{10}	10.5

APPENDIX III Data relating to Figure III

The effect of soil extract on bacterial growth in aerated liquid culture

Substrate	Time in hrs.	Cell counts	Av.	Cells /ml.	Log_{10} Cells /ml.
All cultures	0	Inoculating suspension giving	.	1.24×10^5	5.1
Mineral salts	30	17,19,9,9,13,15,14,15,16,17	14.2	5.68×10^7	7.6
100 ppm.	56	16,12,17,14,15,12,22,13,18,19	15.6	6.24×10^7	7.95
2:4-D	80	16,15,17,13,14,14,17,18,13,17	15.4	6.16×10^7	7.9
	110	20,19,12,13,13,24,12,13,14,16	15.6	6.24×10^7	7.95
		Diluted 1:10			
Soil extract	30	16,14,15,14,18,16,18,17,16,15	16.1	6.44×10^8	8.1
only	56	17,16,17,14,25,18,18,14,25,10	17.5	7.00×10^8	8.45
	80	20,18,16,14,15,17,16,23,18,17	17.4	6.96×10^8	8.43
	110	18,14,13,18,19,13,20,21,20,19	17.5	7.00×10^8	8.45
		Diluted 1:10			
Soil extract	30	16,9,11,10,13,15,16,11,9,15	12.5	5.0×10^8	8.7
and Mineral	56	15,12,13,13,17,14,17,12,14,13	14.0	5.6×10^8	8.75
salts	80	12,15,12,10,16,15,16,15,8,7	12.5	5.0×10^8	8.7
	110	19,14,17,9,12,13,10,18,14,11	13.7	5.48×10^8	8.74
		Diluted 1:10			
Soil extract	30	21,22,20,16,18,23,19,18,17,20	19.4	7.76×10^8	8.9
and mineral		Diluted 1:100			
salts 100ppm.	56	2,2,2,3,6,4,2,3,2,4	3.0	1.2×10^9	9.08
2:4-D	80	3,1,2,4,3,2,2,3,3,2	2.5	1.0×10^9	9.0
	110	5,1,4,4,2,1,1,5,2,3	2.8	1.12×10^9	9.05

APPENDIX III - continued

Phytotoxicity of cultures.

Time taken to detoxicate 100 p.p.m. 2:4-D in cultures containing soil extract.

Time in days	Cress root assay + soil extract. 1	2	3	No soil extract
1	Toxic	Toxic	Toxic	Toxic
2	T	T	T	T
3	T	T	T	T
4	T	T	T	T
5	T	T	T	T
6	T	T	T	T
7	T	T	T	T
8	T	T	T	Detox.
9	T	T	T	Detox
10	T	T	T	-
11	T	T	T	-
12	T	Detox	T	-
13	Detox	Detox	Detox	-
14	Detox	-	Detox	-

APPENDIX IV Data relating to Figure IV

The effect of yeast extract on bacterial growth in cultures containing 100 ppm.2:4-D

Conc.of yeast ext.	Time in hrs.	Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
All cultures	0	Inoculating suspension gives		3.64×10^4	4.56
None	20	4,3,2,4,3,3,2,1,2,1	2.5	1.0×10^7	7.0
	44	11,9,11,8,7,9,7,8,10,10	9.0	3.60×10^7	7.55
	68	9,8,8,7,6,7,8,9,9,8	7.9	3.16×10^7	7.50
	87	8,7,10,11,10,11,7,9,7,8	8.8	3.32×10^7	7.52
	120	10,10,9,10,10,10,9,9,12,10	9.9	3.96×10^7	7.6
0.01%	20	1,1,1,1,2,1,0,1,0,2	1.0	4.0×10^6	6.6
	44	14,14,15,14,15,15,14,16,18,17	15.2	6.08×10^7	7.85
	68	diluted 1:10			
		4,2,3,3,5,5,2,5,2,2	3.1	1.24×10^8	8.1
	87	4,5,5,6,5,5,7,4,5,4	5.0	2.0×10^8	8.3
	120	7,5,6,10,8,9,7,5,6,7	8.0	3.2×10^8	8.5
0.05%	20	5,6,5,5,6,4,7,7,5,6	5.6	2.24×10^7	7.35
		Diluted 1:10			
	44	3,5,3,6,4,2,2,4,3,3	3.5	1.40×10^8	8.15
	68	6,5,5,8,5,7,7,8,6,6	6.3	2.52×10^8	8.4
	87	9,9,7,8,8,7,6,9,8,8	7.9	3.16×10^8	8.5
	120	11,8,9,12,10,9,10,11,9,10	9.9	3.96×10^8	8.6
0.10%	20	6,8,9,6,8,8,5,7,8,5	7.0	2.8×10^7	7.45
		Diluted 1:10			
	44	6,3,5,3,4,6,5,6,4,2	4.4	1.76×10^8	8.25
	68	8,9,7,6,7,8,4,6,8,7	7.0	2.8×10^8	8.45
	87	10,11,10,11,10,12,13,11,9,14	11.1	4.44×10^8	8.65
	120	11,12,11,10,10,12,11,11,12,11	11.1	4.44×10^8	8.65

APPENDIX IVb. Data relating to results recorded on pages 12 and 13

Phytotoxicity of culture solutions assayed by use of cress seedling root test.

Growth of organisms by direct counting under a haemacytometer.

d) The effect of M/1000 calcium chloride and 0.1% calcium carbonate on the detoxication of 100 p.p.m. 2:4-D

Samples diluted to give an initial conc. of 1 p.p.m. 2:4-D

Time days	$CaCl_2$ + 2:4-D	$CaCO_3$ + 2:4-D	2:4-D only	Control cress
	Length of cress roots in mms.			
0	4,3,3,0	4,3,3,2	4,3,2,2	85,82,80,80
1	5,2,2,1	3,2,0,0	3,2,1,1	82,80,80,76
2	3,3,2,2	3,1,1,0	4,2,1,0	86,84,84,80
3	4,3,3,3	5,2,2,1	3,3,3,0	88,86,80,78
4	3,2,2,2	4,3,2,0	3,3,2,1	80,80,78,75
5	4,4,2,0	2,2,1,1	2,2,2,1	88,84,80,77
6	4,1,0,0	3,3,2,0	3,3,2,2	82,80,80,78
7	3,3,3,1	5,1,0,0	4,1,1,0	90,85,80,80
8	4,2,2,1	3,3,3,2	4,2,2,2	85,80,79,75
9	26,10,9,3	82,80,79,78	85,83,80,75	81,80,79,77
10	95,90,88,82	94,87,85,85	92,82,81,76	90,87,80,75
11	88,88,86,79	87,79,76,73	86,85,75,75	78,76,76,70
12	87,85,82,80	84,80,79,74	82,78,74,71	92,86,82,80

APPENDIX IVb - continued

e)i. The effect of Vitamin B_{12} on the growth of B. globiforme in aerated liquid cultures containing 100 p.p.m. 2:4-D

Time - hours	Concentration of Vitamin B_{12}				
	1.0ppm.	0.1ppm.	0.01ppm.	0.005ppm.	None
	Cells/ml.				
0	1.22×10^5	1.22×10^5	1.22×10^5	1.22×10^5	1.22×10^5
24	4.0×10^6	4.4×10^6	4.32×10^6	3.96×10^6	4.12×10^6
48	1.24×10^7	9.2×10^6	1.72×10^7	1.44×10^7	1.42×10^7
72	9.12×10^7	8.76×10^7	8.80×10^7	8.24×10^7	9.16×10^7
96	9.04×10^7	8.80×10^7	8.80×10^7	8.28×10^7	8.88×10^7
120	9.04×10^7	8.76×10^7	8.76×10^7	8.20×10^7	8.92×10^7
144	9.08×10^7	8.84×10^7	8.76×10^7	8.16×10^7	9.08×10^7
168	8.96×10^7	8.80×10^7	8.84×10^7	8.28×10^7	9.12×10^7
192	9.12×10^7	8.76×10^7	8.72×10^7	8.28×10^7	9.12×10^7
216	9.12×10^7	8.84×10^7	8.84×10^7	8.24×10^7	8.96×10^7
240	9.08×10^7	8.80×10^7	8.80×10^7	8.24×10^7	8.92×10^7
264	8.92×10^7	8.76×10^7	8.80×10^7	8.28×10^7	9.12×10^7
288	9.12×10^7	8.72×10^7	8.76×10^7	8.20×10^7	8.92×10^7
336	9.12×10^7	8.80×10^7	8.80×10^7	8.28×10^7	8.92×10^7

APPENDIX IVb - continued

e) ii. Phytotoxicity of cultures. Sample diluted to initial conc.1 ppm.2:4-D

Time days	Conc.of vitamin B_{12} 1.0 ppm.	0.1 ppm.	0.01ppm.	0.005ppm.	None	Control Cress
	Length of cress roots in mms.					
0	3,3,2,2,	3,2,2,1	4,1,1,0	3,3,2,0	3,3,2,2	97,89,85,85
2	4,3,2,2	4,4,3,2	4,2,2,0	4,3,3,2	5,4,4,1	92,88,86,84
4	3,3,1,1	4,3,2,0	4,3,2,1	4,3,2,2	3,3,2,1	87,86,83,80
6	3,2,2,1	3,1,1,0	3,3,2,1	2,2,1,0	3,3,3,0	87,85,80,80
8	4,3,3,2	3,3,2,2	5,3,3,2	4,3,3,3	3,2,2,1	88,85,85,83
9	83,78,75,75	9,7,5,5	4,2,2,0	92,86,83,81	4,3,3,2	89,85,84,82
10	83,83,81,80	87,86,85,80	95,86,84,84	87,87,86,85	85,82,78,76	90,86,84,84
11	86,84,80,80	89,85,80,80	88,88,85,83	88,83,83,82	91,83,80,80	81,80,79,79
12	82,80,78,75	92,90,85,80	97,86,84,84	95,91,86,85	92,84,83,75	91,83,82,78

APPENDIX IVb - continued

f)i. The effect of:-

f. Menaphthone 1 p.p.m. and 0.01 p.p.m.
g. Biotin 0.1 and 0.01 p.p.m. used
h. Casein hydrolysate - non vitamin free and vitamin free material used.
i. Additional phosphate: double quantity of Ammonium phosphate added to medium.

A. On the growth of B. globiforme in aerated liquid cultures containing 100 p.p.m. 2:4-D

Cells per ml.

Time in days	f.1 ppm.	f.0.01 ppm.	g.0.1 ppm.	g.0.01 ppm.	i. 2 grms/ litre
0	1.52×10^5	1.52×10^5	1.52×10^5	1.52×10^5	1.52×10^5
1	3.76×10^6	3.72×10^6	3.92×10^6	3.60×10^6	3.68×10^6
2	2.48×10^7	4.0×10^7	1.08×10^7	1.28×10^7	2.12×10^7
3	7.84×10^7	8.00×10^7	7.76×10^7	7.20×10^7	7.60×10^7
4	7.88×10^7	7.96×10^7	7.80×10^7	7.88×10^7	7.92×10^7
6	7.88×10^7	8.00×10^7	7.80×10^7	8.00×10^7	8.04×10^7
8	7.84×10^7	7.92×10^7	7.76×10^7	8.00×10^7	7.96×10^7
9	7.88×10^7	7.96×10^7	7.84×10^7	8.08×10^7	7.96×10^7
10	7.84×10^7	8.04×10^7	7.80×10^7	7.96×10^7	7.96×10^7
11	7.80×10^7	8.00×10^7	7.88×10^7	7.92×10^7	8.04×10^7
14	7.80×10^7	8.00×10^7	7.84×10^7	7.92×10^7	8.00×10^7

Time in days	h.0.01%	h. 0.01 ppm.	h.Vit. free 0.01%	h.Vit. free 0.01 ppm.	Control no addit:
0	1.52×10^5	1.52×10^5	1.52×10^5	1.52×10^5	1.52×10^5
1	4.80×10^6	3.96×10^6	4.72×10^6	3.84×10^6	3.80×10^6
2	8.48×10^7	2.76×10^7	9.60×10^7	2.48×10^7	2.04×10^7
3	Flakes	6.80×10^7	Flakes	6.24×10^7	7.96×10^7
4	formed in tube	7.96×10^7	formed in tube	7.84×10^7	7.92×10^7
6	Counting not poss:	8.16×10^7	Counting not poss:	8.08×10^7	8.04×10^7
9		8.24×10^7		8.12×10^7	7.96×10^7
10		8.0×10^7		8.04×10^7	7.96×10^7
11		7.96×10^7		7.92×10^7	8.08×10^7
14		7.88×10^7		7.92×10^7	8.00×10^7

B. On the time of detoxication of the culture fluids

Time-days	Length of cress roots in mms.				
	f. 1ppm.	f.0.01ppm.	g.0.1ppm.	g.0.01ppm.	i. 2gm/litre
0	3,3,2,2,	3,2,2,1	3,2,0,0	4,1,1,1	4,2,1,1
1	2,2,0,0	4,2,2,1	3,3,2,2	3,3,2,1	4,2,2,0
2	4,3,3,3	4,2,2,0	3,3,3,1	5,2,2,0	3,3,2,2
3	3,3,2,2	2,2,2,2	4,3,1,1	3,3,3,2	3,3,3,0
4	5,2,1,0	3,2,2,2	4,3,2,2	2,2,2,0	3,3,1,1
6	3,2,2,1	3,3,3,0	3,3,2,1	3,3,3,2	3,3,2,2
8	3,3,3,2	4,4,4,3	4,1,1,0	3,3,2,2	4,2,2,0
9	84,81,79,76	15,8,6,2	39,23,18,10	86,84,83,83	92,87,81,78
10	92,87,83,74	87,85,80,74	86,84,82,80	88,86,82,82	82,80,79,75
11	83,83,80,78	86,79,75,75	83,83,80,74	85,83,81,80	86,84,83,80
14	-	83,81,78,76	87,85,85,82	-	-

APPENDIX IVb. j.

The effect on the time of detoxication of 2:4-D, of adding sterile inert materials to aerated liquid cultures containing initially 100 p.p.m. 2:4-D

Length of cress roots in mms.

Time-days	Brick dust	Cotton wool	Glass wool	Soil	No additions	Control cress No 2:4-D
0	5,3,2,1	3,2,1,1	4,2,1,1	5,3,2,0	3,2,1,1	98,95,89,78
1	2,1,0,0	2,2,1,0	4,3,3,2	3,3,2,2	2,1,1,0	92,87,84,77
2	2,2,1,1	3,2,1,1	3,2,2,1	5,2,2,1	3,3,2,2	84,82,80,78
3	4,3,2,2	3,3,3,2	2,2,0,0	2,2,0,0	3,3,3,0	89,86,84,81
4	3,3,2,2	4,3,3,2	2,2,1,1	4,4,2,1	4,3,3,0	87,85,84,81
5	3,2,2,1	4,2,2,0	4,3,2,2	3,2,1,1	3,3,2,1	88,85,81,79
6	5,3,3,1	3,3,2,1	4,4,2,2	5,2,1,1	4,3,2,1	98,88,83,81
7	4,2,2,0	4,2,2,0	3,3,2,1	3,3,3,2	3,3,3,2	94,88,85,82
8	84,81,78,75	4,2,2,2	2,2,2,0	4,4,2,0	4,3,3,0	89,85,85,78
9	85,80,75,74	92,89,87,81	84,82,80,77	5,5,3,3	88,84,80,70	88,88,82,79
10	89,86,81,78	85,80,80,77	88,87,87,85	87,83,80,80	89,88,81,78	87,87,82,81
11	-	88,85,82,79	86,84,82,81	92,85,82,70	86,86,87,80	91,85,80,77
12	-	-	-	88,82,76,74	-	83,75,75,74

APPENDIX IVb - continued

5. The effect of CO_2 on the growth of B. globiforme in aerated liquid culture containing 100 p.p.m. 2:4-D and on the time of detoxication of the culture medium

Time-days	Normal air		CO_2 free air		Air enriched to 5% CO_2		Control cress no 2:4-D
	Cells/ml.	Lgth. cr's.mms.	Cells/ml.	Lgth. cr's.mms.	Cells/ml.	Lgth. cr's.mms.	Length mms.
0	6.12×10^5	5,3,2,1	6.12×10^5	3,3,3,2	6.12×10^5	4,3,3,0	86,82,79,76
1	7.36×10^6	4,2,2,0	7.40×10^6	5,3,3,2	6.32×10^6	5,2,0,0	87,83,81,80
2	2.48×10^7	3,3,2,1	2.52×10^7	2,2,2,0	3.36×10^7	3,3,3,3	94,90,88,86
4	6.76×10^7	4,3,3,2	6.68×10^7	3,3,2,1	6.56×10^7	3,2,2,0	89,85,83,80
6	6.52×10^7	2,2,0,0	6.72×10^7	4,4,2,2	6.72×10^7	3,2,2,1	82,81,78,76
8	6.64×10^7	3,2,2,1	6.72×10^7	3,3,2,2	6.68×10^7	3,2,2,2	85,81,80,78
9	6.68×10^7	92,89,86,83	6.68×10^7	4,1,1,0	6.72×10^7	68,65,61,58	89,87,82,81
10	6.64×10^7	95,86,84,82	6.68×10^7	3,3,2,1	6.72×10^7	82,78,77,69	84,81,79,78
12	6.64×10^7	88,86,81,78	6.68×10^7	3,3,3,2	6.72×10^7	84,80,80,77	88,84,80,77
14	-	-	-	4,2,2,0	-	92,90,86,83	85,84,83,81
16	-	-	-	4,2,2,2	-	-	88,82,80,76
20	-	-	-	3,3,2,0	-	-	86,83,81,79
24	-	-	-	3,3,2,2	-	-	91,86,84,78

APPENDIX V   Data relating to Figure V.

Each carbon substrate used in concentration of 100 p.p.m.

