ABSTRACT

M.Sc. MEE LEE Microbiology

THE PRODUCTION OF IAA- AND GA-LIKE SUBSTANCES BY AZOTOBACTER VINELANDII

The presence of IAA- and GA-like substances in the culture supernatant of Azotobacter vinelandii was demonstrated using ethyl acetate extraction, followed by chromatography and bioassay. The IAA-like substance was found to be essentially identical with indoleacetic acid, while the GA-like substance was similar to, but not identical with gibberellic acid.

Relatively less IAA-like substance was produced in stationary cultures and in an NH_4^+-containing medium, compared with a nitrogen-free culture medium under shaking conditions. The active substance appeared increasingly from 2 to 6 days incubation, and it remained constant until the end of a 40-day incubation period. A possible intermediate was detected prior to the appearance of IAA.

Positive gibberellin-like responses became irreproducible, perhaps because the organism lost its ability to synthesize gibberellins under the cultural conditions employed.

THE PRODUCTION OF INDOLEACETIC ACID- AND GIBBERELLIN-LIKE SUBSTANCES BY *AZOTOBACTER VINELANDII*

by

MEE LEE

A thesis presented to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Microbiology
McGill University
Montreal

March 1970

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to Professor R. Knowles for his guidance, advice and encouragement throughout this study, and for his assistance in the preparation of this manuscript.

Special thanks are due to Miss S. L. Sim for her enlightening discussions and continued invaluable encouragement in various ways.

Sincere thanks are extended to Dr. M. A. Viswanathan of the Department of Plant Pathology, Macdonald College, for his advice and discussion concerning the problem of plant growth substances; to Dr. C. W. Schaller of the Department of Agronomy, University of California, for donating barley seeds for the bioassay of gibberellins; to Dr. J. Lasik, Mrs. C. Breckenridge and Mr. R. Brouzes for their interest and helpful suggestions; to Miss C. W. Chew and Mr. B. Yeboah-Asuamah for technical assistance in the bioassay of auxin; to Dr. L. A. Fischer and Mr. J. Lefebvre for translating German and French reviews into English.

The co-operation and diligence of the typist, Mrs. M. Couture are greatly appreciated.

The author gratefully acknowledges financial assistance from the Canada Department of Agriculture and from the Faculty of Graduate Studies and Research, McGill University.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF ILLUSTRATIONS

Figure Page

INTRODUCTION

Extensive efforts have been made to demonstrate the production of plant growth substances by microorganisms as well as by plants, and the effects of microorganisms on plant growth. Plant growth substances are organic compounds other than nutrients which, in minute amounts, exert special physiological effects on plants at locations remote from the place of synthesis.

The ability of fungi and bacteria to produce auxin type substances, such as indoleacetic acid (IAA), has long been recognized. Reports on the bacterial synthesis are widely dispersed, but Gruen (1959) has reviewed the literature on auxin production by fungi. Recently, the introduction of refined and improved techniques has given impetus to the search for new gibberellin (GA) or gibberellin-like (GA-like) substances which generally exist only in minute amounts. The bacteria which have been reported as being able to produce these substances are Arthrobacter, Agrobacterium, Azotobacter, Bacillus, Pseudomonas and Rhizobium. Among them, Azotobacter species are of great interest because they have frequently been claimed to influence plant growth and crop yields, and also

to exert a favorable effect on the development of seedlings or germinating seeds. These responses were attributed, by respective authors, to the production of plant growth substances by the cultures.

Several investigations, largely on Azotobacter chroococcum, have been reported since Raznitsyna (1938), using the Avena assay, demonstrated the production of an auxin type substance by pure cultures of Azotobacter chroococcum and Azotobacter agile. Subsequently, workers have shown the production of IAA by cultures grown in nitrogen-free medium. However, variable results were obtained with different strains of the cultures. Vancura and Macura (1960) found IAA in the supernatants of three out of the ten strains of Azotobacter chroococcum tested. Hennequin and Blachère (1966) tested 15 strains of Azotobacter chroococcum and 4 strains of Azotobacter vinelandii but detected IAA in only 6 strains of Azotobacter chroococcum.

Inconsistent reports appear in the literature on the production of GA-like substances by pure cultures of Azotobacter species. Vancura (1961), Lin (1963) and Burlingham (1965) detected GA-like substances in Azotobacter chroococcum cultures, but Hennequin and Blachère (1966) obtained negative results with 19 strains of Azotobacter including those supplied by Vancura and by

Burlingham. Breckenridge (1968) also found a variable result in four Azotobacter cultures.

In the present study, by using chromatography, chromogenic and bioassay methods, Azotobacter vinelandii (Mac.#459) was tested to examine its ability to produce both IAA- and GA-like substances, and to study the effects of certain cultural conditions on the production of these substances. Attention was particularly paid to the synthesis of the IAA-like substance and of possible intermediates.

LITERATURE REVIEW

1. The Use of *Azotobacter* as Bacterial Fertilizer

Bacterial fertilizers are preparations of living bacteria which are applied to soils or seeds to improve crop yields. Azotobacterin, an *Azotobacter* preparation, had long been claimed to stimulate agricultural crop yields in the USSR since 1937, when large-scale field experiments were first established. The conditions for maximum effectiveness have also been studied extensively in the USSR.

In plot and field trials outside the Soviet Union, the effectiveness of Azotobacterin became a very controversial problem. Allison *et al*. (1947), in their greenhouse experiments, found no significant effect on grasses or crucifers with either a Soviet preparation or a stock *Azotobacter* culture. Similar results were reported by Timonin (1948) on tobacco, oats and potatoes using his own *Azotobacter* preparation. Jessen (1949), however, obtained 11% increases in carrot yield and Wichtmann (1952) obtained a 13% increase in green matter of mustard. The increase of spring and winter wheat yields by *Azotobacter* inoculation was demonstrated by Gubanov (1953). Vancura and Macura

(1959) showed an increase of oat yield by inoculating the seeds with Azotobacter culture, but there was no increase in barley yield by the same treatment (Vancura et al. 1959). Rubenchik (1960) investigated the effect of Azotobacter on the germination of stored wheat and he found a greatly increased germination when the grains were allowed to imbibe an Azotobacter culture for 6 hours, but less increase with either longer or shorter imbibition periods. Jackson et al. (1964) observed that inoculation with Azotobacter accelerated the stem and leaf growth of tomato and shortened the time between bud appearance and petal fall. Denarié and Blachère (1966) emphasized in their finding that the growth of potato haulms and stems of tomato was accelerated by some, but not all strains of Azotobacter.

Allison (1947), in his review of the early Soviet work on Azotobacter inoculation of crops, suggested three possible mechanisms for the action of bacterial fertilizers: (1) nitrogen fixation, (2) defence of plants against pathogens and (3) production of plant growth substances.

There is no direct evidence to show that the stimulation of plant growth following inoculation of Azotobacter is due to the contribution of fixed nitrogen. However, it was suggested to be unlikely by Brown et al. (1964). In their experiments, under optimum laboratory

conditions, Azotobacter was found to divide every three hours and the nitrogen content in 10^5 Azotobacter was approximately 1 µg. Thus, they pointed out, providing that 10^5 Azotobacter in the rhizosphere could fix nitrogen at this rate, then the organism could not supply more than approximately 1 µg per gram rhizosphere soil. This amount was negligible compared with the available nitrogen in the agricultural soil. Mishustin and Naumova (1962) also believed that crop responses were not caused by nitrogen fixation, but were more likely to be caused by the bacteria producing heteroauxins or by their antagonisms to harmful microorganisms. Trizno (1954) found that inoculating spring cereals with Azotobacter increased their resistance to lodging. Nevertheless, Mishustin (1966) reported that the antibiotic substances produced by Azotobacter chroococcum (A. chroococcum) inhibited only the growth of fungi but not bacteria.

The third suggestion that Azotobacter influences the development of plants by producing growth substances is generally acceptable. The effects of the Azotobacter inoculation on tomato stem elongation and leaf size were reported to be similar to those of 5 µg gibberellic acid (GA_3) per plant (Jackson et al. 1964). Indoleacetic acid and GA-like substances have been isolated from certain Azotobacter cultures. These reports are tabulated in Table 1.

TABLE 1. Production of GA- and auxin-like substances by *Azotobacter* cultures

Year	Author	*Azotobacter* sp.	GA-like substance	Auxin-like substance
1938	Raznitsyna	A. chroococcum		auxin group
		A. agile		"
1939	Roberts and Roberts	A. chroococcum		heteroauxin
1942	Naundorf and Nilsson	A. chroococcum		"
1957	Smaly and Bershova	A. sp.		"
1958	Burger and Bukatsch	A. chroococcum		IAA
		A. vinelandii		"
		A. indicum		"
		A. beijerinckia		"
"	Krasil'nikov	A. chroococcum		heteroauxin
		A. vinelandii		"
1960	Vancura and Macura	A. chroococcum		IAA
1961	Vancura	A. chroococcum	GA_3	
1963	Lin	A. chroococcum	GA type	
1964	Vintika	A. chroococcum		heteroauxin
"	Zărnescu and Nită	A. chroococcum	gibberellic	auxinic
		A. vinelandii	"	"
"	Burlingham	A. chroococcum	GA-like	
1965	Romanow	A. chroococcum		IAA
"	Brakel and Hilger	A. chroococcum		"
1966	Hennequin and Blachère	A. chroococcum	-'ve	"
		A. vinelandii	-'ve	-'ve
"	Novikova and Irtuganova	A. chroococcum		heteroauxin
1968	Breckenridge	A. chroococcum	GA-like (±'ve)	
		A. vinelandii	" (")	
		A. agile	" (")	
"	Brown and Burlingham	A. chroococcum	3 GA-like	IAA

2. Production of Auxin-like Substances by Bacteria

Auxin, a classic plant growth regulator, has been quantitatively determinable by bioassay since Went (1928) successfully demonstrated the *Avena* curvature phenomenon. The naturally occurring IAA in human urine was first isolated and characterized as an active agent by Kögl and co-workers (1934). Since then many studies have been made of this plant growth substance. Recent investigations showed that the major auxin of higher plants is IAA. Many indole derivatives, other than IAA, have been shown to have biological activity, and thus, following the advent of chromogenic reagents and chromatographic separation, these techniques have become indispensible in conjunction with the bioassays. As many as 35 indole compounds have been distinguished according to their chromatographic behaviour (Stowe and Thimann 1954).

Substances with auxin-like biological activity occur widely among microorganisms (Breckenridge 1968). In the early twentieth century, there was a number of vague reports on the production of IAA or heteroauxin by *Escherichia coli* (Hopkins and Cole 1903), *Proteus vulgaris* (Herter and Broeck 1909), *Bacillus mycoides* (Frieber 1921), *Pseudomonas radiobacter*, *Bacillus subtilis*, *Bacterium* sp. (Boysen-Jensen 1931), root nodule bacteria (Thimann 1936), and *Phytomonas tumefaciens* (Brown and Gardner 1936).

Subsequent reports which appeared in the literature are reviewed chronologically below.

1938 Raznitsyna, using the Avena assay, identified an auxin type substance in a number of bacteria including A. chroococcum and A. agile.

1939 Roberts and Roberts, by the same method, demonstrated the production of heteroauxin by A. chroococcum and a number of actinomycetes, bacteria and molds from Indian soils. They concluded that the active substance was actually indole-3-acetic acid. However, the above bioassay was insufficient evidence for such a conclusion.

1942 Naundorf and Nilsson reported that A. chroococcum produced a heteroauxin and some substance which displayed a favorable effect on the formation of bacteroids in Bacterium radicicola cultures.

1955 Stowe showed that E. coli, Pseudomonas fluorescens and Agrobacterium tumefaciens produced a large quantity of IAA when they were grown in a medium containing either glucose or sucrose and 0.1% of tryptophan. Identifications were based on chromatography and bioassays.

1957 Smaly and Bershova detected the presence of heteroauxin in an A. chroococcum culture, but no experimental detail was given in this report.

1958 Burger and Bukatsch found IAA and an unidentified indole compound in several cultures of Azotobacter species. A. chroococcum and A. vinelandii produced IAA when grown in either tryptophan-containing or nitrogen-free medium, whereas A. indicum and A. beijerinckia produced IAA only when grown in tryptophan-containing medium.
Detailed experimental evidence was presented to support this study, which also indicated that there was a great variation between the Azotobacter species in respect to the appropriate conditions for IAA synthesis.

1958 Kaper and Veldstra showed that Agrobacterium tumefaciens synthesized IAA from tryptophan-containing medium, and tryptophol and 3-indolelactic acid were identified as additional products. From this and subsequent reports it appeared that IAA biosynthesis in microorganisms could occur by pathways different from that already reported for plants.

1958 Krasil'nikov investigated the ability of soil microorganisms to synthesize biotic substances. He indicated that some 16 species of bacteria including A. chroococcum (10 strains), A. vinelandii (2 strains), 3 species of Bacillus, 2 species of Bacterium and Mycobacterium, 4 species of Pseudomonas, a Micrococcus

sp. and 3 Rhizobium sp. were able to synthesize heteroauxin.

1960 Vancura and Macura, using paper chromatography and bioassay, detected IAA in stationary cultures of 3 strains of A. chroococcum which were grown in a nitrogen-free medium. The IAA was found to be converted to a biologically inactive indole-3-carbonic acid in the 40-day culture.

1961 Katznelson and Sirois reported the existence of IAA in 2-day shaking cultures of six species of Arthrobacter, when they tested the culture filtrates with the Avena coleoptile section test. The amount of the active substance was found to be greatly increased when tryptophan was added to the medium.

1962 Larsen et al. obtained IAA and an indole derivative in Acetobacter cultures.

1963 Galachyan found an auxinic substance in the culture filtrates of Xanthomonas beticola and Agrobacterium tumefaciens.

1963 Magie et al. traced the metabolic intermediates of the synthesis of IAA in a culture of Pseudomonas savastanoi which was cultivated with D,L-tryptophan-2-C^{14}. They suggested that the culture synthesized IAA via indoleacetamide as follows:

D,L-tryptophan ⟶ indoleacetamide ⟶ IAA.

1964 Sequeira and Williams suggested that an avirulent mutant of Pseudomonas solanacearum synthesized IAA from tryptophan via indolepyruvic acid.

1964 Zărnescu and Nită proposed that gibberellic and auxinic substances were widely produced by soil micro-organisms. Of the 300 strains tested, they classified them into three groups. Some synthesized only the auxinic substances (Bacillus subtilis, Bacillus megaterium and others), others synthesized only the gibberellic substances (Bacillus cereus, Pseudomonas aeruginosa and others) and the third group produced both auxinic and gibberellic substances (A. chroococcum, A. vinelandii, Pseudomonas fluorescens and some Actinomyces species).

Nevertheless, the bioassays used in their study for identification do not appear to be specific assays according to the literature.

1965 Chailakhyan et al. observed an auxin-like substance in Rhizobium culture.

1965 Brakel and Hilger showed that A. chroococcum produced IAA when tryptophan was added to the medium.

1965 Romanow identified IAA in the cultures of A. chroococcum when it was grown in either tryptophan-containing or NH_4Cl-containing medium. IAA was detected by means of chromatography and biotest.

1966 Chalvignac and Falca demonstrated the presence of 3-indoleacetic, indolacetyl-aspartic, indol-carboxylic and indollactic acid in the 6-day culture filtrate of Pseudomonas fluorescens which was grown in a tryptophan-containing medium.

1966 Hennequin and Blachère found IAA in the cultures of A. chroococcum, Arthrobacter sp., Xanthomonas sp. and Bacillus circulans, but not in A. vinelandii.

1966 Panosyan and Babayan detected a GA-like substance and heteroauxin in a number of phosphorous-mineralizing microorganisms of the water and soil of the Lake Seven area.

1966 Montuelle isolated six bacterial species (Bacillus and Pseudomonas) from various plant tissues. The cultures were found to be capable of synthesizing both auxinic and gibberellic growth factors when grown in synthetic media.

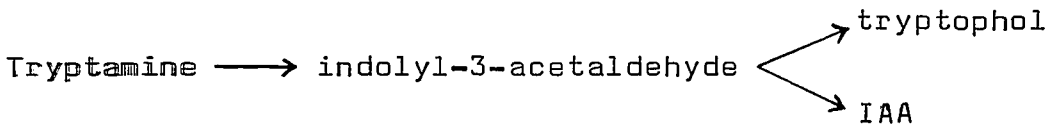
1966 Perley and Stowe demonstrated that tryptamine was an intermediate in the synthesis of IAA from tryptophan by Bacillus cereus.

1966 Novikova and Irtuganova obtained a heteroauxin in the culture filtrates of Bacillus megaterium, A. chroococcum and Chromobacterium aurantiacum. Among them A. chroococcum was found to be the most active and produced about 320 to 420 µg of IAA per 100 ml of the

four-week shaking culture.

Since this work included only chromatographic identification, it might be considered to be not adequate as either qualitative or quantitative evidence.

1968 Rigaud studied the metabolism of tryptamine in a Rhizobium culture. Since IAA and tryptophol were evident in the culture, they speculated that the biosynthesis of these indole compounds occurred as follows:



1968 Brown and Burlingham demonstrated the presence of an IAA in addition to three GA-like substances in a 14-day culture of A. chroococcum.

3. Production of GA-like Substances by Bacteria

A gibberellin was first discovered by Kurosawa (1926) from the fungus, Gibberella fujikuroi. The interest in this growth substance on a worldwide scale occurred in the early 1950's, and since then extensive studies have been made. As improved new techniques are developed, the number of naturally occurring giberellins and GA-like substances found in plants as well as in microorganisms is increasing.

Although gibberellin was first identified in a fungus, it does not seem to be widespread among fungi and actinomycetes. Curtiss (1957) obtained negative results when he searched for GA-like substances in 1000 fungi and 500 actinomycetes. So far, Gibberella fujikuroi remains the best source of gibberellic acid. Recent investigations showed that GA-like substances were produced by a great number of bacteria, as reported in the following literature.

1961 Vancura, using chromatography and bioassay, detected a GA-like substance in the filtrate of a stationary culture of A. chroococcum. The amount of the GA-like substance in a 17-day culture was reported to be as high as 20 µg/ml of the culture.

1962 Katznelson et al. found a minute amount of a GA-like substance in the one-week culture of Arthrobacter globiformis which had been shown to produce IAA in their previous report.

1963 Lin, using chromatography and rice seedling assay, showed the production of a GA-like substance in the supernatants of 5 strains of A. chroococcum.

1963 Galachyan reported the presence of GA-like and auxinic substances in 5-day cultures of Xanthomonas beticola and Agrobacterium tumefaciens.

1964 Nita demonstrated the production of gibberellinic substances by some spore-forming bacteria and by

Pseudomonas fluorescens.

1964 Zărnescu and Nită in their investigation of the stimulating action of microbial metabolites from 300 strains of soil microorganisms, concluded that a GA-like activity was present in the culture filtrates of A. vinelandii, A. chroococcum, Bacillus cereus, Pseudomonas aeruginosa and Pseudomonas fluorescens. The identification of the GA-like activity in their culture extract was conducted by a colorimetric method (Folin reagent) and Pea assay. Neither method is specific for gibberellins.

1964 Montuelle and Cheminais isolated some Bacillus and Pseudomonas species from various plant tissues. Gibberellins were detected in the 45-day culture filtrates of each of the microorganisms.

1964 Burlingham reported GA-like activity in an A. chroococcum culture filtrate.

1965 Katznelson and Cole extended their study of the production of GA-like substances by microorganisms and tested a number of commonly occurring soil bacteria. They found positive responses in the culture filtrates of Arthrobacter, Pseudomonas, Rhizobium, Agrobacterium, Bacillus, Cytophaga and Clostridium. The amount of the GA-like substance produced in shaking cultures ranged from 1 to 14 ug/l.

