BACTERIAL AND CHEMICAL ANTAGONISTS TO

AGROBACTERIUM TUMEFACIENS AND THE

CROWN GALL TUMOR DISEASE

By

ROSALIE K. ~~TRUBY~~ Kelly

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Wilkes Barre, Pennsylvania

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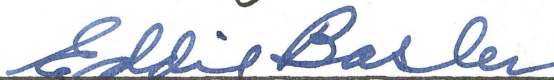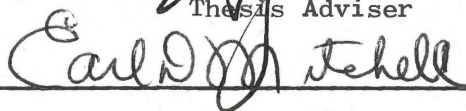December, 1975

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Thesis Approved:

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Thesis Adviser

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Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Interactions between plants and microorganisms can lead to a partial or complete destruction of the host plant or cause non-lethal morphological changes. Galls are the result of the latter type interaction in which the host survives in an adapted form, deviating from the normal by overgrowths (hypertrophy) and cell proliferation (hyperplasy). Crown gall is a nonself-limiting neoplastic disease of gymnosperms and dicotyledonous angiosperms which affects species belonging to one hundred and forty-two genera representing sixty one different families (Elliott, 1951).

Tumors arise when susceptible tissues are infected with virulent strains of Agrobacterium tumefaciens (Braun, 1947; Braun and Stonier, 1958). Once the tumors are formed, the bacteria are no longer needed for continued tumor growth and the abnormal cells proliferate autonomously. Bacteria-free crown gall tissue was isolated (White, 1945; De Ropp, 1947b, c; Hilderbrandt and Riker, 1947, 1949) from primary tumors of many different plant species and upon implantation into a healthy susceptible host, the sterile tumor tissue fragments developed into tumors indistinguishable from those initiated by the bacteria (Braun and Stonier, 1958).

It has been suggested by Braun and Stonier (1958) that a factor of considerable biological interest passes from the bacteria to the host

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cells and brings about a heritable change in the affected cells. This factor, the tumor inducing principle (TIP) may fall into one of five categories: (1) A chemical fraction of A. tumefaciens that is capable of initiating a specific alteration with a resultant continued and in this instance abnormal development of those cells; (2) A metabolic product of A. tumefaciens; (3) A normal host constituent that is converted by the bacteria into a carcinogenic principle; (4) The crown gall bacteria themselves which enter the wounded cells and become so altered in their morphology and physiology as not to be demonstrable by either isolation or staining procedures; or (5) A virus associated with and transmitted by A. tumefaciens. Much investigation of the crown gall disease has centered about the identity of TIP and its possible mechanism of tumor induction.

Thermal inactivation studies on the transformation process done by Braun (1950) indicate that the activation energy for this destruction was more than 80,000 calories per mole, characteristic of protein denaturation, suggesting that either TIP or something intimately associated with the inability of this principle to initiate tumors at a higher temperature may be a factor of complex structure.

Klein (1952, 1953, 1954); Klein and Knupp (1957); Klein, Rasch, and Swift (1953) indicated from their studies that a specific polymer of deoxyribonucleic acid (DNA) may be TIP. It was reported by Klein (1952, 1953) that in tomato plants the level of DNA in the host tissue increased 200% of control values within 24 hrs after inoculation with oncogenic crown gall bacteria and remained at that level for an additional 24 hrs, after which it dropped abruptly, reaching control levels 72 hrs after inoculation. Tissues treated with nononcogenic

cultures showed no DNA peak. When a tomato plant inoculated with the oncogenic strain was held at 30 C, the observed DNA level was less than half that found at 25 C, 24 hrs after inoculation. At the higher temperature, the denaturation curve broke sharply, reaching control levels after 48 hrs suggesting, according to Braun and Stonier (1958), a heat-induced depolymerization of DNA. However, it is difficult to judge whether such a reported rise in DNA is the cause or an effect of the transformation process. It is possible according to Braun and Stonier (1958) that a second DNA peak develops as normal cells are altered to tumor cells.

If TIP is a chemical factor such as DNA postulated by Klein (1952), then one may consider it in terms of the following three possibilities listed earlier: (1) As part of the bacterial cell itself; (2) As a metabolic product of the bacterium; or (3) As a normal host constituent converted by the bacteria into a carcinogenic principle.

The first possibility, that the observed rise in DNA reflects bacterial DNA was not considered likely by Klein (1954) who pointed to the fact that there is no corresponding rise in ribonucleic acid (RNA) as would be expected if bacterial multiplication were involved. The phosphorous content of the crown gall bacteria as calculated from P^{32}-uptake studies is of the order of 2.5 X 10^{-9} µg per bacterium (Stonier, 1956a). In tomato, of the order of 700 µg DNA-phosphorous are produced per gram (dry weight) of tissue (Klein, 1952). On the assumption that 20 percent of the bacterial phosphorous is in the tumor-inducing DNA fraction, such a quantity would represent 1.4 X 10^{12} bacteria. (Should the percentage of such DNA-phosphorous be less, the number of bacteria involved would be correspondingly more.) This

means that 24 hrs post inoculation, a slice of tomato stem 1mm thick

(dry weight approximately 1.5 mg) would contain a minimum of 2.1

billion bacteria, enough to solidly occupy about 1.6 cubic mm [average

bacterial volume is 0.783 μ^3 (Stonier, 1956a)]. This according to

Braun and Stonier (1958) is in contrast to common experience.

Other investigators suggest that bacterial nucleic acids combine

with plant cell nucleic acids and subsequently replicate. Schilperoort

et al. (1967), found a partial homology between A. tumefaciens DNA and

tumor DNA, but found no homology between A. tumefaciens DNA and normal

plant cell DNA. Milo and Srivastava (1969) reported a significant

hybridization between DNA from A. tumefaciens (B_6) and RNA fractions of

tumor tissue. Kovoor (1967) transformed normal tissue culture cells

into tumor cells with A. tumefaciens DNA. Schilperoort (1971) showed

that A. tumefaciens DNA is located in the nuclei of the tumor cells. He

concluded that A. tumefaciens DNA is integrated into the genome of crown

gall tumor cells and that genetic information of the bacterium comes to

expression as crown gall formation. Swain and Rier (1972) reported

that total RNA isolated from oncogenic strains of A. tumefaciens pro-

duced tumors on tomato plants. Beljanski et al. (1974), isolated and

purified two RNA fractions from both oncogenic and nononcogenic strains

of A. tumefaciens. Both RNA fractions were capable of producing tumors

in susceptible host plants. Hamilton and Chopan (1975) reported that

they were able to transfer a plasmid from oncogenic A. tumefaciens to

cured A. tumefaciens (nononcogenic) by injecting the oncogenic strain

into a tumor and then reisolating it. They implied that plasmid DNA

is TIP.

The second possibility, that a much smaller number of bacteria synthesize and release large amounts of nucleic acid was more probable to Braun and Stonier (1958). If this were true, they thought, one might expect that the phosphorous metabolism of oncogenic bacteria would differ from that of nononcogenic crown gall bacteria and that this would be reflected in the amount of phosphorous released by P^{32}-labeled bacteria. However, this was not the case (Braun and Stonier, 1958).

The third possibility, that the normal host constituent becomes converted by the bacteria into a carcinogenic agent presents two alternatives (Braun and Stonier, 1958): (1) The host provides the DNA precursor which is assimilated by the bacteria, altered, and then released, i.e., a special case of TIP being a bacterial metabolite. This is in conflict with P^{32}-release studies (Braun and Stonier, 1958). (2) The host provides the DNA precursor(s) and an alteration is accomplished extracellularly by means of an enzyme or other product released by the bacteria implying that the enzyme or other bacterial product as well as the polymerized host DNA, may induce tumors in the absence of bacteria. In 1954, Klein had 10 to 30 percent success in producing tumors with inoculations of wound sap and bacterial filtrates, however, wound sap from previously heavily wounded plants was essential to its success.

The possibility that the bacteria themselves act as TIP by becoming intimately associated with the host cells was examined by Stonier (1956b) by tracing the fate of P^{32}-labeled bacteria in the host via radioautography. The radioactivity remained localized in the intercellular spaces, except for cells whose walls had been ruptured,

or in the xylem elements. Stonier concluded that the bacteria exert their effect across the host cell membrane. Bogers (1972) showed via electron microscopy that cell walls of Agrobacteria attach to plant cell walls during the tumor initiation process. Using both oncogenic and nononcogenic strains, he found that both bacteria attached to the plant cell walls but only the oncogenic strains produced tumors. He concluded that the nononcogenic Agrobacteria compete for a limited number of specific bacterial attachment sites in the conditioned wounds; and that the attachment of the oncogenic strains to these sites may be an essential stage in tumor initiation.

The possibility of a virus being transmitted by the bacteria as the causative agent in crown gall disease was first seriously advanced by De Ropp (1947b). Camus, Wildman, and Bonner (1951) reported finding a new high molecular weight protein that constitutes about 20 percent of the total protein in crown gall tumor tissue. But as Gautheret (1952) reported, the new protein was noninfectious and absent in normal tissue. Zimmerer, Hamilton, and Pootjes (1966) reported the presence of lytic activity in supernatants from cultures of A. tumefaciens IIBV7 and IIBNV6, oncogenic and nononcogenic respectively, for the induction of crown gall tumors on susceptible plants. Parsons and Beardsley (1968) isolated bacteriophage PS8, which infects several strains of A. tumefaciens originally from sunflower crown gall tumor tissue grown in tissue culture for many years in the apparent absence of the tumor inducing bacteria. These results plus those of Tourneur and Morel (1970) who isolated Agrobacteria phage from several crown gall tissues growing in sterile culture but none from cultures of normal tissue is suggestive of phage involvement in crown gall formation. Leff and Beardsley (1970)

reported that DNA isolated from phage PS8 could induce tumorous
proliferations, sporadically, in pinto bean leaves, sunflower stems,
and tobacco stems. Beiderbeck, Heberlein, and Lippincott (1973)
isolated DNA from A. tumefaciens bacteriophage PS8 and tested whole
phage and phage DNA for tumor-inducing ability on ten species of plants.
The previously reported tumorigenicity of phage PS8 DNA could not be
confirmed and no evidence to implicate phage PS8 is involved in tumor
initiation was obtained.