Substrate	Time in hrs.	Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
All cultures	0	Inoculating suspension		3.47×10^6	6.54
2:4dichloro-phenoxyacetic acid	(64	20,24,17,19,23,22,16,17,24,22	20.4	8.16×10^7	7.91
	(186	28,22,25,20,24,26,23,18,17,26	22.9	9.16×10^7	7.96
4:chlorophenoxy acetic acid	(64	23,25,18,20,22,17,16,17,25,19	20.2	8.08×10^7	7.91
	(186	20,23,16,18,20,23,23,18,19,20	20.0	8.0×10^7	7.90
3:chlorophenoxy acetic acid	(64	16,11,16,13,14,15,13,16,11,14	13.9	5.56×10^7	7.75
	(186	19,10,17,20,13,18,14,18,15,20	16.4	6.56×10^7	7.82
3:4dichloro-phenoxyacetic acid	(64	6,7,5,6,8,6,7,4,6,7	6.2	2.48×10^7	7.39
	(186	10,11,13,12,8,11,10,15,12,14	11.6	4.64×10^7	7.67
2:5dichloro phenoxyacetic acid	(64	2,1,3,1,2,1,4,5,4,3	2.6	1.04×10^7	7.02
	(186	14,6,5,8,7,13,16,4,7,8	8.8	3.52×10^7	7.55
2-chlorophen-oxyacetic acid	(64	8,9,6,4,8,7,8,6,9,7	7.2	2.88×10^7	7.46
	(186	3,4,10,5,7,6,5,6,6,5	5.7	2.28×10^7	7.36
phenoxyacetic acid	(64	3,2,2,4,2,2,0,0,2,0	1.7	6.8×10^7	7.83
	(186	1,1,4,3,2,1,1,2,0,1	1.6	6.4×10^7	7.81
2-4dimethyl phenoxyacetic acid	(64	26,27,29,14,22,23,24,22,19,23	22.9	9.16×10^7	7.96
	(186	9,8,16,14,16,15,25,18,15,19	15.5	6.20×10^7	7.79
o-chloro benzoic acid	(64	14,11,11,8,13,10,11,12,13,10	11.3	4.52×10^7	7.66
	(186	10,24,8,14,18,22,14,10,12,8	14.0	5.6×10^7	7.75
4-iodophen. acetic acid	(64	12,7,6,11,6,9,5,6,8,13	8.3	3.32×10^7	7.52
	(186	7,6,13,5,8,6,9,7,5,6	7.2	2.88×10^7	7.46
-4chlorophen. propionic acid	(64	7,6,8,9,7,10,9,8,8,7	7.9	3.16×10^7	7.50
	(186	9,4,7,6,10,7,10,8,7,6	7.4	2.96×10^7	7.47
2:4:5-trichloro phen.acetic acid	(64	4,3,6,4,2,3,5,5,4,4,	4.0	1.6×10^7	7.20
	(186	5,4,5,3,2,2,4,3,12	3.1	1.24×10^7	7.09
-2:4-dichloro phen.propionic acid	(64	1,1,2,2,4,2,3,1,2,2,	2.0	8.0×10^7	7.90
	(186	1,1,3,0,2,3,1,1,0,0	1.2	4.8×10^7	7.68
2:3:5-tri-iodo benzoic acid	(64	2,1,2,2,0,1,1,2,1,3	1.5	6.0×10^7	7.78
	(186	2,1,1,1,1,1,2,2,1,0	1.2	4.8×10^7	7.68

APPENDIX VI Data relating to Figure VI

Growth of B. globiforme on various concentrations of phenylacetic acid

Conc. of substrate	Time in hrs.	Cell counts	Av.	Cells /ml.	Log_{10}cells/ml.
All concentrations	0	Inoculated with suspension giving		1.64×10^5	5.21
10 p.p.m.	18	3,4,4,2,5,2,4,4,1,2	3.1	1.24×10^7	7.09
	48	5.7.4.3.3.7.4.6.3.5	4.7	1.88×10^7	7.27
	96	10,6,8,14,8,7,10,12,13,18	9.6	3.84×10^7	7.58
	136	9,9,8,12,10,11,10,10,7,11	9.7	3.88×10^7	7.59
	190	9,5,7,6,6,7,5,9,8,10	7.2	2.88×10^7	7.50
100 p.p.m.	18	2,4,1,3,3,2,6,3,5,4	3.3	1.32×10^7	7.12
	48	11,9,10,8,14,7,11,14,12,12	10.8	4.32×10^7	7.64
	96	37,31,40,39,41,36,44,45,49,36	39.8	1.592×10^8	8.20
	136	35,47,42,48,38,49,40,41,39,50	42.9	1.916×10^8	8.28
	190	40,36,41,30,38,39,42,36,41,42	38.5	1.54×10^8	8.19
1,000 p.p.m.	18	1,1,1,1,0,2,2,2,3,1	1.4	5.6×10^6	6.75
	48	10,14,13,17,14,16,15,13,18,19	14.9	5.96×10^7	7.78
		Diluted 1:10			
	96	14,13,12,12,15,14,15,17,10,15	13.7	5.48×10^8	8.74
	136	19,17,18,20,21,16,18,16,22,24	19.1	7.46×10^8	8.87
	190	27,19,19,17,15,16,15,18,14,18	17.8	7.12×10^8	8.85
Control	18	4,4,5,3,4,4,7,4,4,5	4.4	1.76×10^7	7.25
100 ppm.2:4-D	48	12,7,8,3,8,7,5,9,6,5	7.0	2.8×10^7	7.45
	96	11,10,15,12,16,17,10,13,12,15	13.1	5.24×10^7	7.72
	136	13,13,9,15,11,14,16,13,14,14	13.2	5.28×10^7	7.725
	190	14,12,13,15,14,14,16,13,20,18	14.9	5.26×10^7	7.72

APPENDIX VII Data relating to Figure VII

Growth of B. globiforme on various concentrations of sodium benzoate.

Conc. of substrate	Time in hrs.	Cell counts	Av.	Cells/ml.	Log_{10} Cells/ml.
All concentrations.	0	Inoculated with suspension giving		1.64×10^5	5.21
10 p.p.m.	18	1,3,1,2,4,1,1,1,0,3	1.7	6.8×10^6	6.83
	48	14,15,18,10,10,15,11,12,10,14	12.9	5.56×10^7	7.75
	96	22,29,38,33,32,36,30,34,34,38	32.6	1.204×10^8	8.08
	136	38,35,39,41,39,40,42,41,38,39	40.2	1.608×10^8	8.21
	190	34,40,42,32,39,38,43,41,40,42	39.1	1.564×10^8	8.19
100 p.p.m.	18	2,1,2,1,1,2,4,1,2,2,	1.8	7.2×10^6	6.86
	48	29,27, 25,22,23,31,24,27,25,27	26.0	1.04×10^8	8.02
	96	Diluted 1:10			
		8,6,7,5,3,5,3,4,5,7	5.3	2.12×10^8	8.33
	136	9,11,10,10,14,7,12,11,9,11	10.4	4.16×10^8	8.62
	190	5,5,8,6,5,7,7,	6.6	2.64×10^8	8.42
1,000ppm.	18	4,1,3,2,4,3,3,1,2,3	2.6	1.04×10^7	7.02
	48	18,17,14,16,17,20,18,23,17,25	18.5	7.40×10^7	7.87
	96	Diluted 1:10			
		19,17,20,13,18,16,17,20,18,20	17.8	7.12×10^8	8.85
	136	17,21,17,18,18,25,19,20,23,20	20.8	8.32×10^8	8.92
	190	11,12,12,13,16,9,11,14,14,8	13.0	5.2×10^8	8.72
10,000 p.p.m.	18	4,0,0,1,2,0,2,1,2,1	1.3	5.2×10^6	6.72
	48	4,4,3,5,3,1,4,2,4,3	3.3	1.32×10^7	7.12
	96	5,3,2,4,2,2,8,3,4,2	3.5	1.40×10^7	7.15
	136	3,5,6,7,4,8,4,6,3,4	5.0	2.0×10^7	7.30
	190	5,6,7,4,6,5,7,6,8,8	6.2	2.48×10^7	7.39
Control 100 p.p.m. 2:4-D	18	4,4,5,3,4,4,7,4,4,5	4.4	1.76×10^7	7.25
	48	12,7,8,3,8,7,5,9,6,5	7.0	2.8×10^7	7.45
	96	11,10,15,12,16,17,10,13,12,15	13.1	5.24×10^7	7.72
	136	13,13,9,15,11,14,16,13,14,14	13.2	5.28×10^7	7.725
	190	14,12,13,15,14,14,16,13,20,18	14.9	5.26×10^7	7.72

APPENDIX VIII Data relating to Figure VIII

The effect of glucose on the growth of, and detoxication of 2:4-D by, B.globiforme

Media contain mineral salts, 100 p.p.m. 2:4-D together with the stated concentrations of glucose.

1. Growth of bacteria.

Glucose conc.	Time in hrs.	Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
All cultures	0	Inoculating suspension giving		$3.92x10^5$	5.93
None - Control	24	2,2,0,2,3,1,2,4,4,5	2.5	$1.0\ x10^7$	7. 0
	48	6,4,4,6,10,9,10,12,7,11	7.9	$3.16x10^7$	7.5
Culture A	72	10,10,8,8,11,9,9,7,8,8	8.8	$3.52x10^7$	7.55
	96	5,6,6,9,11,8,11,12,9,11	8.8	$3.52x10^7$	7.55
	120	8,5,12,8,9,6,7,7,9,11	8.2	$3.28x10^7$	7.52
	168	8,9,8,12,11,9,6,10,6,9	8.8	$3.52x10^7$	7.55
	240	11,7,7,9,10,6,12,7,8,11	8.8	$3.52x10^7$	7.55
	288	9,7,9,10,8,7,11,9,10,8	8.8	$3.52x10^7$	7.55
0.001%	24	1,2,3,1,6,2,2,2,1,1	2.1	$8.40x10^6$	6.925
	48	9,15,9,17,16,15,13,16,14,17	14.1	$5.64x10^7$	7.75
Culture B	72	20,19,16,15,17,23,16,17,16,19	17.8	$7.08x10^7$	7.85
	96	15,17,19,20,17,23,15,16,17,19	17.8	$7.08x10^7$	7.85
	120	22,11,13,18,13,14,19,23,24,21	17.8	$7.08x10^7$	7.85
	168	19,18,16,16,17,21,18,18,22,17	17.8	$7.08x10^7$	7.85
	240	19,17,16,18,20,22,16,17,16,17	17.8	$7.08x10^7$	7.85
	288	20,15,26,17,25,22,18,13,15,18	18.9	$7.56x10^7$	7.86

continued -

APPENDIX VIII- i. Growth of bacteria - Continued

Glucose conc.	Time in Hrs.	Cell counts	Av.	Cells/ml.	Log$_{10}$Cells/ml.
0.01%	24	10,6,8,8,12,7,11,9,8,9	8.8	3.52×10^{7}	7.55
		Diluted 1:10			
	48	5,4,6,4,4,7,6,5,8,7	5.6	2.24×10^{8}	8.35
	72	7,4,6,9,7,6,9,6,5,7	6.6	2.64×10^{8}	8.42
Culture C	96	6,6,5,5,7,11,8,7,6,9	6.6	2.64×10^{8}	8.42
	120	4,4,3,8,5,10,7,8,6,8	6.6	2.64×10^{8}	8.42
	168	9,8,8,6,7,5,6,5,7,9	7.0	2.80×10^{8}	8.45
	240	7,6,8,9,6,4,8,5,9,8	7.0	2.80×10^{8}	8.45
	288	8,5,7,6,11,10,4,5,6,8	7.0	2.80×10^{8}	8.45
0.1%	24	18,12,14,18,11,16,15,11,19,13	14.7	5.88×10^{7}	7.77
	48	17,20,18,21,18,13,20,24,15,21	18.7	7.48×10^{8}	8.875
	72	Diluted 1:10			
		15,24,28,24,36,17,18,29,23,37	25.0	1.0×10^{9}	9.0
Culture D	96	19,27,28,18,25,22,27,28,22,22	23.8	9.52×10^{8}	8.98
	120	16,22,33,21,30,19,20,23,19,20	22.3	8.92×10^{8}	8.95
	168	21,20,19,24,19,17,19,15,26,19	19.8	7.92×10^{8}	8.90
	240	17,22,24,19,15,17,13,17,17,16	17.7	7.08×10^{8}	8.85
	288	14,17,12,19,12,20,16,17,17,21	16.5	6.60×10^{8}	8.82
1.0%	24	10,9,12,10,11,16,16,14,15,14	12.7	6.8×10^{7}	7.83
	48	Diluted 1:100			
		6,4,4,7,8,6,8,10,7,8	6.8	2.72×10^{9}	9.43
Culture E	72	4,4,4,6,7,5,5,3,3,5	4.6	1.84×10^{9}	9.26
	96	Bacteria solid masses in tubes. Impossible to count.			

APPENDIX VIII - ii. Phytotoxicity of cultures

Cress seedling root test. Samples diluted to give stated original conc. of 2:4-D.

Length of 4 longest roots in each tube measured in mms.

Initial conc.	Culture	Time in days 0	2	4	6	8	10	12
2:4-D	Control Cress Dist.water only	87,81,79,70	90,79,75,70	100,85,83,80	86,85,85,80	90,80,75,75,	87,78,70,70	87,85,81,72
		90,78,75,74	82,82,76,74	87,85,85,80	90,90,80,80	75,72,70,68	90,82,75,73	85,83,71,69
1.0	A	5, 4, 2, 2	4, 3, 2, 2	4, 1, 1, 1	2, 2, 1, 1	4, 4, 3, 3	2, 2, 1, 1	3, 2, 2, 2
0.1		20,17,15,13	21,14,11, 8	20,20,15,15	32,13,12,10	23,18,10, 9	25,25,20,20,	17,17,14,12
0.01		60,50,48,45	64,58,40,40	50,50,45,45	55,50,44,40	65,50,43,41	58,53,46,40	66,57,50,50
1.0	B	3, 3, 3, 3	4, 3, 3, 1	5, 5, 4, 4	1, 1, 1, 1	3, 3, 3, 2	85,82,75,75	79,77,76,70
0.1		25,17,15,15	20,15,12,10	20,20,17,10	19,17,17, 5	21,18,16,13	83,82,78,78	88,75,70,69
0.01		59,57,45,43	60,53,50,44	68,45,45,43	43,40,40,35	63,60,41,33	82,80,78,78	85,78,72,72
1.0	C	4, 3, 3, 2	2, 2, 2, 2	4, 2, 2, 1	95,90,83,75	86,82,76,78	87,86,85,80	100,75,75,72
0.1		13,12,12,10	21,18,10, 9	16,14,13, 9	100,88,83,82	90,80,70,69	80,78,75,71	80,80,77,75
0.01		60,56,50,40	64,59,53,42	61,54,45,39	80,75,73,71	80,76,65,65	85,80,80,73	84,81,76,73
1.0	D	2, 2, 2, 1	3, 2, 2, 1	5, 3, 1, 1	4, 2, 2, 2	3, 3, 2, 2,	85,81,80,79	80,76,76,75
0.1		15,12,12, 9	19,15,15,14	26,16,15,15	15,12,10,10	27,14,11,10	83,80,80,78	82,80,80,75
0.01		57,57,45,40	60,45,45,43	55,52,47,45	60,52,52,40	61,46,39,34	82,78,75,70	91,87,81,68
1.0	E	2, 2, 2, 1	6, 4, 3, 3	3, 2, 2, 1	4, 3, 2, 0	4, 3, 3, 3	88,88,87,70	90,90,80,75
0.1		19,13,12, 9	19,16,13,11	22,17,15,10	27,17,16,11	20,20,18,14	95,90,88,85	85,82,76,70
0.01		62,62,45,38	64,63,58,50	63,63,48,39	52,39,35,34	63,45,41,35	88,87,84,80	86,85,75,72

APPENDIX VIII - iii. Glucose reaction

Test used:

Test described in Methods of Quantitative Micro-analysis by Milton and Waters

and Journal of Biological Chemistry 1915, 20 61.

by Lewis and Benedict.

By this method it is possible to detect down to 40 g of glucose. 2 ml. saturated aqueous picric acid solution is added to the sample followed by 1 ml. of 20% sodium carbonate solution. The mixture is then heated in a boiling water bath for 30 mins. and then allowed to cool.

Reducing sugar is oxidised when heated with picric acid and sodium carbonate giving an orange colouration due to the formation of picramic acid.

Polyphenols, purines, aldehydes and other similar compounds also give coloured solutions.

Initial conc. of glucose.	Time in days					
0	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
0.001%	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
0.01%	Orange	Orange	Yellow	Yellow	--	--
0.1%	Orange	Orange	Orange	Yellow	Yellow	--
1.0%	Orange	Orange	Orange	Tinge of Orange	Yellow	Yellow

APPENDIX IX Data relating to Figure IX

A. Bacterial growth in cultures containing 100 p.p.m. 2:4-D

Time in days.	Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
0	Inoculating suspension gives	.	1.58×10^6	6.20
1	4,4,3,5,2,5,6,7,4,4	4.4	1.76×10^7	7.25
2	9,6,7,11,9,7,8,7,10,5	7.9	3.16×10^7	7.50
3	8,7,9,8,10,8,8,8,6,9	8.1	3.24×10^7	7.51
6	10,7,10,6,9,11,7,9,8,12	8.9	3.56×10^7	7.55
7	6,9,11,8,7,8,10,8,9,12	8.8	3.52×10^7	7.55
8	10,8,12,9,10,11,8,7,10,10	9.5	3.80×10^7	7.58
10	9,7,6,11,5,7,12,10,15,11	9.3	3.72×10^7	7.57
11	10,9,6,11,5,5,11,12,9,10	8.8	3.52×10^7	7.55
14	9,8,12,10,5,12,11,14,9,9	9.9	3.96×10^7	7.6
17	9,8,12,9,11,10,10,8,6,7	9.0	3.60×10^7	7.55

B. Phytotoxicity of culture A. Cress seddling root test.

Time in days.	Length of roots in mms. 1p.p.m. 2:4-D	Av.	0.1p.p.m.	Av.	0.01ppm.	Av.	Control	Av.
0	2,2,1,1,	1.5	18,16,14,11	14.8	59,52,49,48	52	80,80,78,78	79.0
1	3,2,1,1	1.7	17,17,15,12	15.2	61,57,48,47	53.2	82,80,79,79	80.0
2	2,2,2,0	1.5	19,16,15,8	14,5	57,53,48,48	51,5	80.78,78,72	77.0
5	3,3,1,0	1.7	20,15,13,10	14,5	58,51,49,46	51.0	80,78,75,72	76.3
7	2,1,1,1	1.3	16,14,11,10	12.8	60,51,48,48	51.8	85,82,81,79	81.8
10	4,2,1,0	1.7	16,16,15,14	15.2	63,60,57,51	57.7	101,81,76,74	83.0
11	88,88,87,85	87.0	97,90,87,84	89.5	87,85,85,73	82.5	80,79,76,75	77.5

C. Bacterial growth in cultures in which the 2:4-D content is augmented twice before detoxication: at 5 and 10 days.

Time in days	Cell counts	Av.	Cells/ml.	Log_{10} Cells/ml.
0	Inoculating suspension gives		1.58×10^{6}	6.20
1	5,6,3,4,4,3,5,6,4,4	4.4	1.76×10^{7}	7.25
2	7,10,8,11,9,11,12,10,11,10	9.9	3.96×10^{7}	7.6
3	10,12,11,12,10,6,8,11,7,13	10.0	4.00×10^{7}	7.6
5	12,10,9,10,8,13,9,12,6,10	9.9	3.96×10^{7}	7.6
6	31,33,28,34,35,31,31,32,27,33	31.5	1.26×10^{8}	8.1
7	Diluted 1:10			
	2,5,4,3,1,2,2,4,5,4	3.2	1.28×10^{8}	8.1
8	4,4,3,2,4,3,3,4,2,3	3.2	1.28×10^{8}	8.1
10	2,3,4,4,2,2,3,2,5,5	3.2	1.28×10^{8}	8.1
11	11,12,9,13,10,12,12,13,10,9	11.2	4.48×10^{8}	8.65
14	6,9,12,9,8,11,13,12,13,11	10.4	4.16×10^{8}	8.62
17	10,12,14,13,12,15,14,11,12,13	12.6	5.04×10^{8}	8.70

APPENDIX IX continued

D. Phytotoxicity of culture B. Cress seedling root test.

Length of root in mms.

Time in days	1 p.p.m	Av:	0·1 p.p.m.	Av:	0·01 p.p.m.	Av.	Control Cress	Av:
0	3,2,1,1	1.75	18,15,11,10	13.5	63,58,50,41	53	80,77,75,70	75.5
1	2,2,1,1	1.5	16,16,12,9	12.3	59,51,48,36	49	80,75,70,65	72.5
2	3,2,2,0	1.75	21,17,13,9	15.0	65,57,48,42	53	83,78,76,72	77.3
5	2,2,2,0	1.5	19,19,12,10	15.0	61,52,45,42	50	78,76,72,68	73.5
5	1,1,0,0	0.5	12,10,9,6	9.3	53,47,40,33	43.2	88,83,74,65	77.5
7	2,1,1,0	1.0	13,11,7,6	9.2	56,51,43,29	44.7	85,78,78,72	78.2
10	1,1,1,0	0.75	15,11,8,7	10.3	52,50,45,32	44.8	82,80,80,75	79.2
10	2,1,0,0	0.75	14,10,6,4	8.5	62,53,39,21	43.7	90,90,80,75	83.8
11	85,82,81,80	82	90,87,80,75	83	85,82,80,75	80.5	80,77,77,74	77.0

APPENDIX X Data relating to Figure X.