None was detected in the stationary cultures.

1965 Chailakhyan *et al*. detected both auxinic and GA-like substances in a *Rhizobium* culture.

1965 Sobieszczanski, in a study of the role of microorganisms in the life of cultivated plants, observed that *Chromobacterium aurantiacum*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Bacillus polymyxa* produced GA-like and auxin-like substances.

1966 Panosyan and Babayan showed the existence of an auxin and a GA-like substance in the culture filtrates of a number of phosphorous-mineralizing microorganisms of the water and soil of the Lake Seven area.

1966 Montuelle found that most of the bacteria isolated from various plant tissues were able to produce both auxinic and gibberellic growth factors. He believed that the gibberellic growth factors played a part in both bacterial and plant metabolism.

The synthesis of both growth factors *in vitro* by the microorganisms tested was confirmed by chromatography and the conventional *Avena* and dwarf pea assays. However, because of the lack of elaboration, his speculation that the growth factors played a part in both bacterial as well as plant metabolism was not convincing.

1966 Hennequin and Blachère did not detect a GA-like substance in any of the 40 strains of rhizosphere bacteria tested, including the A. chroococcum cultures supplied by Vancura and by Burlingham.

1967 Galsky and Lippincott found a GA-like substance produced by Agrobacterium tumefaciens.

1968 Breckenridge obtained a variable result when she searched for the GA-like substance in the culture supernatants of A. chroococcum (2 strains), A. vinelandii and A. agile.

1968 Brown and Burlingham demonstrated the production of three GA-like substances in the 14-day culture supernatant of A. chroococcum. The concentration of the GA-like substances ranged between 0.01 and 0.1 μg GA_3 equivalent/ml.

MATERIAL AND METHODS

1. Culture

Azotobacter vinelandii (Mac.#459) was used in this work. Cultures were grown in a N-free chemically defined medium, unless otherwise stated. The basal medium was a modified Burk's medium of the following composition (g/l):

Glucose	20.0	$CaSO_4.2H_2O$	0.1
K_2HPO_4	0.2	$MnSO_4.H_2O$	0.05
KH_2PO_4	0.8	$FeSO_4.7H_2O$	0.05
$MgSO_4.7H_2O$	0.2	H_3BO_3	0.05
NaCl	0.2	$Na_2MoO_4.2H_2O$	0.05

pH 6.9

It was autoclaved at 121^{o}C for 20 min. Glucose and potassium phosphates were autoclaved separately and added to the sterile medium, to give concentrations of 2% and 1% (total potassium phosphates) respectively, except in experiments on the effects of glucose and potassium phosphates on the production of plant growth substances. $FeSO_4.7H_2O$ was filter sterilized before adding to the autoclaved medium.

In experiments to study the effect of exogenous nitrogen on the production of plant growth substances,

$(NH_4)_2SO_4$ was autoclaved separately before addition to the basal medium to give a final concentration of 0.1%.

The culture was maintained routinely on agar slants of N-free basal medium. For inoculation, two sub-transfers were made. The culture was first transferred to 10 ml of liquid medium in a 50-ml Erlenmeyer flask, which was then incubated on a rotary shaker at 30°C for 24-36 hours. One milliliter of this culture was then transferred to another 50-ml flask containing 10 ml of medium. After 24 hours incubation, the whole of this inoculum culture was added to 500 ml of medium in a 2-liter flask.

Experimental cultures were incubated either on a rotary shaker (speed 140 rpm) or stationary.

The temperature of incubation in all experiments was 30°C.

Cultures were in general grown for 18 days, except for the study of plant growth substances production in relation to age of cultures.

2. Extraction Procedures

At the end of the incubation period, bacterial cells were spun down by centrifugation at 19,600×g for 30 minutes in a Sorval refrigerated centrifuge.

The culture supernatant was reduced to one-tenth of the original volume in a rotary evaporator at 40°C. The

concentrated supernatant was brought to pH 7.8 using 1N NaOH. It was then extracted twice with an equal volume of ethyl acetate (1 part culture supernatant : 2 parts ethyl acetate in total). The organic fraction was discarded so as to remove neutral and basic organic impurities.

The aqueous fraction was adjusted to pH 2.5 with 20% H_3PO_4. It was then extracted three times with half its volume of ethyl acetate. The three extracts were collected in an amber bottle and evaporated to dryness in a current of warm air from a hair-dryer. The residue was taken up with one-hundredth of the original culture volume of absolute ethanol, i.e., 5 ml of sample was finally obtained from each 500 ml culture.

This sample was re-adjusted to pH 5.4 with 1N NaOH and was then ready to use for chromatography and bioassay.

3. Chromatographic and Chromogenic Methods

Two chromatographic methods were used for purification and detection of plant growth substances. Further identification was carried out by chromogenic and bioassay methods.

3.1. Thin-layer chromatography

A thin layer of silica gel G of 0.5 mm thickness was made on a standard size of TLC glass plate. Before being used, the dried plate was activated for one hour at 85°C.

Test material was spotted on it and separation accomplished by one or more of the following four solvent systems:

A. Isopropanol : NH_4OH : H_2O = 10:1:1

B. Benzene : n-butanol : acetic acid = 70:25:5

C. n-Butanol : acetic acid : H_2O = 4:1:2

D. Butyl acetate saturated with water.

Following chromatography, initial examination was carried out by exposing the chromatogram to ultra-violet light. Occasionally, samples were separated by two-way chromatography. An aliquot of test material was spotted on the right lower corner of a plate, and it was developed in one of the four solvent systems. Subsequently, it was air dried completely and then run in the second direction in another solvent. Fluorescent spots were then examined. For use in the bioassay of gibberellin-like activity, the thin-layer plate was prepared as follows. The test material was streaked on about 3/4 of the plate, and a spot of standard GA_3 was applied on the same plate. The plate was developed and allowed to dry thoroughly, so as to ensure complete evaporation of solvent. The part with test material was divided into 10 horizontal strips according to Rf values. From each strip, the silica gel was scraped off separately and eluted with ethyl acetate. The ethyl acetate eluate was collected in a 50-ml Erlenmeyer flask and dried under vacuum. Bioassay was carried out in the flask to avoid the loss of sample during transfer.

3.2 Chromogenic reaction

Further identification was made by chromogenic reactions. The chromatogram was sprayed with one of the following reagents to detect both gibberellin-like and IAA-like substances.

1. Salkowski reagent.
2. Ehrlich reagent.
3. Ferric chloride in methanolic sulphuric acid.

Salkowski reagent was 1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$, diluted 1:1 with absolute ethanol (Nitsch and Nitsch 1955). It is dangerously corrosive and was thus preferably applied to thin-layer plates rather than to papers. Immediately after spraying, IAA gave a pale pink colour, which after heating at 60°C for 3 to 5 min, became brighter.

Ehrlich reagent was 2% p-dimethyl-aminobenzaldehyde in 2N HCl in 80% ethanol. After being sprayed, the chromatogram was heated at 60°C for 3 to 5 min. IAA was identified as a purple spot. According to Bentley (1961) and Stowe and Thimann (1954), this reagent specifically gave a rather red, purple or blue colour with indole derivatives.

Reagent 3 was methanol containing 0.05% $FeCl_3$ and 3% H_2SO_4. A characteristic fluorescence in sulphuric acid reagents has been used for detection of gibberellins

(MacMillan 1963; Bird and Pugh 1958). Addition of $FeCl_3$ to the methanolic sulphuric acid enables the reagent to be used for detecting both IAA and GA_3 (Vancura 1961). Following spraying, the chromatogram was heated at 60°C for 5 min. IAA appeared as a pink spot which did not fluoresce and GA_3 gave a blue-green fluorescence.

3.3. Paper Chromatography

Test materials were separated on Whatman No. 1 chromatography paper with two solvent systems which gave fairly satisfactory separation of GA_3, IAA, indole-3-acetamide (IAM), and indole-3-acetonitrile (IAN). Chromatograms were run descendingly in one of the following solvent systems:

A. Isopropanol : NH_4OH : H_2O = 10:1:1

D. Butyl acetate saturated with water.

Initial observation was made by fluorescent reaction as in the case of thin layer separation, and the same chromogenic reagents were used to locate GA-like and IAA-like substances. For use in bioassays, chromatograms, after drying thoroughly, were divided into 10 equal horizontal strips representing the sequence of 10 Rf values. The strips were used directly for bioassays.

4. Bioassay Methods

4.1. Barley endosperm α-amylase bioassay

The use of barley endosperm has been suggested for bioassay of gibberellins, which are capable of inducing the release of reducing sugar from barley endosperm (Paleg 1960). The quantity of reducing substances released is proportional to the logarithm of gibberellin concentration applied, and this proportionality has been confirmed to be satisfactory for assaying gibberellic acid (Nicholls and Paleg 1963).

The release of reducing substance is a result of gibberellin-dependent synthesis and secretion of α-amylase by the aleurone layer (Varner and Chandra 1964). On this basis, Jones and Varner (1967) suggested a direct measurement of α-amylase activity released by the barley endosperm as a gibberellin assay. They pointed out that the production of α-amylase was one step nearer to the primary site of action of gibberellin, therefore, measurement of α-amylase might be expected to be less susceptible to non-specific interference than would be measurement of reducing sugar released.

In the present work, Jones and Varner's method was found to be relatively simple and fairly reproducible for assaying GA_3. The range of this assay for GA_3 was 1×10^{-3} to 1×10^{-1} μg/ml. The procedure for measuring GA_3-induced α-amylase released is outlined below:

Preparation of endosperm.--Barley seed (White Naked Atlas) was kindly donated by Dr. C. W. Schaller of the Department of Agronomy, Agricultural Experimental Station, University of California, Davis, U.S.A. Barley seed was cut in half transversely to separate the embryo from the endosperm. The embryo-halves were discarded. The endosperm-halves were surface sterilized by soaking in a 2% sodium hypochlorite solution for 20 min. They were washed 3 to 4 times with sterile distilled water. The sterilized endosperm-halves were imbibed for three days on moist and sterile sand contained in a petri dish in a refrigerator.

Starch solution.--Starch solution was prepared by mixing 150 mg of potato starch, 600 mg KH_2PO_4 and 29.4 mg of $CaCl_2.2H_2O$ with distilled water to a total volume of 100 ml. The mixture was boiled for 1 min, cooled, then centrifuged for 10 min in a clinical centrifuge. The clear supernatant was decanted off and used for assay.

Iodine solution.--Iodine stock solution was prepared by mixing 6 g of potassium iodide and 600 mg of iodine in a volume of 100 ml with distilled water. Iodine solution used for stopping α-amylase reaction was prepared by adding 1 ml of the stock solution to 99 ml of 0.05 N hydrochloric acid.

Following the 3-day imbibition period, 10 half seeds were transferred to a 25 ml Erlenmeyer flask which contained

either the dried eluate from a strip of thin-layer chromatogram or a strip of paper chromatogram. Prior to the addition of seeds, 2 μM of acetate buffer (pH 4.8) and 20 μM of $CaCl_2$ in a final volume of 2 ml were added to each flask. The half seeds were then incubated for 36 hr at 25°C with continuous shaking in a water bath shaker.

At the end of the incubation period, the medium was decanted into a centrifuge tube, and washings totalling another 3 ml of distilled water were added. The suspension was then centrifuged for 5 min in a clinical centrifuge. For the assay of α-amylase activity, 0.2 ml of the test supernatant fraction was taken and made up to 1 ml with distilled water. The reaction was started by adding 1 ml of the starch substrate to it. After a period of 10 min, the reaction was stopped by adding 1 ml of iodine reagent. Five milliliters of distilled water was added to this final reaction mixture. The optical density was read at 655 mμ. The decrease in OD was directly proportional to the quantity of α-amylase present in the reaction mixture, and to the concentration of GA_3 applied.

4.2. Lettuce hypocotyl and radicle assay

Lettuce hypocotyl assay is one of the commonly used methods for detecting GA_3. There is a linear relationship between hypocotyl elongation and the logarithm of the GA_3 concentration (Frankland and Wareing 1960). Though there

is some doubt as to the specificity of this assay for GA_3, the merit of this test lies in its simplicity and the seedlings are small enough to be grown directly on the test solution.

Radicle growth of lettuce had also been reported to be promoted by gibberellins. Especially active was GA_3 which, at a concentration of 100 parts per billion, caused a 40% increase in root growth (Paleg et al. 1964).

The sensitivity to auxin is generally higher in young roots than in young stems and coleoptiles, therefore several attempts have been made to develop an auxin test using roots as an assay system. In general, roots are known to respond to very low concentrations of auxin by a slight increase in growth rate, and inhibition occurs at higher concentration. Thus the prevalent response of intact root to exogenous auxin is either a retardation or a stimulation in elongation, depending on the species. Burström (1950, 1957) presented an extensive study on the applicability in general of roots as a test object for auxin. He emphasized that cell elongation proceeded via two phases. The first was characterized by a dissolution of cell wall material; the second by the intussusception of new cell wall material. In roots, exogenous auxin stimulated the first phase, but its effect on the second phase, if any, was a retardation, the magnitude of which was dependent on

the concentrations of auxin, Ca^{++}, and other factors. If the effect on the second phase was slight, the overall response might be a stimulation; otherwise, there might be a retardation. Nevertheless, if response of growth in roots of a species is indeed caused by the added auxin, it may serve, in principle, as a test object for auxin activity irrespective of the mechanism and physiological significance of the response.

In this work, lettuce seeds (Lactuca sativa, Grand Rapid) were used for assaying GA- and IAA-like substances.

Seeds were germinated for 48 hours on moist filter paper, and seedlings with uniform radicles (6-8 mm in length) were then selected for assay. Each strip of paper chromatogram was placed in the center of a petri dish containing a piece of filter paper (dia = 7 cm) moistened with 2 ml of distilled water. Ten selected seedlings were transplanted and arranged in a row on each strip of paper chromatogram. They were then grown in the growth chamber with continuous illumination (light intensity 1500 ft. candle) at $25^{o}C$. One milliliter of distilled water was added per day to maintain the water content.

After 72 hours, lengths of radicles and hypocotyls were measured to the nearest mm.

4.3. Avena coleoptile straight growth test

IAA is characterized mainly by its ability to influence the rate of cell elongation. The etiolated coleoptile of Avena sativa is a classical test object in IAA study. Several modifications of Avena coleoptile straight growth tests have been used since the method was worked out by Bonner (1933). Generally speaking, IAA stimulates the elongation of the coleoptile proportional to the concentration of IAA within a certain range, but inhibition results from higher concentrations of IAA.

This had long been considered to be a specific test for IAA, until the existence of gibberellins was recognized, which also show auxinic activity in this assay. However, this is still regarded as a conventional test for IAA, providing the test material is subjected to chromatographic separation prior to the test. The Avena coleoptile straight growth test is technically simple and less influenced than is the Avena coleoptile curvature test by differences in polar transportability of the various auxins.

Cultivation of test plant.--Brighton oat (Avena sativa), supplied by the Central Experimental Farm, Ottawa, was used in the present work.

The hull-less Brighton oats were surface sterilized with a 2% NaOCl solution for 30 min. After washing thoroughly 3 to 4 times with sterile water, they were

soaked for 2 hours in sterile water. Pre-soaked seeds were then lined up on sterile and moistened vermiculite, with embryo up and pointing in the same direction. They were germinated in darkness at 25°C. At this stage, the seedlings were illuminated with 3 hours of red light in each 24 hours, in order to suppress the elongation of the internode and stimulate the growth of the coleoptile. After two and a half days, seedlings with a uniform length (25-30 mm) were selected for assay.

Test procedure.--All procedures, except the final measurement, were carried out under red light.

Different lengths of coleoptile sections have been used in published assays. Since long sections show pronounced geotropic curvature, they are difficult to measure. Sections of 4-5 mm, cut 3 mm below the tip, were reported to show greatest response to IAA and the geotropic curvature of these sections was negligible (Nitsch and Nitsch 1956).

For the present assay, test material was separated by paper chromatography. Each strip of chromatogram was cut into pieces and placed in a 25-ml Erlenmeyer flask containing 0.5 ml of 0.02 M phosphate buffer (pH 5.1), 0.5 ml of 4% sucrose solution and 1 ml of distilled water.

A 5-mm section of coleoptile was used which was cut 3 mm below the tip. Ten coleoptile sections were added to each flask and the flasks were placed in a rotary shaker.

Sections were allowed to grow for 24 hours in the dark at $25^{o}C$.

Lengths of coleoptiles were measured by means of an overhead projector. Results were expressed as elongation over control in mm and in some cases as equivalent IAA concentration.

RESULTS AND DISCUSSION

PART I - PRODUCTION OF IAA-LIKE SUBSTANCE BY *AZOTOBACTER VINELANDII* (MAC. #459)

1. Production of IAA-like Substance in 18-day Shaking Culture

The ethyl acetate extract was examined by chromatographic methods for an IAA-like substance which was further identified by bioassays.

1.1. Preliminary detection of IAA-like substance by thin-layer chromatography and chromogenic reaction

A spot of the ethyl acetate extract (80-100 µl) was co-chromatographed with the standard IAA on a thin-layer plate. Four solvent systems were used (Table 2). The test material showed a very distinct fluorescent spot at Rf 0.37 after development in solvent A, and at Rf 0.85 in solvent B. These Rf values corresponded to those of the reference IAA in the same solvent systems. The unknown fluorescent spot located similarly to the reference in the other two solvent systems. It located at Rf 0.88 in solvent C, in which IAA gave an Rf 0.90. It fluoresced at Rf 0.40 in solvent D, while IAA located at Rf 0.18 to 0.40.

TABLE 2. Thin-layer chromatographic and chromogenic behaviour of the extract of the 18-day culture and of reference compounds

	Rf in solvent systems*				Fluor-escence	Chromogenic reagents**		
	A	B	C	D		1	2	3
Test material	0.37	0.85	0.88	0.40	bluish-purple	mauve	purple	pink
IAA***	0.37	0.85	0.90	0.18-0.40	pale purple	pink	purple	pink
IAM	0.90	0.45	0.78	0.15	-	purple	violet	purple
IAN	0.95	0.85	0.92	0.90	-	greenish-blue	pinkish-orange	blue

*Solvent systems:

A Isopropanol : NH_4OH: H_2O = 10:1:1

B Benzene : n-butanol : acetic acid = 70:25:5

C n-Butanol : acetic acid : H_2O = 4:1:2

D Butyl acetate saturated with water

**Chromogenic reagents:

1. Salkowski reagent
2. Ehrlich reagent
3. Ferric chloride in methanolic sulphuric reagent

***Reference compounds:

IAA indoleacetic acid

IAM indoleacetamide

IAN indoleacetonitrile

Gibberellins do not fluoresce, whereas IAA gives a bluish-purple fluorescence. However, this preliminary test is used with reservation, since Azotobacter vinelandii has been reported to produce fluorescent pigments (Johnstone and Fishbein 1956).

For further identification, the chromatograms were sprayed with chromogenic reagents (Figure 1). It was observed that the spot showed a mauve color when sprayed with Salkowski reagent, and appeared purple with Ehrlich reagent. Furthermore, it was as pink as IAA when sprayed with the less specific Fe Cl_3-methanolic sulphuric reagent. The results are summarized in Table 2.

On the basis of the above evidence, therefore, the unknown spot was likely to be an IAA-like substance.

1.2. Preliminary detection of IAA-like substance by paper chromatography and chromogenic reaction

A paper loaded with spots of IAA and the test material was developed in solvent A (Figure 2). The test material gave a bluish-purple fluorescent spot at Rf 0.35 which corresponded to that of the IAA. This fluorescent spot became visible after spraying with one of the three chromogenic reagents. It appeared mauve with Salkowski, purple with Ehrlich and mauve with $FeCl_3$-methanolic sulphuric reagent.