The most prominent theories that have emerged on TIP suggest that
it is bacterial nucleic acid in origin. Nuclear DNA (Klein, 1952, 1953,
1954; Klein and Knupp, 1957; Klein et al., 1953; Kovoor, 1967;
Schilperoort et al., 1967; Quetier et al., 1969; Milo and Srivastava,
1969), plasmid DNA (Hamilton and Chopan, 1975; Watson et al., 1975),
and nuclear RNA (Swain and Rier, 1972; Beljanski et al., 1974) have all
been implicated as possibilities for elaboration of the crown gall tumor
disease.

The best evidence for the continued longevity of the crown gall
tumor is DNA continuity; and associated with DNA is RNA. DNase
inactivates DNA and RNase will inactivate RNA. Braun and Wood (1966)
found that when RNase was added to the wound site prior to bacterial
inoculation, there was a marked inhibition of tumor formation. Since
many things can happen to pure enzymes in foreign tissue, bacteria that
produced DNase and RNase were used in a portion of this study. Part of
this investigation was to look for bacteria antagonistic to A.
tumefaciens which would inactivate TIP when inoculated into a
susceptible host along with the tumor inducing bacteria. In addition,
the effects of certain chemical antagonists such as antibiotics,

pantoyl lactone, and natural plant hormones which are associated with the process of wound healing, were also determined both on <u>A. tumefaciens</u> and the initiation of the crown gall tumor disease.

CHAPTER II

MATERIALS AND METHODS

Tumor Inducing Bacterium

A virulent subculture of Lippincott's <u>Agrobacterium</u> <u>tumefaciens</u> B_6, a Gram negative motile aerobic rod (0.5 to 1.0 μ by 1.5 to 2.0 μ) was utilized in all tests.

Growth Medium

Mannitol-yeast agar consisting of 5.0 g mannitol, 0.2 g magnesium sulfate, 0.2 g dipotassium phosphate, 0.2 g sodium chloride, 0.1 g calcium sulfate, 15.0 g agar, and 100 ml of 10% yeast-water in 1 liter of twice deionized distilled water was utilized as the growth medium. Its pH was adjusted to 7.0 with KOH prior to sterilization by auto-claving at 121 C with 15 pounds pressure per square inch for 15 min.

Growth

<u>A.</u> <u>tumefaciens</u> was grown on slants of the mannitol-yeast agar at 25 C for 24 hrs for all tests unless otherwise noted. Stock cultures were transferred monthly and maintained at 4 C. Periodically <u>A.</u> <u>tumefaciens</u> was run thru the 3-keto-lactose test (Bernaerts and DeLey, 1963) to check on its pathogenicity. Growth assays were performed in Kimax test tubes (18 X 150 mm) containing a total volume of 5 ml (growth

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medium plus test chemicals). Cells grown on slants of mannitol-yeast agar were the source of inocula. The cells were harvested, washed with sterile twice deionized distilled water, and resuspended in sterile twice deionized distilled water to an optical density (540 nm) of 0.8 (Coleman Junior Spectrophotometer). Two drops (0.1 ml) of this suspension was used to inoculate each tube. Each testing situation was performed in triplicate for the growth assays. Tubes were incubated at 25 C with reciprocal shaking. Growth was determined by following the increase in optical density of the culture at 540 nm. Aseptic conditions were maintained at all times.

Inoculation Procedure

Twenty-four hour cultures of A. tumefaciens grown on mannitol-yeast agar slants at 25 C were harvested, washed with sterile twice deionized distilled water and resuspended in sterile twice deionized distilled water to an optical density (540 nm) of 0.8. Most test organisms were grown on nutrient agar slants for 24 hrs at 25 C, harvested, washed with sterile twice deionized distilled water, resuspended in sterile twice deionized distilled water, and adjusted to an optical density of 0.8 (540 nm). Other agrobacteria used as test organisms were grown on mannitol-yeast agar slants. Anaerobes were grown in freshly steamed thioglycollate broth while cellulolytic bacteria were grown in cellulose minimal medium (1.0 g K_2HPO_4, 0.5 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 liter of distilled water; pH to 7.5, with a 1.0 X 9 cm strip of filter paper per tube). Each was harvested by centrifugation and resuspended in sterile twice deionized distilled water. Chitinolytic bacteria were grown in chitin minimal salts medium

[0.03% each of K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and NaCl; sterilized by autoclaving at 121 C with 15 pounds pressure per square inch for 15 minutes prior to adjusting the pH to 7.4 and saturating with chitin flakes (from crab shells, lot 93C-2740, Sigma Chemical Company, St. Louis, Mo.) prior to inoculation] for 3 days at 37 C with reciprocal shaking. Five ml portions of each culture were harvested by centrifugation and re-suspended in sterile twice deionized distilled water. Resuspended *A. tumefaciens* cells were added to resuspended test organism cells immediately prior to inoculation into the host plants. When utilized, test chemicals were added to the resuspended *A. tumefaciens* cells immediately prior to inoculation into host plants. A sterile disposable 1 cc syringe with a 27 gauge needle was used to wound the plant and to inoculate 2×10^8 cells of *A. tumefaciens* and 2×10^8 cells of the test organism or 2×10^8 cells of *A. tumefaciens* and the test chemicals together into the host plant.

Host Plant Growth Conditions

Both sunflowers (*Helianthus annus* L.) and tomatoes (*Lycopersicon esculentum* Mill., var. *spring giant*) were used as host plants.

Sunflower seeds (Northrup King & Co., Minneapolis, Minn.) were planted in lots of 15 per flat in a soil mixture containing garden soil, peat moss, and sand in a ratio of 2:1:1. Flats were kept under green-house conditions; and were watered daily with tap water. Germination took 1 to 2 weeks. Four week old seedlings of approximately the same size were used for all test inoculations. Twenty-four hours prior to inoculation, flats of seedlings were transported to a 25 C 50% humidity room with 100 foot candles of light for a period of 16 hrs

followed by a period of darkness for 8 hrs. Plants remained under
these growth conditions for the duration of their use as hosts.

Tomato seeds (Ferry-Morse Seed Company, Inc., Fulton, Ky.) were
treated with arasan (tetra-methylthiuramdisulfide, complements of
Dr. F. Gough, O.S.U.) an anti-fungicide, prior to planting in flats
containing Perl-gro (Grace and Co., Cambridge, Mass.) in lots of about
18 to 24 seeds (the usual contents of each packet). Flats were kept in
the greenhouse and were watered daily with tap water. After germination
which usually occurred from 7 to 14 days, the plants were watered with
Hoagland's nutrient solution containing Fe-EDTA once a week and with
tap water each of the remaining 6 days. When the seedlings developed
2 sets of secondary leaves, they were transplanted into 4 inch diameter
clay pots containing Perl-gro and were maintained in the greenhouse
under the above irrigation conditions. At about 4 to 5 weeks of age,
seedlings of approximately the same size were transported to a growth
chamber (Sherer-Gillet Co., Marshall, Mich.) which was maintained at
25 C, 90% humidity and 12 hr day lengths in preparation for test
inoculations. Plants were generally inoculated after 1 day in the
growth chamber, kept for another 5 days for possible tumor trans-
formation to occur, subirrigated daily with tap water, then transported
back to the greenhouse where they were maintained under the usual
greenhouse conditions except that subirrigation was continued for the
duration of the 5 week testing period.

Tumor Development

Tumor development was monitored weekly. Final observations
were made at 5 weeks post wounding and inoculation. Plants

were then either discarded or utilized for reisolation experiments.

Tumor size was recorded as small if the tumor resulting from inoculation of A. tumefaciens plus the test organism was smaller than the control (1.9 cm on the average), i.e., a tumor produced by inoculation of A. tumefaciens into the host plant, and large if the resulting tumor was larger than the control.

In the tomato stem tumor system, the tumors were measured with a metal drafting kit (Riefler). Both the diameter of the tumor inside the plant stem (when the stem was split) and the diameter of the outer tumor (the entire tumor including the epidermal stem layer) were measured and recorded.

Test Organisms

Isolations were made from soil, air, water, insects, and birds. Soil samples were taken from open fields, nurseries, greenhouses, orchards, suburban areas, and whenever possible from areas around plant roots. One gram of soil was suspended in 10 ml of sterile distilled water and 0.1 ml aliquots of the soil suspension were inoculated into tubes of steamed thioglycollate broth, cellulose minimal medium, and streaked onto nutrient agar plates for isolation. Cultures were incubated at 25 C. All growth was restreaked onto nutrient agar plates for isolation and colonial morphology was recorded. Cellulolytic isolates were maintained in cellulose minimal medium. All other pure cultures isolated from the soil were maintained on nutrient agar slants. All isolates were kept at 4 C unless otherwise noted.

Air isolates were made by exposing sterile nutrient agar plates to the surroundings in open fields, nurseries, greenhouses, orchards, and

suburban areas. Colonies were restreaked for isolation. All pure cultures isolated from air were maintained on nutrient agar slants.

Water samples taken from streams, creeks, rivers, ponds, and lakes were inoculated into cellulose minimal medium and streaked onto nutrient agar plates. Incubation followed at 25 C. Positive cellulose medium cultures and colonies from nutrient agar plates were restreaked for isolation. Cellulolytic isolates were maintained in cellulose minimal medium at 25 C while other water isolates were kept on nutrient agar slants.

Isolations made from insects were made by streaking grasshopper intestinal contents onto nutrient agar plates and incubating them at 30 C. Colonies were restreaked for isolation. Pure cultures were maintained on nutrient agar slants.

Chitinolytic cultures isolated from birds, frog, and fish intestines, ponds, and a compost pile were grown in chitin minimal salts medium at 37 C with reciprocal shaking for several days. Positive chitinolytic cultures were maintained on chitin minimal salts agar.

Several miscellaneous laboratory cultures were also utilized as test organisms. Subcultures were graciously donated by various co-workers. These were grown on nutrient agar slants.

Anaerobes of the genus <u>Clostridium</u> were subcultured from stock cultures into freshly steamed thioglycollate broth, grown at 25 C for 24 hrs and maintained in thioglycollate broth.