Bacterial growth in cultures in which an additional charge of 100 p.p.m. 2:4-D added after detoxication of the first charge.

Time in days.	Cell counts	Av:	Cells/ml	Log_{10} cells/ml.
0	Inoculating suspension gives		$7.78 x 10^5$	5.85
1	1,2,1,0,1,0,2,1,0,0	0.8	$3.2 x 10^6$	6.5
2	6,7,6,4,5,6,5,7,4	5.0	$2.0 x 10^7$	7.3
3	4,5,7,6,5,6,4,5,7,5	5.6	$2.24 x 10^7$	7.35
4	5,6,5,5,6,7,5,6,4,6	5.5	$2.20 x 10^7$	7.34
8	6,7,4,5,5,8,5,8,7,6	6.0	$2.40 x 10^7$	7.38
11	7,4,8,6,5,4,5,4,7,6	.5.6	$2.24 x 10^7$	7.35
14	15,6,9,9,8,10,8,12,7,11	9.5	$3.80 x 10^7$	7.58
15	12,16,18,15,11,12,12,14,8,13	13.1	$5.24 x 10^7$	7.72
18	12,10,11,13,11,10,12,7,9,12	10.7	$4.28 x 10^7$	7.63
20	10,12,15,10,14,11,13,10,12	12.1	$5.04 x 10^7$	7.7
22	18,10,16,13,17,11,21,15,20,19	16.0	$6.40 x 10^7$	7.8
27	13,16,14,16,12,13,12,17,17,13	15.7	$6.28 x 10^7$	7.8

APPENDIX X - continued

Phytotoxicity of cultures. Cress seedling root test.

Length of roots in mms.

Time in days.	1.0 p.p.m. 2:4-D	Av.	0.1 p.p.m.	Av.	0.01p.p.m.	Av.	Control	Av.
0	3,2,1,0	1.75	21,19,14,11	16.2	68.54,52,48	55.5	87,86,82,80	83.8
1	2,2,2,1	1.75	22,18,15,15	17.5	65,61,55,49	57.5	89,85,83,80	84.2
2	3,2,2,2	2.3	31,26,13,10	20	76,65,61,54	64	91,89,86,81	86.8
3	6,4,4,3	4.2	34,30,28,22	28.5	83,78,73,60	73.5	92,89,87,78	86.5
4	2,2,2,1	1.8	21,20,19,15	18.8	68,57,54,51	57.5	83,80,76,71	77.5
8	3,2,1,1	1.8	23,19,15,11	17.0	70,58,47,45	55.0	81,77,75,73	76.5
9	12,10,8,7	9.2	46,42,37,27	40.5	86,80,77,72	78.8	86,83,79,75	80.8
10	86,83,81,78	82	87,83,80,78	42	100,94,90,82	91.5	85,84,82,79	82.5
11	89,84,80,76	82.2	89,86,84,81	85	88,84,81,78	87.8	88,86,83,81	84.5
13	2,2,1,1,	1.5	19,17,14,10	15	71,56,43,42	53	85,83,80,78	79
15	3,2,2,1	2.0	22,21,16,14	18.2	70,69,49,43	57.8	86,82,79,74	80.2
18	3,1,1,1	1.5	17,16,13,13	14.2	67,53,43,41	51	81,77,75,70	75.8
19	4,4,4,3	3.8	33,31,24,16	26	68,56,44,44	53.0	83,79,76,76	78.5
20	86,81,78,76	80.2	86,83,81,79	82.2	91,88,86,83	87	81,77,75,70	75.8
22	91,86,81,78	84	89,83,80,71	80.8	88,86,80,75	82.2	81,78,76,71	76.5
27	81,79,76,72	76.5	79,77,73,70	74.8	85,81,80,78	81	89,86,82,80	83.8

APPENDIX Xa.

Elution of 2:4-D from chromatograms

Methods attempted

1. Sections of paper soaked for 12 hours in:

    a. Developing solvent

    b. Ether

    c. Acidified ether

    d. Water

    e. Concentrated ammonia solution

    f. Chloroform

    g. Acidified chloroform

    h. Acid alcohol

    i. Carbon tetrachloride

    j. Acidified carbon tetrachloride.

2. Paper pulped in boiling water filtered through glass wool to remove the fragments and the filtrate acidified and extracted with ether.

3. 2 above repeated using hot caustic soda in place of boiling water

4. 2 above repeated using hot concentrated HCl in place of boiling water. This was the only process whereby sufficient 2:4-D was eluted to give a positive colour.

APPENDIX XI - Data relating to Figure XI.

Phytotoxicity recorded on chromatograms. Measured as inhibition of growth of pea root sections. Samples taken during detoxication in culture solutions containing initially 100 p.p.m. 2:4-D. Chromatograms divided into 10 equal sections between the origin and solvent front lines. Control section taken from above the origin.

0 hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	20.4	20.6	19.4	18.2	18.4	19.8	18.0	21.6	19.2	22.2	20.4
Final wt. mgs.	40.7	40.1	40.0	38.0	38.7	40.6	19.9	44.2	38.8	44.4	40.8
% of cont. increase	100	95	102.8	109	108.6	105	12	104.1	102	100	100

48 hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	19.8	24.4	22.0	23.4	22.2	24.2	23	23	25	23.6	22.2
Final wt. mgs.	34.8	43.8	42.2	43.4	43.6	39.0	24.6	35.4	44.4	40.0.	42.8
% of cont. increase	81.5	86.4	100	97	98.5	65.8	9	57	83.5	93	100

APPENDIX XI - continued

96 hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	18.8	20.0	20.4	19.0	19.8	20.8	21.6	20.4	19.8	19.4	20.4
Final wt. mgs.	32.4	36.0	40.0	36.2	35.4	33.6	25.2	26.8	37.4	33.4	37.6
% of cont. increase	86.1	95	114.4	108.0	93.5	73.2	19.8	37.4	106.0	81.0	100

120hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	19.4	17.4	18.2	21,8	19.0	22.0	18.2	20.8	20.0	21.4	18.4
Final wt. mgs.	31.2	34.6	31.8	38.4	33.8	38.0	20.6	36.8	36.0	36.8	35.8
% of cont. increase	65	105	79.5	81.0	83.0	67.5	14.05	41.0	63.8	76.5	100

APPENDIX XI - continued

142 hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	19.6	18.6	18.6	17.8	17.6	19.0	18.4	18.0	17.8	20.4	17.8
Final wt. mgs.	32.0	36.4	40.4	38.2	34.4	41.2	22.0	23.8	33.6	27.8	25.4
% of cont. increase	62.9	96.8	118.2	110.2	96.5	117.8	18.9	21.1	89.8	36.4	100

180 hrs.

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	21.4	18.8	18.4	18.0	16.2	21.0	19.4	16.8	17.8	17.8	18.6
Final wt. mgs.	39.0	35.4	35.6	37.4	31.6	41.4	27.6	20.2	32.8	30.4	34.4
% of cont. increase	85.8	103.8	110.0	127.0	111.7	114.0	49.8	51.8	101.7	79.7	100

APPENDIX XIV. Data relating to Figure XIV

Growth of 10 2mm. pea root sections on chromatogram after development of unknown substance from 40 day culture of B. globiforme grown on 1,000 p.p.m. 2:4-D

Section	1	2	3	4	5	6	7	8	9	10	Control
Init.wt. mgs.	22.8	24.6	23.0	22.8	24.4	23.4	22.2	21.2	24.6	22.6	20.8
Final wt. mgs.	34.2	45.4	45.6	40.0	44.0	39.2	27.2	26.8	38.8	38.6	42.8
% of increase	45.4	80	89.2	68.5	73	61.4	20.4	24.0	52.4	80.2	100

APPENDIX XIVa. Data relating to results described on page 35.

Growth measured by direct counting under a haemacytometer. Phytotoxicity of the solutions by growth of cress seedling roots in samples of the culture fluid sterilised and diluted to give an initial concentration of 1 p.p.m. 2:4-D.

Organism 1.

Time in days	Cell counts	Cells/ml.	Length of cress mms.
0	Inoculating suspension gives 4.3×10^6 cells/ml.	4.3×10^6	2,2,1,1
1	1,2,1,0,1,1,0,3,1,2	4.8×10^6	3,2,2,1
2	3,2,1,1,0,2,3,0,1,1	5.6×10^6	4,1,1,0
4	2,1,0,2,2,1,2,1,1,0	4.8×10^6	3,3,2,1
6	4,0,0,2,2,0,1,0,3,1	5.2×10^6	2,2,0,0
10	3,1,1,2,1,1,0,1,1,4	6.0×10^6	4,3,2,1
14	1,2,2,1,0,0,1,2,2,2	5.2×10^6	3,3,2,0
19	1,0,2,2,2,0,2,1,3,2	6.0×10^6	2,2,2,2
24	1,0,0,1,2,0,3,2,3,1	5.2×10^6	5,1,1,1
29	3,0,2,2,0,1,1,0,2,3	5.6×10^6	3,2,2,2
33	2,2,0,0,1,2,1,2,2,1	5.2×10^6	3,3,3,1
40	2,2,0,1,2,1,1,2,2,1	5.6×10^6	4,2,1,1

APPENDIX XIVa - continued

Organism 2.

Time in days	Cell counts	Cells/ ml.	Length of cress mms.
0	Inoculating suspension gives	9.6×10^5	3,3,1,1
1	6,4,5,3,2,4,4,2,4,5	1.56×10^7	3,3,2,2
2	10,15,14,12,12,9,10,10,10,12,12	$4,52 \times 10^7$	4,2,2,2
3	14,14,13,13,12,15,14,13,11,14	$5,32 \times 10^7$	4,4,3,3
4	15,12,15,14,12,13,14,12,15,14	$5,44 \times 10^7$	3,3,2,1
6	14,15,14,11,12,14,14,12,13,14	$5,32 \times 10^7$	3,3,3,1
8	15,14,12,12,13,11,13,15,14,14	$5,32 \times 10^7$	4,3,3,2
9	16,17,9,11,12,14,14,15,12,15	$5,40 \times 10^7$	86,83,82,80
10	14,13,16,10,12,15,13,17,11,13	$5,36 \times 10^7$,	85,83,79,78
12	14,16,14,14,11,12,11,15,13,15	$5,36 \times 10^7$	86,84,81,77

Organism 3

Time in days	Cell counts	Cells/ ml.	Length of cress mms.
0	Inoculating suspension gives	3.312×10^5	4,3,2,2
1	2,4,2,3,3,3,2,2,1,2	9.6×10^6	3,3,3,2,
2	2,5,2,4,3,3,4,5,7,4	1.56×10^7	3,2,2,2
3	7,6,8,6,7,6,10,5,9,6	2.80×10^7	2,2,0,0
4	11,14,11,9,10,13,9,8,8,10	4.12×10^7	2,1,0,0
6	13,11,10,12,9,10,14,11,10,14	4.56×10^7	4,4,3,3
7	14,13,13,15,17,14,16,15,17,13	$5,88 \times 10^7$	4,3,3,2
9	16,13,14,18,19,16,18,15,16,18	6.52×10^7	3,3,2,1
11	Counting impossible		3,1,1,0
14	Bacteria forming flakes		4,2,1,1
19	in the tubes		2,2,1,1
24			2,2,2,1
30			4,2,2,1
36			3,2,1,0
42			4,2,1,0

APPENDIX XIVa- continued

Phytotoxicity (measured by growth of cress seedling roots) of aerated liquid culture solutions containing 100 p.p.m. 2:4-D inoculated with Organism 2 which had not previously been grown in the presence of 2:4-D i) in pure culture ii) in a mixture with organism 3.

Samples diluted to give an initial concentration of 1 p.p.m.2:4-D

Time in days	Length of cress roots in mms.		Control cress No 2:4-D
	i) Organism 2 only	ii) Organisms 2 & 3	
0	3,3,2,2,	5,3,2,2	88,88,85,83
1	3,3,3,1	4,3,1,0	88,84,84,82
2	5,4,3,3	4,4,2,1	86,83,80,77
3	4,4,3,3	3,3,2,2	83,82,82,74
4	3,3,2,0	4,3,3,2	93,85,82,81
6	4,4,3,2	3,3,1,1	92,83,83,77
8	3,3,2,0	3,3,2,1	87,87,85,81
10	4,3,1,1	3,2,0,0	83,82,81,80
12	3,3,2,1	3,2,2,2	88,77,77,74
14	3,3,3,3	4,3,1,0	85,82,81,80
16	2,2,1,1	83,80,78,78	90,85,80,78
18	3,3,2,2	85,84,81,79	83,82,82,81
20	4,3,1,0	87,84,80,77	86,83,82,79
22	4,2,2,2	-	85,84,80,78
24	3,3,2,2	-	86,82,80,80
26	3,2,2,1	-	83,80,80,79
28	83,80,79,78	-	81,80,80,80
30	83,82,80,79	-	91,88,82,82
32	85,84,82,80	-	80,78,78,77

APPENDIX XV Data relating to Figures XVa and XVb.

The effect of removal of samples of perfusate from the perfuser on the duration of the lag phase before detoxication in the removed perfusate.

Sample = Time, in days, of removal of the aliquot from the perfuser. Sample 0 being taken at the time of addition of 2:4-D to the perfuser.

Time of assay measured in days from the addition of 2:4-D to the perfuser.

Samples assayed at 1 p.p.m. only using inhibition of growth in length of 2 mm. pea root sections growing in petri dishes containing 10 mls. of 0.5% sucrose solution, together with the berbicide sample. The sections are grown in the dark at 25°C for 14 hours and aerated by rocking. Initial length of 10 sections is 20 mms. The cutting blades are set to cut 2 mm. sections starting 1.5 mms. back from the root tip.

APPENDIX XV - continued

XVa. Soil samples from Bedford College Botany Garden

TIME	Sample Perfuser	0	1	2	3	6	7	8	10	Control
0	23.0	26.6	-	-	-	-	-	-	-	41.20
1	22.5	25.36	23.6	-	-	-	-	-	-	40.50
2	24.86	24.43	23.91	24.4	-	-	-	-	-	39.52
3	24.2	25.5	22.49	23.7	24.53	-	-	-	-	40.06
6	22.48	26.2	27.0	26.40	25.36	26.0	-	-	-	42.78
7	24.88	25.0	26.40	24.82	25.71	24.82	23.5	-	-	41.9
8	25.40	25.2	25.50	25.22	26.30	23.00	22.26	23.64	-	39.80
9	23.7	25.41	24.90	25.45	26.10	24.20	24.55	24.55	-	39.75
10	25.61	39.87	42.32	43.00	24.00	24.41	23.00	25.12	40.51	40.80
11	40.15	41.66	42.60	41.83	24.17	25.20	24.71	23.63	40.00	42.10
12	40.28	-	-	-	25.8	25.25	22.85	25.18	-	39.20
14	-	-	-	-	42.40	25.60	24.75	23.6	-	39.31
17	-	-	-	-	39.41	25.30	24.82	24.72	-	42.42
20	-	-	-	-	-	23.85	25.62	25.15	-	41.56

APPENDIX XV - continued

XVb. Soil samples from King's College Botany Department

Time	Sample Perfuser	0	1	2	3	4	5	6	8	10	Control
0	26.33	25.61	-	-	-	-	-	-	-	-	44.0
1	23.96	26.40	24.80	-	-	-	-	-	-	-	44.76
2	26.24	24.67	25.72	24.50	-	-	-	-	-	-	45.15
3	25.20	25.25	24.12	25.9	24.63	-	-	-	-	-	43.52
4	22.31	24.68	25.49	24.36	26.21	23.7	-	-	-	-	44.30
5	23.75	24.93	24.12	27.67	26.75	25.83	26.60	-	-	-	44.34
6	24.06	25.35	26.34	25.5	24.29	24.25	24.22	25.7	-	-	44.32
8	26.22	25.95	23.28	24.51	23.89	24.43	23.63	25.91	23.85	-	43.86
10	44.70	43.8	24.27	26.20	41.53	23.48	26.17	25.85	24.30	42.12	42.24
12	44.35	42.60	42.69	43.59	44.68	26.0	25.08	24.0	24.95	44.63	44.26
14	-	-	43.71	45.62	-	24.42	24.81	25.24	24.34	-	44.17
16	-	-	-	-	-	24.22	23.59	24.0	44.12	-	45.95
18	-	-	-	-	-	25.75	22.0	23.12	43.71	-	43.58
21	-	-	-	-	-	24.13	24.52	24.45	-	-	44.99
23	-	-	-	-	-	42.65	25.31	-	-	-	45.78
26	-	-	-	-	-	39.78	24.39	-	-	-	43.10
30	-	-	-	-	-	-	25.62	-	-	-	43.96

APPENDIX XVb

Time in days	0	1	2	3	4	5	6	7	8	Control Cress No 2:4-D
	Time of removal of perfusate in days									
	Length of cress roots in mms.									
0	2,2,1,1	-	-	-	-	-	-	-	-	85,83,82,80
2	3,3,1,0	2,2,2,1	2,2,0,0				-	-	-	86,81,79,77
4	2,2,1,1	4,2,1,1	5,3,2,2	2,2,1,1	3,2,2,2	-	-	-	-	77,77,76,75
6	4,2,2,0	5,21,	3,3,3,3	3,2,2,0	4,2,2,1	3,3,2,2	4,2,2,0	-	-	83,82,79,78
8	3,2,2,1	2,2,0,0	4,3,2,2	14,10,2,2	1,1,0,0	3,3,2,2	4,3,3,2	11,5,3,2	7,3,3,1	87,86,81,78
10	81,78,62,58	59,48,42,33,	17,15,10,9	86,82,79,77	36,28,15,11	72,61,58,52	48,36,19,16	74,63,59,50	68,52,49,45	91,84,83,82
12	83,83,79,78	82,82,80,77	86,82,79,69	81,78,78,74	87,82,80,79	83,81,76,72	80,76,62,13	83,80,80,74	87,86,86,83	93,89,86,86
14	-	-	-	-	83,82,82,76	[illegible]	88,84,80,76			85,84,82,73

Time in days	9	10	11	12	13	14	15	16	17	Control Cress No 2:4-D
10	43,38,25,15	15,10,7,3	-	-	-	-	-	-	-	-
12	88,84,83,80	81,76,63,62	63,60,58,46	36,32,24,19	-	-	-	-	-	-
14	85,85,81,76	83,81,78,74	83,80,76,75	86,84,80,77	3,3,2,1	2,2,1,1	-	-	-	-
16	-	-	-	-	21,19,15,12	3,3,2,2	2,2,1,0	5,2,1,1	-	
18	-	-	-	-	76,72,68,40	13,10,10,4	5,3,2,2	3,3,2,2	4,4,2,2	86,84,81,80
20	-	-	-	-	86,85,83,79	82,80,76,73	4,3,3,1	13,10,7,3	5,3,3,1	88,88,76,74
25	-	-	-	-	-	-	18,14,9,5	21,20,15,13	2,2,2,1	87,85,81,80
30	-	-	-	-	-	-	82,80,76,74	40,32,10,9	3,3,3,1	92,90,86,84
38	-	-	-	-	-	-		79,73,68,59	2,2,2,0	88,85,81,78
47	-	-	-	-	-	-		83,81,77,73	4,3,1,1	83,81,80,77
										83,82,77,75

APPENDIX XVI Data relating to Figure XVI

Growth of a mixture of organisms in soil perfusate containing 100 p.p.m. 2:4-D as an added carbon substrate

1. Cultures aerated with normal air

Time in hrs.	Cell counts	Av.	Cells/ml.	Log_{10} cells/ml.
0	22,21,23,24,20,22,20,18,25,20	21.5	8.60×10^7	7.94
	Diluted 1:10			
25	12,11,19,14,11,16,13,14,17,16	13.3	5.34×10^8	8.73
50	12,11,14,15,11,14,10,15,11,12	12.5	5.00×10^8	8.70
75	12,13,12,19,12,14,10,12,15,13	13.2	5.28×10^8	8.72
100	10,17,14,11,10,12,16,15,16,14	13.5	5.40×10^8	8.73
126	Second application of 2:4-D 100 p.p.m.			
148	12,9,11,13,10,11,14,16,19,9	12.4	4.96×10^8	8.70
176	15,14,15,16,15,15,17,10,14,15	14.6	5.84×10^8	8.76

2. Cultures aerated with carbondioxide free air

Time in hrs.	Cell counts	Av.	Cells /ml.	Log_{10} Cells/ml.
0	22,21,23,24,20,22,20,18,25,20	21.5	8.60×10^7	7.94
	Diluted 1:10			
25	13,10,12,10,11,9,10,10,9,12	10.6	4.24×10^8	8.63
50	13,11,16,12,11,15,12,10,15,14	12.9	5.16×10^8	8.71
75	14,16,14,13,15,10,9,13,12,13	12.9	5.16×10^8	8.71
100	12,15,19,11,9,13,12,13,11,13	12.8	5.12×10^8	8.70
148	13,10,10,11,11,14,12,15,16,12	12.4	4.96×10^8	8.70
176	13,13,12,15,19,13,14,12,20,22	15.3	6.12×10^8	8.78

APPENDIX XVII Data relating to Figure XVII

Growth of pea root sections on chromatograms. 2 mm. sections grown for 24 hrs. on portion of chromatogram moistened with 0.75ml. of 0.5% sucrose solution. Length of 10 pea root sections measured in mms. Chromatograms divided into 10 equal portions.