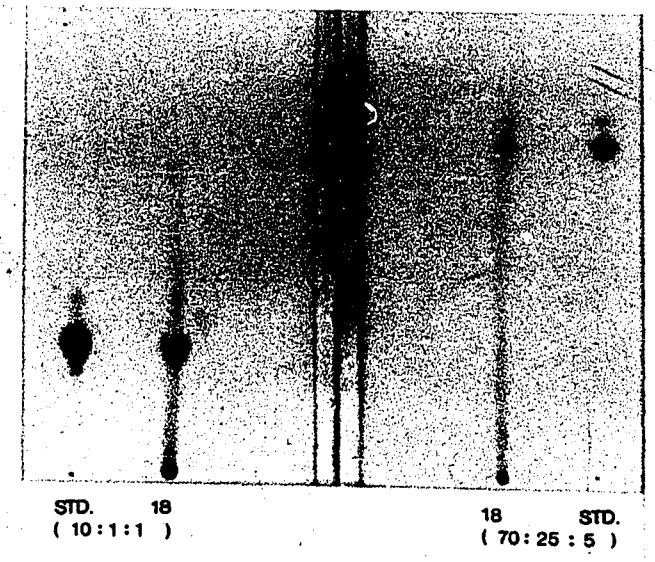
Figure 1. Thin-layer chromatograms of the extract of the 18-day culture (18) in four solvent systems. Standard IAA (STD) was co-chromatographed as reference.

Solvent A Isopropanol:NH_4OH:water = 10:1:1

Solvent B Benzene:n-butanol:acetic acid = 70:25:5

Solvent C n-Butanol:acetic acid:water = 4:1:2

Solvent D Butyl acetate saturated with water = Butyl - H_2O


A
B
STD. 18
(10 : 1 : 1)
18 STD.
(70 : 25 : 5)
C
D
STD. 18
(4 : 1 : 2)
18 STD.
(Butyl – H2O)

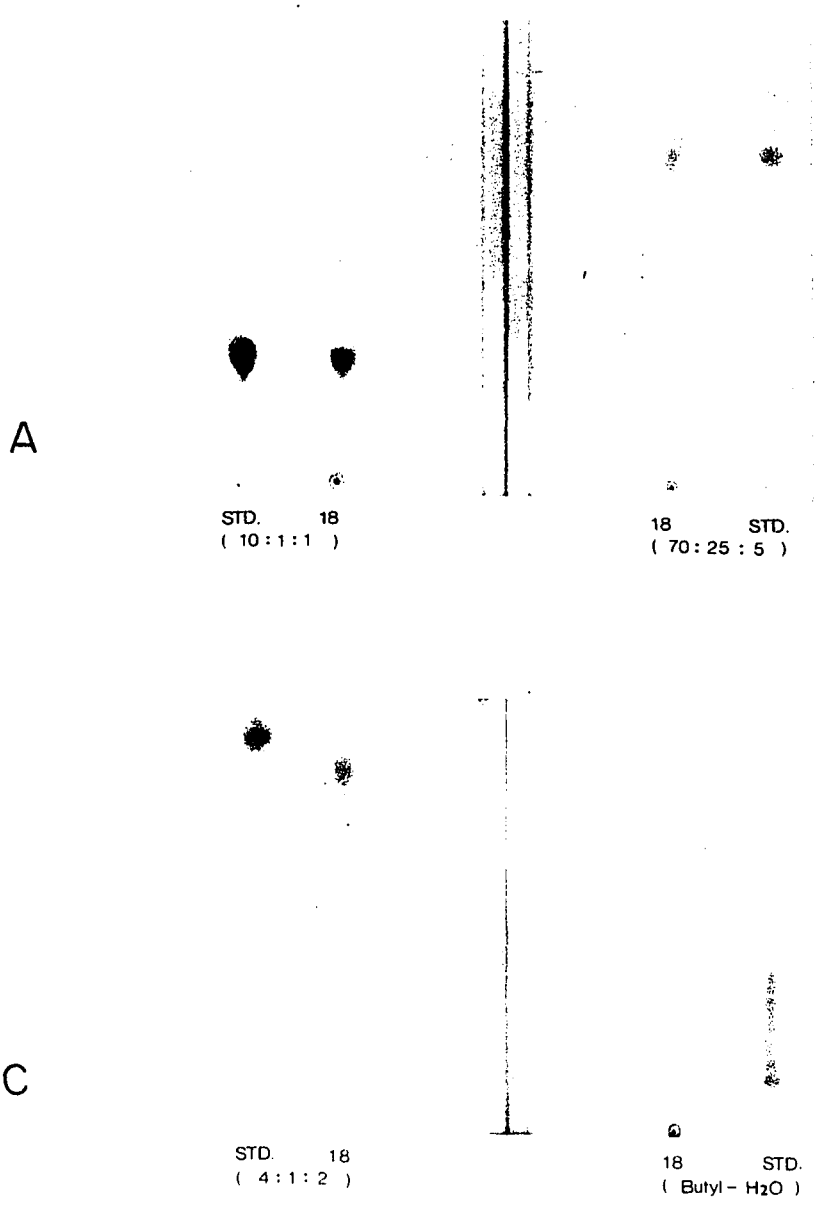
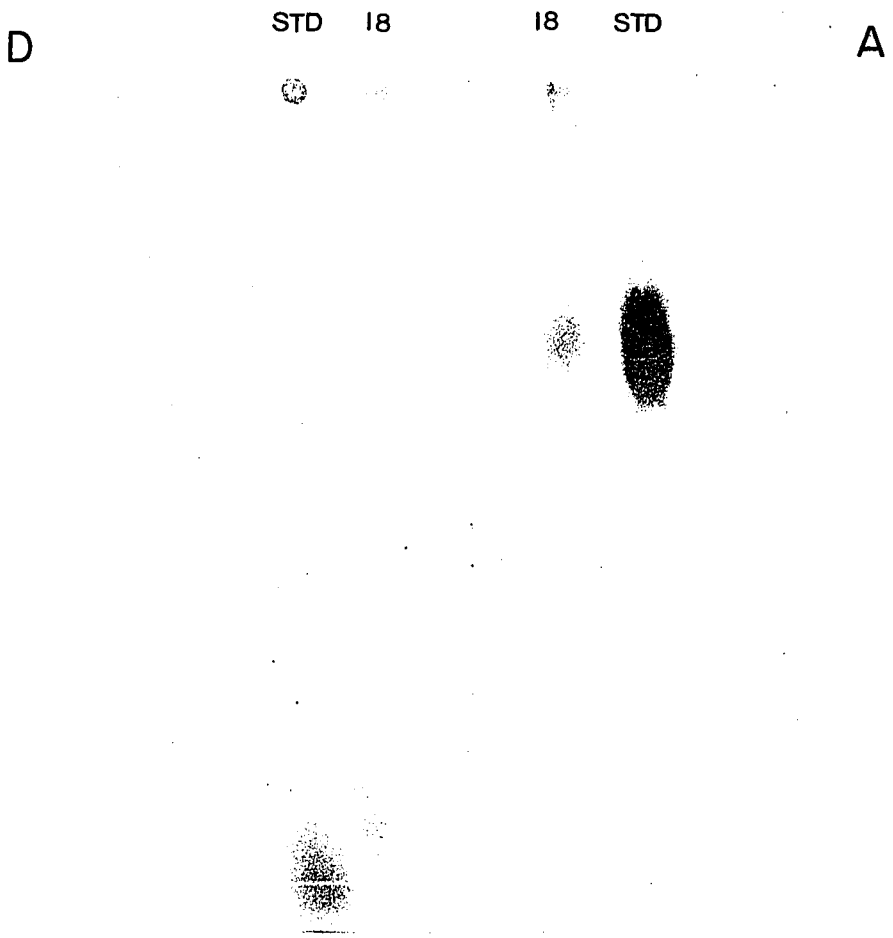

A
STD. 18
(10 : 1 : 1)
B
18 STD.
(70 : 25 : 5)
C
STD. 18
(4 : 1 : 2)
D
18 STD.
(Butyl – H2O)

Figure 2. Paper chromatograms of the extract of the 18-day culture (18) in two solvent systems. Standard IAA (STD) was co-chromatographed as reference.

Solvent A Isopropanol:NH_4OH:H_2O = 10:1:1

Solvent D Butyl acetate saturated with water = Butyl - H_2O


D
STD
18
18
STD
A

When solvent D was used to separate the test material (Figure 2), the fluorescent spot was observed to have an Rf value of 0.9 and IAA located at Rf 0.95. The fluorescent spot gave a chromogenic reaction identical to that of the spot which appeared at Rf 0.35 in solvent A.

Results from the paper chromatography, summarized in Table 3, were in general agreement with those from the thin-layer chromatography.

1.3. Paper chromatographic separation and further characterization by bioassays

1.3.1. Lettuce assay of sample

A paper chromatogram loaded with 1 ml of the ethyl acetate extract was developed in solvent A. Horizontal strips representing 10 Rf values were used directly for assay. It was observed that there were stump-like shortenings of roots with remarkable ramifications in the seedlings growing on the regions (Rf 0.35) carrying the fluorescent spot which was suspected to be an IAA-like substance on the basis of its chromogenic reaction (Figures 2 and 3A). No effect on the lettuce hypocotyls was observed. In an experiment using half the amount of the test material, inhibition of root growth was still highly significant. The variations of this assay in each experiment were too great to permit quantitative estimation of the active substance.

TABLE 3. Paper chromatographic and chromogenic behaviour of the extract of the 18-day culture and of reference compounds

	Rf in solvent systems*		Fluor-escence	Chromogenic reagents**		
	A	D		1	2	3
Test material	0.35	0.90	bluish-purple	mauve	purple	mauve
IAA***	0.35	0.95	purple	pink	violet	mauve
IAM	0.70	0.85	-	purple	violet	violet
IAN	0.86	0.98	-	blue	mauve	grey-blue

*Solvent systems:

A Isopropanol : NH_4OH : H_2O = 10:1:1

B Benzene : n-butanol : acetic acid = 70:25:5

C n-Butanol : acetic acid : H_2O = 4:1:2

D Butyl acetate saturated with water

**Chromogenic reagents:

1. Salkowski reagent
2. Ehrlich reagent
3. Ferric chloride in methanolic sulphuric reagent

*** Reference compounds

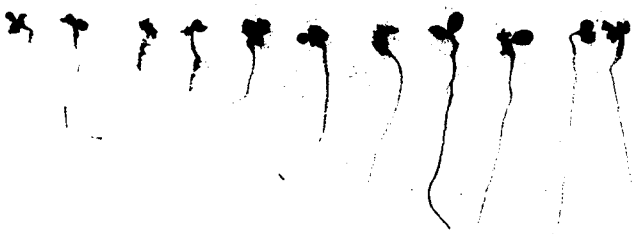
IAA indoleacetic acid

IAM indoleacetamide

IAN indoleacetonitrile

Figure 3. Effects on shortening of lettuce (Lactuca sativa, Grand rapid) roots (A) and elongation of oat (Avena sativa, Brighton) coleoptiles (B) by the extract of the 18-day culture separated by paper chromatography in solvent system A (Isopropanol: NH_4OH : H_2O = 10:1:1).

LETTUCE ASSAY

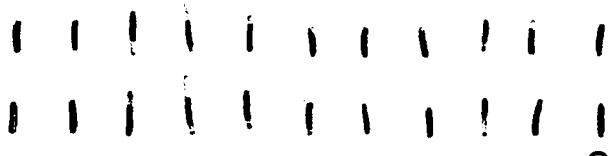


A

Rf 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 C

PC Solvent 10 : 1 : 1

AVENA COLEOPTILE ASSAY



B

Rf : 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 C

PC : Solvent 10 : 1 : 1

The fact that the same phenomenon was observed when IAA solutions of 10^{-4} to 10^{-6} M were used for assay, further indicated that the unknown spot showed auxinic activity.

1.3.2. *Avena* coleoptile assay of standard IAA

Solutions of IAA with concentrations from 10^{-2}M to 10^{-8}M were assayed as reference (Figure 4). The dosage response curve reached a peak at a concentration of about 10^{-4} to 10^{-5}M and showed inhibition at higher concentrations.

1.3.3. *Avena* coleoptile assay of sample

One milliliter of the ethyl acetate extract was separated in solvent system A. The chromatogram being cut into 10 equal strips was used for assay. Significant elongation of the coleoptiles was induced by strips corresponding to the region of the fluorescent spot (Figures 2 and 3B). This result was in accord with those using chromatographic and chromogenic methods. It was concluded, therefore, that an IAA-like substance was produced by *A. vinelandii*. When a blank chromatogram was assayed, simultaneously no positive response was observed (Figure 5).

For further confirmation of the auxinic activity of the test material, it was separated in solvent D. If the positive response in *Avena* assay was an artifact due to an

Figure 4. Response of oat (*Avena sativa*, Brighton) coleoptiles to IAA.

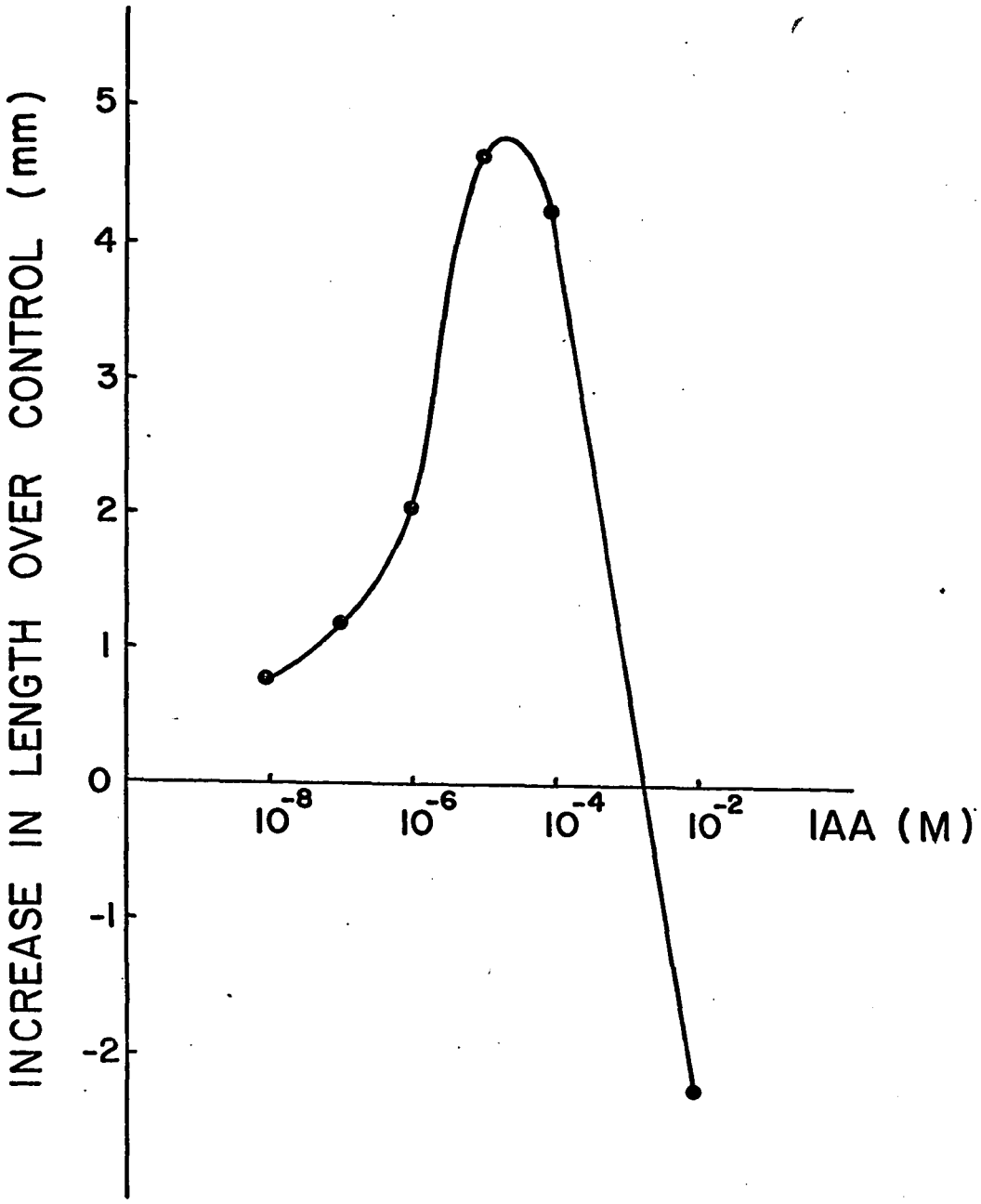
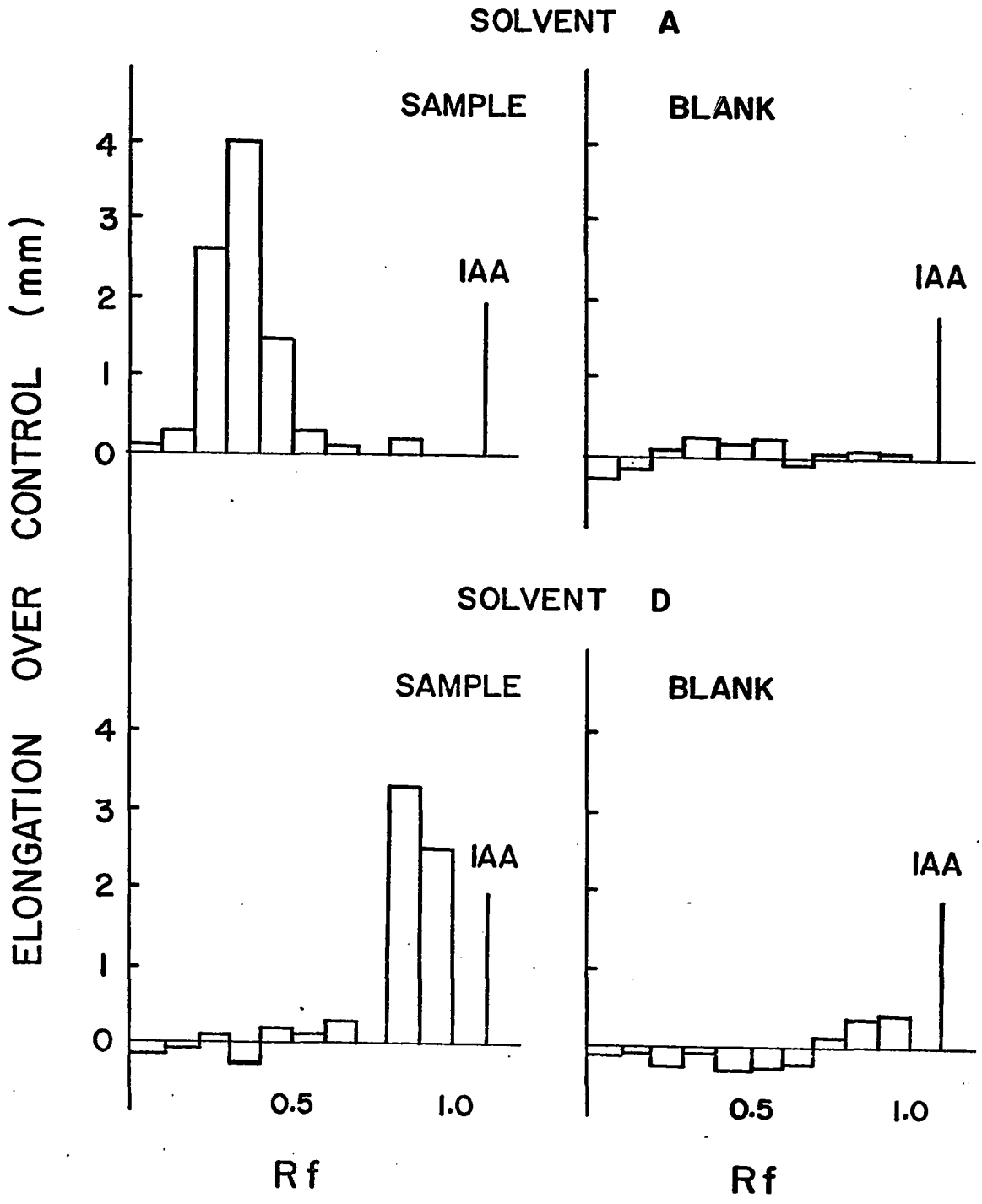

INCREASE IN LENGTH OVER CONTROL (mm)
5
4
3
2
1
0
-1
-2
10^{-8}
10^{-6}
10^{-4}
10^{-2}
IAA (M)

Figure 5. Histograms showing activities in the oat (*Avena sativa*, Brighton) coleoptile assay of segments from paper chromatograms of the 18-day culture extract, separated in two solvent systems.