Agrobacteria other than <u>tumefaciens</u> B_6, contributed by Dr. Lee A. Bulla, Research Leader, Stored Products Insect Research, Grain Marketing Research Center, Manhattan, Kansas, were also utilized as

test organisms. These were grown on mannitol-yeast agar slants,

incubated at 25 C for 24 hrs, and maintained on the same medium.

Nuclease Screening Procedure

Each isolate was screened for production of DNase and RNase prior

to selection as a test organism. Bacteria that produced DNase, RNase,

DNase and RNase, and neither DNase nor RNase were chosen as test

organisms. The screening procedure consisted of streaking isolates

onto plates of a variation of the Bacto-DNase test agar with methyl

green with DNA (sperm, control no. 5268, Nutritional Biochemicals Corp.,

Cleveland, Ohio) and RNA (sodium nucleate, Nutritional Biochemicals

Corp., Cleveland, Ohio) according to Difco Chemicals : 20 g Difco Bacto

tryptose, 2.0 g DNA, 5.0 g NaCl (J. T. Baker Chemical Co., Phillipsburg,

N. J.), 30 g Difco Bacto agar, 0.5 g methyl green (Ethylated hexamethyl-

pararosaniline, double green SF, methylanaline green, No. B347, the

Matheson Co., Inc., East Rutherford, N. J.) in 1 liter of distilled

water. To prepare the RNase medium, 2.0 g of RNA were substituted for

the DNA and methyl green content was increased to 0.75 g. Sterilization

was accomplished at 121 C with 15 pounds pressure per square inch for

15 min. A clearing around the streak was indicative of nuclease

production by the bacterium under test.

Bacterial Reisolations

Aniline-blue yeast-water mannitol medium, a variation of Pastel's

medium (1926): 5.0 g mannitol, 0.2 g magnesium sulfate, 0.2 g dipotas-

sium phosphate, 0.2 g sodium chloride, 0.1 g calcium sulfate, 0.1 g

aniline-blue, 15.0 g agar, 100 ml of 10% yeast water, 900 ml distilled

water, adjusted to pH 7.0 prior to sterilization was originally tried as a selective medium. <u>A.</u> <u>tumefaciens</u> produces abundant growth and absorbs aniline-blue whereas <u>A.</u> <u>radiobacter</u> and other agrobacteria do not absorb aniline-blue. However, all of the test organisms streaked onto the agar pick up the aniline-blue also, thereby making the medium useless as a selective medium for these particular laboratory purposes.

Sodium selenite yeast-water glucose agar medium: 5.0 g glucose, 0.1 g sodium selenite, 15.0 g agar in 1 liter of 1 percent yeast water (Hendrickson, Baldwin, and Riker, 1934) was investigated as a differential medium. <u>A.</u> <u>tumefaciens</u> grows abundantly with a distinct red color due to the presence of selenite while <u>A.</u> <u>radiobacter</u> does not grow on it. However all of the test organisms also grew abundantly on the medium and picked up the red color of the free selenite. This formulation was also useless as a selective medium.

Various combinations of carbon sources and pH indicators were tried in an effort to find a selective and/or a differential medium for use in these investigations. None were successful.

Suspensions of macerated tumors used for reisolation studies were streaked onto mannitol-yeast agar. The several colonies growing on it were each restreaked onto fresh mannitol-yeast agar plates for isolation. Each isolated colony was then subjected to the 3-keto-lactose test, wet mount, and a Gram stain for identification. All positive 3-keto-lactose cultures were reinoculated into a host plant to check for tumor production, a conclusive positive test for <u>A.</u> <u>tumefaciens.</u>

Reisolation Procedures

In the tube technique, the tumor was excised with a scalpel and placed about 5 cm down into a test tube which was held at an angle. The scalpel was resterilized (by flaming it after dipping into methanol) after each cut and the cut away epidermal cells were removed from the tube. The tumor was shaken into 2 ml distilled water already in the tube, incubated at 25 C for several minutes, mashed with a 5 ml pipette, and incubated at 25 C for an additional 5 minutes. Plates of mannitol-yeast agar were streaked with the water suspension using cotton swabs. This technique was utilized throughout the investigation.

In the mortor and pestle technique, the tumor was excised with a scalpel and placed into a mortor. Two ml distilled water was added and the contents were ground using a pestle. Plates of mannitol-yeast agar were streaked with the water suspension using cotton swabs. This technique was tried several times but was not utilized since the previous one gave faster and more defined results.

Preliminary Antibiotic Screening

Low concentration Difco Bacto-sensitivity antibiotic disks: chloromycetin, 5 μg; erythromycin, 2 μg; kanamycin, 5 μg; neomycin, 5 μg; novobiocin, 5 μg; penicillin, 2 units; streptomycin, 2 μg; and tetracycline, 5 μg; were utilized in the initial sensitivity screening. These experiments were done to determine antibiotic sensitivity which could be utilized as markers in the event plasmid transfer experiments were initiated. Plates of mannitol-yeast agar were evenly inoculated with A. tumefaciens. Four disks were placed on each plate and

incubation followed at 25 C for 48 hrs. Zones of inhibition indicative

of sensitivity of **A. tumefaciens** to the particular antibiotic were

observed, measured, and recorded.

Tetracycline

Tetracycline (hydrochloride, crystalline, No. T-3383, Sigma

Chemical Co., St. Louis, Mo.) was dissolved in phosphate buffer (pH 7.0)

to a concentration of 1 mg/ml and filter sterilized (HAWG 04700,

HA 0.45 μ). Growth assays were performed on **A. tumefaciens** with tetra-

cycline final concentrations of 20, 16, 10, 6, 2, and 1 μg/ml. All of

these concentrations at which growth was inhibited were then injected

into host plants along with 2 X 10^8 cells of **A. tumefaciens.**

Rifampicin

Rifampicin [3-(4-methylpiperazinyliminomethyl) rifamycin SV,

No. R-3501, Sigma Chemical Co., St. Louis, Mo.] was dissolved in

phosphate buffer (pH 7.0) to a concentration of 1 mg/ml and filter

sterilized. Growth assays were performed on **A. tumefaciens** with

rifampicin final concentrations of 20, 16, 10, 6, 2, 1, 0.2, and 0.1

μg/ml. Several concentrations at which growth was inhibited were then

injected into host plants along with 2 X 10^8 cells of **A. tumefaciens.**

Pantoyl Lactone

Pantoyl lactone (No. P-2750, lot 101c-2291, Sigma Chemical Co.,

St. Louis, Mo.) was dissolved in twice deionized distilled water,

adjusted to pH 7.0 with NaOH, filter sterilized, and diluted to give

final concentrations of 0.22, 0.11, 0.062, 0.055, and 0.027 M. The

varying concentrations were added to resuspended 24 hour cells of

A. tumefaciens and the combinations were then inoculated into host

plants.

Plant Hormones

Indole acetic acid (IAA), purified 4/9/75 by Dr. E. Basler, O.S.U.;

zeatin [6-(trans-4-hydroxy-3-methylbut-2-enylamino) purine, No. Z-0125,

Sigma Chemical Co.]; gibberellic acid (GA_3) which was purchased from

Sigma Chemical Co., No. G-3250; and abscisic acid (ABA) also from Sigma

Chemical Co., No. A-7383 were used in growth assay studies using

A. tumefaciens. A preliminary screening was done on a plate of

mannitol-yeast agar that was heavily streaked with A. tumefaciens.

A small amount of the powdered hormone was added to the surface of each

inoculated plate.

Each plant hormone was made up into a stock solution of 1 mg/ml

using phosphate buffer (pH 7.0), filter sterilized, and varying

dilutions were added to tubes of freshly inoculated cultures of A.

tumefaciens to give final concentrations of 20, 16, 10, 6, 2, 1, 0.2,

and 0.1 μg/ml. IAA, zeatin, GA_3, and ABA and all together at a

concentration of 20 μg/ml were inoculated into host plants along with

2×10^8 cells of A. tumefaciens.

CHAPTER III

RESULTS AND DISCUSSION

Antagonisms of Test Organisms

Soil Isolates

Seventeen different soil isolates were used as test organisms, 2 of which produced DNase only (Table I). One of the DNase producing soil isolates allowed A. tumefaciens to form small tumors while the other allowed it to form large tumors thereby showing no complete tumor antagonism and no general positive correlation in tumor inhibition or accentuation.

No soil isolate produced only RNase. Of the 11 soil isolates that produced both DNase and RNase, 5 allowed formation of small tumors and 6 allowed formation of large tumors. None of the soil isolates producing both nucleases showed complete tumor antagonism and nearly equal amounts of small and large tumors were formed by A. tumefaciens and these test organisms. Therefore, no general correlation can be made.

Of the 4 soil isolates that produced neither nuclease, 2 allowed formation of small tumors while the remaining 2 allowed formation of large tumors. None of these isolates exhibited total antagonism toward tumor formation by A. tumefaciens; and no positive correlation was found between tumor size and lack of nuclease production.

TABLE I

TEST ORGANISMS: SOURCE, NUCLEASE PRODUCTION, AND TUMOR ANTAGONISMS IN THE PRESENCE OF <u>A.</u> <u>TUMEFACIENS</u>

Source	Isolated	Nuclease Produced			Number & Percent Producing			
					Small Tumors		Large Tumors	
			#	%	#	%	#	%
Soil Isolates	17	DNase	2	12	1	6	1	6
		DNase & RNase	11	64	5	29	6	35
		Neither	4	24	2	12	2	12
		Total:			8	47	9	53
Air Isolates	71	DNase	23	32	14	20	9	13
		DNase & RNase	38	54	12	16	26	37
		Neither	10	14	4	6	6	8
		Total:			30	42	41	58
Water Isolates	10	DNase	4	40	3	30	1	10
		DNase & RNase	4	40	1	10	3	30
		Neither	2	20	0	0	2	20
		Total:			4	40	6	60
Insects Isolates	28	DNase	2	7	1	4	1	4
		DNase & RNase	14	50	11	39	3	11
		Neither	12	43	6	21	6	21
		Total:			18	64	10	36
Miscellaneous Laboratory Cultures	23	DNase	3	13	1	4	2	9
		DNase & RNase	15	65	7	30	8	35
		Neither	5	22	3	13	2	9
		Total:			11	47	12	53
Anaerobic Isolates	9	Not tested	--	--	3	33	6	67
Cellulolytic Isolates	11	DNase	1	9	1	9	0	0
		DNase & RNase	10	91	5	45.5	5	45.5
		Total:			6	54.5	5	45.5
Chitinolytic Isolates	10	RNase	4	40	3	30	1	10
		DNase & RNase	6	60	2	20	4	40
		Total:			5	50	5	50
Agrobacteria Other than <u>tumefaciens</u> B$_6$	7	DNase	3	42	1	13	2	29
		DNase & RNase	2	29	2	29	0	0
		Neither	2	29	2	29	0	0
		Total:			5	71	2	29

Twelve percent of the soil isolates produced only DNase, 64% produced DNase and RNase, and 24% produced neither nuclease (Table I). Of these, 8 or 45% (of the total soil isolates) seemed to behave somewhat antagonistically by allowing A. tumefaciens to form small tumors, while 9 or 53% allowed A. tumefaciens to form tumors of increased size. Of the DNase producing soil isolates utilized as test organisms, 6% allowed formation of small tumors while another 6% allowed formation of large tumors. No soil isolate produced only RNase. Of the DNase and RNase producing test organisms, 29% allowed formation of small tumors while 35% allowed formation of large tumors. Of the test organisms producing neither nuclease, 12% behaved somewhat antagonistically and allowed formation of small tumors while an equal amount allowed formation of large tumors.