Section 1 = Rf 0-0.1 Section 2 = Rf 0.1-0.2 etc.

Time in hrs.	Section 1	2	3	4	5	6	7	8	9	10	Control
	Final length of pea sections in mms.										
0	43.72	37.9	43.48	34.21	33.19	40.66	26.96	33.0	34.88	42.3	41.42
26	38.17	38.12	41.68	37.10	36.00	39.70	24.00	36.88	41.16	38.24	37.79
50	35.84	40.3	39.61	36.72	38.95	36.29	24.50	31.2	35.22	36.20	36.92
74	38.28	39.68	41.29	40.12	42.42	43.7	25.64	33.34	41.94	37.14	40.66
100	36.68	36.45	39.24	38.92	31.02	34.37	25.02	38.20	37.21	37.33	40.10
126	38.62	40.1	39.03	42.80	37.30	35.10	25.12	35.70	37.22	38.42	42.25
148	39.78	36.98	40.00	39.61	42.96	42.6	27.48	38.42	41.79	41.10	43.82
172	43.67	44.72	44.00	36.92	42.67	40.66	44.4	42.67	46.54	44.00	42.00

APPENDIX XVIII Data relating to Figure XVIII

As XVII but cultures aerated in CO_2 free air

Time	Section 1	2	3	4	5	6	7	8	9	10	Control
in hrs.	Final length of pea sections in mms.										
0	43.91	39.0	42.45	35.39	33.40	40.12	25.4	33.41	39.42	40.80	41.63
26	35.28	41.25	41.42	40.45	44.67	41.7	23.96	41.30	41.62	38.98	41.82
50	39.44	39.62	42.04	37.32	42.00	34.2	24.81	36.7	39.64	41.46	38.50
74	40.22	43.3	40.00	40.78	42.44	39.10	25.42	43.61	47.20	44.1	42.71
100	36.80	40.00	36.82	36.89	41.97	37.28	24.96	34.32	36.20	36.70	40.79
126	39.70	40.84	41.26	38.67	38.00	37.30	24.61	32.42	38.92	40.36	41.48
148	36.0	41.36	39.15	38.00	31.72	31.42	26.70	40.00	44.01	36.69	42.24
172	43.6	42.32	42.59	45.06	43.38	40.62	28.16	33.18	41.98	36.78	43.00
195	40.65	47.94	38.68	49.00	46.72	41.85	39.74	47.46	45.48	44.92	46.00

APPENDIX XIXa.

Composition of media used in the tests applied in the characterisation of 2:4-D decomposing organism isolated from soil.

Nutrient broth

Beef extract	1 grm
Yeast extract	2 grms.
Bacteriological peptone	5 grms.
Sodium chloride	5 grms.
Glass distilled water	1000 mls.

Nutrient agar

As "Nutrient broth" plus 2% agar

Inorganic broth

Ammonium phosphate	1 grm.
Potassium chloride	0.2 grm.
Magnesium sulphate	0.2 grm.
Calcium carbonate	1 grm.
Glass distilled water	1000 grms.

Inorganic agar

As "Inorganic broth" plus 2% agar

Nutrient gelatin

Bacteriological nutrient gelatin supplied by "Difco" containing:-

Bacto-beef extract	3 grms.
Bacto-peptone	5 grms.
Bacto-gelatin	120 grms.
Glass distilled water	1 litre

Starch broth

Ammonium phosphate	1 grm..
Potassium chloride	0.2 grms.
Magnesium sulphate	0.2 grm.
Soluble starch	10 grms.
Glass distilled water	1000 mls.

Carbohydrate fermentation media

Bacteriological peptone	10 grms.
Carbohydrate under test	10 grms.
Glass distilled water	1000 mls.
pH 7	
Bromocresol purple	1%

Durham tubes inserted into each culture tube

Nitrate reduction medium

Bacteriological peptone	5 grms.
Potassium chloride	0.2 grms.
Magnesium sulphate	0.2 grms.
Potassium nitrate	1.0 grms.
Glass distilled water	1000 mls.

+ Durham tubes

APPENDIX XIXa - continued

Amino acid medium

Casein hydrolysate	10 grms.
Magnesium sulphate	0.2 grms
Potassium chloride	0.2 grms
Ammonium phosphate	1.0 grms.
Glass distilled water	1000 mls.

Dorset's Egg Medium - as described in Mackie and McCartney "Handbook of Practical Bacteriology" 8th Edition.

2 Eggs

12.5 mls. glass distilled water

Solidified at 75°C

Sterilised at 90°C

Litmus milk - as described in Mackie and McCartney.

Fresh milk steamed and cream removed

Litmus - neutral solution

Phenol

Ammonium phosphate	1 grm.
Potassium chloride	0.2 grm.
Magnesium sulphate	0.2 grm.
Phenol	0.1 grm.
Glass distilled water	1 litre

Acid fast stain

Method of Ziehl-Neelsen described in Mackie and McCartney.

Gram stain

Both Jensen's Modification and Kopeloff and Beerman's Modification and the Gram stain were used. These are also described in Mackie and McCartney.

APPENDIX XIXb Data relating to Figure XIX

The effect of initial concentration of 2:4-D on its detoxication

All cultures inoculated with equal inocula from the same stock culture. Time for complete detoxication assayed i.e. samples diluted to give concentration of 1 pp.m. 2:4-D only. Inhibition of growth in length of 2 mm. pea root sections used as assay of phytotoxicity of samples.

Time in hrs.	Con- trol	Initial conc. 2:4-D in p.p.m. 10	50	100	200	400	600	800	1,000
0	44.78	25.46	26.1	26.28	24.58	25.15	24.84	26.43	26.00
34	43.75	24.99	24.74	24.92	25.92	26.39	25.5	25.74	25.42
70	43.18	41.26	42.79	24.95	25.31	26.12	25.33	26.16	25.95
160	44.56	44.30	42.57	42.45	23.28	25.90	24.60	26.18	24.99
185	44.81	-	-	44.26	25.32	24.80	25.04	24.82	25.15
326	44.58	-	-	-	43.76	24.97	25.86	25.27	25.34
360	42.64	-	-	-	43.85	26.00	26.27	25.14	24.27
480	44.39	-	-	-	-	24.93	25.13	25.26	25.19

APPENDIX XX Data relating to Figure XX

The effect of soil extract and agar dialysate on the detoxication of 2:4-D

Phytotoxicity assayed by inhibition of growth of 10 2mm. pea root sections in 10 mls. of 0.5% sucrose together with sample. Grown in petri dishes in the dark at 26°C for 14 hours. Aerated by rocking. Sample diluted to give a concentration in the sucrose equal to an initial concentration of 1 p.p.m. 2:4-D.

Culture containing initially 1,000 p.p.m. 2:4-D

Time in days	Final lgth.mms.	Control lgth.mms.	Time in days	Final lgth.mms.	Control lgth.mms.
0	25.88	42.82	49	(Soil extract, Agar dialysate added to samples.)	
6	26.62	44.50	49	24.81	42.27
9	23.34	43.95	56	26.13	44.78
11	22.39	42.85	58	23.82	43.96
14	26.00	43.25	63	24.92	42.76
18	24.48	42.48	67	25.50	44.10
21	23.65	43.71	71	26.00	44.64
23	(Sample diluted to 100 p.p.m. 2:4-D)		82	23.12	41.98
28	25.58	43.26	114	24.90	43.83
32	26.00	44.00	139	24.84	44.51
39	25.12	43.61			
42	24.91	43.04			

Culture containing initially 100 p.p.m. 2:4-D

Time in days	0	1	2	3	4	5	6	7	9	11	14	15
Final lgth.mms.	25.82	25.80	24.10	24.95	25.38	43.41	42.00	26.06	25.87	25.32	43.49	44.26
Control lgth.mms.	42.58	44.81	44.12	43.65	43.12	44.17	43.42	42.96	43.67	42.98	44.67	43.25

APPENDIX XX continued

Sample diluted to 100 p.p.m. from 1,000 p.p.m. 2:4-D at 23 days from inoculation

Time in days	23	28	32	36	41	45	49	50
Final lgth.mms.	23.86	24.24	24.50	24.52	24.79	25.79	44.22	43.12
Control lgth.mms.	44.85	44.31	42.39	44.16	43.78	44.86	43.76	45.00

Sample containing initially 1,000 p.p.m. 2:4-D to which soil extract added 49 days from inoculation

Time in days	49	50	54	58	63	67	70	71
Final lgth.mms.	23.70	24.49	24.80	25.55	24.00	39.65	43.64	44.72
Control lgth.mms.	44.22	42.83	44.28	43.35	42.63	44.85	43.87	43.77

Sample containing initially 1,000 p.p.m. 2:4-D to which agar dialysate added at 49 days from inoculation

Time in days								
Final lgth.mms.	24.29	24.00	24.81	25.16	25.69	29.22	31.16	30.04
Control lgth.mms.	43.49	44.26	43.61	42.44	45.12	41.83	44.27	43.82

APPENDIX XXb

The effect of soil extract and agar dialysate on the detoxication of 2:4-D

Soil extract and agar dialysate used in various concentrations separately and in combination.

Phytotoxicity of cultures assayed as before by inhibition of growth in length of 2mm. pea root sections.

Culture containing initially 100 p.p.m. 2:4-D

a) Concentration of soil extract

Time in days	None	5% A	5%	2.5%	1.0%	0.5%	0.25%	Control Sections
	Final length of 10 sections in mms.							
0	24.74	24.63	24.52	24.85	25.34	23.49	24.67	42.91
2	24.81	25.12	24.76	24.96	24.68	25.34	24.18	43.62
4	25.13	25.42	24.69	25.76	26.00	25.19	25.08	41.48
6	24.92	25.36	24.82	23.74	25.18	24.63	24.56	44.29
8	39.68	40.42x	42.00x	24.63	26.62	28.62	43.87x	43.57
10	45.26x	23.95	24.61	44.70x	41.98x	43.29x	25.26	42.54
14	23.66	25.49	29.67	24.36	25.05	24.61	23.65	43.00
21	42.82x	41.33x	44.01x	43.05x	45.66x	44.82x	40.98x	42.13
28	44.14x	43.87x	45.28x	43.63x	42.35x	44.40x	44.49x	43.32
35	22.17	25.62	43.32x	44.07x	44.67x	43.50x	43.31x	45.10
42	24.29	25.00	41.98x	27.76	44.49x	44.71x	42.15x	44.10
49	23.13	24.17	42.81x	43.75x	45.00x	43.00x	43.87x	43.31
56	24.98	24.06	45.65x	41.29x	32.17	42.42x	43.30x	42.86
63	24.78	24.90	42.52x	44.66x	44.33x	42.76x	41.98x	43.51
70	24.26	25.19	43.50x	41.59x	43.45x	45.10x	39.83	44.87
77	24.81	23.76	45.18x	40.28x	25.69	44.62x	44.30x	43.52
84	24.59	24.85	22.67	26.74	22.64	40.92	43.02	44.71

x 2:4-D to restore conc. to 100 p.p.m. appropriate quantity of soil extract added to each culture on the day following assay shewing no phytotoxicity in the culture except 5%A. Here 2:4-D only added - no soil extract.

APPENDIX XXb continued

b) Concentration of agar dialysate

Time in days	None	100%A	100%	50%	25%	10%	5%	Control Sections
	Final length of 10 sections in mms.							
0	23.46	23.66	24.25	24.08	24.42	23.34	24.05	42.86
2	24.22	24.09	24.62	24.54	24.54	24.17,	24.69	43.00
4	25.03	24.76	23.77	25.74	24.52	24.47	24.49	42.15
6	24.18	25.28	23.60	24.46	23.75	25.25	24.72	43.62
8	23.18	24.32	43.76^{x}	43.50^{x}	24.88	44.95^{x}	43.38^{x}	44.23
10	44.62^{x}	41.98^{x}	24.61	24.46	42.87^{x}	26.12	25.17	42.95
14	23.05	25.10	25.67	29.78	25.00	39.76	25.10	42.87
21	43.50^{x}	42.83^{x}	43.71^{x}	44.00^{x}	43.16^{x}	43.50^{x}	42.92^{x}	43.77
28	42.95^{x}	43.72^{x}	44.15^{x}	43.79^{x}	44.76^{x}	37.62	41.74^{x}	43.49
35	23.38	24.56	44.22^{x}	43.71^{x}	43.42^{x}	44.84^{x}	24.83	44.26
42	25.14	24.10	28.29	42.12^{x}	42.75^{x}	41.76^{x}	23.15	43.61
49	24.50	25.75	43.41^{x}	43.80^{x}	43.30^{x}	36.23	24.65	42.44
56	25.64	25.30	43.34^{x}	43.00^{x}	42.65^{x}	45.00^{x}	25.67	43.25
63	23.49	24.81	44.14^{x}	42.65^{x}	43.71^{x}	44.49^{x}	25.17	44.13
70	26.24	24.35	45.32^{x}	23.82	44.32^{x}	44.50^{x}	23.84	45.06
77	24.76	23.58	42.78^{x}	44.96^{x}	43.75^{x}	44.88^{x}	24.51	43.83
84	24.25	24.29	43.61	43.68	44.20	43.42	24.76	44.65

100% A. Fresh agar dialysate not added when 2:4-D charge renewed.

APPENDIX XXb continued

Culture containing initially 1,000 p. .m. 2:4-D

a) Concentration of soil extract

Time in days	None	5%A	5%	2.5%	1.0%	0.5%	0.25%	Control Sections
	Final length of 10 sections in mms.							
0	24,76	24.12	23.56	23.82	24.05	23.81	24.96	43.30
2	23.65	24.45	24.60	24.59	23.98	24.00	25.00	44.15
4	24.71	23.88	24.68	24.16	24.26	23.60	24.36	43.63
6	23.00	24.41	23.43	24.95	23.46	25.51	23.61	42.90
8	25.31	25.41	24.40	29.76	25.27	24.82	29.51	43.18
10	24.62	42.15x	40.00x	42.89x	44.20x	41.23x	43.67x	43.12
14	23.50	22.36	24.12	25.06	24.50	21.81	25.76	44.16
21	25.11	24.19	42.15x	41.72x	42.30x	43.51x	44.26x	43.55
28	24.20	24.07	43.81x	40.95x	44.18x	42.51x	28.15	42.82
35	23.90	25.18	42.46x	41.34x	42.63x	43.93x	44.92x	43.59
42	24.49	24.35	33.87	42.00x	43.59x	41.45x	43.12x	44.13
49	25.10	25.57	44.19x	43.85x	44.86x	42.16x	44.38x	45.00
56	26.20	24.14	43.65x	46.12x	41.72x	43.00x	42.95x	43.18
63	24.32	24.23	44.29x	44.33x	36.19	44.93x	41.86x	44.59
70	23.49	23.83	41.76x	40.71x	43.45x	43.19x	43.13x	43.73
77	24.79	24.97	40.18x	42.10x	42.00x	42.99x	42.70x	42.91
84	24.65	24.40	36.74	41.63	43.72	43.72	43.91	42.75

P.T.O. for agar dialysate

5% A. Fresh soil extract not added when 2:4-D charge renewed.

APPENDIX XXb continued

b) Concentration of agar dialysate

Time in days	None	100%A	100%	50%	25%	10%	5%	Control Sections
	Final length of 10 sections in mms.							
0	22.05	24.67	23.25	23.75	23.20	24.34	25.09	44.16
2	24.35	23.96	25.16	23.95	23.50	22.21	23.22	43.84
4	23.20	24.25	24.68	24.40	23.55	25.05	24.56	43.50
6	24.00	22.96	22.21	23.79	25.18	25.22	23.93	44.49
8	23.37	23.79	24.73	24.51	24.95	22.56	22.39	42.51
10	23.80	43.18x	43.00x	42.78x	44.11x	43.90x	24.85	43.11
14	24.61	24.32	22.34	24.00	27.15	23.49	23.00	43.65
21	23.92	23.69	44.33x	42.52x	44.10x	43.66x	24.76	42.35
28	23.75	24.50	43.63x	42.98x	43.50x	26.91	23.05	43.68
35	24.99	23.82	42.46x	43.26x	42.58x	44.07x	24.92	42.35
42	23.63	23.63	29.87	42.34x	43.92x	43.24x	25.01	44.91
49	24.12	24.10	44.17x	39.56	36.72	44.27x	24.39	43.75
56	23.38	24.35	42.45x	44.80x	42.28x	42.86x	24.34	44.62
63	25.22	24.33	31.69	45.00x	43.10x	43.07x	23.81	43.66
70	23.13	24.25	44.78x	42.81x	42.22x	44.91x	22.89	42.19
77	24.51	22.72	43.45x	43.66x	43.50x	28.62	23.37	43.78
84	24.56	23.90	44.80x	28.74	41.62	34.18	24.89	44.15

100% A. Fresh agar dialysate not added when 2:4-D charge renewed.

APPENDIX XXI Data relating to Table IV

The effect of adding small quantities of toxic substances to aerated solutions containing 100 p.p.m. 2:4-D

Additional substrates:

A. 2-4dichlorophenol
B. Chloro-p-benzoquinone
C. 1,2,4-trihydroxybenzene
D. 2,4-dihydroxyphenoxyacetic acid
E. 3-5dichlorocatechol
F. Quinhydrone
G. No additions.

a) Additional substrate conc. 10 p.p.m.