Solvent A Isopropanol: NH_4OH : H_2O = 10:1:1

Solvent D Butyl acetate saturated with water

Blank chromatograms developed in the corresponding solvent systems are shown as control.
Vertical line represents the response of 10^{-6} M IAA in the corresponding assay.


SOLVENT A
SAMPLE
BLANK
IAA
IAA
4
3
2
1
0
ELONGATION OVER CONTROL (mm)
SOLVENT D
SAMPLE
BLANK
IAA
IAA
0.5
1.0
0.5
1.0
Rf
Rf

ammonialysis or any other reaction with solvent A, the same response should not have been obtained using another solvent system. As shown in Figure 5, responses were similar when the extract was separated in the two solvent systems. A blank of solvent D was also run as control.

1.4. Quantitative estimation of the active substance

Since the chromatograms were cut into ten equal strips, an active substance might have located in more than one strip. For the quantitative estimation of the active substance in the Avena assay, the responses in the areas of the histogram from Rf 0.3 to 0.5 were summed up and the total response then calculated from the dosage response curve (Figure 4). The active substance was equivalent to 8.0×10^{-6}M IAA.

2. Production of IAA-like substance under Different Conditions

Experiments were carried out to examine the effect of the following cultural conditions on the production of the IAA-like substance.

1. Shaking and stationary culture
2. N-free and NH_4^+-containing medium
3. Content of potassium phosphates
4. Illumination

In this section, test materials were exclusively separated by paper chromatography, developed in solvent system A and bioassayed using largely the conventional Avena coleoptile method. The lettuce assay was used occasionally.

2.1 Shaking and stationary culture

In the chromatogram (Figure 6), the IAA-like substance of the stationary culture appeared as a very dim spot with Salkowski spray. Its Rf value corresponded to that of the IAA, but it did not fluoresce. This could have been due to a low concentration of the active substance which was not sufficient to give a fluorescence or sharp color reaction.

In extracts from both stationary and shaking cultures, a positive response was obtained in the Avena coleoptile test. Less active substance was produced by the stationary culture if compared to the shaking culture (Figure 7).

2.2. N-free and NH_4^+-containing medium

In general, exogenous combined nitrogen is utilized preferentially by Azotobacter while nitrogenase is repressed. No pathway of IAA biosynthesis in Azotobacter has been reported. An experiment was established to study the effect of exogenous NH_4^+ on the production of the IAA-like substance.

Figure 6. A thin-layer chromatogram of the extracts of 18-day cultures under different conditions. The shaking culture (18), stationary culture (Sta), shaking culture in low potassium phosphates medium (PK), and shaking culture in NH_4^+ medium (N). References are indoleacetic acid (IAA), indoleacetamide (IAM) and indoleacetonitrile (IAN).

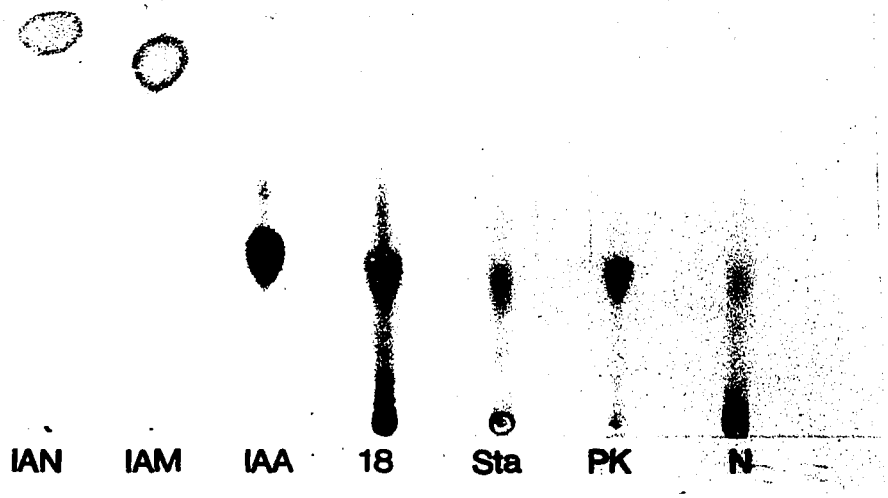
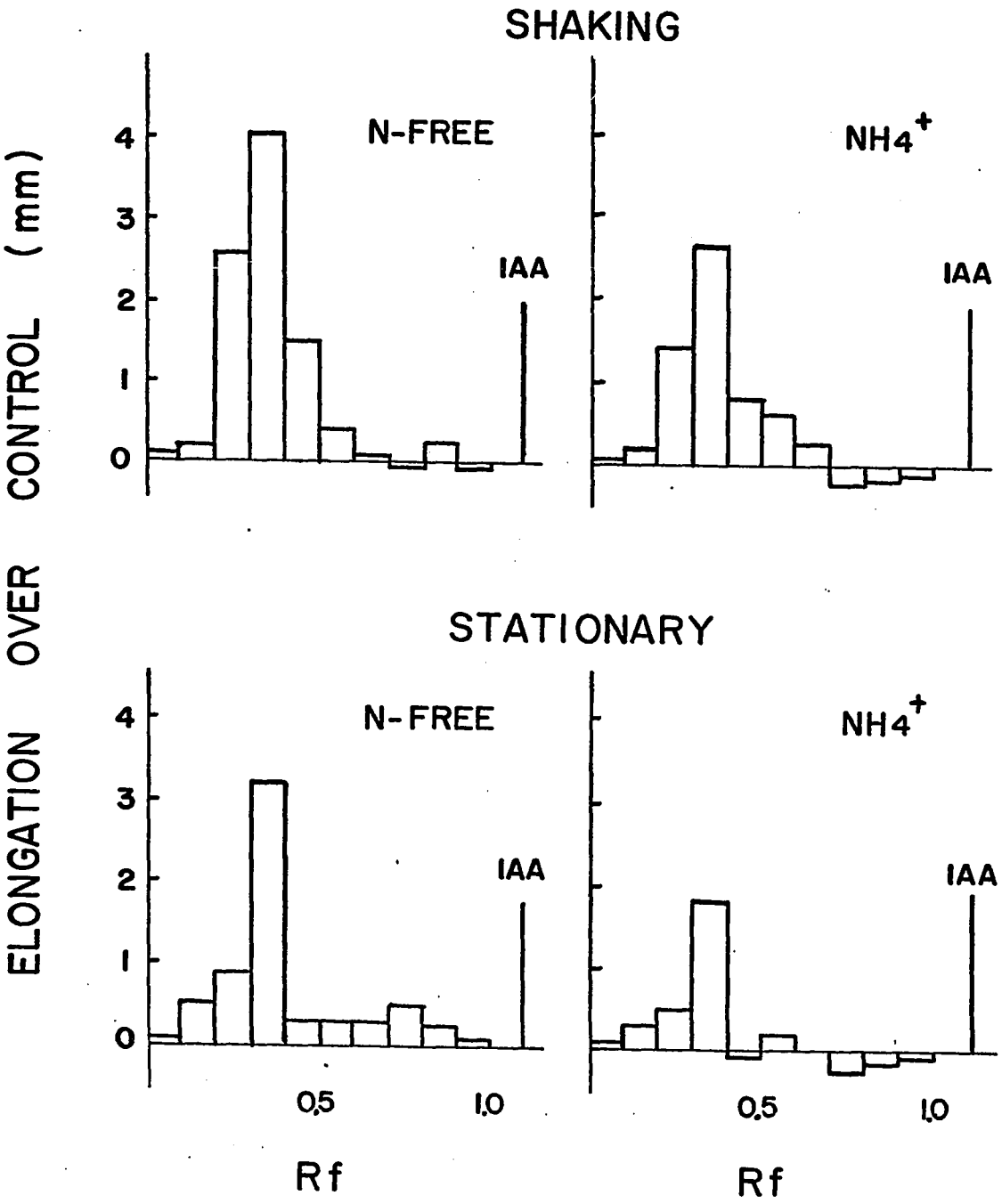

IAN
IAM
IAA
18
Sta
PK
N
TLC Solvent 10 : 1 : 1
Salkowski Reagent

Figure 7. Histograms showing comparative activities in the oat (Avena sativa, Brighton) coleoptile assay of segments from paper chromatograms of the extracts of the 18-day shaking and stationary cultures grown in N-free and in NH_4^+-containing media.

Vertical line represents the response of 10^{-6}M IAA in the corresponding assay.


SHAKING
N-FREE
NH4+
IAA
IAA
4
3
2
1
0
ELONGATION OVER CONTROL (mm)
STATIONARY
N-FREE
NH4+
IAA
IAA
4
3
2
1
0
0.5
1.0
0.5
1.0
Rf
Rf

In the extract from a shaking culture grown in the NH_4^+-containing medium, a very dim grey spot at Rf 0.37 was observed after spraying with Salkowski reagent (Figure 6). It was suspected that it might not be an IAA-like substance, however, it showed a positive response in the Avena coleoptile assay (Figure 7). The response was about one-sixth that of the sample from the culture growing in N-free medium.

In stationary culture (Figure 7), the same phenomenon was obtained. The active substance was detectable only by the Avena assay and was relatively less in an NH_4^+-containing culture than in a N-free culture.

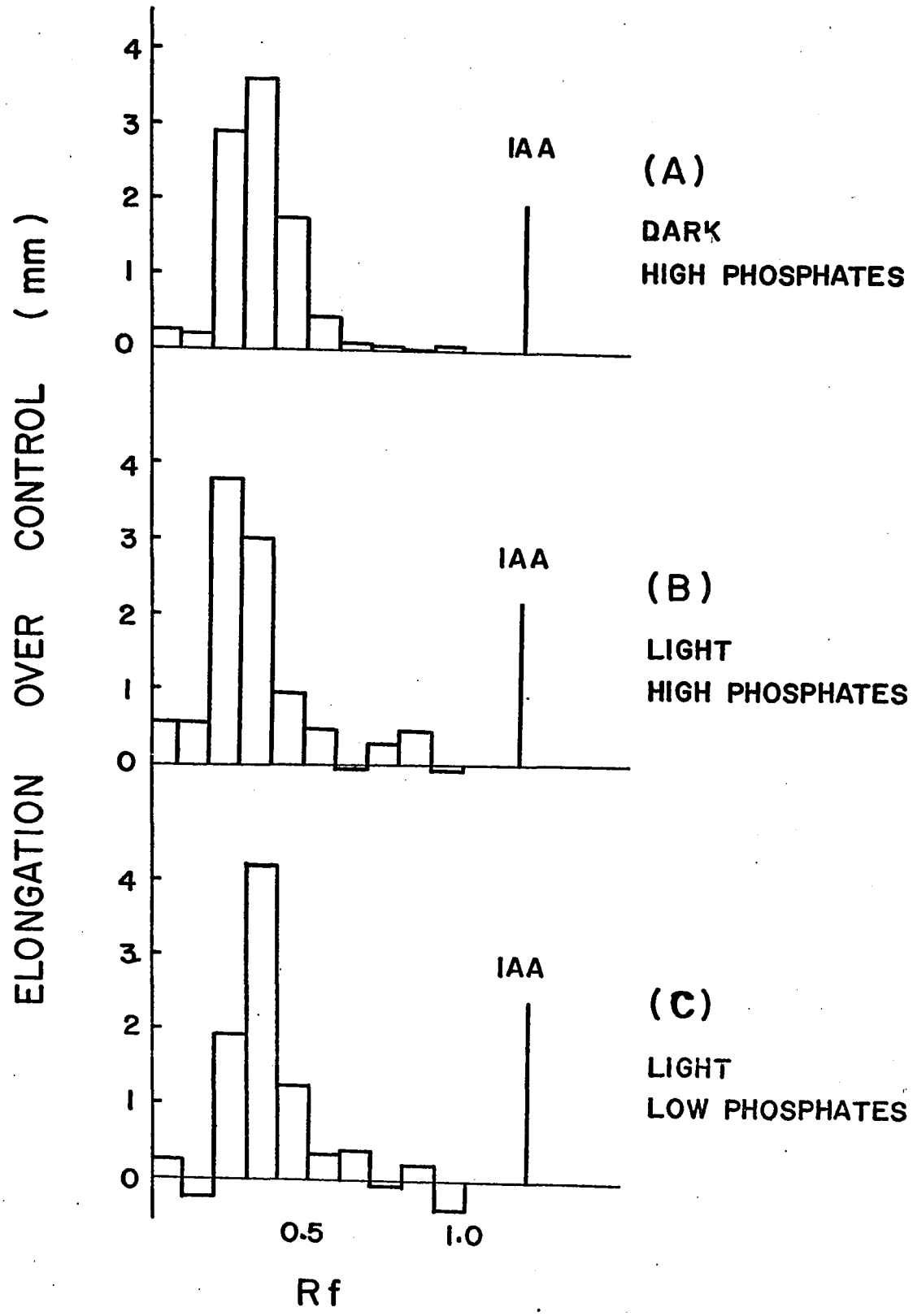
2.3. Content of potassium phosphates

The potassium phosphates in the medium played a role in buffering as well as in providing a phosphate supply. Cultures were grown in the basal medium containing 0.2 and 1 g/l of potassium phosphates respectively.

An IAA-like substance, judged by its Rf value, fluorescence and chromogenic reaction with Salkowski reagent (Figure 6), was detected in both cases. Samples from both high and low potassium phosphates media showed similar responses in the lettuce assay. Moreover, the same results were obtained in the Avena test (Figures 8A and 8C).

Figure 8. Histograms showing activities in the oat (*Avena sativa*, Brighton) coleoptile assay of segments from paper chromatograms of the extracts from dark-grown (A) and light-grown (B) cultures, and from a culture in low potassium phosphates medium (C).

Vertical line represents the response of 10^{-6}M IAA in the corresponding assay.


ELONGATION OVER CONTROL (mm)
4
3
2
1
0
IAA
(A)
DARK
HIGH PHOSPHATES
IAA
(B)
LIGHT
HIGH PHOSPHATES
IAA
(C)
LIGHT
LOW PHOSPHATES
0.5
1.0
Rf

2.4. Illumination

Gibberellins have been reported to be insensitive to light, but naturally occurring IAA has been observed to be light sensitive.

An experiment was therefore established to study whether there was any quantitative difference in the production of IAA-like substance between dark-grown and light-grown cultures. Extraction and chromatography were done in a normally illuminated laboratory. There was no detectable increase in the production of IAA-like substance when the culture was grown in darkness (Figures 8A and 8B).

2.5. Comparative estimation of IAA-like substance produced under different conditions

Table 4 summarizes the results obtained from cultures grown for 18 days under different conditions. The concentration of the IAA-like substance was higher in the shaking culture than in the stationary culture. Furthermore, the production of the IAA-like substance was reduced by exogenous NH_4^+. No difference was observed when the potassium phosphates content of the medium was decreased from 1.0 to 0.2 g/l. Also, the active substance did not appear to be light sensitive.

TABLE 4. Amounts of the active substance produced by 18-day cultures under different conditions

Culture condition		Amount equivalent to IAA ($\times 10^{-6}$M) in the extract	
		Shaking	Stationary
N-free medium	light	7.75	2.5
	dark	8.0	
NH_4^+-medium*		1.50	0.8
Low potassium phosphates**		7.8	

*Medium containing 0.1% of $(NH_4)_2SO_4$

**Medium containing 0.2 g/l of potassium phosphates

3. Production of IAA-like Substance in Relation to Age of Shaking Culture

Reports of the production of auxin or IAA by *Azotobacter* species have generally been based on cultures of one or a few weeks. Vancura and Macura (1960) detected IAA in 15-day stationary cultures and it was degraded with age to a biologically inactive indol-3-carbonic acid, no IAA being detected at the end of a 40-day incubation period. Romanow (1965) found IAA in 10-day shaking cultures. Novikova and Irtuganova (1966) and Hennequin and Blachère (1966) were able to demonstrate IAA in one- to four-week cultures. IAA was reported by Brown and Burlingham (1968) to appear in 14-day cultures.

In order to study the relation between the production of this active substance and the age of the culture, a series of experiments was performed. The ethyl acetate extracts were prepared from cultures grown for 1, 2, 3, 5, 6, 7, 12, 18 and 40 days respectively. A further objective was to detect possible intermediates in the young cultures or IAA degradation products in the old cultures.

3.1. Thin-layer chromatographic and chromogenic behaviour of test materials

The ethyl acetate extracts from different aged cultures were cc-chromatographed with synthetic IAA, IAM

and IAN on the same plate. They were separated using four different solvent systems. The Rf values and chromogenic behaviour of the three indole compounds in the respective solvent system were used as references (Table 2, page 34).

3.1.1. Solvent system A (10:1:1)

An IAA-like substance, determined by its Rf value and mauve color with Salkowski spray, was observed in the 3, 5, 18 and 40-day samples (Figure 9A). However, in the 2-day sample, a spot at the corresponding region appeared grey-yellow. No indole compound was present in the one-day culture. Since only one color spot was detected from cultures grown for from 3 to 40 days, it is probable that the IAA-like substance which appeared in the 3-day culture was not degraded during the course of a 40-day incubation period.

Regarding the grey-yellow spot in the 2-day sample, it might be the same IAA-like substance, in a very low concentration, or an indole compound other than IAA. Neither intermediate nor degradation product of IAA was observed.

3.1.2. Solvent system B (70:25:5)

A bluish-purple fluorescent spot with an Rf identical to that of IAA was detected in extracts of the 6-day and older cultures. It appeared pink with $FeCl_3$-methanolic-

Figure 9. Thin-layer chromatograms of the extracts of different aged cultures in four solvent systems and chromogenic sprays as illustrated. Digit indicates the age of culture in days.