No soil isolate was entirely antagonistic to tumor formation by A. tumefaciens. Forty-seven percent of the soil isolates did exhibit somewhat of an antagonistic effect toward tumor formation by A. tumefaciens in allowing the formation of tumors smaller than controls. Fifty-three percent of the soil isolates seemed to accentuate the ability of A. tumefaciens to form tumors in allowing it to form tumors of similar size and/or larger than the controls. No general positive correlation in tumor antagonism to A. tumefaciens is evidenced by the soil isolates.

Air Isolates

Seventy-one different air isolates were used as test organisms in search of bacterial antagonists to A. tumefaciens and the crown gall tumor disease. Twenty-three produced DNase and 14 of these showed some

antagonism to A. tumefaciens in that they allowed it to form small

tumors whereas 9 allowed A. tumefaciens to form large tumors (Table I).

No air isolates produced only RNase. Thirty-eight air isolates produced

DNase and RNase, 12 of which allowed formation of small tumors while 26

allowed formation of large tumors. Ten air isolates produced both

nucleases. Four of these allowed formation of small tumors while 6

allowed formation of large tumors.

Thirty-two percent of the air isolates produced DNase, 54% produced

both DNase and RNase, and 14% produced neither nuclease (Table I). Of

these, 30 or 42% seemed to behave somewhat antagonistically in allowing

A. tumefaciens to form small tumors, while 41 or 58% permitted formation

of large tumors. Of the DNase producing test organisms, 20% (of the

total air isolates) allowed formation of small tumors while 13% allowed

formation of large tumors. No air isolate produced RNase only. Of the

DNase and RNase producing test organisms, 16% allowed A. tumefaciens to

form small tumors and 37% allowed formation of large tumors. Of the

air isolates that produced neither nuclease, 6% allowed A. tumefaciens

to form small tumors while 8% allowed formation of large tumors.

No air isolate was entirely antagonistic to A. tumefaciens. Forty-

two percent of the air isolates did exhibit somewhat of an antagonistic

effect toward tumor formation by A. tumefaciens in allowing the

formation of tumors smaller than the controls. Fifty-eight percent of

the air isolates seemed to accentuate ability of A. tumefaciens to

form tumors (similar size and/or larger than the controls). More air

isolates allowed for large tumor formation than for tumor antagonism,

and more than twice as many large tumors were formed by A. tumefaciens

and air isolates producing both nucleases than were small tumors.

However, no general positive correlation can be made between nuclease production and either tumor antagonism or tumor accentuation and air isolates utilized as test organisms.

Water Isolates

Ten different water isolates were utilized, 4 of which produced DNase (Table I). Three of the DNase producing water isolates allowed formation of small tumors while 1 allowed formation of large tumors. No water isolate produced RNase only. Of the 4 water isolates that produced DNase and RNase, 1 allowed formation of small tumors while the other 3 allowed formation of large tumors. Both of the water isolates that produced neither nuclease allowed formation of large tumors.

No water isolate was entirely antagonistic to formation of tumors by A. tumefaciens. Forty percent of the water isolates produced DNase, 40% produced DNase and RNase, and 20% produced neither nuclease (Table I). Of these, 4 or 40% seemed to behave somewhat antagonistically in allowing formation of small tumors, while 6 or 60% allowed formation of tumors of increased size. Three of the DNase producing water isolates or 30% (of the total water isolates used as test organisms) allowed formation of small tumors while 1 or 10% allowed formation of large tumors. Of the DNase and RNase producing water isolates, 1 or 10% allowed formation of small tumors while 3 or 30% allowed formation of large tumors. All or 20% of the water isolates producing neither nuclease allowed formation of large tumors by A. tumefaciens.

More large tumors were formed with water isolates and A. tumefaciens than were small tumors. As a whole, the water isolates accentuated tumor formation rather than acting antagonistically. However, the

DNase producing water isolates allowed A. tumefaciens to form 3 times as many small tumors as large tumors indicating somewhat of an antagonistic effect of these isolates on A. tumefaciens and its ability to cause tumor formation. The water isolates that produced both DNase and RNase allowed A. tumefaciens to produce 3 times as many large tumors as small tumors showing a greater degree of tumor accentuation by the presence of RNase along with DNase. Of the water isolates that produced neither nuclease, only tumor accentuation was observed. No general positive correlation can be made between tumor antagonism or tumor accentuation and nuclease production in the water isolates utilized as test organisms.

Insect Isolates

Twenty-eight different insect isolates were used, 2 of which produced DNase (Table I). One of the DNase producing insect isolates allowed formation of small tumors while the other allowed formation of large tumors. No insect isolate produced only RNase. Fourteen insect isolates produced DNase and RNase. Of these, 11 seemed to behave somewhat antagonistically by allowing formation of small tumors, while 3 of the insect isolates producing both nucleases seemed to accentuate ability of A. tumefaciens to form tumors by allowing it to make large tumors. Twelve insect isolates produced neither nuclease. Half of these allowed formation of small tumors while the other half allowed formation of large tumors. No general positive correlation can be made with both the DNase producing insect isolates and those producing neither nuclease, as half of each allowed for small tumor formation while the other half allowed for large tumor formation. There may be

an antagonism to ability of <u>A</u>. <u>tumefaciens</u> to make tumors in those

isolates producing both nucleases. It would seem that the added

production of RNase may somehow alter ability of <u>A</u>. <u>tumefaciens</u> to

carry out TIP to the fullest extent, thereby producing tumors smaller

than <u>A</u>. <u>tumefaciens</u> can produce without the antagonistic effect of these

test organisms. However, 3 of the insect isolates producing both

nucleases did allow formation of large tumors by <u>A</u>. <u>tumefaciens</u>.

Seven percent of the insect isolates produced DNase, 50% produced

both DNase and RNase, and 43% produced neither nuclease (Table I). Of

these, 18 or 64% seemed to behave somewhat antagonistically in allowing

formation of small tumors, while 10 or 36% seemed to accentuate ability

of <u>A</u>. <u>tumefaciens</u> to form tumors by allowing the organism to produce

large tumors. Of the DNase producing insect isolates, 4% (of the total

insect isolates) allowed formation of small tumors while another 4%

allowed formation of large tumors. No insect isolate produced RNase

only. Thirty-nine percent of the insect isolates producing DNase and

RNase allowed formation of small tumors while 11% allowed formation of

large tumors. This is more than 3 times as many small tumors as large

tumors and may be due to an antagonistic effect created by perhaps the

added production of RNase in most of these isolates. Twenty-one percent

of those producing neither nuclease allowed production of small tumors

while the remaining 21% allowed formation of large tumors.

No insect isolate was entirely antagonistic to <u>A</u>. <u>tumefaciens</u> and

tumor formation. The most antagonism seen in the insect isolates is in

those producing both nucleases where more than 3 times as many test

organisms allowed formation of small tumors by <u>A</u>. <u>tumefaciens</u>. Also, if

one looks at the total of small tumor formation versus large tumor

formation, nearly twice as many isolates allowed A. tumefaciens to form small tumors as large tumors. Perhaps there is a slight tumor antagonism shown in the DNase and RNase producing insect isolates. However no general positive correlation between nuclease production and tumor formation can be made.

Miscellaneous Laboratory Cultures

Twenty-three different miscellaneous laboratory cultures were used as test organisms, 3 of which produced DNase (Table I). One of the DNase producing cultures allowed formation of small tumors while 2 allowed formation of large tumors. None of these cultures produced only RNase. Of the 15 laboratory cultures that produced both DNase and RNase, 7 allowed formation of small tumors while 8 allowed formation of large tumors. Three of the cultures producing neither nuclease allowed formation of small tumors while two allowed formation of large tumors. No general positive correlation can be made on the miscellaneous laboratory cultures pertaining to tumor formation and nuclease production. Even though twice as many DNase producing cultures allowed accentuation of tumor formation by forming large tumors, this number represents only 2 out of 3 DNase producers and is probably not significant.

Of the 23 laboratory cultures, 13% produced DNase, 65% produced DNase and RNase, and 22% produced neither DNase nor RNase (Table I). Eleven or 47% allowed formation of small tumors while 12 or 53% allowed formation of large tumors. Of the DNase producing cultures, 4% (of the total miscellaneous laboratory cultures) allowed for small tumor formation, while 9% allowed for large tumor formation. No laboratory culture produced only RNase. Thirty percent of the cultures producing

both nucleases allowed formation of small tumors while 35% allowed

formation of large tumors. Of the cultures that produced neither

nuclease, 13% allowed formation of small tumors while 9% allowed

formation of large tumors.

No laboratory culture was entirely antagonistic to ability of A.

tumefaciens to form tumors. Although there were twice as many large

tumors formed by the DNase producing cultures, this category repre-

sented a total of 3 cultures thereby perhaps making the results not

entirely significant and no general positive correlation can be made

from these results. No laboratory culture produced only RNase, and the

other groups of nuclease producing test organisms allowed A. tumefaciens

to stimulate production of approximately equal amounts of small and

large tumors. No general positive correlation can be made between

nuclease production and ability of A. tumefaciens to form tumors for

the miscellaneous laboratory cultures used as test organisms.