Time in days	A	B	C	D	E	F	G	Control peas
	Final length of 10, 2 mm. pea sections							
0	23.89	24.70	24.18	22.12	23.80	24.45	24.19	43.98
1	23.84	23.81	23.85	22.67	24.10	24.48	23.97	42.82
2	24.75	24.00	24.13	24.14	24.31	25.00	25.12	42.45
3	24.17	24.32	24.50	23.91	23.79	23.71	24.23	43.70
4	23.83	24.29	23.50	24.04	24.25	23.00	25.01	42.71
5	24.42	23.70	22.31	23.87	23.40	24.25	24.50	43.78
6	24.63	24.80	23.36	23.13	24.32	23.90	23.66	44.00
7	23.81	23.62	24.29	22.68	23.86	23.56	23.82	44.33
8	25.22	24.19	24.10	23.97	23.78	23.80	24.61	43.82
9	24.17	23.20	24.31	23.36	24.51	24.76	31.62	43.91
10	25.14	24.30	24.52	24.19	23.15	23.61	43.83	42.42
11	23.62	22.70	24.11	23.34	22.76	24.10	43.76	43.31
12	24.12	24.66	23.14	24.14	23.91	23.23	-	44.42
13	24.75	23.94	22.98	23.87	24.71	24.25	-	43.21
14	24.31	23.69	25.12	23.94	24.36	23.75	-	42.52
15	23.25	24.23	22.95	24.37	24.14	23.71	-	43.20
16	39.68	23.86	23.75	23.91	23.48	23.68	-	42.32
17	44.13	24.24	24.04	22.47	24.44	24.15	-	43.81
18	42.68	23.28	23.81	23.39	23.52	23.94	-	42.85
19	-	24.27	24.56	24.49	24.15	23.50	-	43.50
20	-	23.81	23.19	23.16	23.61	24.79	-	42.94
21	-	43.57	24.65	24.53	24.50	44.47	-	44.11
22	-	43.45	23.76	23.88	23.62	44.16	-	43.67
23	-	-	24.55	23.69	23.44	-	-	43.50

b) <u>Additional substrate conc. 1 p.p.m.</u>

Time in days	A	B	C	D	E	F	G	Control peas
	Final length of 10, 2 mm. pea sections							
0	23.78	24.74	24.00	24.30	23.65	23.60	24.17	44.80
1	24.59	25.00	23.73	24.50	24.62	23.57	24.28	44.16
2	23.60	23.89	24.24	24.55	24.61	24.06	24.51	43.81
3	24.59	23.85	24.71	22.00	23.26	23.85	24.26	43.79
4	24.09	24.06	24.44	23.74	23.73	24.85	23.35	44.26
5	24.45	24.20	24.15	24.25	24.00	23.62	23.92	43.42
6	23.37	23.95	24.76	23.73	23.59	24.61	24.25	44.23
7	24.50	24.10	23.50	23.50	22.86	23.47	23.68	43.66
8	23.61	23.42	23.89	24.11	23.48	24.12	24.81	44.79
9	24.25	24.39	24.50	23.69	23.65	24.55	23.10	43.93
10	23.61	23.74	24.31	24.46	23.14	24.72	44.95	44.58
11	23.51	23.72	24.50	23.77	24.62	24.50	43.87	43.31
12	23.53	24.73	23.90	24.16	22.95	23.23	-	43.55
13	24.11	23.16	24.08	23.77	24.44	23.84	-	44.23
14	24.31	24.71	23.84	23.65	24.21	24.63	-	43.52
15	41.67	44.18	24.15	23.64	24.74	23.46	-	43.99
16	44.39	43.66	24.43	23.63	24.45	24.35	-	44.50
17	-	-	23.95	24.25	24.02	23.98	-	44.35
18	-	-	24.38	22.94	23.71	24.29	-	43.72
19	-	-	23.25	24.68	24.83	24.12	-	43.75
20	-	-	24.76	24.55	38.13	24.83	-	44.15
21	-	-	25.10	23.29	44.38	43.27	-	43.80
22	-	-	23.16	24.62	-	44.08	-	42.92
23	-	-	23.63	24.18	-	-	-	43.77

APPENDIX XXII

The effect of 1 p.p.m. glutamic acid on 2:4-D detoxication by Nocardia in aerated liquid culture.

Cress root assay. Samples diluted to 1 p. .m.2:4-D

Time in days	Initial conc. of 2:4-D in p.p.m. 100	400	1,000	No glutamic acid 100 ppm.2:4-D
0	Toxic	Toxic	Toxic	Toxic
1	T	T	T	T
2	T	T	T	T
4	T	T	T	T
6	T	T	T	T
8	T	T	T	T
10	Detox	T	T	Detoxicated
12	D	T	T	D
16	-	T	T	-
20	-	T	T	-
30	-	T	T	-

APPENDIX XXIII Data relating to Figure XXIII

Measurement of phytotoxicity and radioactivity of chromatographed samples of 2:4-D enriched perfusate taken at intervals throughout the detoxication of 100 p.p.m. 2:4-D labelled with C^{14} in the methylene group of the side chain.

Chromatograms developed in isopropanol/ammonia/water solvent, dried, radioactivity estimated and sectioned into 10 equal portions. Section 1 = Rf0-01, section 2 = Rf0.1-0.2 etc. 2 mm.pea root sections grown in 0.75ml. of 0.5% sucrose on each portion and length measured after 24 hours growth at 26°C in the dark.

Time from inoculation in hours

C'gram section	0	4¼	28	51	80	101	129	157½	180
	Final length of 10, 2mm. pea root sections								
1	32.15	30.00	37.92	37.30	45.21	44.24	37.54	41.90	41.29
2	36.96	33.68	35.20	37.52	38.00	51.42	43.57	44.51	43.34
3	35.47	32.53	37.91	34.00	44.62	48.23	44.15	42.00	42.60
4	27.78	25.89	37.20	36.63	38.84	49.00	43.00	47.02	45.64
5	27.80	26.40	39.42	35.22	45.23	42.74	41.10	40.52	41.23
6	31.79	30.00	32.63	35.00	39.70	36.64	37.36	41.22	41.65
7	29.75	28.72	25.04	26.23	28.91	25.62	38.27	43.13	44.12
8	31.81	29.00	26.82	28.40	24.00	30.72	25.03	40.00	41.00
9	40.61	35.30	33.20	38.00	38.00	44.69	40.52	42.75	45.51
10	29.58	28.00	41.00	34.98	36.00	40.00	42.96	43.80	43.62
Control	38.40	34.82	37.60	39.00	41.01	48.50	42.22	44.00	42.13

Radioactivity P.T.O.

Radioactivity counts recorded on chromatograms

Each position represents a distance of 0.5cm. travelled down the chromatogram from the origin. Width of exposed portion is also 0.5cm. Each position counted for 5 minutes and any region recording a count of 18 per minute or more is recounted for 20 minutes on the first 4 chromatograms. Later samples - each position is counted for 1 minute and any region recording a count of 18 per min. or more is counted for 5 minutes. Counts given here as gross counts per minute.

Time from inoculation in hours	0	4¼	28	51	80	101	129	157½	180
Position	Gross counts per minute								
1	13.2	14.6	12.0	14.2	13	38.4	140.2	105.2	144.6
2	11.8	14.2	12.0	17.0	16.3	14.8	68.2	53.8	64.8
3	14.6	12.6	13.8	9.8	9	13	13	11	12
4	15.8	10.8	13.4	14.6	15	12	14	15	15
5	12.8	12.6	10.8	12.8	8	10	12	12	9
6	14.8	13.4	11.6	11.2	12	12	15	12	12
7	13.6	10.5	10.4	14.4	16	18	9	13	13
8	13.4	12.2	13.4	14.6	5	14	11	17	15
9	13.4	12.0	11.6	14.4	14	9	15	13	17
10	13.8	10.2	11.8	12.8	10	8	10	14	9
11	15.6	10.8	12.8	8.8	14	12	14	7	8
12	12.6	12.5	10.2	12.6	10	10	13	15	11
13	13.8	10.8	10.0	13.6	10	17	17	10	12
14	14.8	11.6	11.0	12.4	16	12	10	15	12
15	12.8	11.0	12.0	13.0	12	18	13	10	10
16	13.2	14.0	11.6	13.4	14	15	13	17	18
17	14.2	12.8	12.6	13.2	10	12	17	13	13
18	13.0	17.6	14.6	8.6	9	13	9	17	9
19	10.8	13.2	15.4	11.4	14	14	15	17	14
20	13.8	16.2	14.6	13.2	6	13	8	10	14
21	14.2	12.8	15.6	12.4	18	18	17	11	13
22	12.2	13.6	16.8	12.6	17	14	17	8	12
23	13.2	10.8	45.6	13.8	17	10	16	9	7
24	14.0	14.2	240.1	12.2	18	13	12	11	16
25	10.0	11.2	376.4	12.6	13	13	16	9	5
26	14.2	15.4	167.6	14.2	18	13	13	16	8
27	12.3	15.2	25.4	13.0	11	16	18	7	16
28	39.6	67.5	14.4	26.5	16	14	12	10	7
29	252.4	348.8	14.7	138.1	60.3	14	11	12	11
30	398.0	337.25	14.4	292.0	248.6	5	17	17	12
31	47.8	93.8	13.2	217.0	166.3	13	13	14	13
32	16.2	21.0	10.0	64.6	38.9	8	14	12	11
33	14.4	12.25	13.2	15.8	9	22	19.2	17	10
34	13.8	14.4	16.4	12.6	16	79	53.4	5	12
35	15.0	12.0	14.4	15.2	6	155	83.2	16	10
36	14.6	13.4	10.20	16.8	19	140	76.8	6	9
37	13.8	11.4	10.80	12.4	13	43	19.4	13	7
38	14.2	12.2	14.2	12.2	11	14	14	16	15
39	10.8	11.8	14.6	12.6	16	12	14	14	7
40	10.6	14.4	Front	13.4	15	15	13	11	18
41	17.0	13.7		15.2	12	9	12	10	15
42	11.2	12.8		10.4	11	14	12	10	6
43	15.2	13.8		10.6	13	17	17	10	Front
44	13.0	10.6		13.4	14	10	17	14	
45	14.4	14.2		12.2	16	17	12	15	
46	14.6	12.6		14.6	8	16	7	14	
47	13.8	13.2		13.8	14	9	11	15	
48	Front	Front		14.4	Front	10	18	13	
49				Front		16	13	Front	
50						12	15		
51						14	13		
52						15	11		
53						12	16		
						Front	Front		

APPENDIX XXIV. Data relating to Figure XXIV

Methylene labelled 2:4-D

1. Radioactivity measured on chromatograms during detoxication of 2:4-D. The data from which this graph was constructed is given in Appendix XXVI.

2. Evolution of $C^{14}O_2$ during detoxication of 2:4-D

   0.1 ml. trap fluid dried onto 2 cm. planchette. Background counts taken with the planchette in position before the addition of the active sample. Each sample counted for 20 minutes. The trap fluid is renewed at each sampling.

Time in hours	Background count	Count/mm.	Sample Count	Count/mm.
0	251	12.55	248	12.4
24	364	18.2	4,324	216.2
48	302	15.1	31,456	1572.8
76	268	13.4	11,172	558.6
99	304	15.2	2,432	121.6
120	252	12.6	1,650	82.5
140	276	13.8	2,474	123.7
172	296	14.8	1,522	76.1
194	250	12.5	578	28.9
220	204	10.2	507	25.35
240	253	12.65	462	23.1

APPENDIX XXV Data relating to Figure XXV

Growth of bacteria during detoxication of 100 p. .m. 2:4-D labelled with C^{14} in:-

a) The methylene group of the side chain

Time in hrs.	Cell counts	Av.	Cells /ml.	Log_{10} cells/ml.
0	Inoculating suspension giving:		2.69×10^6	6.43
24	12,10,11,9,12,9,10,10,8,11	10.2	4.08×10^7	7.61
50	17,15,18,16,16,17,15,15,14,15	15.8	6.32×10^7	7.80
74	15,16,18,14,15,15,19,14,16,15	15.7	6.28×10^7	7.80
97	14,13,13,12,15,21,17,20,14,17	15.4	6.16×10^7	7.79
140	12,15,13,15,16,14,15,13,14,13	15.1	6.04×10^7	7.78
194	14,19,15,16,18,17,13,14,15,13	15.4	6.16×10^7	7.79
218	14,17,15,13,13,15,21,14,15,18	15.5	6.20×10^7	7.79
234	18,14,15,17,16,17,15,13,13,16	15.4	6.16×10^7	7.79

b) The carboxyl group of the side chain

Time in hrs.	Cell counts	Av.	Cells/ ml.	Log_{10} cells/ml.
0	Inoculating suspension giving:		2.19×10^6	6.34
24	6,3,4,5,3,1,4,3,2,2	3.3	1.32×10^7	7.12
50	15,16,14,10,12,11,12,13,10,15	12.8	5.12×10^7	7.71
74	14,16,12,14,11,12,15,15,15,13	13.7	5.48×10^7	7.74
97	14,13,16,15,12,14,16,15,14,17	14.5	5.80×10^7	7.76
124	14,13,10,14,16,14,16,15,17,15	14.4	5.76×10^7	7.76
150	14,14,13,17,15,16,13,14,13,12	14.1	5.64×10^7	7.75
174	14,15,17,14,13,16,12,19,17,14	15.1	6.04×10^7	7.78
197	15,14,16,14,16,17,17,16,15,18	15.8	6.32×10^7	7.80
223	17,14,15,17,16,17,15,16,15,16	15.8	6.32×10^7	7.80
245	13,16,14,16,17,15,17,16,18,15	15.7	6.28×10^7	7.80
270	18,15,17,14,16,14,16,15,15,17	15.7	6.28×10^7	7.80

APPENDIX XXVI. Data relating to Figure XXVI

Measurement of phytotoxicity and radioactivity of chromatographed samples of media, inoculated with organisms of the effective Nocardia strain, and containing initially 100 p.p.m. 2:4-D labelled with C^{14} in the methylene group of the sidechain, taken at intervals throughout the detoxication of the 2:4-D.

Chromatograms developed in isopropanol/ammonia/water, solvent dried, and radioactivity and phytotoxicity estimated as before.

Phytotoxicity

Ch'gram section	Time from inoculation in hours									
	0	24	49	74½	99	140½	172	194	219	233
	Final length of 10.2mm. pea root sections									
1	37.18	37.00	38.78	43.00	40.41	40.00	48.86	40.32	40.30	34.90
2	37.38	43.66	39.00	32.56	38.01	40.52	43.16	36.19	31.18	39.89
3	37.35	42.40	34.72	40.92	44.28	43.38	33.74	41.78	40.84	35.96
4	38.01	48.31	38.75	40.72	41.38	40.37	45.09	40.28	40.20	42.33
5	37.90	44.77	41.46	42.00	40.37	40.83	44.62	39.46	42.27	40.44
6	36.39	43.86	39.95	42.64	41.00	40.00	42.60	36.89	39.94	40.57
7	26.81	25.02	25.87	29.83	33.19	33.00	32.34	28.56	30.96	32.18
8	37.08	42.38	39.64	43.26	41.12	40.92	45.01	41.63	35.93	36.13
9	37.34	43.68	41.52	41.01	41.86	40.98	46.26	41.18	40.38	40.46
10	37.00	44.37	40.46	31.83	41.00	40.50	46.50	35.30	34.42	39.11
Control	38.00	43.94	41.35	41.08	41.93	41.58	46.00	42.71	41.32	40.45

APPENDIX XXVI. Data relating to Figure XXVI - continued

Radioactivity

Time from inoculation in hours

Position	0	24	49	74½	99	140½	142	194	219	233
0 origin-	15	37	363.4	645.4	401	333.4	22.58	221.4	221.8	165
1	16	23.8	415.2	326.6	544	368.8	230.8	189.2	147.2	155
2	16	18	57.5	12	41.6	29.2	24.5	13	12	55.4
3	14	16	17.7	15	13	16	18	18	15	12.5
4	15	16	16	13	15	16	19	17	11	17
5	18	15	16	20	18	17	18	16	18	17
6	15	14	12	13	14	10	13	12	17	12
7	10	19	16	10	15	11	14	11	14	18
8	17	17	14	16	10	16	10	14	16	11
9	15	16	14	15	14	10	15	13	17	17
10	10	14	13	12	15	18	13	12	10	16
11	16	15	9	17	14	17	15	10	17	13
12	14	17	17	18	11	10	19	10	14	12
13	17	10	19	10	15	17	18	16	10	12
14	14	18	13	14	13	12	9	15	15	16
15	18	14	14	13	12	14	14	15	19	18
16	14	14	16	16	11	18	17	16	17	14
17	19	15	16	19	15	12	16	16	13	14
18	18	15	14	18	12	10	17	15	16	15
19	18	14	14	20	13	12	19	14	14	16
20	11	15	10	17	11	19	14	17	15	13
21	13	15	13	16	18	19	16	16	19	17
22	15	15	13	15	17	14	10	17	15	15
23	19	17	11	13	13	16	9	13	18	13
24	15	15	11	10	14	16	19	18	17	12
25	14	18	9	13	11	15	11	16	14	11
26	15	17	17	11	10	13	13	14	12	18
27	18	16	14	15	12	19	13	14	9	16
28	19	14	14	14	17	15	14	15	13	17
29	15	18.63	16	15	14	13	15	18	12	14
30	10	62.2	28	17	12	13	11	8	18	16
31	16	334	69	17	14	17	13	16	15	13
32	17	291	44.2	12	15	20	17	10	14	15
33	24.1	97.4	20	14	12	19	13	13	184	11
34	96.4	29.6	15	13	16	9	15	12	12	16
35	392	17	16	24	15	11	11	16	15	15
36	449.4	16	14	21	15	17	19	18	13	13
37	156.4	14	15	15	9	14	14	17	14	17
38	47.6	15	16	20	10	18	16	18	16	18
39	16.6	18	14	15	18	14	16	12	14	14
40	15	17	15	16	19	18	15	17	19	16
41	18	19	16	20	14	17	19	16	14	21
42	14	13	14	10	19	13	13	14	15	19
43	11	17	15	16	13	11	15	19	18	17
44	12	16	20	13	19	18	15	19	19	14
45	15	17	10	16	17	17	13	12	10	15
46	18	17	16	15	16	12	11	11	17	19
47	18	13	19	16	15	16	17	18	15	18
48	15	19	12	11	18	15	11	11	16	10
49	17	15	17	16	8	19	19	17	10	12
50	17	Front	Front	13	15	14	10	14	13	17
51	11			17	14	12	7	18	12	14
52	19			19	10	18	13	16	17	11
53	16			10	13	14	Front	13	15	12
54	18			12	13	Front		18	14	12
55	10			Front	Front			17	Front	Front
56	13							15		
	Front							Front		

APPENDIX XXVII. Data relating to Figure XXVII

Radioactivity and phytotoxicity recorded on chromatogram. Acid ether extract at 99 hours from inoculation of culture solution.