Solvent systems:

Solvent A Isopropanol : NH_4OH : H_2O = 10:1:1

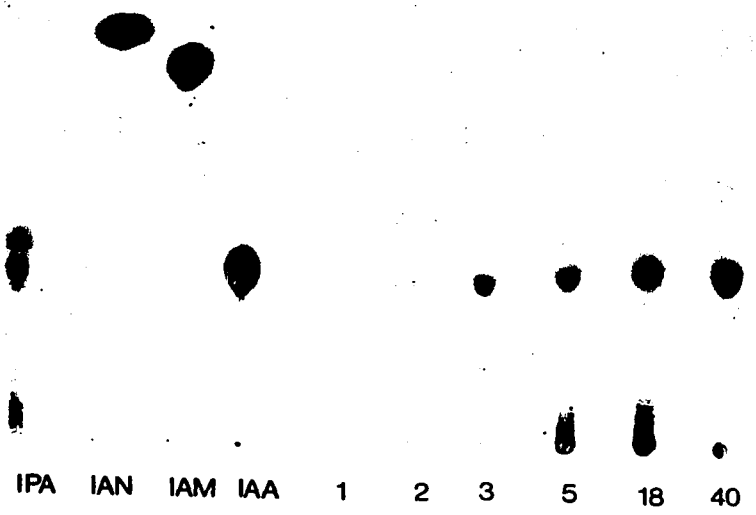
Solvent B Benzene : n-butanol : acetic acid = 70:25:5

Solvent C n-Butanol : acetic acid : H_2O = 4:1:2

Solvent D Butyl acetate saturated with water = Butyl acetate-H_2O

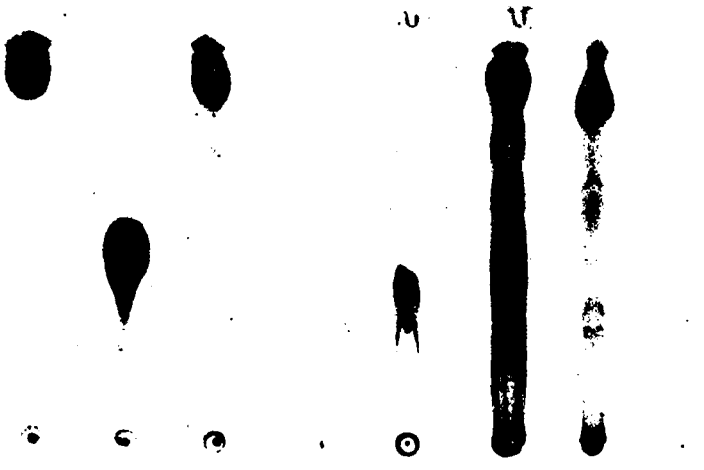
References:

IAA = Indoleacetic acid
IAM = Indoleacetamide
IAN = Indoleacetonitrile
IPA = Indolepyruvic acid



9A

TLC Solvent 10 : 1 : 1
Salkowski Reagent



9B

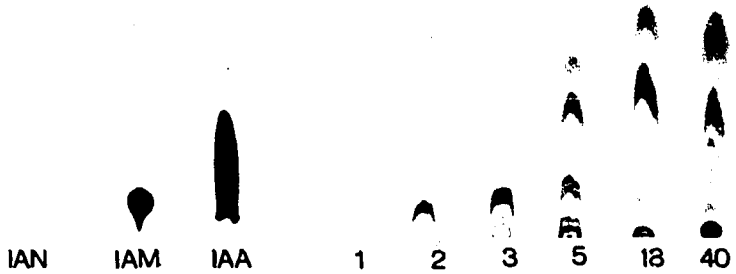
TLC Solvent 70 : 25 : 5
Salkowski Reagent

9C

IPA IAN IAM IAA 1 2 3 5 18 18N

TLC Solvent 4 : 1 : 2

Ehrlich Reagent



9D

IAN IAM IAA 1 2 3 5 18 40

TLC Solvent Butyl acetate — H_2O

Ehrlich Reagent

sulphuric reagent and became mauve with Salkowski spray (Figure 9B). In this solvent system, although IAN located at the same position, there was no agreement in the chromogenic reactions between IAN and the fluorescent spot.

Another spot, having an Rf value similar to that of the IAM appeared in the 3, 6 and 12-day cultures, but it was not detected in the 18 or 40-day sample. It appeared prior to IAA and disappeared in the old cultures, and it might thus be an intermediate in the synthesis of the IAA-like substance by Azotobacter vinelandii. Although it became purple with either Salkowski or $FeCl_3$-methanolic-sulphuric reagent, there was some doubt whether it could be IAM, a possible precursor of IAA, in view of its absence in chromatography in solvent system A (Figure 9A). Another unidentified band at Rf 0.28, showing characteristic violet color with Salkowski reagent, could have been another indole intermediate.

3.1.3. Solvent system C (4:1:2)

On the basis of the Rf value and purple color, an IAA-like substance was located with Ehrlich spray in the 5, 18 and 40-day cultures (Figure 9C). Similarly, another purple spot was found to be in accord with IAM in the 2, 3, and 5-day cultures. No color spot was detected in the one-day culture. Recults from solvent systems B and C,

but not A showed that an indole compound other than IAA appeared in the young cultures. This compound was suspected to be IAM, a possible precursor of the IAA-like substance.

3.1.4. Solvent system D (Butyl acetate saturated with water)

Solvent system D was not ideal for the separation of IAA, since the latter usually appeared to be a very big spot extending from Rf 0.18 to 0.25, but the three references still could be distinguished. Therefore, the test materials were subjected to this solvent system and then visualized with Ehrlich spray.

A purple spot which appeared in the same region as did the IAM was observed in the 2, 3 and 5-day cultures. An IAA-like substance was detected in the 5-day and older cultures. In addition, there was an unidentified spot located at about Rf 0.4 which was pink in the 5 and 18-day cultures, and orange in the 40-day culture. It is doubtful if this was a degradation product of the IAA-like substance.

3.2. Two-way chromatography

In the one-way chromatography, a possible intermediate of the IAA-like substance was viewed in three out of the four solvent systems and it was suspected to be IAM. Since only one spot was detected in solvent A, the indole

spots which appeared in the other chromatograms could have been artifacts resulting from a reaction with the solvent systems. The ethyl acetate extract of the 5-day culture was therefore tested using two-way chromatography.

A fluorescent spot which was detected at Rf 0.37 in solvent A, was re-separated into two indole compounds in solvent D, one of which behaved like IAA and the other like IAM, as judged by their Rf values and colors with Salkowski spray.

Furthermore, if the sample was first run in solvent B, two spots were obtained which were later found, on running in solvent A, to have the same Rf value.

It is concluded that no artifact was formed in the solvent systems used.

3.3. Paper chromatographic and chromogenic behaviour of test materials

The ethyl acetate extracts of different aged cultures were separated by paper chromatography and then sprayed with Salkowski reagent.

3.3.1. Solvent system A (10:1:1)

According to its Rf value and chromogenic reaction, an IAA-like substance was detected in the 2 to 40-day cultures, but that in the 2-day culture gave a pale yellow color instead of mauve (Figure 10A). No color spot was detected in the one-day culture.

Figure 10. Paper chromatograms of the extracts of different aged cultures in two solvent systems and chromogenic sprays as illustrated. Digit indicates the age of culture in days.

Solvent systems:

Solvent A Isopropanol : NH_4OH : H_2O = 10:1:1

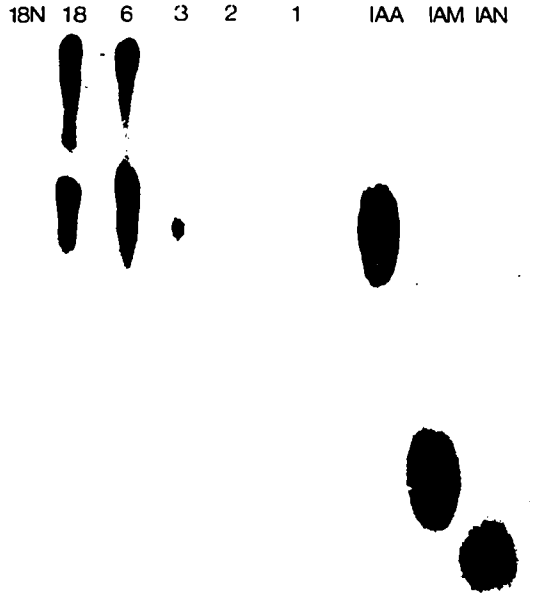
Solvent B Butyl acetate saturated with water = Butyl acetate-H_2O

References:

IAA = Indoleacetic acid
IAM = Indoleacetamide
IAN = Indoleacetonitrile

PC Solvent 10 : 1 : 1
Salkowski Reagent

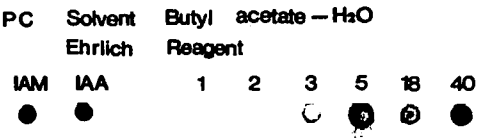
18N 18 6 3 2 1 IAA IAM IAN



A

PC Solvent Butyl acetate — H_2O
Ehrlich Reagent

IAN IAM IAA 1 2 3 5 18 40



D

3.3.2. Solvent system D (butyl acetate - H_2O)

Similarly, an IAA-like substance was detected in the 5 to 40-day cultures (Figure 10B). On the other hand, a spot having an Rf value identical to that of the IAM appeared in the 2, 3, 5 and 18-day cultures.

It can be concluded from the chromatographic studies that an IAA-like substance was produced by A. vinelandii. It appeared in the 5-day culture and remained to the end of the 40-day experimental period studied.

3.4. Avena coleoptile assay of test materials

The responses of Avena coleoptiles to the IAA-like substance produced by different aged cultures are shown in Figure 11. The peak of the activity in all cases occurred in the area carrying the color spot which was believed to be an IAA-like substance. There was no auxinic activity in the one-day sample, thus confirming the negative chromogenic reactions in both thin-layer and paper chromatography.

Positive response of the Avena coleoptile elongation was very slight in the 2 and 3-day samples. The concentrations of the active substance in the extracts were equivalent to 10^{-8} and 3×10^{-7}M IAA respectively. That of the 5-day culture gave a response equivalent to 3×10^{-6}M IAA. Significant elongation of the Avena coleoptiles was induced by the IAA-like substance extracted from the 6 to 40 days

Figure 11. Effects on elongation of oat (Avena sativa, Brighton) coleoptiles by the extracts of different aged cultures separated by paper chromatography in solvent A (Isopropanol : NH_4OH : H_2O = 10:1:1).

Vertical line represents the response of 10^{-6}M IAA in each experiment.

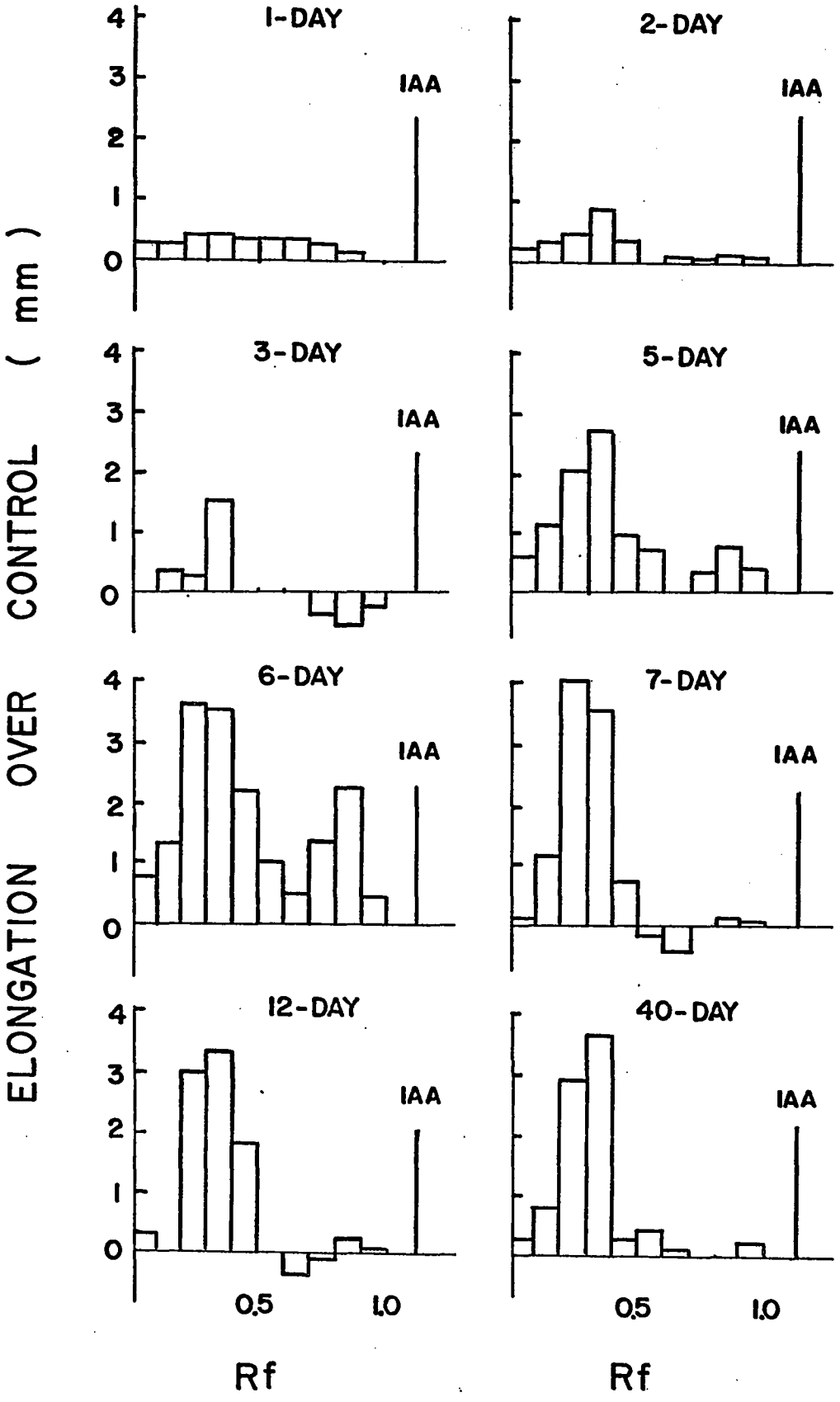
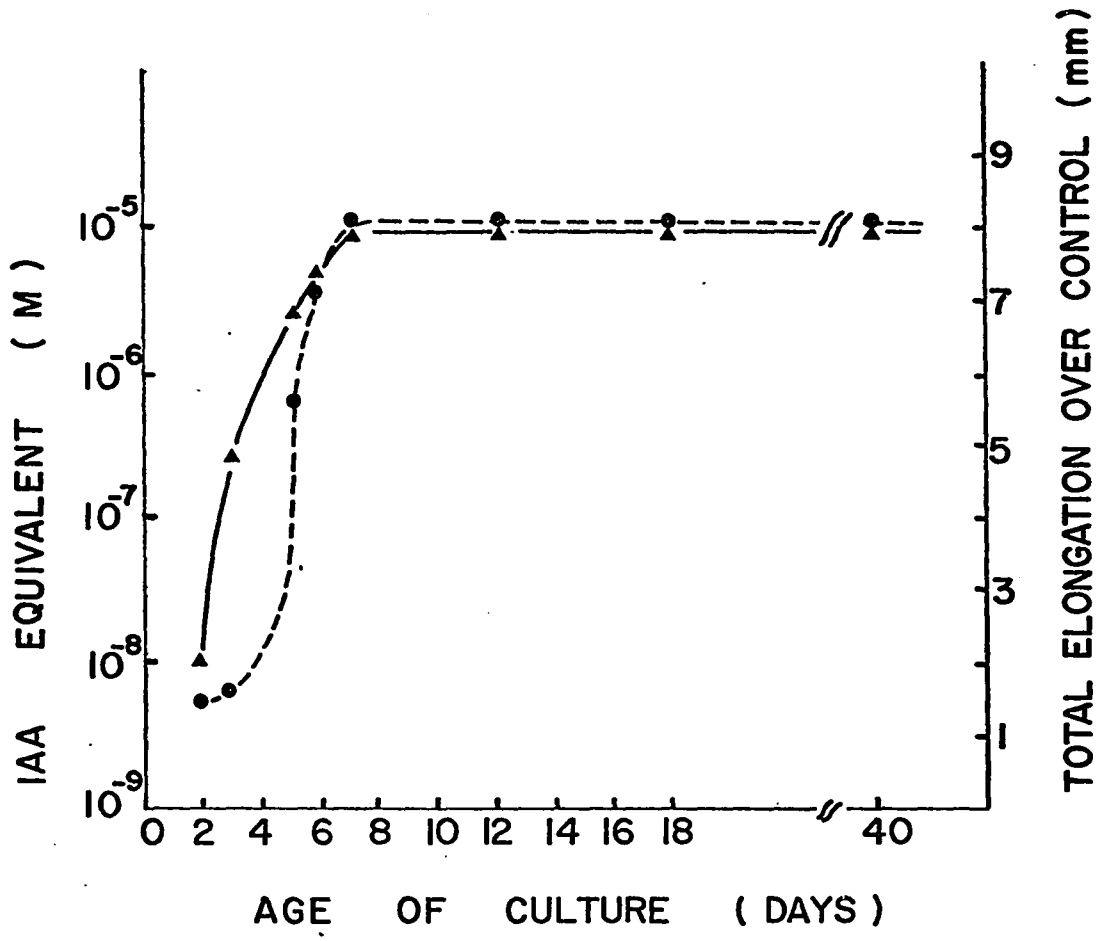

1-DAY
2-DAY
3-DAY
5-DAY
6-DAY
7-DAY
12-DAY
40-DAY
IAA
4
3
2
1
0
0.5
1.0
Rf
ELONGATION OVER CONTROL (mm)

Figure 12. Comparative activities of the IAA-like substance on oat (Avena sativa, Brighton) coleoptiles produced by different aged cultures. The amounts are expressed as IAA equivalent (—▲—) and total elongation over control (---●---).


IAA EQUIVALENT (M)
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
0 2 4 6 8 10 12 14 16 18 40
AGE OF CULTURE (DAYS)
TOTAL ELONGATION OVER CONTROL (mm)
9
7
5
3
1

cultures, the amounts being in the range 5×10^{-6} to 8×10^{-6}M IAA equivalent.

It may be concluded that the auxinic activity appeared increasingly from 2 to 6 days and remained constant from then onwards.

Comparative activities of the amounts of the active substance in cultures of different ages expressed in terms of both the IAA equivalent and the total elongation of coleoptile over control are shown in Figure 12.

Another auxinic activity at about Rf 0.9 was observed in the 5-day culture, and it increased remarkably in the 6-day culture (Figure 11), but was no longer detectable in the 7-day and older cultures. Since chromatographic and chromogenic results did not show the existence of an indole compound at Rf 0.9, the interpretation of this activity is difficult on the basis of the present evidence.

PART II - PRODUCTION OF GIBBERELLIN-LIKE SUBSTANCE BY *AZOTOBACTER VINELANDII* (MAC. #459)

The problem of the production of gibberellin-like substances by pure cultures of *Azotobacter* species has been as controversial as has been the effectiveness of *Azotobacter* to be utilized as a bacterial fertilizer. In order to investigate further the inconsistent evidence, the culture of *Azotobacter vinelandii* (Mac. #459), used in the present study, was one of the cultures which had been tested by Breckenridge (1968) when she obtained a variable result. The methods of extraction and identification were adapted from those applicable for the investigation of gibberellins from plants. When standard gibberellic acid was treated identically, the procedure was shown to be reliable.

In the first trial, under stationary conditions, the culture which had been maintained routinely on agar slants of the basal medium containing 0.2 g/l of potassium phosphates, was used as the inoculum and it was grown for a period of 18 days in the same medium. Negative results were obtained consistently in several repetitions. Neither did the experiments with shaking cultures succeed in detecting the presence of any GA-like substance.

Shortly after the above preliminary experiments had been performed, the presence of a GA-like substance was detected in a culture medium which contained 1 g/l of potassium phosphates. The culture was then kept routinely on agar slants of the same medium. The following experiments were then performed, in which the cultures were grown under stationary conditions. Unless otherwise stated, the nitrogen-free basal medium containing 1 g/l of potassium phosphates and 2% glucose was used.