Anaerobes

Nine different anaerobes were used (Table I). None of these could

be tested for nuclease production. Three anaerobes allowed formation

of small tumors while six allowed formation of large tumors.

Thirty-three percent of the anaerobes behaved antagonistically

allowing A. tumefaciens to produce small tumors (Table I). Sixty-six

percent allowed A. tumefaciens to accentuate its tumor production and

form large tumors.

No general positive correlation can be made between nuclease

production and tumor formation for the anaerobes utilized as test

organisms. However, the anaerobic organisms allowed A. tumefaciens to

produce twice as many large tumors as small tumors. Perhaps these data indicate that the anaerobes accentuated the ability of A. tumefaciens to allow TIP to manifest itself to a greater degree.

Cellulolytic Isolates

Eleven different cellulolytic isolates were utilized, one of which produced DNase (Table I). This DNase-producing cellulolytic isolate allowed only formation of small tumors by A. tumefaciens. No cellulolytic isolate produced RNase only. Of the 10 DNase and RNase-producing cellulolytic isolates, 5 allowed formation of small tumors while the other 5 allowed formation of large tumors. No cellulolytic organism tested produced neither nuclease.

Nine percent of the cellulolytic organisms produced DNase and 91% produced both DNase and RNase (Table I). Six or 54.5% allowed formation of small tumors while 5 or 45.5% allowed formation of large tumors. Nine percent (of the total cellulolytic isolates) allowed formation of small tumors. No cellulolytic organism (1) producing DNase only allowed formation of large tumors.

No cellulolytic organism tested produced only RNase, and no cellulolytic organism produced neither nuclease. Of the DNase and RNase producing cellulolytic isolates, 5% allowed formation of small tumors while another 5% allowed formation of large tumors.

No cellulolytic isolate allowed A. tumefaciens to behave entirely antagonistically and yield no tumors. No cellulolytic organism produced only RNase, and none produced neither DNase nor RNase. Only 1 cellulolytic organism isolated, and consequently utilized as a test organism, allowed formation of small tumors, whereas the cellulolytic

organisms that produced both nucleases allowed formation of equal
amounts of small and large tumors.

Even though the DNase producing cellulolytic organism (1) allowed
formation of small tumors, no significant general positive correlation
can be made regarding nuclease production, tumor formation, and
antagonism. No general positive correlation can be made with the DNase
and RNase producing cellulolytic organisms since they allowed A.
tumefaciens to produce equal amounts of small and large tumors.

Chitinolytic Isolates

Ten different chitinolytic isolates were utilized (Table I). None
of the chitinolytic isolates produced DNase only and none produced no
nuclease. The chitinolytic organisms were the first group of isolates
to produce only RNase. Of the 4 RNase producing chitinolytic organisms,
3 allowed formation of small tumors by A. tumefaciens while the
remaining 1 allowed formation of large tumors. No complete tumor
antagonism was shown by the RNase producing chitinolytic organisms but
perhaps partial antagonism to tumor formation by A. tumefaciens was
shown by 3 out of the 4 RNase producing test organisms. Of the 6 DNase
and RNase producing chitinolytic organisms, 2 allowed formation of
small tumors while 4 allowed formation of large tumors.

Forty percent of the chitinolytic organisms produced RNase and
60% produced DNase and RNase. None produced only DNase and neither
DNase nor RNase (Table I). Five or 50% allowed formation of small
tumors thereby appearing to behave somewhat antagonistically toward
ability of A. tumefaciens to elaborate TIP. The remaining 5 or 50%
allowed formation of large tumors or accentuated ability of A.

<u>tumefaciens</u> to elaborate TIP. Of the RNase producing chitinolytic organisms, 30% allowed formation of small tumors while 10% allowed production of large tumors. Of the DNase and RNase producing chitinolytic organisms, 20% allowed formation of small tumors while 40% allowed formation of large tumors.

No chitinolytic organism was entirely antagonistic to tumor formation by <u>A</u>. <u>tumefaciens</u>. No chitinolytic organism produced only DNase and none produced neither DNase nor RNase. Fifty percent of the total chitinolytic isolates allowed production of small tumors while the remaining 50% allowed formation of large tumors. Three times as many small tumors were formed by <u>A</u>. <u>tumefaciens</u> and chitinolytic organisms producing RNase than were large tumors. Twice as many large tumors were produced by <u>A</u>. <u>tumefaciens</u> and chitinolytic organisms producing both DNase and RNase. Even though RNase producing chitino-lytic organisms allowed <u>A</u>. <u>tumefaciens</u> to produce more small tumors, and the DNase and RNase producing chitinolytic organisms allowed for production of twice as many large tumors as small tumors, no overall general positive correlation can be made between nuclease production and antagonism or accentuation of tumor production by <u>A</u>. <u>tumefaciens</u>.

<u>Agrobacteria other than tumefaciens B</u>$_6$

Seven different agrobacteria other than <u>tumefaciens</u> B$_6$ were utilized, 3 of which produced DNase (Table I). Of the DNase producing cultures, 1 allowed formation of large tumors. None produced only RNase. Of the 2 DNase and RNase producing cultures, both allowed formation of small tumors. The 2 cultures that produced neither nuclease also allowed formation of small tumors.

Of the 7 agrobacteria cultures, 42% produced DNase, 29% produced
DNase and RNase, and 29% produced neither nuclease (Table I). Of these,
5 or 71% seemed to behave somewhat antagonistically in allowing
formation of small tumors while 2 or 29% allowed formation of large
tumors. Of the DNase producing cultures, 13% (of the total agrobacteria
other than <u>tumefaciens</u> B_6) allowed formation of small tumors while 29%
allowed formation of large tumors. All of the DNase and RNase producing
cultures, 29%, allowed formation of small tumors and all of the cultures
producing neither nuclease, 29%, also allowed formation of small tumors.
No general positive correlation can be made between nuclease production
and tumor antagonism or tumor accentuation as the same type of tumor
antagonism is seen with and without the presence of nucleases in these
test organisms.

No organism in this group was entirely antagonistic to tumor
formation by <u>A</u>. <u>tumefaciens</u> (B_6). Seventy-one percent of these
organisms exhibited somewhat of an antagonistic effect toward formation
of tumors by allowing the formation of tumors smaller than controls.
Twenty-nine percent of the cultures seemed to accentuate ability of
<u>A</u>. <u>tumefaciens</u> (B_6) by forming tumors of similar size and/or larger than
the controls. No general positive correlation in tumor antagonism to
<u>A</u>. <u>tumefaciens</u> (B_6) is evidenced by the agrobacteria other than
<u>tumefaciens</u> (B_6) utilized as test organisms.

Nuclease Production and Antagonisms

One hundred and seventy-seven of the isolates were screened for
nuclease production. Thirty-eight test organisms produced DNase,
4 produced RNase, 100 produced both, and 35 produced neither (Table II).

TABLE II

NUCLEASE PRODUCTION AND TUMOR ANTAGONISMS

Nuclease	Nuclease Produced by Test Organisms		Small Tumors		Large Tumors	
	#	% of Total	#	%*	#	%*
DNase	38	22	22	58	16	42
RNase	4	2	3	75	1	25
Both	100	56	45	45	55	55
Neither	35	20	17	49	18	51
TOTAL	177		87	49	90	51

*Percent of total DNase producing test organisms or percent of total RNase producing organisms, etc.

No test organism behaved entirely antagonistically towards A. tumefaciens and inhibited tumor production. Partial tumor inhibition as well as tumor accentuation were observed. Tumors smaller than controls (those produced by A. tumefaciens alone and 1.9 cm in diameter on the average) were designated small while tumors of the same size as the controls or larger were termed large.

Of the DNase producing test organisms, 22 allowed A. tumefaciens to form small tumors while 16 allowed A. tumefaciens to yield large tumors. Thus no general positive correlation was found between DNase production and tumor inhibition.

Very few (4) isolates produced RNase only. Several of the chitinolytic organisms elaborated this enzyme. Three of these allowed A. tumefaciens to form small tumors while the remaining 1 allowed A. tumefaciens to make large tumors.

Most (100) of the test organisms produced both DNase and RNase. However, no general positive correlation can be made regarding tumor production since nearly equal amounts of small and large tumors were produced by A. tumefaciens in their presence. Forty-five test organisms producing both nucleases allowed A. tumefaciens to make small tumors while 55 allowed A. tumefaciens to make large tumors. The combination of both nucleases did not inhibit tumor production.

Since many isolates produced neither nuclease, these were selected as test controls. Seventeen of these organisms allowed A. tumefaciens to form small tumors while 18 producing neither DNase nor RNase allowed A. tumefaciens to form large tumors. No general positive correlation can be made since nearly equal amounts of both small and large tumors were produced regardless of the nuclease capability of the test

organisms. The only positive observation is that even with DNase and RNase present, A. tumefaciens was able to cause production of tumors.

As a whole, 87 or 49% of the test organisms utilized allowed formation of small tumors by A. tumefaciens while 90 or 51% allowed formation of large tumors (Table II). Twenty-two percent produced DNase, 2% produced RNase, 56% produced both DNase and RNase, and 20% produced neither nuclease. Of the DNase producing test organisms, 58% allowed formation of small tumors by A. tumefaciens and 42% allowed formation of large tumors. No general positive correlation can be made with DNase production and tumor formation as both slight tumor antagonism and tumor accentuation were caused by these organisms. Since the presence of DNase in the test organism did not inhibit A. tumefaciens from forming tumors, and since both A. tumefaciens and the test organism were reisolated from tumors in equal proportions in reisolation studies, they had the opportunity to behave antagonistically and it appears that TIP is not naked DNA from A. tumefaciens.