Radioactivity								Phytotoxicity	
Position	Count	Position	Count	Position	Count	Position	Count	Section	Final length pea roots
1	14	16	14	31	12	46	13	1	38.22
2	15	17	18	32	14	47	10	2	34.90
3	12	18	14	33	16	48	17	3	31.38
4	5	19	14	34	15	49	11	4	40.04
5	15	20	13	35	19	50	15	5	40.23
6	18	21	19	36	16	51	19	6	28.15
7	14	22	14	37	19	52	13	7	32.24
8	13	23	14	38	17	53	14	8	32.43
9	15	24	18	39	15	54	15	9	39.94
10	13	25	16	40	15	55	17	10	39.12
11	17	26	13	41	16	56	16	Control	40.28
12	16	27	13	42	18	57	10		
13	16	28	14	43	18	Front			
14	12	29	14	44	14				
15	14	30	14	45	12				

APPENDIX XXVIII. Data relating to Figure XXVIII

Radioactivity recorded on chromatograms during the detoxication of 100 p.p.m. 2:4-D labelled with C^{14} in the carboxyl group of the sidechain

Chromatograms developed and radioactivity estimated as in previous experiments

Radioactivity

Position	Time from inoculation in hours: 0	25	49½	76	94	121½	149½	173½	200	223	246	275
0 origin	16	21.2	22.2	29	30.2	166.9	157.2	90.2	58.4	44.6	43.6	45.9
1	13	18.6	16.63	56.4	30.6	94.0	90.0	46.8	41.6	26.4	24.0	33.1
2	14	16	11	15	18	16	18	15	17	15	14	14.6
3	17	15	18	13	15	9	13	16	12	15	16	12
4	12	19	13	16	10	13	15	11	15	14	13	14
5	12	14	14	10	18	11	15	16	16	11	18	16
6	18	15	10	13	15	18	17	13	9	13	17	15
7	19	13	14	12	14	15	16	15	15	9	17	18
8	14	17	16	15	12	16	16	16	19	16	14	9
9	16	18	7	14	16	15	13	10	14	14	12	19
10	9	14	19	14	17	16	16	15	14	15	16	13
11	19	17	14	12	9	12	15	14	14	17	11	19
12	14	16	14	18	7	14	15	16	15	10	9	9
13	12	14	13	11	11	15	18	13	11	18	16	16
14	18	11	16	16	15	18	15	16	13	15	9	7
15	15	16	17	19	14	14	16	17	15	16	18	13
16	17	11	9	12	14	13	16	14	17	15	13	16
17	15	15	11	14	17	15	15	9	18	15	12	7
18	15	19.	16	15	11	19	12	12	14	13	14	14
19	15	18	16	14	12	17	17	19	15	9	12	19
20	19	10	12	13	10	17	14	17	12	9	15	12
21	15	13	14	13	14	12	16	12	18	16	14	14
22	17	14	12	14	16	10	17	15	17	15	14	15
23	11	18	14	13	16	16	14	14	13	15	14	16
24	19	16	15	10	17	16	15	17	15	8	19	15
25	18	14	16	15	15	16	17	12	10	19	15	17
26	16	15	16	19	13	15	15	8	11	17	16	16
27	16	12	15	11	15	13	14	16	14	12	17	14
28	17	10	15	16	19	14	16	18	15	13	12	12
29	13	15	18	11	17	22.6	16	16	15	17	17	17
30	13	20.2	37.6	16	9	66.8	18	13	16	14	14	9
31	14	133.2	322.0	106.4	19.0	90.2	11	18	16	12	13	17
32	21.6	520.4	416.4	438.8	29.1	27.8	14	11	14	14	11	17
33	83.0	372.0	192.2	370.4	162.2	10	18	10	11	16	15	11
34	482.6	104.0	42.2	108.4	347.0	14	15	12	11	15	10	13
35	546.0	22.0	17	24.0	173.6	17	11	15	10	15	18	14
36	89.6	11	12	13	48.4	16	16	11	18	12	15	13
37	15	16	16	16	14	14	11	9	11	15	13	15
38	18	12	15	15	16	13	15	15	6	10	16	14
39	16	16	15	13	11	14	15	17	15	15	15	17
40	11	15	11	12	18	16	16	17	18	13	17	18
41	18	7	13	9	16	10	18	14	14	18	18	16
42	16	15	13	14	12	15	17	16	10	11	17	14
43	15	18	14	9	17	17	13	15	Front	14	10	10
44	7	17	10	18	11	14	17	12		12	12	15
45	17	16	15	13	15	18	18	8		15	10	12
46	14	18	18	16	16	15	16	16		13	Front	17
47	11	9	13	18	17	Front	14	14		9		15
48	15	17	15	12	13		12	Front		14		16
49	14	16	9	14	14		Front			15		Front
50	16	13	Front	15	11					Front		
51	16	Front		Front	12							
52	12				12							
	Front				Front							

APPENDIX XXVIII. Data relating to Figure XXVIII

2. Evolution of $C^{14}O_2$ during detoxication of 2:4-D labelled with C^{14} in the carboxyl group of the side chain.

0.1ml. trap fluid dried onto a 2cm. planchette. Background counts taken with planchette in position before addition of the active sample. Each sample counted for 20 minutes. The trap fluid is renewed at each sampling.

Time in hours	Background		Sample	
	Count	Count/min.	Count	Count/min.
0	270	13.5	280	14
25	292	14.6	108	25.4
50	274	13.7	1,170	58.5
76	274	13.7	2,348	167.4
96	310	15.5	6,348	317.2
122	368	16.4	23,616	1,180.8
150	306	15.3	13,180	659.0
174	312	15.6	1,362	68.1
200	262	13.6	2,464	123.2
224	278	13.9	1,108	55.4
246	282	14.1	528	26.4
275	326	16.3	536	26.8

APPENDIX XXIX. Data relating to Figures XXIX and XXXI

The production of phenolic substances during the detoxication of 100 p.p.m. 2:4-D

The presence of phenolic material in solution may be detected using Folin and Ciocaltin's Phenol Reagent. Quantities of the order of 1 p.p.m. per ml. can be detected.
To a 1 ml. sample is added 0.5ml. of the reagent followed by a 1.5 ml. saturated sodium carbonate solution. The colour is then allowed to develop for 20 minutes at a temperature of 30°C and the sample made up to 5 mls. G.D.W. The depth of colour is estimated by use of a Unicam colorimeter at wave-length 485. A curve shewing the percentage transmission of light under these conditions by solutions containing various quantities of 2:4-dichlorophenol was prepared and the experimental results compared with this.

% transmission of light recorded in:-

Time hrs.	Culture medium	Alkali trap
0	100	100
22	100	91
64	98	91.6
86	100	90
116	100	91
158	97	88.5
190	99.5	88
230	95	81
256	99	80.5
280	100	81
306	100	81

APPENDIX XXX. Data relating to Figures XXX and XXXI

The release of free chloride ions into the culture medium during the detoxication of 2:4-D by the effective strain of Nocardia was estimated using a standard N/500 silver nitrate solution.

5 ml. samples of culture medium were removed aseptically after vigorous shaking and a few drops of saturated potassium chromate solution added as an indicator. N/500 silver nitrate was run in from a burette until a faint permanent red colour of silver chromate became visible. An attempt to standardise the colour from day to day was made by using a standard solution of silver nitrate and sodium chloride with an equal quantity of indicator as comparator.

5mls. culture medium titrated against N/500 $AgNO_3$

Time hrs.		mls. $AgNO_3$ needed	Chloride liberated	% Chloride liberated
0	(1	5.1	0)	0
	(2	5.1	0)	
22	(1	5.18	0.08)	4
	(2	5.19	0.09)	
62	(1	5.27	0.17)	8
	(2	5.27	0.17)	
86	(1	5.95	0.85)	40
	(2	5.95	0.85)	
116	(1	7.20	2.10)	96
	(2	7.25	2.15)	
158	(1	7.35	2.25)	108
	(2	7.40	2.3)	
190	(1	7.40	2.3)	108
	(2	7.35	2.25)	
230	(1	7.40	2.3)	108
	(2	7.40	2.3)	

APPENDIX XXXIII. Data relating to Figure XXXIII

	Radioactivity in CO_2 trap			Chloride in medium	
Time in hrs.	Gross count/ 10 mins.	Back-grd. /min.	Gross count/ min.	mls N 500AgNO$_3$ ≡ 5 mls. medium	AgNO$_3$ per 1ml. medium
0	201	19	20.1	16.75	3.95
3	253	14	25.3	15.0	3.0
6	250	14	25	13.5	2.7
23	1495	16	149.5	11.5	2.3
30	2170	19	217	11.0	2.2
47	3093	13	309	11.0	2.2
54	3604	18	360.4	10.0	2.0
71	4255	14	425.5	9.0	1.8
119	7218	19	721.8	9.5	1.9
126	8344	12	834.4	10.0	2.0
133	8596	16	859.6	9.0	1.8
157	8731	17	873.1	10.0	2.0

APPENDIX XXXIV. Data relating to Figure XXXIV

Radioactivity in CO_2 trap				Chloride in medium	
Time in hrs.	Gross count /10 mins.	Back-grd./ min.	Gross count/ min.	mls. N/500 $AgNO_3$ ≡ 5 mls. medium	$AgNO_3$ per ml. medium
0	168	16	16.8	7.5	1.5
17	432	14	43.2	7.5	1.5
23	1114	19	111.4	7.0	1.4
41	1188	18	118.8	6.5	1.3
47	1804	15	180.4	5.0	1.0
65	3494	17	349.4	4.25	0.85
71	4172	16	417.2	4.00	0.80
91	5568	12	556.8	2.00	0.40
137	6000	17	600.0	2.50	0.50
161	6038	15	603.8	2.35	0.47

APPENDIX XXXV.

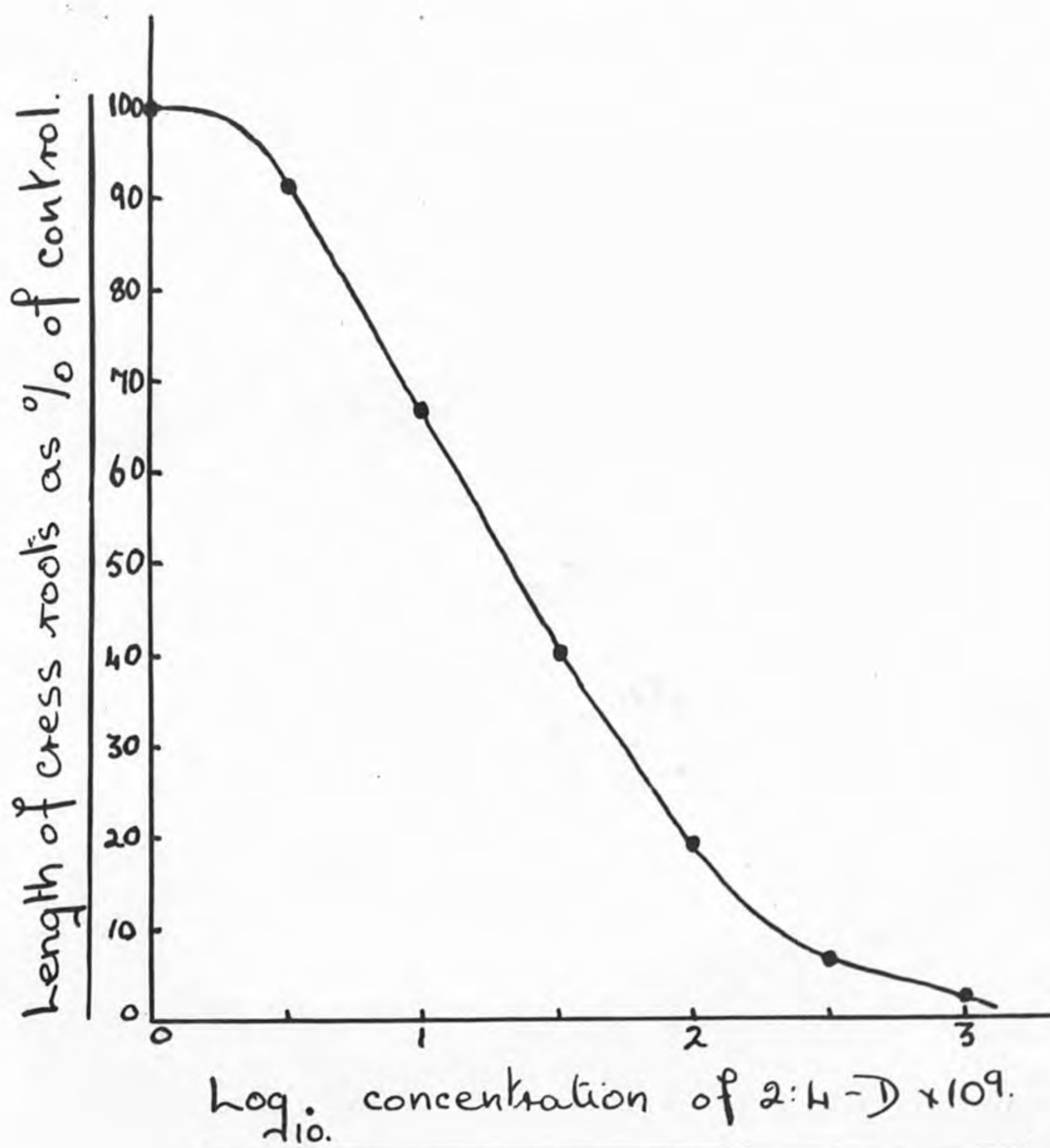
Calibration graphs

1. Cress root test for phytotoxicity
2. Pea root section test for phytotoxicity - using weight of sections.
3. Pea root section test using length of sections.
4. Phenol test.
5. Radioactivity C^{14} on planchettes
6. Radioactivity C^{14} on developed chromatograms
7. Phytotoxicity-pea test on developed chromatograms.

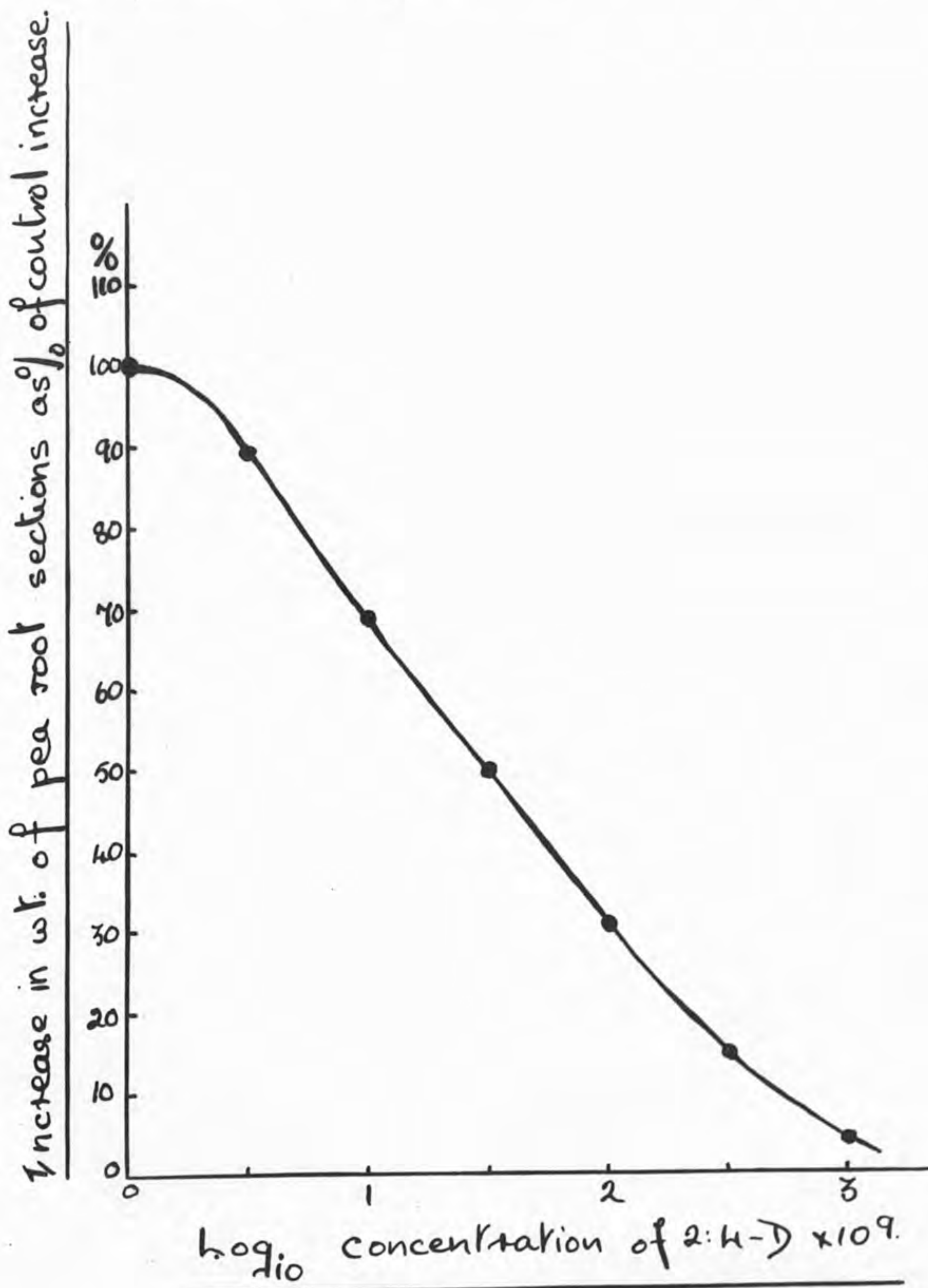
Length of cress seedling roots grown at 26°C in the dark for 4 days in test tubes on filter paper moistened with glass distilled water containing the stated quantities of 2:4-D. (5 mls. solution).

Carter's Plain Cress: variety 4015.

4 longest roots in each tube measured.

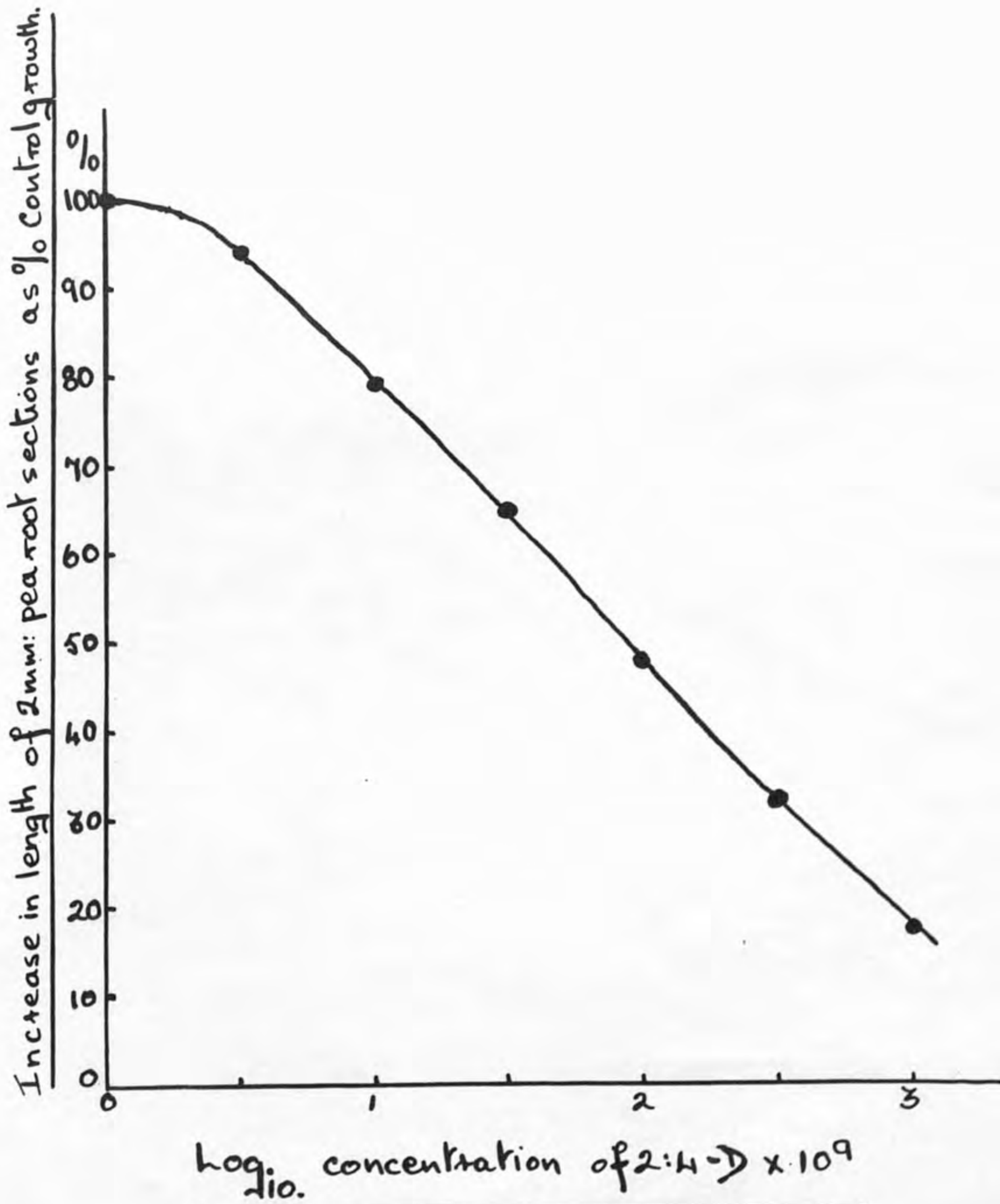


Increase in weight of 2mm. pea root sections during 24 hrs. growth at 26°C in the dark on filter paper moistened with 0·75 mls. of 0·5% sucrose solution together with the stated concentration of 2:4-D.