1. Production of GA-like Substance in 18-day Stationary Culture

Supernatants of 18-day cultures were tested for GA-like substances. The following culture conditions were varied:

1. Potassium phosphates concentration
2. Glucose concentration
3. Illumination

For preliminary trials, the ethyl acetate extract of each of the cultures was separated by thin-layer chromatography, developed in the solvent system B (Benzene : n-butanol : acetic acid = 70:25:5). The chromatogram was sprayed with $FeCl_3$-methanolic-sulphuric reagent and exposed to UV light, so as to determine the fluorescent reaction of the sample. Further, chromatogram sections were screened by the barley endosperm and lettuce hypocotyl assays.

Gibberellic acid, which gave a greenish blue fluorescence with the $FeCl_3$-methanolic-sulphuric reagent at Rf 0.6 was co-chromatographed with the sample in each trial.

1.1. Barley endosperm assay of GA_3

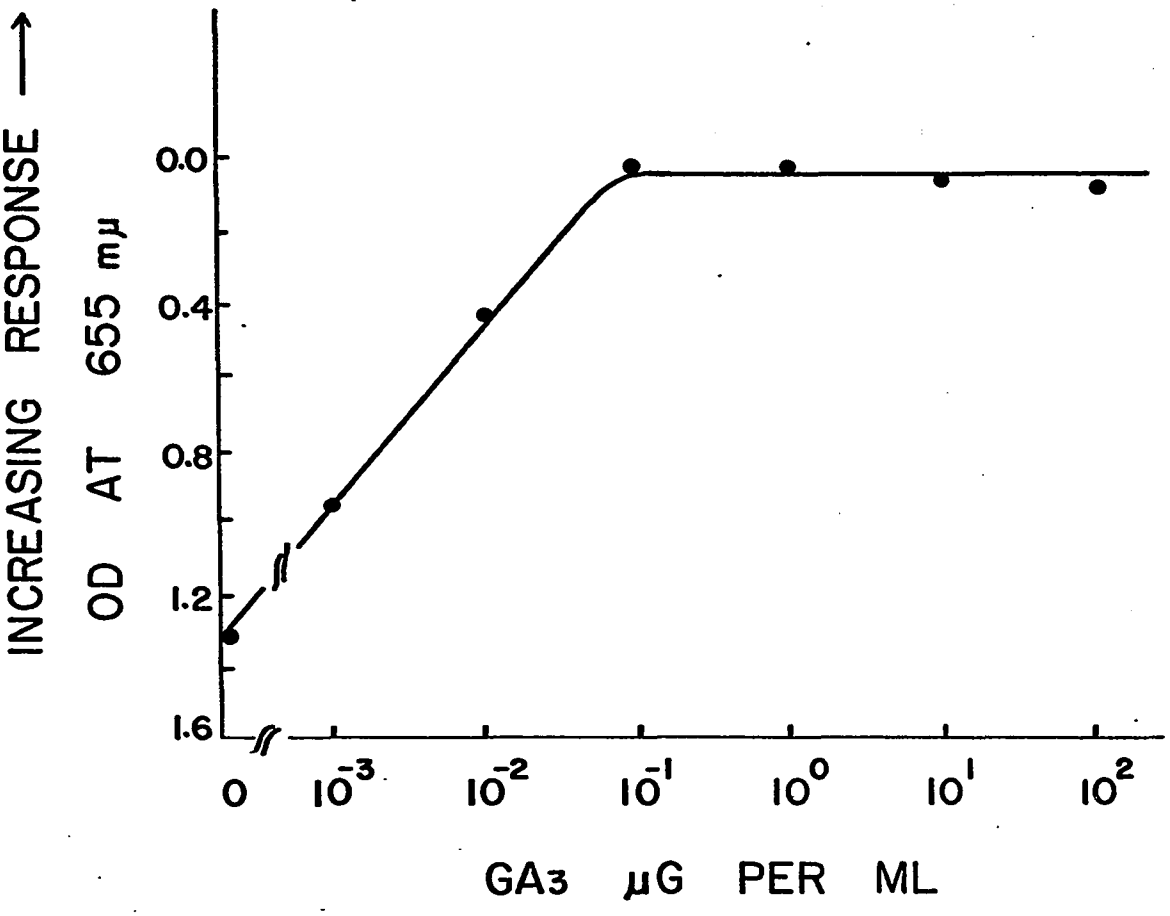
According to the positive reports in the literature, the GA-like substance produced by an Azotobacter culture was believed by Vancura (1961) to be actually GA_3, and of the three GA-like substances obtained by Brown and Burlingham (1968), the dominant one was identified very close to GA_3.

For reference, solutions of GA_3 with concentrations from 100 to 0.001 µg/ml were chromatographed identically. The strip of the chromatogram at Rf 0.6 to 0.7 was assayed with barley endosperms. A dosage response curve is shown in Figure 13.

1.2. Potassium phosphates concentration

The culture was grown in the basal medium containing 0.2, 0.6 and 1 g/l of potassium phosphates (K_2HPO_4:KH_2PO_4 = 4:1) respectively. After being chromatographed and sprayed, no fluorescent reaction at the Rf corresponding to that of GA_3 was observed in any extract. However, a pink spot was visible at Rf 0.85 in all cases, which was evidently an IAA-like substance on the basis of lettuce radicle and Avena coleoptile assays.

Figure 13. A dosage response curve of GA_3 in the barley endosperm bioassay. Each level of GA_3 was chromatographed by thin-layer chromatography in solvent system B (Benzene : n-butanol : acetic acid = 70:25:5).


INCREASING RESPONSE ⟶
OD AT 655 mμ
0.0
0.4
0.8
1.2
1.6
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
GA3 μG PER ML

For further study, 0.5 ml of each of the ethyl acetate extracts was co-chromatographed with a spot of GA_3. The part of the chromatogram carrying GA_3 was sprayed and exposed to UV light, so as to check the Rf value in each trial, while the part loaded with the ethyl acetate extract was bioassayed with barley endosperms. A positive result was shown only in the extract from the culture medium containing 1 g/l of potassium phosphates (Figures 14A and 14B) and the area of the response was in accord with the Rf of the GA_3. The activity of the active substance was approximately equivalent to 3.0×10^{-1} µg GA_3/ml of the extract. For control, a blank chromatogram was run and assayed identically, and the effect of solvent on barley endosperms was shown to be negligible (Figure 14C).

All extracts which showed either positive or negative response with barley endosperms were also tested with lettuce hypocotyls, but no response was obtained in all cases.

It can be concluded that a substance having an Rf value in solvent system B and an effect on barley endosperm identical to those of the GA_3 was produced by A. vinelandii. However, it behaved differently from GA_3 in the fluorescence inducing spray and did not show a response in the hypocotyl test.

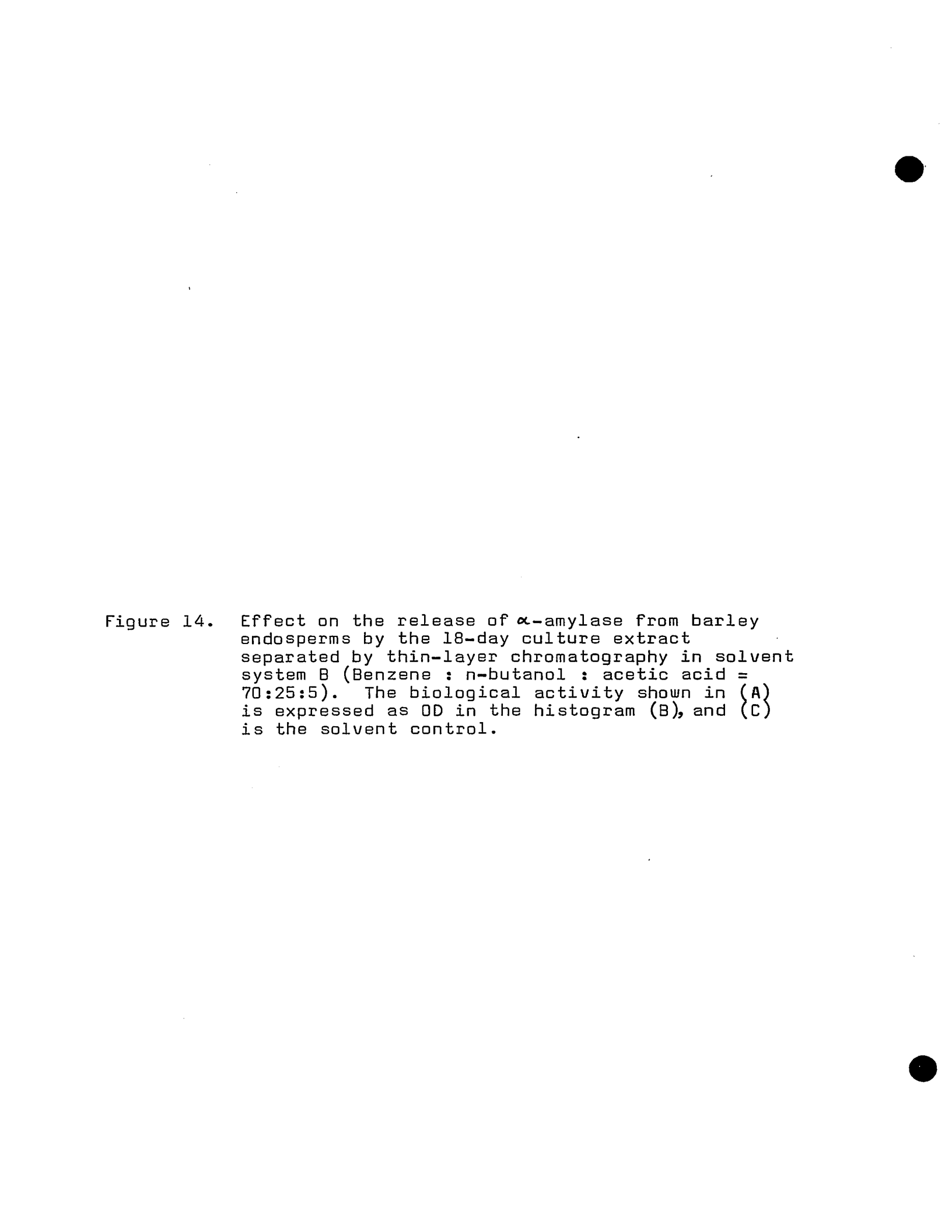
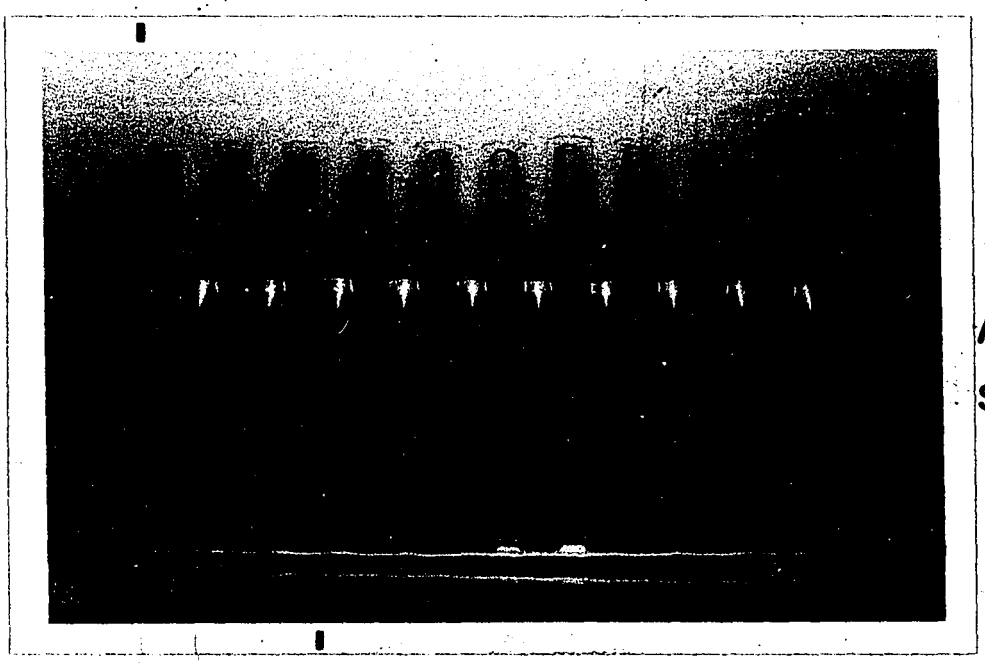
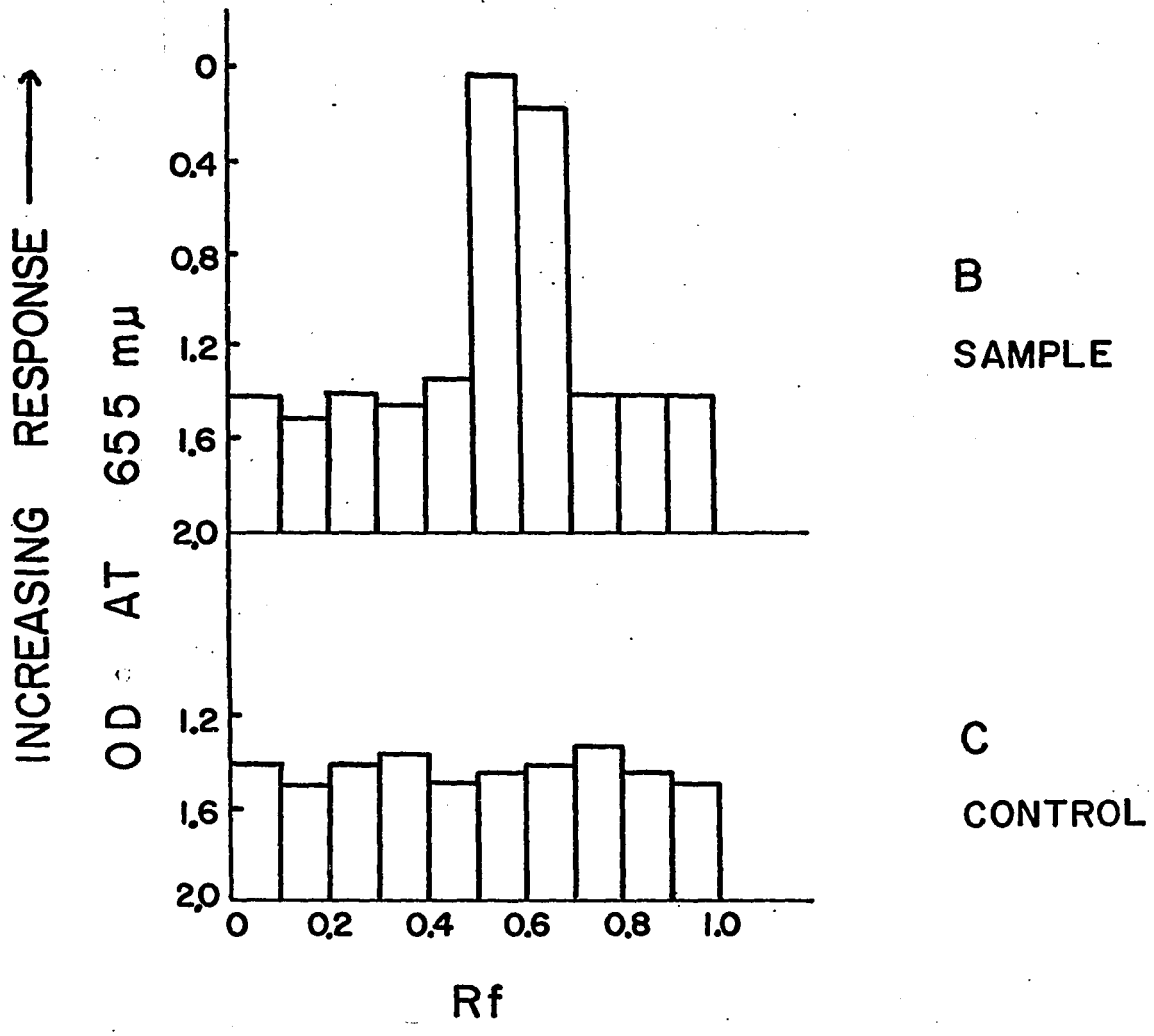


Figure 14. Effect on the release of α-amylase from barley endosperms by the 18-day culture extract separated by thin-layer chromatography in solvent system B (Benzene : n-butanol : acetic acid = 70:25:5). The biological activity shown in (A) is expressed as OD in the histogram (B), and (C) is the solvent control.



A

SAMPLE

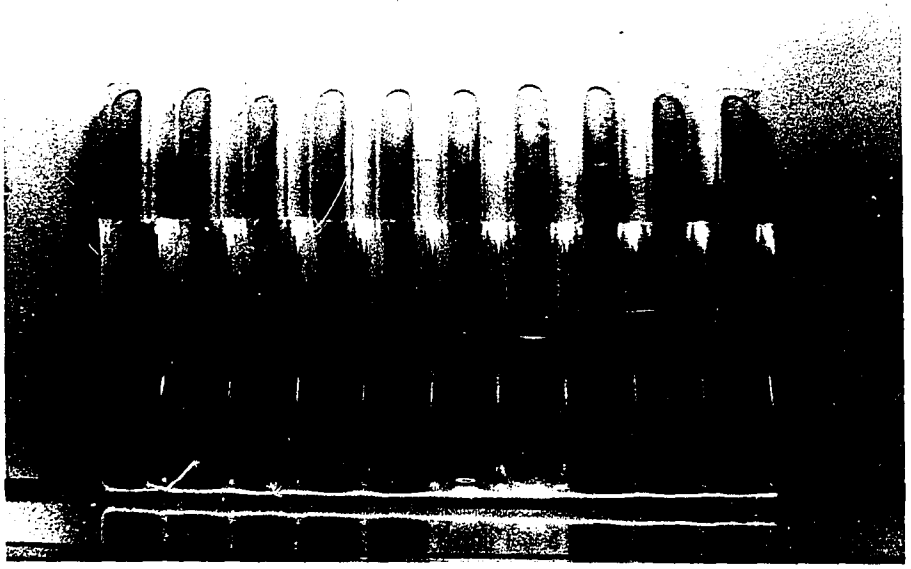


B

SAMPLE

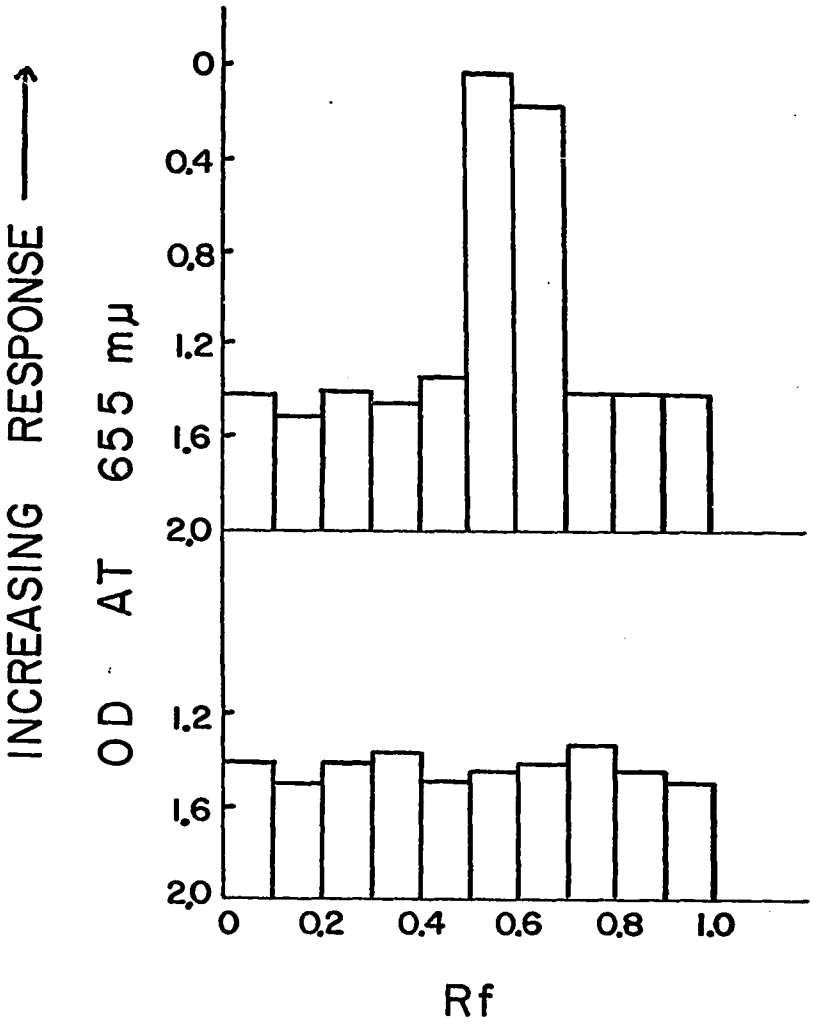
C

CONTROL



A

SAMPLE



B

SAMPLE

C

CONTROL

1.3. Glucose concentration

An experiment was established to study the effect of different concentrations of carbon source on the production of the GA-like substance. The culture was grown in the basal medium containing 0, 0.01, 0.1, 1 and 2% of glucose respectively. After 18 days incubation, no growth was detected in the medium containing either 0% or 0.01% of glucose.