It was surprising that only 2% of the entire lot of isolates utilized as test organisms produced only RNase. Only several chitino-lytic organisms tentatively identified as Gram negative, motile, green pigmented short rods from varied sources of isolation (yellow-billed cuckoo, frog, chicken gut, and compost pile) were able to elaborate only this nuclease. Of these, 75% allowed formation of small tumors by A. tumefaciens while 25% allowed formation of large tumors (Table II). None of the RNase producing test organisms were entirely antagonistic to A. tumefaciens. Even though 3 times as many RNase producing test organisms allowed A. tumefaciens to form small tumors, no general positive correlation can be made regarding nuclease production and tumor

inhibition since only a few of these test organisms were available. However, one can say that since the RNase-producing test organisms and A. tumefaciens could be reisolated in equal amounts from the tumors, they did perhaps act antagonistically, and the majority of them somehow either partially inhibited elaboration of TIP by A. tumefaciens or caused its destruction after elaboration and prior to the time it exerts its effects on the plant cells. From these results it would follow that TIP is most likely not naked A. tumefaciens RNA.

Fifty-six percent of all the test organisms screened for nuclease production produced both DNase and RNase (Table II). None of the test organisms producing both nucleases inhibited A. tumefaciens from producing tumors. However, nearly equal amounts of small and large tumors were made by A. tumefaciens in their presence. Forty-five percent allowed formation of small tumors by A. tumefaciens while 55% allowed formation of large tumors. No general positive correlation can be made about production of both DNase and RNase by test organisms and ability of A. tumefaciens to make tumors in their presence. Since both the test organisms and A. tumefaciens were reisolated from tumors in equal proportions, they had the opportunity to behave antagonistically and it appears that TIP is not A. tumefaciens DNA nor RNA.

Many isolates produced neither nuclease. These were also selected as test organisms and they made up 20% of the total isolates screened for nuclease production. Of these, none was entirely antagonistic toward A. tumefaciens since none inhibited tumor formation by A. tumefaciens. Nearly equal amounts of small tumors (49%) and large tumors (51%) were made by A. tumefaciens and these test organisms (Table II). The lack of nuclease production didn't seem to matter to

ability of <u>A. tumefaciens</u> to elaborate TIP and produce tumors. Again both slight tumor antagonism and tumor accentuation were observed with these test organisms. These results complement the three previous observations, i.e., TIP is not naked DNA, RNA, nor DNA and RNA from <u>A. tumefaciens</u>, still the organism was capable of causing production of small and large tumors in the presence of test organisms that produced neither nuclease.

Again, as with the other test organisms, these cultures were reisolated along with <u>A. tumefaciens</u> in equal proportions from tumors. Therefore, they remained alive and had the opportunity to behave perhaps antagonistically toward <u>A. tumefaciens</u>. Since these test organisms also allowed <u>A. tumefaciens</u> to form tumors, it would seem then that TIP may not be naked DNA or RNA from <u>A. tumefaciens</u> but some other entity. It is possible that this entity may even be composed of bacterial DNA or RNA somehow sheltered from the attack of DNase and RNase produced by the antagonistic organisms in the entire mileu of wound rehealing, TIP elaboration, and tumor formation.

Antibiotic Antagonisms

Antibiotics are known to inhibit specific areas of bacterial cell growth and division. Penicillin, vancomycin, ristocetin, bacitracin, and novobiocin inhibit cell wall synthesis at various steps during the membrane associated assembly of mucopeptide from uridine nucleotide precursors (Burrows, 1973). D-Cycloserine competitively inhibits two successive steps, i.e., the racemization of L-alanine to form D-alanine and the following dipeptidization reaction to form D-alanyl-D-alanine, in the formation of the pentapeptide (Davis et al., 1973).

A variety of antibiotics are known to inhibit protein synthesis. Tetracycline is bacteristatic and blocks protein synthesis by interacting with the 30S subunit of the ribosome (Burrows, 1973). Rifampicin (rifampin) selectively inhibits bacterial RNA polymerase (Davis et al., 1973).

Antibiotic effects on A. tumefaciens were studied and those showing any bactericidal or bacteriostatic activity were utilized in plant inoculation studies.

Of all the antibiotics used in the preliminary sensitivity screening: chloromycin, neomycin, erythromycin, kanamycin, novobiocin, penicillin, streptomycin, and tetracycline, only tetracycline produced a zone of inhibition 18mm in diameter (Table III).

TABLE III

EFFECT OF ANTIBIOTIC SENSITIVITY
DISCS ON A. TUMEFACIENS

Antibiotic	Concentration	Zone of Inhibition
Chloromycin	5 µg	none
Neomycin	5 µg	none
Erythromycin	2 µg	none
Kanamycin	5 µg	none
Novobiocin	5 µg	none
Penicillin	2 units	none
Streptomycin	2 µg	none
Tetracycline	5 µg	18 mm

Tetracycline

When added to viable A. tumefaciens cells in final concentrations

of 20, 16, 10, 6, 2, and 1 μg/ml, tetracycline appeared to inhibit

growth of the cells (Figure 1). The highest concentration of tetra-

cycline, 20 μg/ml, inhibited growth of A. tumefaciens the greatest, and

each successively lesser concentration inhibited growth of A.

tumefaciens to a lesser degree. The lowest concentration tested

(1 μg/ml), inhibited the least. Microscopic examination of the cells at

0, 5, 9, 12, 15, and 24 hrs of growth revealed no morphological changes

had occurred.

All of the concentrations of tetracycline utilized in the growth

assay were added with A. tumefaciens in plant inoculations. No con-

centration of the antibiotic was entirely antagonistic toward ability of

A. tumefaciens to make tumors. However, all concentrations of tetra-

cycline allowed formation of small tumors (0.2 to 0.4 cm in diameter)

as compared to controls (1.0 cm) (Table IV). Therefore, it appears

that tetracycline behaved antagonistically toward A. tumefaciens. The

highest concentration of tetracycline, 20 μg/ml, inhibited A.

tumefaciens the greatest (Figure 1), and allowed it to form tumors of

0.7 cm on the average (0.2 cm corrected for stem diameter). Other

concentrations allowed for formation of tumors of decreasing size.

Although the effect is not linear, a gradual decrease in tumor size is

evident. The exceptions may simply be the result of possible human

error in calculation and/or exact measurement of the proper concen-

trations of tetracycline. Errors in inoculation of exactly 2×10^8

cells of A. tumefaciens each time are also likely to occur.

Figure 1. Effect of Tetracycline on Growth of <u>A. tumefaciens</u> in
mannitol-yeast broth at 25 C.   ● , control;
⬡ , 1 μg/ml tetracycline; ▲ , 2 μg/ml tetracycline;
▢ , 6 μg/ml tetracycline; ○ , 10 μg/ml tetracycline;
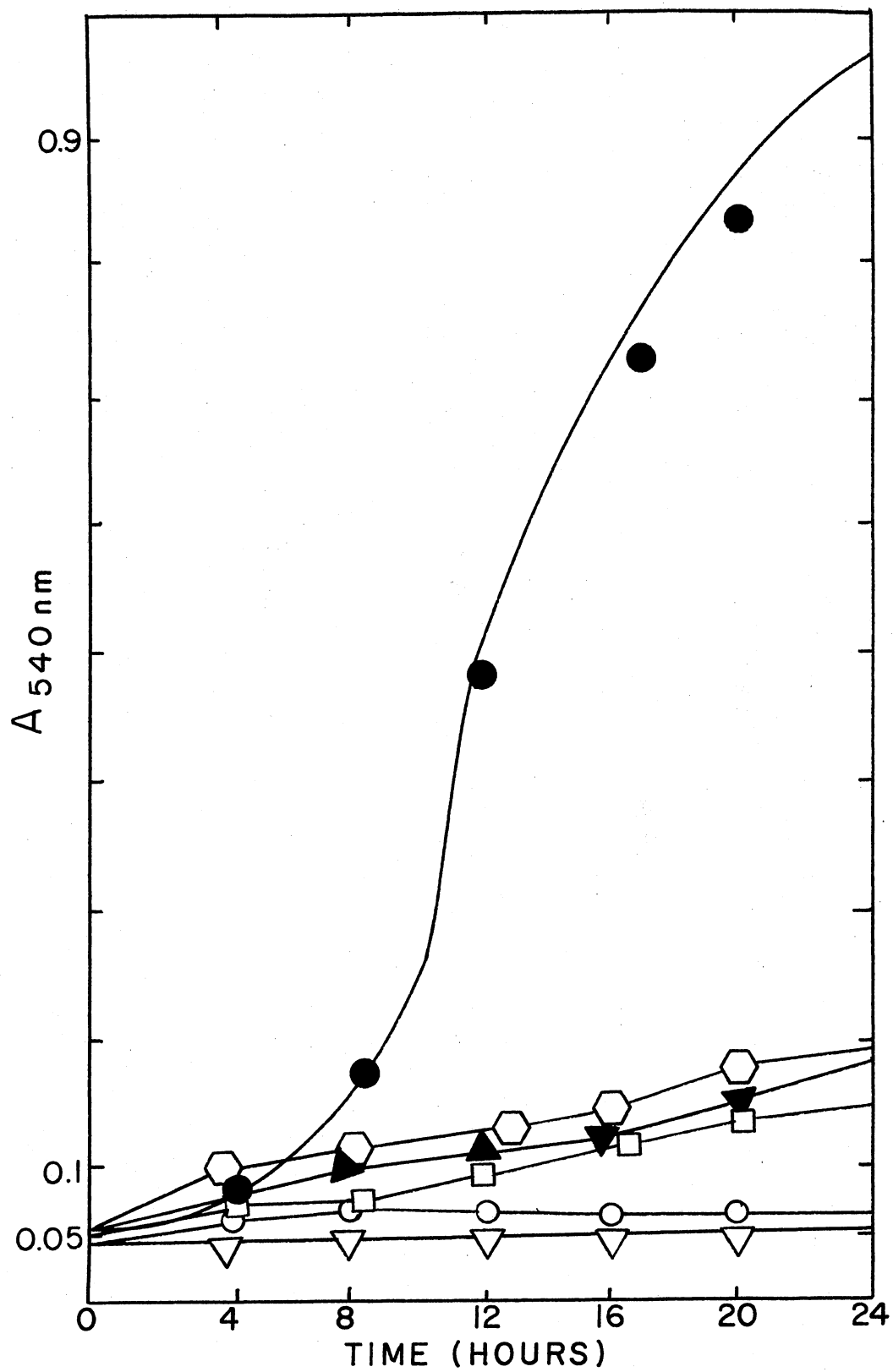▽ , 16 and 20 μg/ml tetracycline.