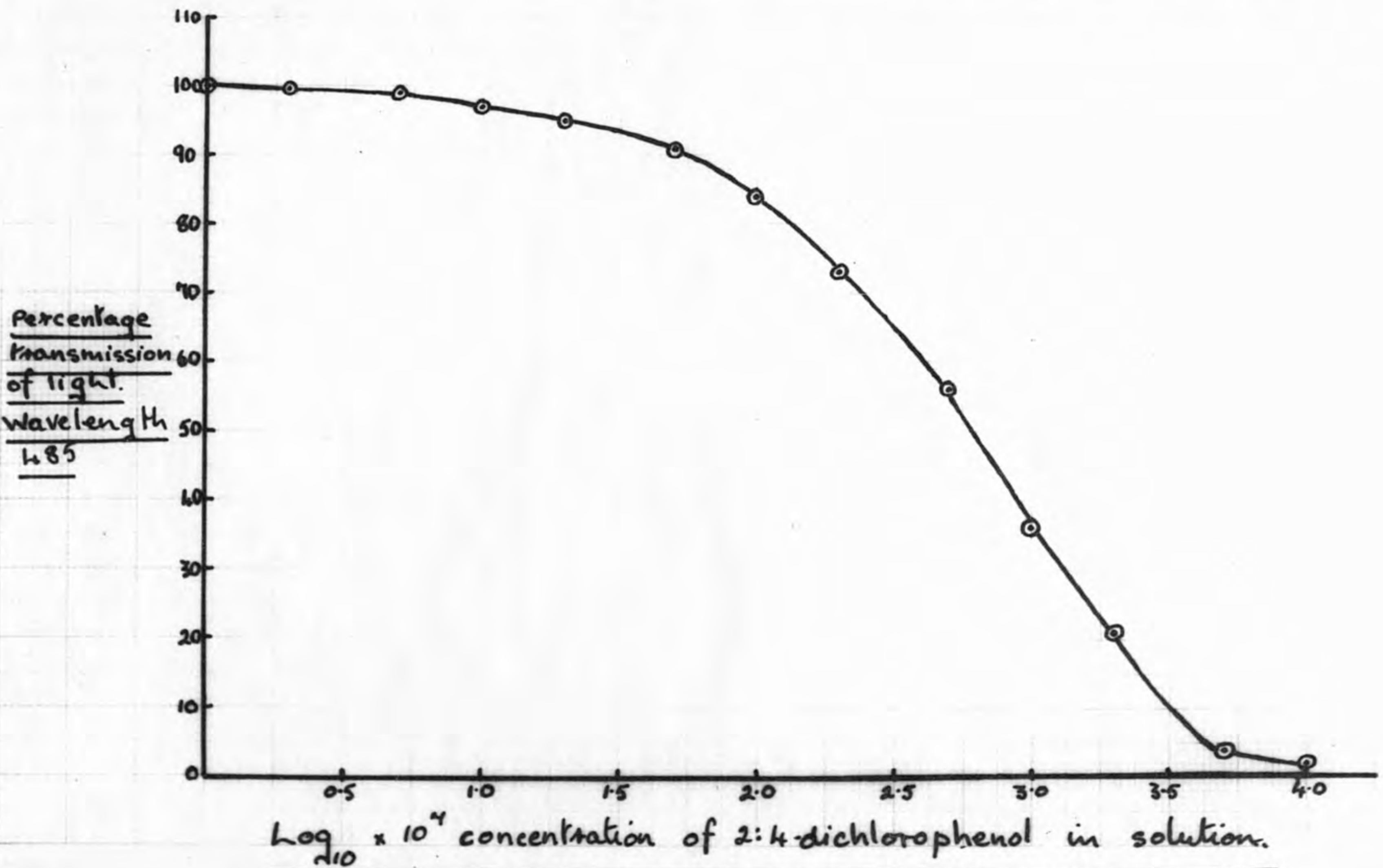


Increase in length of 2mm. pea root sections during 14 hours growth at 26°C in petri dishes containing 10 mls. of 0·5% sucrose solution together with the stated concentration of 2:4-D.

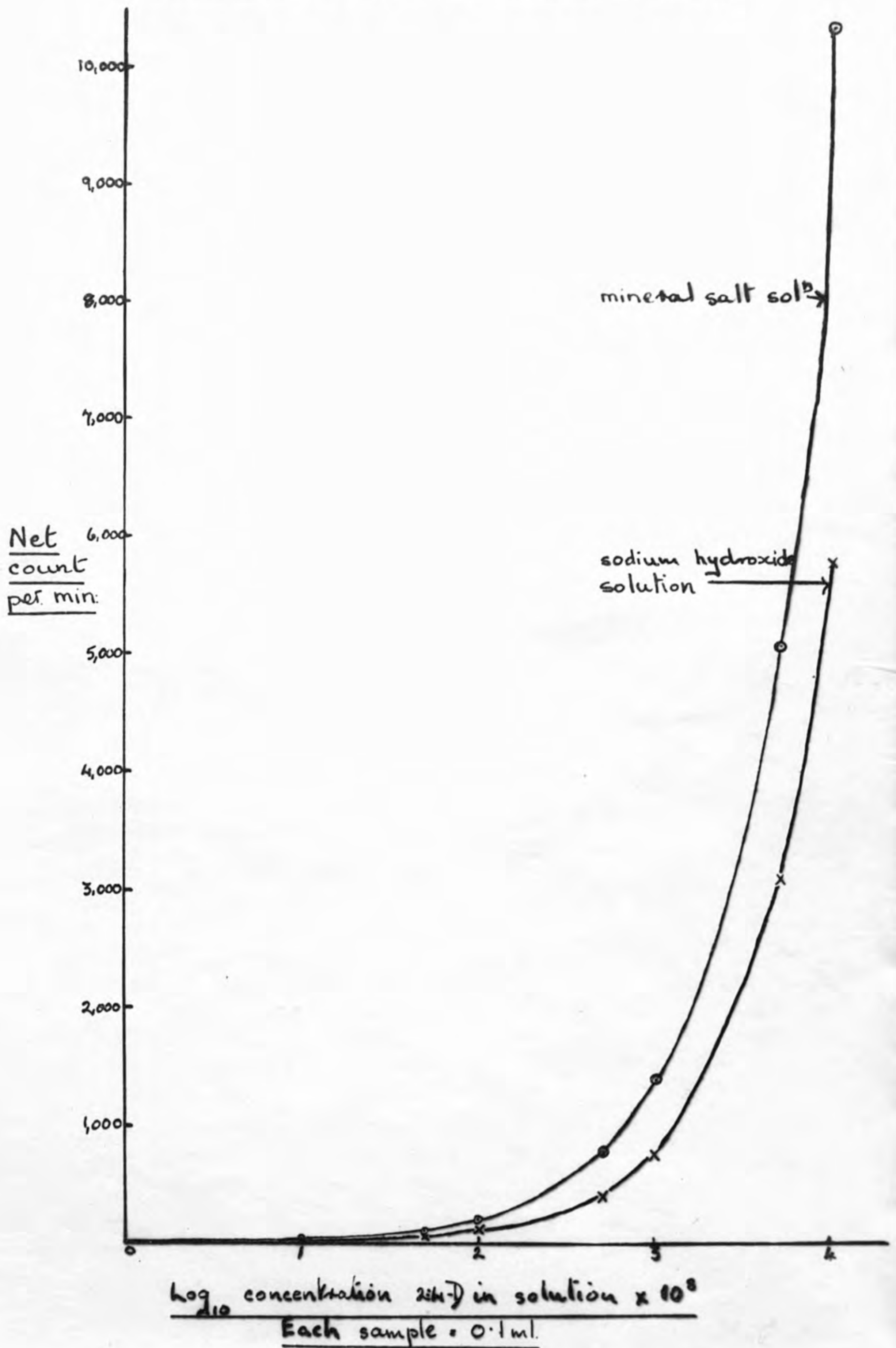
Solutions aerated by rocking.



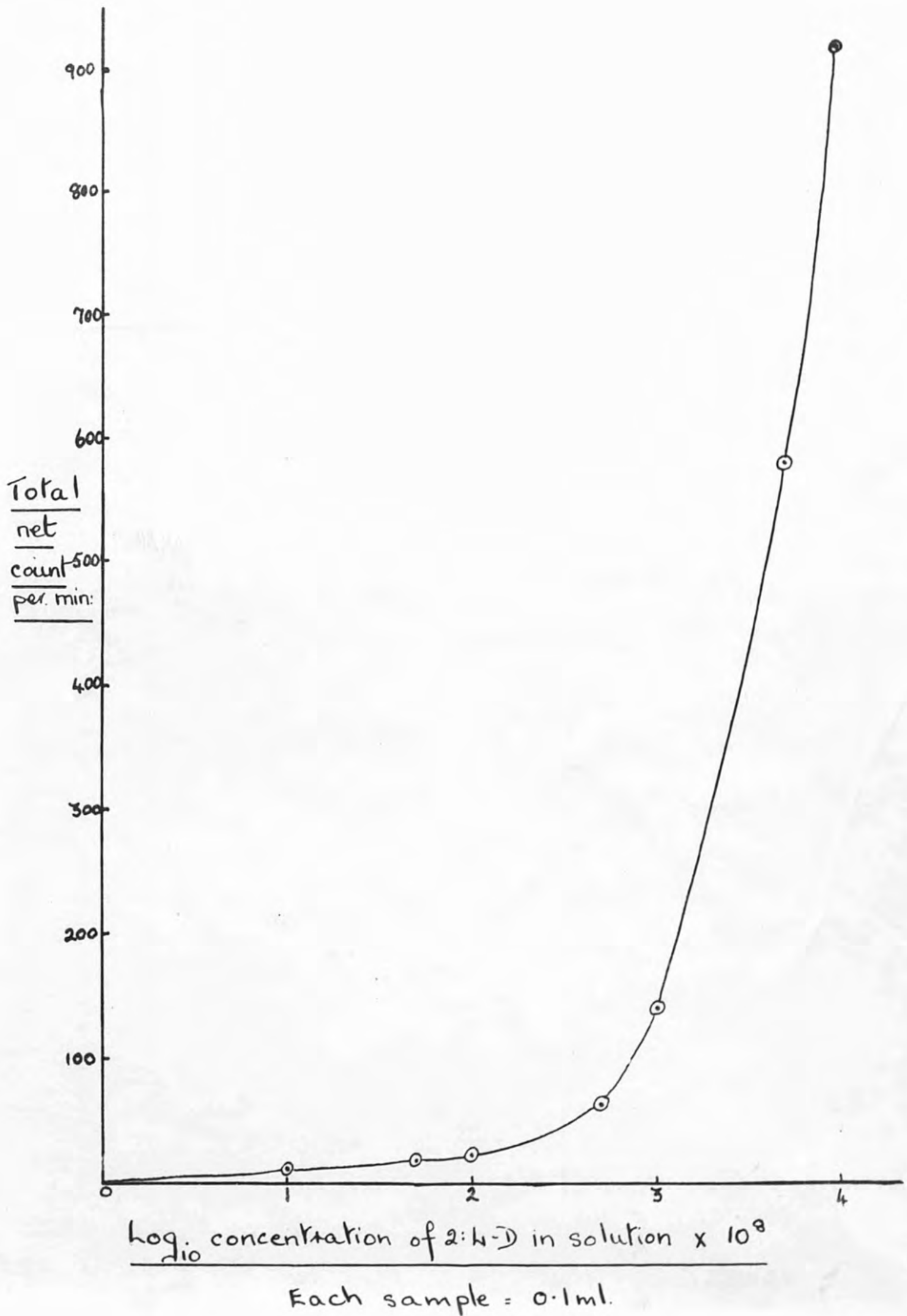
Calibration Curve. To measure the concentration of 2:4-dichlorophenol in a solution after its reaction with Folin and Ciocalteu's Phenol Reagent using percentage transmission of light.



Net counts per minute on planchette from dried nutrient salt and NaOH solutions.

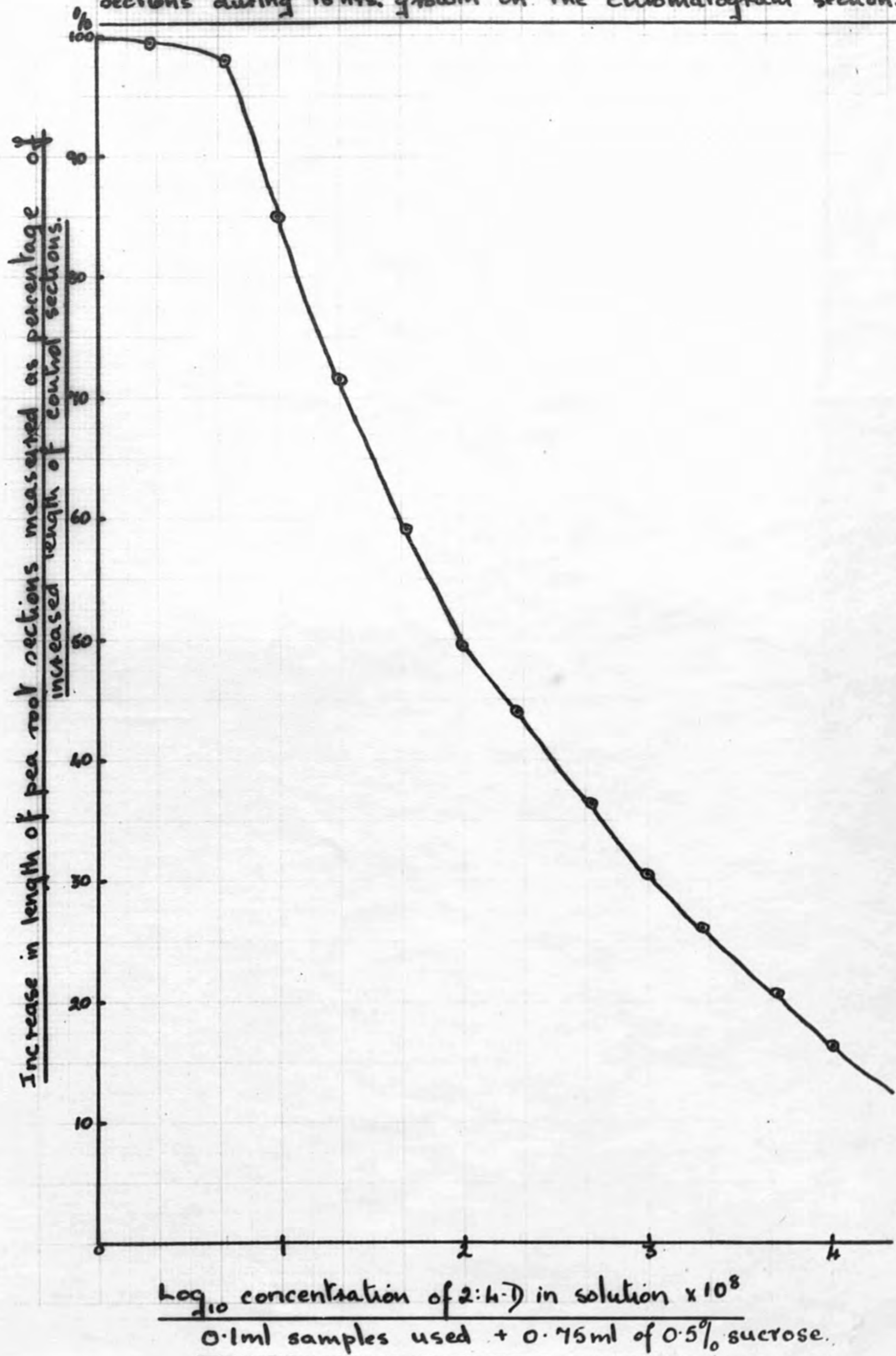


Total net counts per minute on chromatograms after development in propanol/ammonia/water, solvent



Phytotoxicity of 2:4-D on chromatograms after development.

Assayed by measurement of increase in length of 2mm. pea root sections during 18 hrs. growth on the chromatogram section.



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FURTHER STUDIES ON THE BREAKDOWN OF 2:4-DICHLOROPHENOXYACETIC ACID BY A SOIL BACTERIUM

By L. J. AUDUS and K. V. SYMONDS
Botany Department, Bedford College, University of London

(With 4 Text-figures)

Bacterial growth rates and the kinetics of detoxication have been followed during the course of breakdown of 2:4-dichlorophenoxyacetic acid in a liquid mineral salt medium by cultures of *Bacterium globiforme* isolated from garden soil. It was found that toxicity persisted apparently unchanged long after bacterial growth had ceased, and finally disappeared suddenly after a lag period comparable in duration with those obtained in soil perfusion experiments. These results can best be explained by the production of highly phytotoxic intermediates in the first stages of 2:4-D breakdown. Preliminary experiments involving paper partition chromatography indicate that there may be at least two such intermediates.

Introduction

Previous investigations on the breakdown of 2:4-dichlorophenoxyacetic acid (2:4-D) and other hormone herbicides of similar molecular structure in soil using the perfusion technique of Lees & Quastel (1946) have demonstrated clearly that the kinetics of the breakdown and its sensitivity to bacterial poisons indicate an attack on the molecule by an adapted soil micro-organism (Audus, 1949). From 'enriched' soils such an organism has been isolated on a mineral salt-2:4-D-agar medium in which the herbicide was the only source of organic carbon (Audus, 1950). The organism was shown to belong to the *B. globiforme* group. Continued subculturing on this solid medium results in a steady loss of vigour. Growth also takes place in a mineral salt solution (Thornton's solution) containing 2:4-D, if it is vigorously aerated; then there is no apparent loss of vigour. By continued subculturing into this liquid medium, with an occasional short period on agar, the organism has been kept growing and capable of decomposing 2:4-D for a period of about 4 years.

Growth, however, is never vigorous and all attempts to improve it by the addition to the culture medium of various accessory growth factors (i.e. yeast extract, casein hydrolysate, Menaphthone*, vitamin B_{12} and soil extracts) have met with little success. Growth on standard nutrient media is always luxuriant but the organism becomes immediately de-adapted. There seemed therefore little immediate prospect of

* 2-Methyl-1:4-naphthaquinone.

cultivating the adapted organism in quantity for studies of 2:4-D breakdown in bulk. It was therefore decided to follow growth of the bacterium in the mineral salt—2:4-D solution and to observe simultaneously toxicity changes in the medium as a first step towards elucidation of the mechanism of breakdown in liquid culture.

Growth and toxicity changes

(1) *Methods*

The culture medium has been a simple inorganic salt solution (Thornton's solution) containing the ammonium salt of 2:4-D at concentrations of either 0·1 % or 0·01 %. of acid equivalent. Thornton's solution has the following constitution: $(NH_4)H_2PO_4$, 0·1 %; KCl, 0·02 %; $MgSP_4$, 0·02 %. 25 ml. of this medium were placed in 100 ml. boiling tubes, sterilized and innoculated with 0·1 ml. of suspension of the adapted form of *B. globiforme* from previous liquid cultures. The cultures were then incubated at 25° C. in the dark and air from activated carbon filters was blown continuously through them. At suitable intervals (usually every 24 hr.) small samples (0·8 ml.) of the culture fluid and suspended bacteria were removed under sterile conditions and total cell counts made on a portion of the sample. A small quantity of methylene blue was added to 0·5 ml. of the suspension, and the bacteria allowed to take up the strain for at least ½ hr. This allowed easy counting in a haemacytometer. Total cell numbers were estimated per 25 ml. of the original culture.