The extract from each sample was examined by chromatography and by the barley endosperm test. Positive response in the barley endosperm assay was shown by the extract of the culture medium containing either 1% or 2% of glucose. Their activities were very similar (Figure 15). It was thus shown that the increase of glucose from 1% to 2% increased the growth of the culture, but not the production of the active substance. Both culture growth and assay response increased markedly from 0.1 to 1.0% glucose.

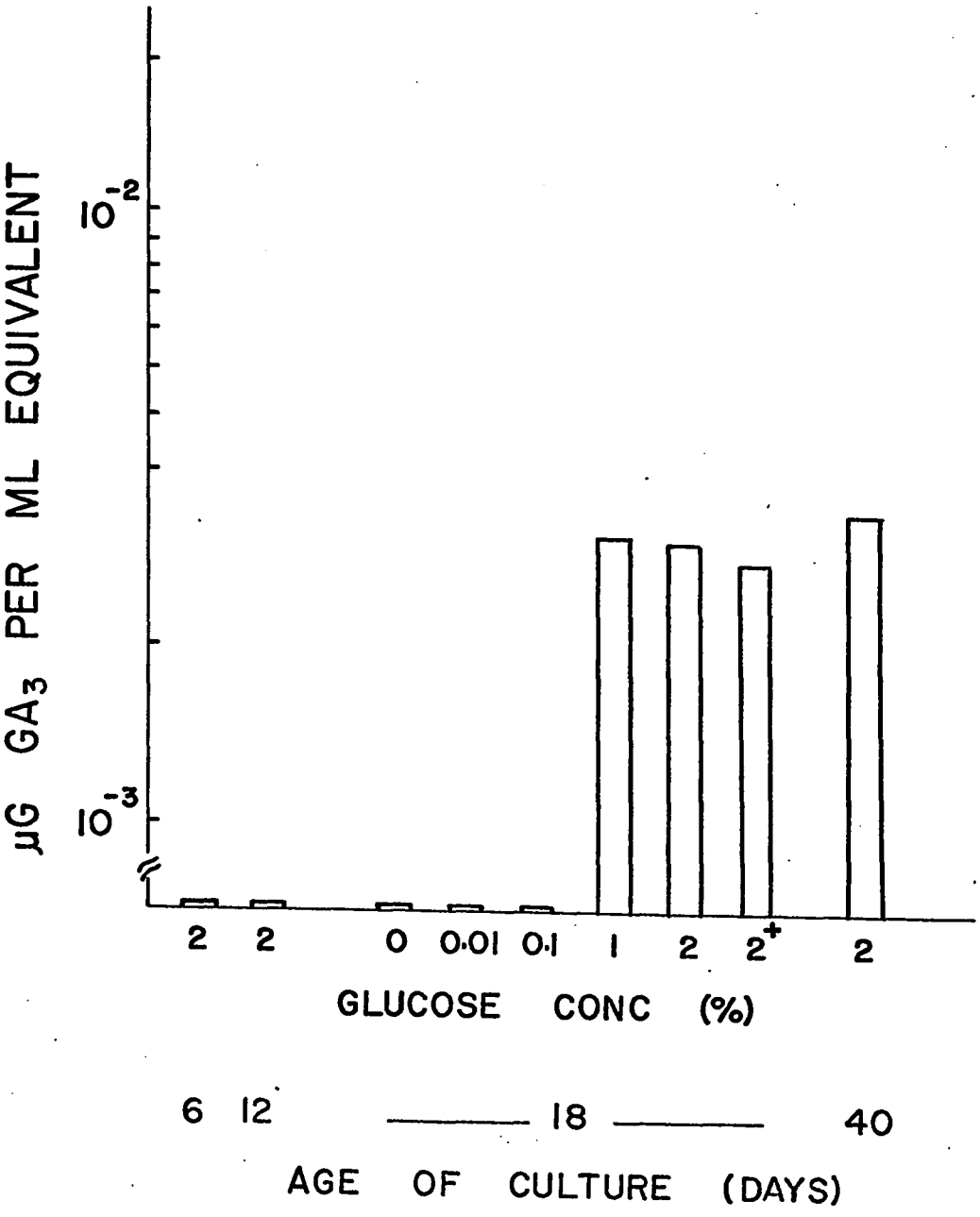
1.4. Illumination

Extracts from the dark-grown and light-grown cultures were tested to study whether the production of GA-like substance was affected by light.

The results from the barley endosperm assay showed that there was no difference in the amount of active substance produced, when the culture was grown either in illumination or in the dark (Figure 15).

Figure 15. Comparative activity in the barley endosperm assay of the GA-like substance produced by the cultures under different conditions. The activity is expressed as μg/ml GA_3 equivalent in the culture.

+ indicates dark-grown culture. The rest are light-grown cultures.


µG GA3 PER ML EQUIVALENT
10-2
10-3
2 2 0 0·01 0·1 1 2 2+ 2
GLUCOSE CONC (%)
6 12 18 40
AGE OF CULTURE (DAYS)

2. Age of Culture

Ethyl acetate extracts were prepared from the 6, 12, 18 and 40-day cultures. Absence of the active substance was shown in the 6 and 12-day extracts. By the barley endosperm assay, the amount of the GA-like substance detected in both the 18-day and 40-day cultures was about 3×10^{-3} µg/ml of the culture (Figure 15).

3. Problem in Reproducibility

Clearly, the GA-like substance was produced by the culture under certain conditions. However, subsequent experiments failed completely to reproduce any of the above results. On the other hand, there was a consistent demonstration of auxinic activity in any trial.

Exactly the same experiments were then run in which a new freeze-dried culture of the same strain was used. Again, negative results were obtained in the detection of GA-like substances, while the presence of an IAA-like substance was shown. This implied that the production of the GA-like substance was influenced in some unknown way by the culture conditions, or that after being maintained in culture, the organism lost its ability to synthesize such a substance.

GENERAL DISCUSSION

Most of the investigations on endogenous auxin, auxin-like substances, gibberellin and gibberellin-like substances have been conducted using solvent extraction methods, so as to concentrate the active substance which generally exists only in minute amounts. With chromatographic techniques, plant growth substances in either plant or microbial extracts can be separated, at least to some extent, from interfering substances in the extracts, and consequently, their biological activities can be reflected more efficiently by bioassays. In general, the identification of a naturally occurring plant growth substance in an extract meets the following criteria:

1. chromatographic behaviour
2. reaction with fluorescence-inducing spray
3. reaction with chromogenic reagent
4. biological activity from the eluate of the chromatogram

Many of the indole auxins can be made visible on chromatograms by several chromogenic reagents which give characteristic color reactions. Two of the most prevalent reagents employed for this purpose are the Salkowski and Ehrlich reagents. As many as 35 indole compounds have been

shown to be distinguishable, according to their Rf values in different solvent systems and to the color developed with chromogenic reagents (Stowe and Thimann 1954).

On the basis of the above criteria, the capacity of the culture to produce IAA- and GA-like substances was examined. In the ethyl acetate extract of an 18-day culture, a fluorescent spot which behaved similarly to IAA, in four thin-layer chromatograms and in two paper chromatograms, was visualized with three chromogenic sprays. Results indicated that the colors developed in all reagents were exclusively identical to those of IAA. These provided circumstantial evidence that the spot was an IAA-like substance, and its auxinic activity was affirmed by two bioassays. In addition, our observation, in certain extracts, that a substance having an Rf value in thin-layer chromatography and a positive response in the barley endosperm assay similar to those of the GA_3, suggested that it is likely to be a GA-like substance. However, it behaved negatively in the fluorescence-inducing spray and in the lettuce hypocotyl elongation assay.

Various bioassay systems have been used to determine the existence of a plant growth substance. Nevertheless, the specificity of each of the bioassay systems is still doubtful, or incompletely understood. Several indole compounds other than IAA exhibit auxinic activity (Wightman

1962; Larsen 1961). Solvent and chemical impurities also have been claimed to be sources of GA-like growth promoting activity (Hartley, Pegg and Thomas 1969; Briggs 1966). In the present work, the possibility of a false positive caused by solvent residues was checked with control chromatograms. The effect of solvent residues on each of the bioassay systems was found to be negligible.

Two methods were used for screening the auxinic activity of the IAA-like substance. Although the variation of the lettuce radicle assay did not permit quantitative evaluation, the merit of its simplicity and the high susceptibility of the radicles made the method useful. Results were similar to those of Vancura and Macura (1960), who reported a stump-like shortening of roots induced by a substance extracted from an A. chroococcum culture. They believed that the active substance was actually IAA.

Using the Avena straight growth test, we found that the quantity of IAA-like substance per ml of the culture was equivalent to 8×10^{-8}M IAA (i.e., 0.014 µg/ml). The amount of the auxinic substance produced by Azotobacter cultures has been reported variably. Burlingham (1964), using the Avena test, reported that in a 30-day old culture of A. chroococcum, the quantity of IAA produced was 1 µg/ml which was in accord with the finding of Hennequin and Blachère (1966) in a 7-day culture of A. chroococcum. An

amount of 10^{-3}% (i.e., 10 µg/ml) of IAA equivalent was obtained in a 10-day culture of A. chroococcum by Romanow (1965) who determined the active substance with the barley, alfalfa and sugar beet bioassays. According to the data of Vintika (1964), an A. chroococcum culture produced up to 6 µg of heterauxin per ml of medium. So far, no report has been made on the quantity of the auxinic substance produced by A. vinelandii.

A similar situation has appeared in the literature concerning the production of GA-like substances by Azotobacter cultures. Vancura (1961), using the etiolated section of maize leaves and mesocotyls as test materials, detected a relatively high amount of GA_3 (20 µg/ml) in a 17-day old stationary culture. Brown and Burlingham (1968) reported that an A. chroococcum culture grown under shaking conditions produced 3 GA-like substances, of which the dominant one had an Rf value similar to that of GA_3, and the average concentration of GA/ml culture was 0.03 µg GA_3 equivalent. Using the agar plate method and the barley endosperm assay, Breckenridge (1968) in her report of the production of GA-like substances by A. vinelandii (Mac. #459) showed a quantity equivalent to 0.05 µg GA_3/ml and 2.02 10^{-3} µg GA_3/ml.

Katznelson and Cole (1965) detected 1-14 µg of GA_3 per liter in a number of bacteria grown for a period of one

week under shaking conditions, whereas none was detected in the stationary cultures. Moreover, they found that an increase in glucose concentration increased the growth of the culture, but did not affect the amount of the active substance produced. Under our conditions, a level of the active substance of approximately 3×10^{-3} µg GA_3 equivalent per ml of the 18-day culture was obtained as determined by the barley endosperm assay. The amount of the active substance remained fairly constant over a period of 40 days incubation. In addition, we found in agreement with the data of Katznelson and Cole (1965) that increase of glucose concentration from 1% to 2% in the medium did not increase the amount of the active substance produced.

A considerable variability might reasonably be expected with the different long procedures of extraction, chromatography and bioassay involved, especially the various bioassays. Stowe and Yamaki (1957) indicated the variability in quantitation of active substance obtained with the same extract but with different assay systems. Katznelson and Cole (1965) found GA-like substances in an Arthrobacter globiformis culture. By means of the lettuce hypocotyl test, they obtained an amount equivalent to 2 µg GA_3/l, but using the lettuce seed germination test, a value of about 100 µg GA /l was obtained. Furthermore, the different cultural conditions also introduced great variables. For

instance, we obtained a quantity as high as 8×10^{-8}M IAA equivalent per ml in an 18-day shaking culture grown in the nitrogen-free medium, whereas the amount of the active substance in an 18-day stationary culture grown in the NH_4^+-containing medium (about 8×10^{-9}M IAA equivalent/ml) was unable to be detected by the chromatographic method with the extract employed.

Various reports exist on the production of GA-like substances by microorganisms. Mowat (1963) has reported the presence of powerful inhibitors which interfere with the bioassays in algal extracts. Katznelson and Cole (1965) found that in a Bacillus polymyxa culture, the anti-gibberellins which had an Rf similar to that of the GA_3 were produced simultaneously with and obscured the presence of the GA_3. The presence of interfering substances which rendered variable results was also claimed by Breckenridge (1968) in the detection of GA-like substances in Azotobacter cultures.

In the present work, there was no evidence to show any influence of interfering substances or impurities. Positive gibberellin-like responses and subsequently reasonable results were obtained in a series of experiments before the onset of irreproducibility. It is quite unlikely that interfering substances could have been the cause of irreproducibility. It is possible that the microorganism

produced the active substance only under certain critical conditions, and after being maintained in culture it lost its ability to synthesize the active substance.

Divergent reports have appeared in the literature not only on the production of GA-like substances by Azotobacter cultures, but also on the synthesis of IAA-like substances or auxins. Of the ten analysed strains of A. chroococcum, Vancura and Macura (1960) demonstrated that only three were conclusively positive. Hennequin and Blachère (1966) obtained IAA in six out of the 15 tested strains of A. chroococcum. It is generally believed that the ability of Azotobacter species to synthesize plant growth substances depends not only on the species, but also on the strain. Furthermore, our result suggests that it also depends on the culture conditions.

The naturally occurring IAA in plants is light-sensitive and is degraded non-enzymatically by visible light in the presence of sensitizing pigments. Nevertheless, the IAA-like substance produced by the culture does not seem to be light sensitive. There is a considerable number of compounds which inhibit the sensitized photodestruction of IAA. For example, polyphenols and ascorbic acid are highly effective, possibly by competing with IAA for oxidation (Brauner and Brauner 1954; Goldacre 1954). Various components of plant extracts have also been shown to protect

IAA (Brauner and Brauner 1954), and the inhibitory activity was attributed to their polyphenolic character. It is meaningful to refer to the work of Hennequin and Blachère (1966) who demonstrated the synthesis of phenolic compounds by A. chroococcum and A. vinelandii. Accordingly, phenolic compounds in Azotobacter cultures may protect IAA from photodestruction.

The amount of the IAA-like substance produced by the stationary culture was less than that of the shaking culture. This suggested that the synthesis of the active substance was not inhibited by oxygen. Since the biosynthetic pathway of the IAA-like substance is still unknown, further information would be required to determine the effect of oxygen on its production. However, it should be noted that aeration facilitates the growth of Azotobacter and brings about an increase in growth rate and in final yield of the bacterial mass (Rubenchik 1963; Schmidt-Lorenz and Rippel-Baldes 1958). Thus it is reasonable that more active substance could be produced.

Extensive researches have shown that tryptophan is a primary precursor of IAA in the metabolism of a number of organisms. IAA has been shown to be effectively synthesized by several bacteria grown in tryptophan-containing medium (Stowe 1955; Kaper and Veldstra 1958; Larsen et al. 1962). By using tracer experiments, Magie et al. (1963) were able

to demonstrate the conversion of L-tryptophan to IAA by a cell free extract of Pseudomonas savastanoi. Burger and Bukatsch (1958) detected IAA in the tryptophan-containing medium of four Azotobacter cultures, but when they were grown in a nitrogen-free medium, only two were able to synthesize IAA. These observations were later confirmed by Romanow (1965) who found the addition of tryptophan stimulated the production of IAA which was readily identified by paper chromatography in a 24-hr culture of A. chroococcum and the spot of the IAA became more pronounced with age. However, Romanow observed that when an amount of NH_4Cl-nitrogen which was equivalent to that of the tryptophan-nitrogen was substituted in the medium, the IAA spot on the paper chromatogram was detectable only after 14 days incubation, although the supernatant of the 5-day culture showed auxinic activity by bioassay.

We found that in either shaking or stationary conditions, relatively less IAA-like substance was produced in the culture medium containing 0.1% of $(NH_4)_2SO_4$. Exogenous combined nitrogen is utilized preferentially by an Azotobacter culture and the nitrogenase is repressed. Since NH_4^+ is one of the primary products in the nitrogen fixation process, it is unlikely that the exogenous NH_4^+ would inhibit the synthesis of indole compounds. On the other hand, there may be some relation between the biosynthesis

of the IAA-like substance and the nitrogen fixation mechanism. Although the present evidence is not sufficient to interpret this phenomenon, it would appear promising for further investigation.

As mentioned above, studies of the production of auxin by Azotobacter cultures have generally been based on cultures of one to a few weeks. Results in a series of our experiments showed that the IAA-like substance was produced increasingly from 2 to 6 days and it remained constant from then onward. An indole compound, which appeared to be something other than IAA, separated by solvent D in thin-layer chromatography was seen in the old cultures (from 12 to 40 days incubation). It was doubtful if this was a degradation product of the IAA-like substance, since no sign of any degradation product occurred in the other three chromatographic separations. Moreover, the same auxinic activity, as judged by the Avena coleoptile assay, was detected in the cultures from 6 days up to 40 days of incubation. It is thus concluded that no important degradation of the IAA-like substance occurred, which is in agreement with the suggestion of Montuelle (1966), in which he found no degradation of the auxinic growth factors produced by Bacillus and Pseudomonas cultures. On the other hand, Vancura and Macura (1960) reported that the IAA produced by A. chroococcum was degraded with age to a biologically

inactive indole-3-carbonic acid, and that IAA was no longer detectable at the end of a 40-day incubation period.

There have been many studies on the biogenesis of the indole auxins and each group of organisms utilizes widely different pathways in the synthesis of IAA from L-tryptophan. Several possible pathways have been suggested for the conversion of tryptophan to IAA in microorganisms. 3-indole-pyruvic acid has been shown to be a possible intermediate in Rhizopus suinus (Thimann 1935) and Pseudomonas solanacearum (Sequeira and Williams 1964), and tryptophol has been considered to be an additional product or intermediate of the same pathway in Agrobacterium tumefaciens (Kaper and Veldstra 1958) and Acetobacter xylinum (Larsen et al. 1962). A convincing elucidation of IAA synthesis from tryptophan via tryptamine was made in Bacillus cereus (Perley and Stowe 1966) and in the blue green algae, Chlorogloea fritschii (Ahmad and Winter 1969). Moreover, Magie et al. (1963) demonstrated that IAM is the intermediate precursor of IAA in Pseudomonas savastanoi. Burger and Bukatsch (1958) were aware that when tryptophan was added to the Azotobacter culture medium, an intermediate appeared in addition to IAA, but no detailed report was made. Our finding, according to the chromatographic behaviour and chromogenic reaction in three out of the four solvent systems tested, showed that the indole compound which

appeared prior to the IAA-like substance is evidently IAM. Although its absence in the solvent system A is unexplainable, it appears likely that similar biosynthetic pathways occur in the present Azotobacter culture and in Pseudomonas savastanoi (Magie et al. 1963). Direct evidence of the conversion of IAM to IAA by Azotobacter must wait further investigations employing isotopically labelled intermediates.