TABLE IV

EFFECT OF TETRACYCLINE ON TUMOR FORMATION BY A. TUMEFACIENS IN LYCOPERSICON ESCULENTUM

Plant #*	Amount of Antibiotic	Site**	Tumor Size***		Stem Size***	
49	None	(1)	1.7		0.6	
49	"	(3)	1.5	1.6	0.6	0.6
			Corrected Value:			1.0
50	20 μg/ml	(1)	0.7		0.5	
50	"	(3)	0.7		0.5	
51	"	(1)	0.7		0.5	
51	"	(3)	0.7	0.7	0.5	0.5
			Corrected Value:			0.2
52	16 μg/ml	(1)	1.0		0.6	
52	"	(3)	0.8		0.5	
53	"	(1)	0.9		0.6	
53	"	(3)	0.8	0.9	0.5	0.6
			Corrected Value:			0.3
54	10 μg/ml	(1)	1.0		0.6	
54	"	(3)	0.8		0.5	
55	"	(1)	1.0		0.6	
55	"	(3)	0.9	0.9	0.6	0.6
			Corrected Value:			0.3
56	6 μg/ml	(1)	0.7		0.5	
56	"	(3)	0.7		0.5	
57	"	(1)	0.8		0.5	
57	"	(3)	0.8	0.8	0.5	0.5
			Corrected Value:			0.3
58	2 μg/ml	(1)	1.0		0.6	
58	"	(3)	1.1		0.6	
59	"	(1)	1.4		0.6	
59	"	(3)	1.0	1.1	0.8	0.7
			Corrected Value:			0.4
60	1 μg/ml	(1)	0.9		0.5	
60	"	(3)	0.9		0.6	
61	"	(1)	1.2		0.6	
61	"	(3)	1.0	1.0	0.7	0.6
			Corrected Value:			0.4

*All plants received 2×10^8 cells of A. tumefaciens.

**Site: (1) and (3) denote internode 1 and 3 respectively beginning with the one directly above the cotyledons.

***Tumor, stem, and average sizes given as diamters in cm. Corrected Value: denotes the average tumor size minus the average stem size in cm.

Rifampicin

When added to viable A. tumefaciens cells in final concentrations of 10, 6, 2, and 1 µg/ml, rifampicin appears to inhibit growth of the cells, while concentrations of 0.2 and 0.1 µg/ml of the antibiotic allowed A. tumefaciens to follow a natural growth pattern (Figure 2). Microscopic examination of the cells at 0, 5, 9, 12, 15, and 24 hrs of growth revealed no morphological changes and very few lysed cells.

Rifampicin concentrations of 6, 2, 1, and 0.2 µg/ml were utilized with A. tumefaciens cells in plant inoculations. No concentration of the antibiotic was entirely antagonistic toward ability of A. tumefaciens to make tumors. All concentrations of rifampicin allowed formation of tumors smaller than without the antibiotic, thereby exhibiting an antagonistic effect on tumor formation. The control formed tumors of 1.8 cm in diameter on the average [1.2 cm in diameter corrected size (Table V)]. The highest concentration of rifampicin used, 6 µg/ml, allowed formation of tumors 0.7 cm in diameter (0.2 cm corrected), while the rest of the concentrations of rifampicin utilized permitted tumor formation of decreasing size.

Pantoyl Lactone Antagonisms

Pantoyl lactone (PL) has been utilized in cell division studies (Grula and Grula, 1962a) on Erwinia species and on Micrococcus lysodeikticus in this laboratory. Effects of PL as a chemical antagonist to the crown gall tumor disease were investigated.

Final concentrations of 0.22, 0.11, 0.062, 0.055, and 0.022 M (28.6, 14.3, 8.1, 7.2, and 3.6 µg/ml, respectively) PL were injected

Figure 2. Effect of Rifampicin on Growth of <u>A. tumefaciens</u> in mannitol-
yeast broth at 25 C. ● , control; ▲ , 0.1 μg/ml
rifampicin; ⬡ , 0.2 μg/ml rifampicin; O, 1 μg/ml
rifampicin; ■ , 2, 6, and 10 μg/ml rifampicin.
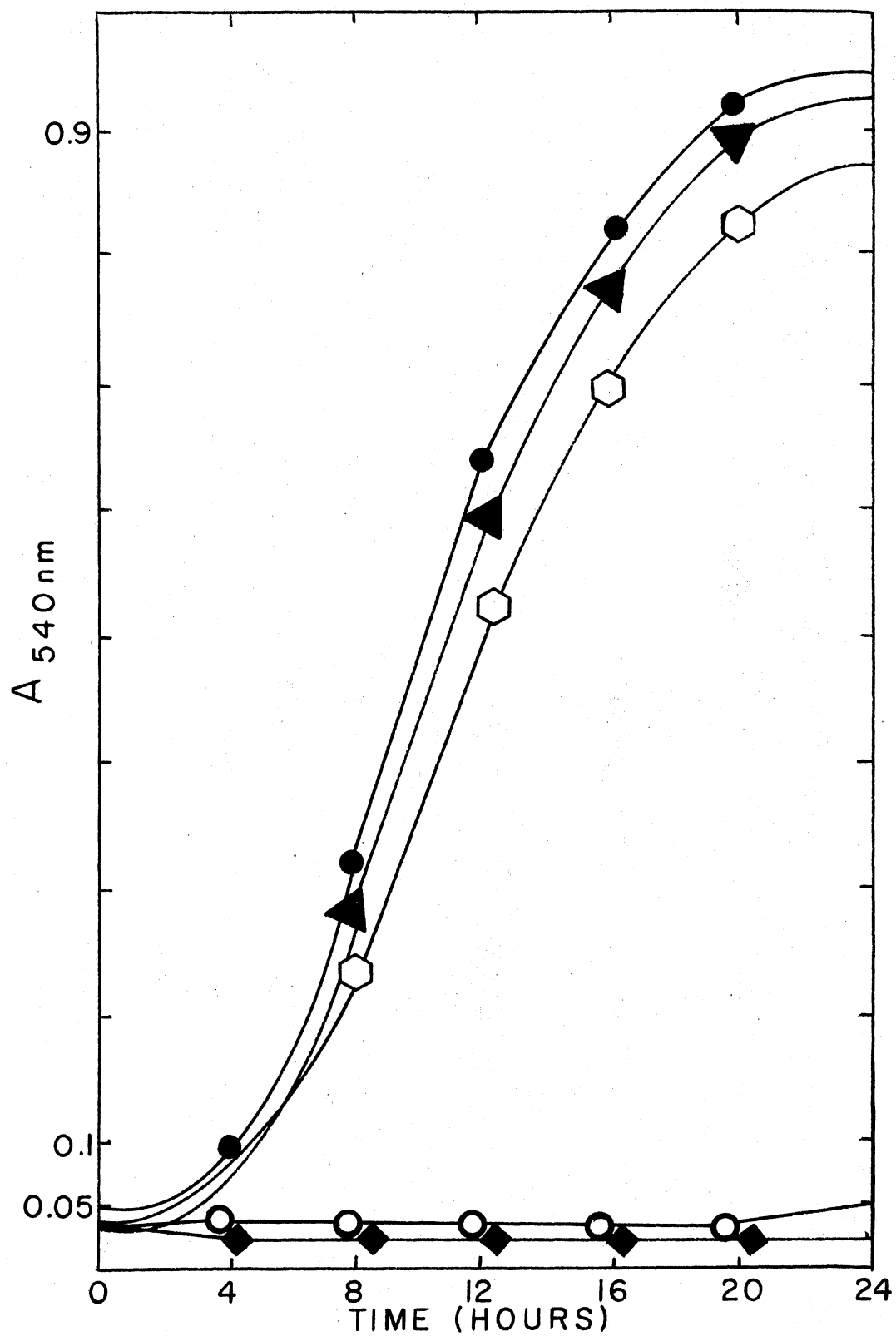
TABLE V

EFFECT OF RIFAMPICIN ON TUMOR FORMATION BY
<u>A</u>. <u>TUMEFACIENS</u> IN <u>LYCOPERSICON</u> <u>ESCULENTUM</u>

Plant #*	Amount of Antibiotic	Site**	Tumor Size***		Stem Size***	
62	None	(1)	1.8		0.6	
62	"	(3)	1.8		0.6	
63	"	(1)	1.8		0.6	
63	"	(3)	1.8	1.8	0.6	0.6
			Corrected Value:			1.2
64	6 µg/ml	(1)	0.6		0.5	
64	"	(3)	0.8		0.5	
65	"	(1)	0.6		0.5	
65	"	(3)	0.8	0.7	0.5	0.5
			Corrected Value:			0.2
66	2 µg/ml	(1)	1.7		0.6	
66	"	(3)	1.5		0.6	
67	"	(1)	1.5		0.6	
67	"	(3)	1.5	1.6	0.6	0.6
			Corrected Value:			1.0
68	1 µg/ml	(1)	1.3		0.5	
68	"	(3)	1.5		0.6	
69	"	(1)	1.3		0.5	
69	"	(3)	1.5	1.4	0.6	0.6
			Corrected Value:			0.8
70	0.2 µg/ml	(1)	1.9		0.6	
70	"	(3)	1.3		0.6	
71	"	(1)	1.6		0.6	
71	"	(3)	1.3	1.5	0.6	0.6
			Corrected Value:			0.9

*All plants received 2×10^8 cells of <u>A</u>. <u>tumefaciens</u>.

**Site: (1) and (3) denote internode 1 and 3 respectively beginning with the one directly above the cotyledons.

***Tumor, stem, and average sizes given as diameters in cm.
  Corrected Value: denotes the average tumor size minus the average stem size in cm.

into susceptible host plants along with 2 X 10^8 cells of <u>A.</u>

<u>tumefaciens</u>.

No concentration of PL was entirely antagonistic toward ability of

<u>A. tumefaciens</u> to form tumors. At a concentration of 0.22 M (28.6 µg/ml)

PL allowed formation of tumors of 1.5 cm diameter on the average while a

concentration of 0.11 M (14.3 µg/ml) allowed formation of tumors 1.9 cm

in diameter (Table VI). Lower concentrations of PL allowed for

formation of tumors of smaller size.