Another aliquot (0·2 ml.) of the sample was also taken, sterilized by heating on a boiling water-bath for ½ hr. and then tested for phototoxicity by the cress root-growth test used in the previous perfusion work (Audus, 1951).

In this way a complete record was kept of the growth of bacterial numbers and changes in the toxicity of the medium for considerable periods under pure culture conditions.

Results

Fig. 1 shows the results of an experiment in which were studied two concentrations of 2:4-D (0·1 and 0·01 %). Two controls were also studied, i.e. one without 2:4-D and the other without mineral salts. Both these controls showed very little growth over the experimental period. There seems little doubt from this that the considerable growth in the presence of 2:4-D is due to the utilization of this compound as a source either of carbon or of energy for atmospheric carbon fixation.

Comparison of the growth curves in the two 2:4-D concentrations shows that the initial bacterial growth rate is independent of the herbicide concentration, but that growth ceases after about 3 days in the low concentration and continues up to about 15 days in the high concentration, although at a much slower rate. The maximum number of cells produced when growth ceases is approximately proportional to the initial concentration of 2:4-D in the medium, which is strong support for the contention that 2:4-D is being directly utilized as a fuel or carbon source.

However, toxicity changes present a less clear picture. Thus in the low 2:4-D concentration there was little significant change in toxicity detectable by the cress root test for the first 9 days, i.e. for 6 days after bacterial growth had apparently ceased; then suddenly all toxicity disappeared within 24 hr. This is the type of detoxication sequence observed in the soil perfusion experiments but there is no close correlation, as would have been expected, with bacterial proliferation. In the

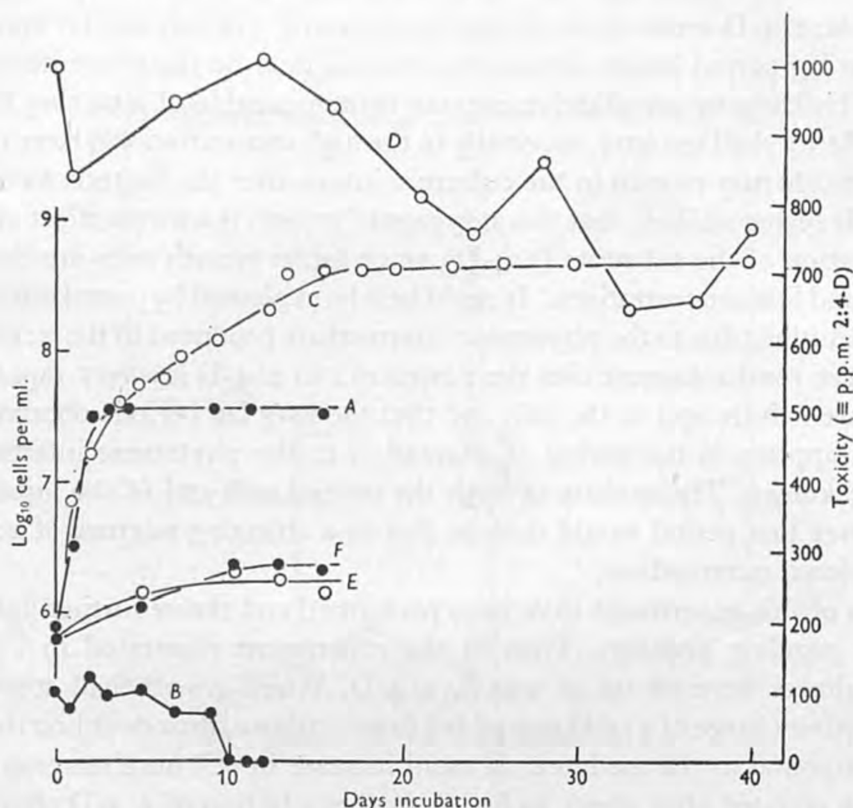

Fig. 1. Bacterial growth and 2:4-D detoxication curves for cultures of *B. globiforme* growing in a mineral salt solution containing two concentrations of 2:4-D. *A*, growth in 0·01 % 2:4-D; *B*, detoxication curve for 0·01 % 2:4-D; *C*, growth in 0·1 % 2:4-D; *D*, detoxication curve for 0·1 % 2:4-D; *E*, control growth with no mineral salts; *F*, control growth with no 2:4-D.

higher concentration (0·1 %) there was no significant change in toxicity during the phase of bacterial proliferation (0–16 days), but when growth had ceased a slow drop in toxicity became apparent and continued until the experiment was stopped after 40 days.

These results are susceptible of two explanations. The first, which we can dismiss as unlikely, is that the organism is growing on a non-phytotoxic impurity in the 2:4-D, the consumption of which does not affect the toxicity levels. Adaptation to 2:4-D breakdown then takes place over the observed lag periods (i.e. about 4 days in 0·01 % and about 16 days in 0·1 %). It would seem probable, from the relatively

smooth form of the growth curves, that this adaptation takes place in non-growing bacteria. These explanations seem unlikely, since the 2:4-D used was a specially pure sample. Any impurities could not have been present in sufficient amounts to give the not inconsiderable growth of Fig. 1.

The second and, it is felt, more plausible explanation is that the initial immediate growth is at the expense of the 2:4-D itself and that the absence of any marked change of toxicity during growth is due to the fact that the initial products of the attack on the 2:4-D molecule is an intermediate with a closely similar toxicity. The subsequent lag period before detoxication would then be the phase during which the cell is building up an adaptive enzyme system capable of attacking this intermediate. As we shall see later, especially in the high concentrations, large quantities of the herbicide may remain in the culture solution after the bacteria have stopped growing. It seems unlikely that this stoppage of growth is a direct effect of too high a concentration of the substrate (2:4-D), since initial growth rates are the same in both high and low concentrations. It could best be explained by postulating bacterial growth inhibitions due to the phytotoxic intermediate produced in the 2:4-D breakdown. These results suggest that the adaptation to 2:4-D is a very rapid process, both in pure culture and in the soil, and that the long lag periods observed before toxicity disappears is the period of adaptation to the phytotoxic intermediate of 2:4-D breakdown. The toxicity of both the treated soils and of the liquid culture medium over this period would then be due to a changing mixture of 2:4-D and this breakdown intermediate.

Variants of this experiment have been performed and throw further light on this somewhat puzzling problem. Thus in the experiment illustrated in Fig. 2 two parallel cultures were set up in 0·01 % 2:4-D. When growth had stopped after 5 days a further charge of 2:4-D was added to one culture, thus doubling the amount of this compound in the medium. A rapid increase in cell numbers was observed but growth stopped after about 24 hr. A further addition of 2:4-D after 10 days, before rapid detoxication had set in, gave a further similar increase in bacterial numbers. The interesting feature of this experiment is that the rapid disappearance of toxicity took place in both cultures at the same time, starting about 12 days after the commencement of incubation. Thus further additions of 2:4-D during the process of adaptation to the intermediate do not seem, with the concentrations used, to affect the course of the adaptation.

If, however, additional 2:4-D is not added to the culture medium until after detoxication is completed then quite a different picture is obtained. Thus, in the experiment of Fig. 3, a culture, started with 'adapted' organisms on a concentration of 0·01 % 2:4-D, completed its initial growth in just over 2 days. Detoxication commenced between the seventh and eighth days and was complete in between 9 and 10 days. On the fourteenth day a further quantity of 2:4-D was supplied to bring the concentration up to the original level of 0·01 %. There was a subsequent small but significant increase in bacterial numbers. The toxicity, which was

re-established on this further addition of 2:4-D, remained uncharged, however, for a further lag period of 4 days (cf. initial 7 to 8-day lag) before detoxication started. It was complete within a further 2 days.

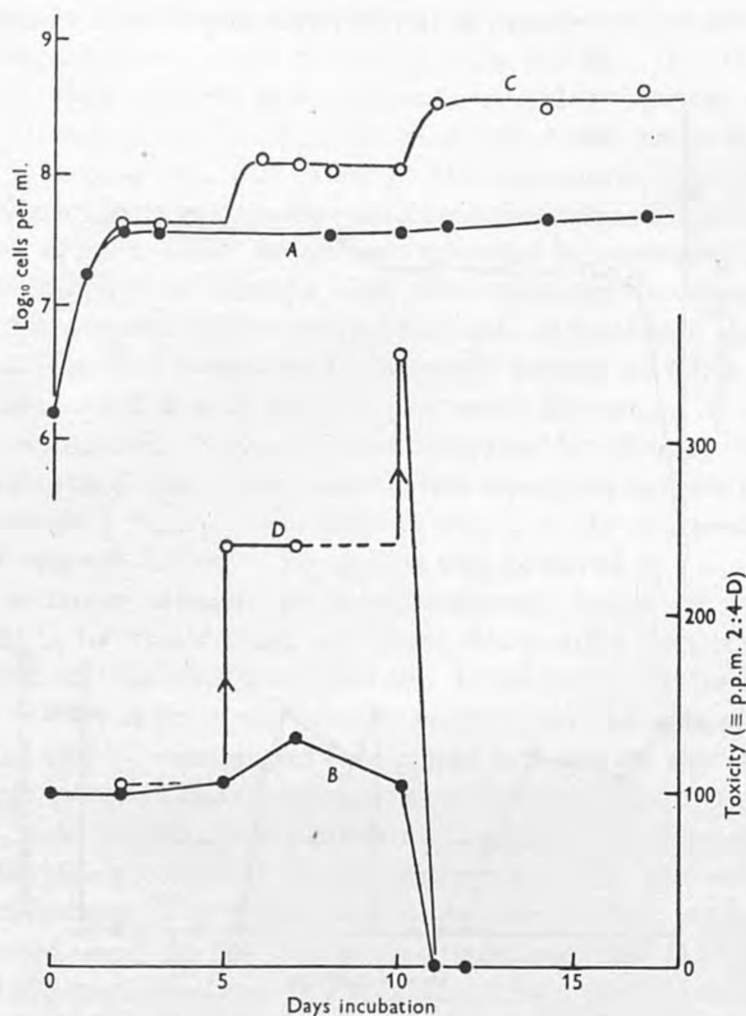

Fig. 2. Bacterial growth and 2:4-D detoxication curves for cultures of *B. globiforme* growing in a mineral salt solution containing 2:4-D. *A*, Growth curve corresponding to culture medium *B* receiving one charge of 2:4-D at the commencement of the experiment; *C*, growth curve corresponding to culture medium *D*, in which 2:4-D concentration was augmented twice before detoxication set in.

The appearance of this second lag after a first detoxication in pure culture is very surprising, since it does not happen in soil perfusion experiments. There, when a soil population has once become adapted to the breakdown of 2:4-D, this adaptation may be retained in the soil for very considerable periods of weeks or months, although it may eventually disappear (unpublished results). The differences may be due to breakdown in soil being caused by a complex population of more than one

type of organism of which *B. globiforme* is only one component. The maintenance of adaptation in the soil may be a property of one of the other bacterial components not yet isolated.

Whatever the situation in soil, one possible explanation of these liquid culture results is that the enzyme system of *B. globiforme* adapted to the breakdown of the

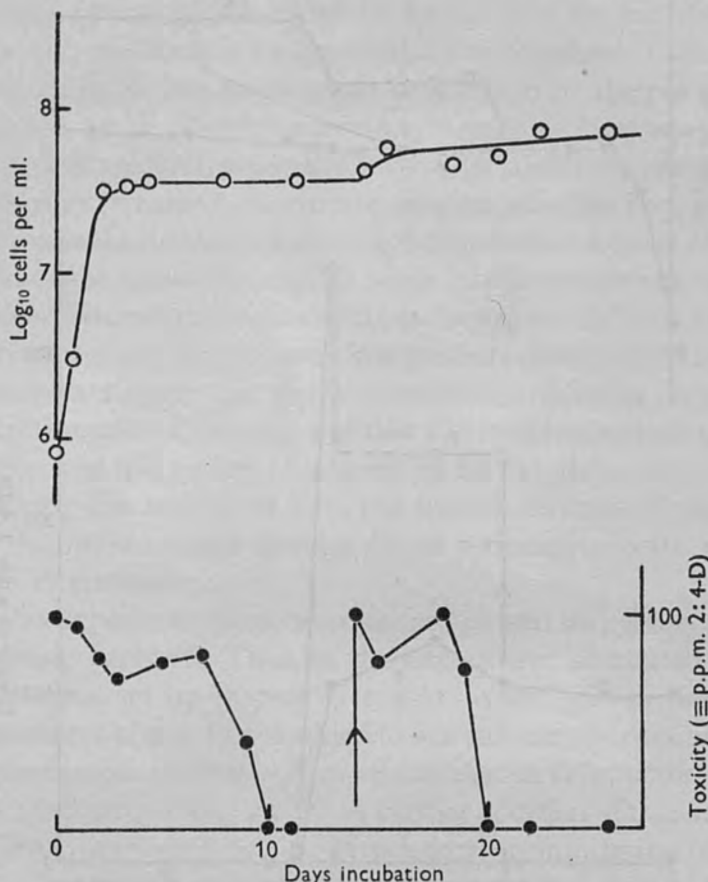

Fig. 3. Bacterial growth and 2:4-D detoxication curves for cultures of *B. globiforme* growing in a mineral salt solution containing 2:4-D, where a second quantity of 2:4-D was added to the culture medium after the first complete detoxication.

intermediate is unstable in the absence of its substrate, rapidly disappears after detoxication, and has to be re-formed when more intermediate arrives from the breakdown of 2:4-D.

This explanation of toxicity changes during growth in pure culture calls for direct experiments to isolate and identify this hypothetical, highly phytotoxic intermediate. Attempts have been made to do so by using the methods of paper partition chromatography.

CHROMATOGRAPHIC EXPERIMENTS

One major experiment has been performed using 750 ml. of a 0·1 % 2:4-D culture medium containing the usual mineral salts. This solution was sterilized and then innoculated with 15 ml. of bacterial suspension from a previous growth experiment. The suspension contained about 30 million cells per ml. It was subsequently incubated in the dark at 25° C. and vigorously aerated by blowing air through it. Bacterial growth and toxicity were measured as before and results similar to those obtained in Fig. 1 were obtained. After 40 days incubation, when toxicity of the medium still showed little change, the culture solution was acidified by the addition of about 10 ml. of conc. HCl. It was then extracted by successive shakings with ether. The combined ether extracts were then evaporated to dryness in partial vacuum until a brown crystalline residue remained. It was found that this residue consisted of at least two components, one easily soluble in warm 30% ethanol (presumably unchanged 2:4-D) while a very small fraction, a brown crystalline component, was insoluble. Separation was carried out by allowing the hot solution in 30% ethanol to cool. The brown material first separating out was filtered off and air dried. It weighed 0·3 mg., equivalent to 0·04% of the total amount of 2:4-D present in the original culture. This residue was dissolved in a small quantity of concentrated ammonia solution, the excess ammonia boiled off and a standard solution of 0·01 % by weight made up. From this a series of dilutions were prepared and a cress root toxicity test carried out. It was found that the brown residue had a toxicity at least as great and possibly greater than that of 2:4-D itself.

0·5 ml. of the 0·01 % solution was then placed as a spot on the starting line of a one-way paper partition chromatogram using Whatman No. 2 paper. A similar spot of 0·01 % 2:4-D solution was placed on a similar strip in the same tank. After suitable equilibrium precautions the chromatograms were run using propanol–ammonia–water solvent. The papers were subsequently dried, cut into transverse strips and assayed using the pea root section technique (Audus & Thresh, 1953). The results of the assay are shown in Fig. 4 where the growth of test root sections, as a percentage of the growth of control sections on untreated filter-paper, is plotted against R_F.

It will be seen that under these conditions 2:4-D runs with an R_F of about 0·75 and that it gives a very sharp spot.* No other growth response reaching significance level is seen on the chromatogram. With the unknown inhibitor, however, three probable spots are seen. First, there is one at R_F 0·75 corresponding to 2:4-D and indicating that approximately half of the brown material was unchanged 2:4-D. Then there is another marked inhibition at R_F 0·85. This is in advance of the

* In running 2:4-D on a chromatogram for this biological assay amounts much greater than this (i.e. 50 μg.) are not advisable. Such high amounts give complete inhibition at the centre of the spot and even the slightest tail (a fraction of a percent of the total) may give a high inhibition and thus a very broad spot.

2:4-D spot and therefore could not be explained by tailing. There can be little doubt that this is a phytotoxic compound quite distinct from 2:4-D. There is a third spot retained on the starting line and this again is taken to be a toxic compound distinct from 2:4-D. It might be suggested that this last spot is due to 2:4-D prevented from moving on the paper by some binding material extracted by the ether from the bacterial cell. This is most unlikely, since extracts of cultures made in the first few days of incubation do not show such a spot.

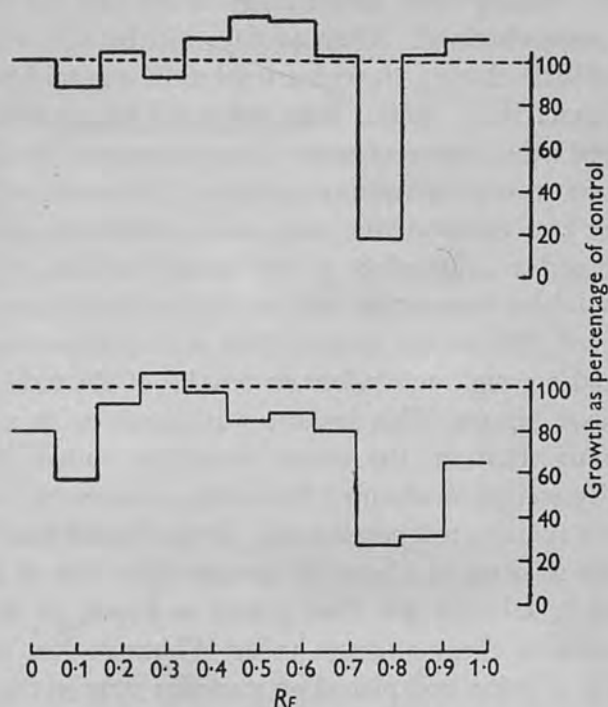

Fig. 4. Paper chromatograms of 2:4-D (top) and of the unknown substance (bottom) obtained from 40-day-old cultures of *B. globiforme* grown on a mineral salt solution containing 0·1 % of 2:4-D.

We are faced with the possibility of at least two phytotoxic intermediates in 2:4-D breakdown, and the next step is to attempt their identification and to study the kinetics of their production. Some preliminary observations of the latter have already been made by running small samples (0·5 ml.) of culture fluid from the standard experimen (25 ml. of culture medium at a concentration of 0·01 % 2:4-D) at intervals on similar chromatograms. Results so far are incomplete but they indicate that at this lower concentration both intermediates accumulate slowly at the expense of 2:4-D over the whole of the lag phase and then disappear rapidly during the short detoxication period.

It is very probable that the disappearance of phytotoxicity does not mark the complete breakdown of the 2:4-D molecule, presumably by oxidation to CO_2, water and ionic chlorine. The detection and identification of these smaller intermediate

fragments of the breakdown must rely on other sensitive non-biological methods. Such methods are at present being developed. An indication of the persistence of such compounds is given by the chromatropic acid test (Freed, 1948). This test is extremely sensitive and can detect quantities of 2:4-D of the order of 0·1 μg./ml.; but it is not specific to 2:4-D, being given by a wide range of other organic compounds of similar constitution. But culture solutions which have been completely detoxicated have been shown to give a definite colour reaction with this test, pointing to the persistence of non-toxic products.

In conclusion it should be stressed that these results are very largely preliminary, and the theories to explain them have been put forward more as working hypotheses than as established beliefs. They do suggest, however, that the picture previously depicted of the course of 2:4-D breakdown in soil and the implications of cross-perfusion experiments with its homologues will have to be reconsidered in the light of these new findings.

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