SUMMARY

1. Azotobacter vinelandii (Mac. #459) was shown to produce both IAA- and GA-like substances in the culture supernatant. Identification was made by chromatography, chromogenic reaction and fluorescence-inducing spray, and was affirmed by bioassay.

2. Characteristics of the IAA-like substance were essentially identical to those of indoleacetic acid in all tests.

3. The amount of the IAA-like substance produced in an 18-day shaking culture was equivalent to 8×10^{-8}M IAA, and that in a stationary culture was 2.5×10^{-8}M. Relatively less was produced when 0.1% of $(NH_4)_2SO_4$ was added to the nitrogen-free culture medium.

4. Both the IAA- and the GA-like substances did not appear to be light sensitive.

5. The IAA-like substance was produced increasingly from 2 to 6 days of incubation, and it remained constant to 40 days of incubation, while no degradation of the active substance was evident. Another indole compound, which behaved similarly to indoleacetamide in chromatography, was detected prior to the appearance of IAA.

6. Chromogenic and bioassay evidence showed that the GA-like substance was similar to but not identical with gibberellic acid.

7. The GA-like substance was not detected in the culture medium containing less than 0.1% of potassium phosphates.

8. Increase of glucose concentration from 1% to 2% in the medium did not increase the amount of GA-like substance produced by the culture.

9. A quantity of GA-like substance equivalent to 3×10^{-3} μg GA_3/ml was obtained in an 18-day stationary culture, and it remained constant through 40 days of incubation.

10. Irreproducibility of the GA responses occurred with the culture used. The culture lost and did not regain its ability to synthesize the GA-like substance under the culture conditions employed. It is suggested that this phenomenon may be responsible for the conflicting reports which have appeared in the literature.

REFERENCES

Ahmad, M. R., and A. Winter. 1969.
Hormonal relationships of algae in pure culture. III. Tryptamine is an intermediate in the conversion of tryptophan to indole-3-acetic acid by the blue green alga Chlorogloea fritschii.
Planta 88: 61-66.

Allison, F. E. 1947.
Azotobacter inoculation of crops: I. Historical.
Soil Sci. 64: 413-429.

Allison, F. E., V. L. Gaddy, L. A. Pinck, and W. H. Armiger. 1947.
Azotobacter inoculation of crops: II. Effect on crop under greenhouse conditions.
Soil Sci. 64: 489-497.

Bentley, J. A. 1961.
Chemical determination of auxins.
In: Encycl. Pl. Physiol. 14: 513-520.
Springer-verlag, Berlin.

Bird, H. L., and C. T. Pugh. 1958.
A paper chromatographic separation of GA_3 and GA_1.
Pl. Physiol. 33: 45-46.

Bonner, J. 1933.
The action of the plant growth hormone.
J. gen. Physiol. 17: 63-76.

Boysen-Jensen, P. 1931.
Über Wachstumregulatoren bei Bakterien.
Biochem. Z. 236: 205-210.

Brakel, J., and F. Hilger. 1965.
Etude qualitative et quantitative de la synthèse de substances de nature auxinique par Azotobacter chroococcum in vitro.
Bull. Inst. agron. Stns. Rech. Gembloux. 33: 469.
Cited from: Brown, M. E., and S. K. Burlingham. 1968.
Production of plant growth substances by Azotobacter chroococcum.
J. gen. Microbiol. 53: 135-144.

Brauner, L., and M. Brauner. 1954.
Untersuchungen über die Photolyse des Heteroauxins.
Z. Bot. 42: 83-124.

Breckenridge, C. 1968.
Production of gibberellin-like substances by Azotobacter.
M.Sc. Thesis (Dept. of Microbiology, Macdonald College), McGill University.

Briggs, D. E. 1966.
Residues from organic solvents showing gibberellin-like biological activity.
Nature 210: 419-421.

Brown, M. E., and S. K. Burlingham. 1968.
Production of plant growth substances by Azotobacter chroococcum.
J. gen. Microbiol. 53: 135-144.

Brown, M. E., S. K. Burlingham, and R. M. Jackson. 1964.
Studies on Azotobacter species in soil. III. Effects of artificial inoculation on crop yields.
Plant Soil 20: 194-214.

Brown, N. A., and F. E. Gardner. 1936.
Galls produced by plant hormones, including a hormone extracted from Bacterium tumefaciens.
Phytopathology 26: 708-713.

Burger, K., and F. Bukatsch. 1958.
Über die Wuchsstoffsynthese im Boden frei lebender, stickstoffbindender Bakterien.
Zentbl. Bakt. ParasitKde. 3: 1-28.

Burlingham, S. K. 1964.
Growth regulators produced by Azotobacter in culture media.
Ann. Rep. Rothamsted experimental station 92.

_____. 1965.
Production of gibberellin-like substances by Azotobacter in culture.
Ann. Rep. Rothamsted experimental station 86.

Burström, H. 1950.
Studies on growth and metabolism of roots.
IV. Positive and negative auxin effects on cell elongation.
Physiol. Plant. 3: 277-292.

Burström, H. 1957.
On the adaptation of roots to β-indolylacetic acid.
Physiol. Plant. 10: 187-197.

Chailakhyan, M. Kh., and J. Falcou. 1966.
Bactérisation des graines et physiologie de la plante.
Ann. Inst. Pasteur 111, No. 3: 49-56.

Chailakhyan, M. Kh., A. A. Megrebyen, N. Karapetyan, and N. L. Kaladzhyan. 1965.
Growth substances in the secretion of Rhizobium sp.
Dokl. Akad. Nauk. Arm. SSR. 40: 307-314.
Cited from: Chem. Abst. 63: 16628e. 1965.

Curtiss, R. W. 1957.
Survey of fungi and actinomycetes for compounds possessing gibberellin-like activity.
Science 125: 646.

Denarié, J., and H. Blachère. 1966.
Inoculation de graines de végétaux cultivés à l'aide de souches bactériennes.
Ann. Inst. Pasteur 111, No 3: 57-74.

Frankland, B., and P. F. Wareing. 1960.
Effect of gibberellic acid on hypocotyl growth of lettuce seedlings.
Nature 185: 255-256.

Frieber, W. 1921.
Beitrage zur Frage der Indolbildung und der Indolreaktionen sowie zur Kenntnis des Verhaltens indolnegativer Bakterien.
Centbl. Bakt. 87: 254-277.
Cited from: Roberts, J. L., and E. Roberts. 1939.
Auxin production by soil microorganisms.
Soil Sci. 48: 135-139.

Galachyan, R. M. 1963.
Effect of amino acids and age of culture on the synthesis of growth substances.
Izv. Akad. Nauk. Arm. SSR. Biol. Nauki. 16: 37-40.
(Russian)

Galsky, A. G., and J. A. Lippincott. 1967.
Production of a gibberellin-like substance by strains of Agrobacterium tumefaciens.
Pl. Physiol. 42: S-29.

Goldacre, P. L. 1954.
The photochemical inactivation of indoleacetic acid sensitized by non-protein components of plant tissues.
Aust. J. Biol. Sci. 7: 225-250.

Gruen, H. E. 1959.
Auxins and fungi.
Annu. Rev. Pl. Physiol. 10: 405-411.

Gubanov, Y. V. 1953.
The effect of Azotobacterin on the growth, development and yield of wheat in the Krasnodar territory.
Author's thesis, Krasnodar.
Cited from: Brown, M. E., R. M. Jackson, and S. K. Burlingham. 1968.
Growth and effects of bacteria introduced into soil.
In: The ecology of soil bacteria. 531-551.
Liverpool University Press, Liverpool.

Hartley, R. D., T. A. Hill, G. F. Pegg, and G. G. Thomas. 1969.
Solvent and chemical impurities as sources of gibberellin-like growth-promoting activity.
J. Exp. Bot. 20: 276-287.

Hennequin, J. R., and H. Blachère. 1966.
Recherches sur la synthèse de phytohormones et de composés phénoliques par Azotobacter et des bactéries de la rhizosphère.
Ann. Inst. Pasteur 111, No. 3: 89-102.

Herter, C. A., and C. T. Broeck. 1911.
A biochemical study of Proteus vulgaris Hauser.
J. Biol. Chem. 9: 491-511.

Hopkins, F. G., and S. W. Cole. 1903.
A contribution to the chemistry of proteids.
II. The constitution of tryptophan and the action of bacteria upon it.
J. Physiol. 29: 451-466.

Jackson, R. M., M. E. Brown, and S. K. Burlingham. 1964.
Similar effects on tomato plants of Azotobacter inoculation and application of gibberellins.
Nature 203: 851-852.

Jessen, W. 1949.
Effect of inoculants on non-leguminous plants.
Z. PflErnahr. Dung. Bodenk. 44: 206-208.

Johnstone, D. B., and J. R. Fishbein. 1956.
Identification of Azotobacter species by fluorescence and cell analysis.
J. gen. Microbiol. 14: 330-335.

Jones, R. L., and J. E. Varner. 1967.
The bioassay of gibberellins.
Planta 72: 155-161.

Kaper, J. M., and H. Veldstra. 1958.
On the metabolism of tryptophan by Agrobacterium tumefaciens.
Bioshim. Biophys. Acta 30: 401-420.

Katznelson, H., and S. E. Cole. 1965.
Production of gibberellin-like substances by bacteria and actinomycetes.
Can. J. Microbiol. 11: 733-741.

Katznelson, H., and J. C. Sirois. 1961.
Auxin production by species of Arthrobacter.
Nature 191: 1323-1324.

Katznelson, H., J. C. Sirois, and S. E. Cole. 1962.
Production of a gibberellin-like substance by Arthrobacter globiformis.
Nature 196: 1012-1013.

Kögl, F., A. J. Haagen-Smit, and H. Erxleben. 1934.
Uber ein neues Auxin (Heteroauxin) aus Harn.
Z. Physiol. Chem. 228: 90-103.

Krasil'nikov, N. A. 1958.
Microorganisms synthesizing biotic substances.
In: Soil organisms and higher plants. 234-251.
The Academy of Sciences of the USSR., Moscow.

Kurosawa, E. 1926.
Experimental studies on the action of Fusarium heterosporum on rice plants.
Trans. Nat. History Soc. Formosa 16: 213-227.

Larsen, P. 1961.
Biological determination of natural auxins.
In: Encycl. Pl. Physiol. 14: 521-582.
Springer-verlag, Berlin.

Larsen, P., A. Harbo, S. Klungsoyr, and T. Aasheim. 1962.
On the biogenesis of some indole compounds in Acetobacter xylinum.
Physiol. Plant. 15: 552-565.

Lin, J-Y. 1963.
The production of gibberellin by Azotobacter chroococcum.
M.Sc. Thesis (National Taiwan Univ., Taipei, Taiwan).

MacMillan, J., and P. J. Suter. 1963.
Thin-layer chromatography of the gibberellins.
Nature 197: 790.

Magie, A. R., E. E. Wilson, and T. Kosuge. 1963.
Indoleacetamide as an intermediate in the synthesis of indoleacetic acid in Pseudomonas savastanoi.
Science 141: 1281-1282.

Mishustin, E. N. 1966.
Action d'Azotobacter sur les végétaux supérieurs.
Ann. Inst. Pasteur 111, No.3: 121-135.

Mishustin, E. N., and A. N. Naumova. 1962.
Bacterial fertilizers, their effectiveness and mechanism of action.
Microbiology (A Translation of Mikrobiologiya) 31: 442-452.

Montuelle, B. 1966.
Synthèse bactérienne de substances de croissance intervenant dans le métabolisme des plantes.
Ann. Inst. Pasteur 111, No.3: 136-146.

Montuelle, B., and L. Cheminais. 1964.
Synthèse de substances du groups des gibberellines par des bacteries.
C. R. Acad. Sc. Paris 258: 6016.

Mowat, J. A. 1963.
Gibberellin-like substances in algae.
Nature 200: 453-455.

Naundorf, G., and R. Nilsson. 1942.
Über formbildende Wirkstoffe bei Azotobacter chroococcum und der Einfluss dieser formativan Wirkstoffe auf die Bakteroid-enbildung von Bacterium radicicola.
Naturwissenschaften 30: 753.
Cited from: Vancura, V., and J. Macura. 1960.
Indole derivatives in Azotobacter cultures.
Folia microbiol. 5: 293-295.

Nicholls, P. B., and L. G. Paleg. 1963.
A barley endosperm bioassay for gibberellins.
Nature 199: 823-824.

Nita, A. Z. 1964.
The action of the metabolic products of microorganisms on higher plants.
Abstr. of paper presented at the VIII Inter. Congr. Soil Sci. Commission 111, Bucharest 106-107.

Nitsch, J. P., and C. Nitsch. 1955.
The separation of natural plant growth substances by paper chromatography.
Beitr. Biol. Pflanz. 31: 387-408.
Cited from : Bentley, J. A. 1961.
Chemical determination of auxins.
In: Encycl. Pl. Physiol. 14: 513-520.
Springer-verlag, Berlin.

_____. 1956.
Studies on the growth of coleoptile and first internode sections. A new, sensitive straight-growth test for auxins.
Pl. Physiol. 31: 94-111.

Novikova, A. T., and L. D. Irtuganova. 1966.
Heterauxin production by soil microorganisms.
Microbiology (A Translation of Mikrobiologiya) 35: 598-601.

Paleg, L. G. 1960.
Physiological effects of gibberellic acid.
(I) On carbohydrate metabolism and amylase activity of barley endosperm.
Pl. Physiol. 35: 293-299.

Paleg, L. G., D. Aspinall, B. Coombe, and P. B. N. Nicholls. 1964.
Physiological effects of gibberellic acid.
(VI) Other gibberellins in three test systems.
Pl. Physiol. 39: 286-290.

Panosyan, A. K., and G. S. Babayan. 1966.
Intensity of synthesis of auxin and GA-like substances by phosphcrous-mineralizing microorganisms of water and soil of Lake Seven area.
Biol. Zh. Armenii 19: 78-84. (Russian)
Cited from: Chem. Abst. 65: 4290c.

Perley, J. E., and B. B. Stowe. 1966.
The production of tryptamine from tryptophan by Bacillus cereus (KVT).
Biochem. J. 100: 169-174.

Phelps, R. H., and L. Sequeira. 1967.
Synthesis of indoleacetic acid by cell-free systems from virulent and avirulent strains of Pseudomonas solanacearum.
Phytopathology 57: 1182-1190.

Raznitsyna, E. A. 1938.
The formation of growth factors of the auxin group by bacteria. (Russian)
Dokl. Akad. Nauk. SSSR. 17: 353.

Rigaud, M. J. 1968.
Sur le métabolisme de la tryptamine par Rhizobium.
C. R. Acad. Sc. Paris t267: 2216-2219.

Roberts, J. L., and E. Roberts. 1939.
Auxin production by soil microorganisms.
Soil Sci. 48: 135-139.

Romanow, I. 1965.
Recherches sur la synthèse de l'acide indole-3-acétique par les Azotobacter.
Ann. Inst. Pasteur 109, No.3: 280-292.

Rubenchik, L. I. 1963.
In: Azotobacter and its use in agriculture.
Academy of Sciences of the Ukrainian SSR. English edn. Jerusalem. Israel Programme for Scientific translations.

Schmidt-Lorenz, W., and A. Ripple-Baldes. 1958.
Wirkung des Sauerstoffpartial druckes auf Wachstum und stickstoffbindung von Azotobacter chroococcum, Beij.
Arch. mikrobiol. 28: 45-68.

Sequeira, L., and P. H. Williams. 1964.
Synthesis of indoleacetic acid by Pseudomonas solanacearum.
Phytopathology 54: 1240-1246.

Smaly, V. T., and O. I. Bershova. 1957.
Formation of heteroauxin in Azotobacter cultures.
Microbiology (A Translation of Mikrobiologiya) 26: 526.

Sobieszczanski, J. 1966.
Studies on the role of microorganisms in the life of cultivated plants. III. Origin of the bacterial substances stimulating the growth of plants.
Acta Microbiol. Pol. 15: 67-84.

Stowe, B. B. 1955.
The production of indoleacetic acid by bacteria.
Biochem. J. 61: IX-X.

Stowe, B. B., and K. V. Thimann. 1954.
The paper chromatography of indole compounds and some indole-containing auxins of plant tissues.
Arch. Biochem. 51: 499-516.

Stowe, B. B., and T. Yamaki. 1957.
The history and physiological action of the gibberellins.
Annu. Rev. Pl. Physiol. 8: 181-216.

Thimann, K. V. 1935.
On the plant growth hormone produced by Rhizopus suinus.
J. Biol. Chem. 109: 279-291.

_____. 1936.
On the physiology of the formation of nodules on legume roots.
Proc. Nat. Acad. Sci. 22: 511-514.

Timonin, M. I. 1948.
Azotobacter preparation (Azotogen) as a fertilizer for cultivated plants.
Soil Sci. Soc. Am. Proc. 13: 246-250.

Trizno, S. I. 1954.
Inoculation of seeds with Azotobacter as a method of preventing lodging of spring cereals on peat-bogs.
Trudy Inst. Melior. vod. bolot. Khoz. 4: 36-42.
Cited from: Brown, M. E., S. K. Burlingham, and R. M. Jackson. 1964.
Studies on Azotobacter species in soil.
III. Effects of artificial inoculation on crop yields.
Plant Soil 20: 194-214.

Vancura, V. 1961.
Detection of gibberellic acid in Azotobacter cultures.
Nature 192: 88-89.

Vancura, V., and J. Macura. 1959.
The development of Azotobacter in wheat rhizosphere and its effect on the yield.
Folia microbiol. 4: 200-202.

_____. 1960.
Indole derivatives in Azotobacter cultures.
Folia microbiol. 5: 293-295.

Vancura, V., J. Macura, O. Fischer, and J. Vondracek. 1959.
The relation of Azotobacter to root system of barley.
Folia microbiol. 4: 110-129.

Varner, J. E., and G. R. Chandra. 1964.
Hormonal control of enzyme synthesis in barley endosperm.
Proc. Nat. Acad. Sci. 52: 100-106.

Vintika, Y. 1964.
Collection: Bacterial fertilizers (Russian).
Izd. Kolos., Moscow.
Cited from: Novikova, A. T., and L. D. Irtuganova. 1966.
Heteroauxin production by soil microorganisms.
Microbiology (A Translation of Mikrobiologiya) 35: 598-601.

Went, F. W. 1928.
Wuchsstoff und Wachstum.
Recl. Trav. bot. néerl. 25: 1-116.
Cited from: Bentley, J. A. 1961.
Extraction and purification of auxins.
In: Encycl. Pl. Physiol. 14: 501-512.
Springer-verlag, Berlin.

Wichtmann, H. 1952.
Studies on the relationship between Azotobacter content and soil fertility.
Arch. mikrobiol. 17: 54-78.

Wightman, F. 1962.
Metabolism and biosynthesis of 3-indoleacetic acid and related indole compounds in plants.
Can. J. Bot. 40: 689-718.

Zărnescu, A., and L. Nită. 1964.
Acțiunea produșilor de metabolism ai microorganismelor asupra plantelor superioare.
Am. Inst. Cercet. Pentru Cereale Plante Teh-Fundulea ser. B. 32: 443-455.