No general positive correlation can be made between tumor inhibi-

tion nor tumor accentuation and PL concentration. If PL does act

antagonistically toward <u>A. tumefaciens</u> this antagonism does not appear

to have any effect on the ability of <u>A. tumefaciens</u> to make tumors.

Even at a concentration of about 0.062 M, i.e., 8.1 µg/ml, (at which PL

reverses induced cell division inhibition in a species of <u>Erwinia</u>) no

morphological changes were observed in <u>A. tumefaciens</u> and there was no

effect on the formation of tumors by <u>A. tumefaciens</u> in its presence.

Plant Hormone Antagonisms

Indole acetic acid (IAA), the major plant auxin, is responsible for

cell enlargement in higher plants (Jablonski and Skoog, 1954) and normal

plant tissue requires auxin for growth. Crown gall tumor tissue

requires no auxin to grow and is inhibited by auxin (Braun and Stonier,

1958). Kaper and Veldstra (1958) reported that <u>A. tumefaciens</u> causes

plant IAA to break down into indole acidic aldehyde and indole lactic

acid.

Cytokinins such as zeatin which affect cell division are thought

to possess important regulatory activity in tRNA due to their location

TABLE VI

EFFECT OF PANTOYL LACTONE ON TUMOR FORMATION BY
A. TUMEFACIENS IN LYCOPERSICON ESCULENTUM

Plant #*	Amount of PL	Site**	Tumor Size***	Avg. Size***
25	None	(1)	1.7	
25	"	(3)	1.9	
26	"	(1)	2.0	
26	"	(3)	1.9	1.9
27	0.22 M	(1)	1.4	
27	"	(3)	1.2	
28	"	(1)	1.8	
28	"	(3)	1.7	1.5
29	0.11 M	(1)	1.9	
29	"	(3)	2.2	
30	"	(1)	2.1	
30	"	(3)	1.4	1.9
31	0.062 M	(1)	2.1	
31	"	(3)	2.2	
32	"	(1)	1.8	
32	"	(3)	1.4	1.9
33	0.055 M	(1)	2.0	
33	"	(3)	1.6	
34	"	(1)	1.8	
34	"	(3)	1.5	1.7
35	0.022 M	(1)	1.8	
35	"	(3)	1.6	
36	"	(1)	2.4	
36	"	(3)	2.1	2.0

*All plants received 2 X 10^8 cells of A. tumefaciens.

**Site: (1) and (3) denote internode 1 and 3 respectively beginning
with the one directly above the cotyledon.

***Tumor and Avg (tumor) sizes given as diameters in cm. Avg: average.

at the 3' end of the anticodon loop (Fox, 1969). Cytokinin activity

has been detected in tomato (Fox, 1969), sunflower root exudates

(Kende, 1964), and tobacco tumor tissue (Braun, 1956). Zeatin occurs

naturally not only as the free base but also in the nucleoside and

nucleotide form (Miller, 1965; Letham, 1966), and as an integral part

of corn RNA (Hall et al., 1966).

Sachs, Bretz, and Lang (1959) found gibberellic acid (GA_3) to

directly activate cell division in the subapical meristem in the

longitudinal direction visible by stem elongation. This process leads

to flowering in rosette plants and GA_3 has thus been associated with

floral initiation. Gibberellic acid plays an essential role in seed

germination (Paleg, 1960; Yomo and Jinoma, 1966) and in the reversal of

dormancy in plants (Donaho and Walker, 1957; Eagles and Wareing, 1964).

Plant hormones must often interact to yield a needed result.

Wareing, Hanney, and Digby (1964) suggest that the complete phenomenon

of normal wood formation, including cambial division, transversal

enlargement of the daughter cells, and typical lignification requires

interaction of auxin with gibberellin and cytokinin.

Auxins, cytokinins, and gibberellins play an active role in plant

growth but this growth is modified (Thimann, 1969) by certain

naturally occurring inhibitors such as abscisic acid (ABA). Abscisic

acid increases permeability of plant cell membranes (Glinka and

Reinhold, 1972), produces dormancy in plants (Phillips and Wareing,

1958a; 1959; Robinson, Wareing, and Thomas, 1963; Robinson and Wareing,

1964), and inhibits RNA and DNA synthesis in a variety of plant

tissues (Villiers, 1968; Walton, Soofi, and Sondheimer, 1970; Shih and

Rappaport, 1970).

In the preliminary screening, all of the hormones seemed to pro-
duce the same kind of slight inhibitory zone of about 6.0 to 6.5 mm
around the point of hormone application (3.0 to 3.5 mm). There were no
morphological changes observed in A. tumefaciens at the end of the 3
day incubation period. Only small whole cells and some lysed cells
were observed.

Indole acetic acid seemed to slightly inhibit A. tumefaciens from
growing at the same rate as the control when concentrations of 20 and
16 μg/ml of the hormone were added to viable cells (Figures 3, 4;
Table VII). The length of the lag phase was 4 hrs for the control
cells while the cells to which 20 μg/ml IAA was added remained in the
lag phase for 6 hrs. There was no change in cellular morphology during
growth. IAA seemed to slow down the process of cell division in A.
tumefaciens without causing any morphological changes to the cells.

Zeatin, the cytokinin which stimulates plant cell division did not
stimulate cell division in growing cells of A. tumefaciens (Figures 5,
6; Table VII). The length of the lag period of growth for both the
control cells and the cells to which 20 μg/ml of zeatin was added was
2 hrs. There was no increase nor decrease in this initial growth phase
with the addition of the naturally occurring cytokinin. Periodic
microscopic examination of the cells during growth revealed no
morphological changes.

Gibberellic acid, the natural plant growth hormone which activates
cell division (stem elongation) in plants also seemed to activate cell
division in A. tumefaciens (Figures 7, 8; Table VII). The length of
the lag period of growth was reduced from 2 hrs for the control to
0 hrs in the cells containing 20 μg/ml GA_3. This 2 hrs decrease in the

Figure 3. Effect of IAA at Different Concentrations on Growth of
A. tumefaciens in mannitol-yeast broth at 25 C.
● , control; ■ , 0.1, 0.2, 1, and 2 µg/ml IAA;
⬡, 6 µg/ml IAA; □ , 10 µg/ml IAA; O , 16 µg/ml
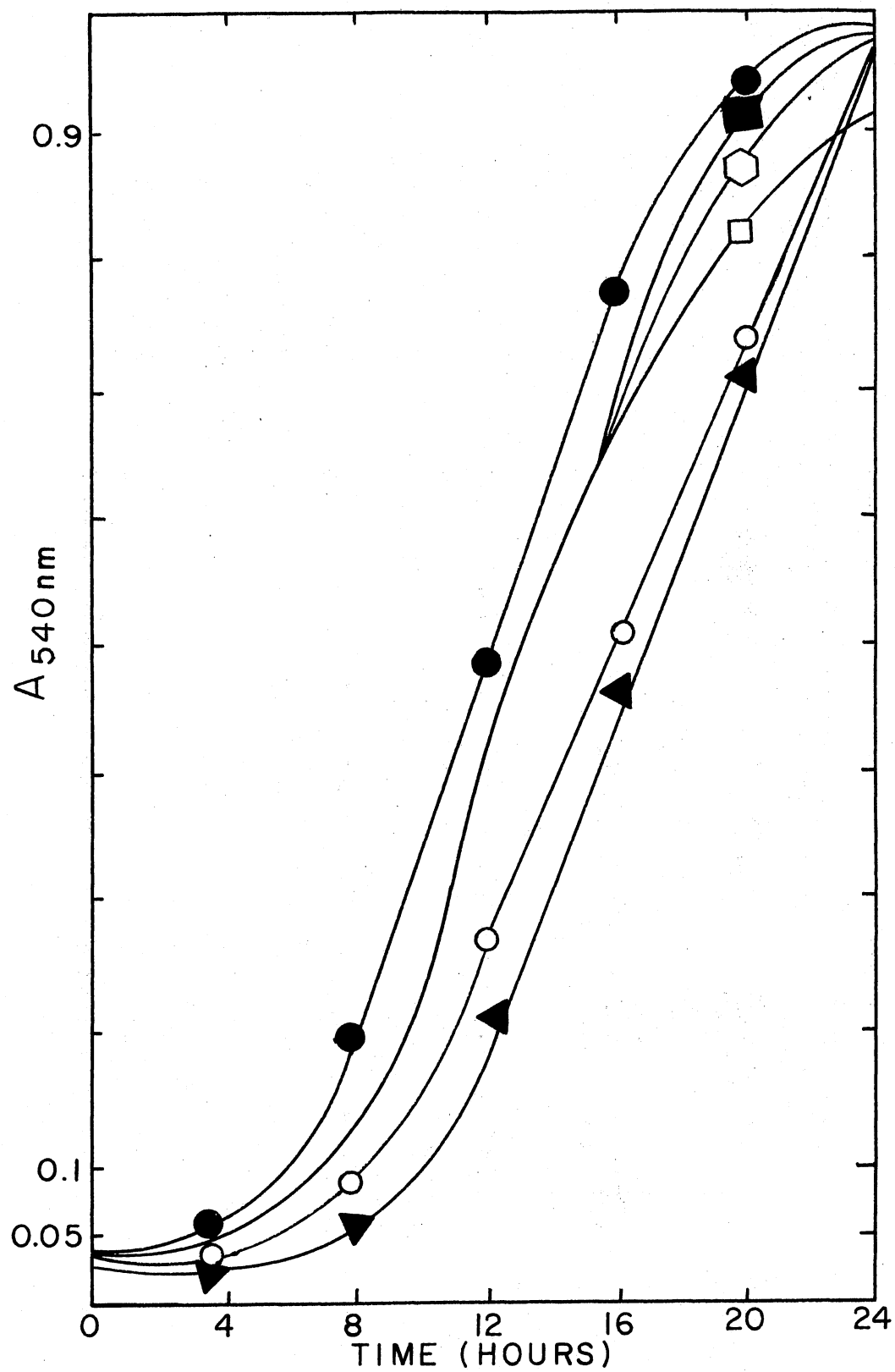IAA; ▲ , 20 µg/ml IAA.

Figure 4. Effect of IAA on Growth of A. tumefaciens in mannitol-yeast broth at 25 C. ● , control; O , 20 µg/ml IAA.
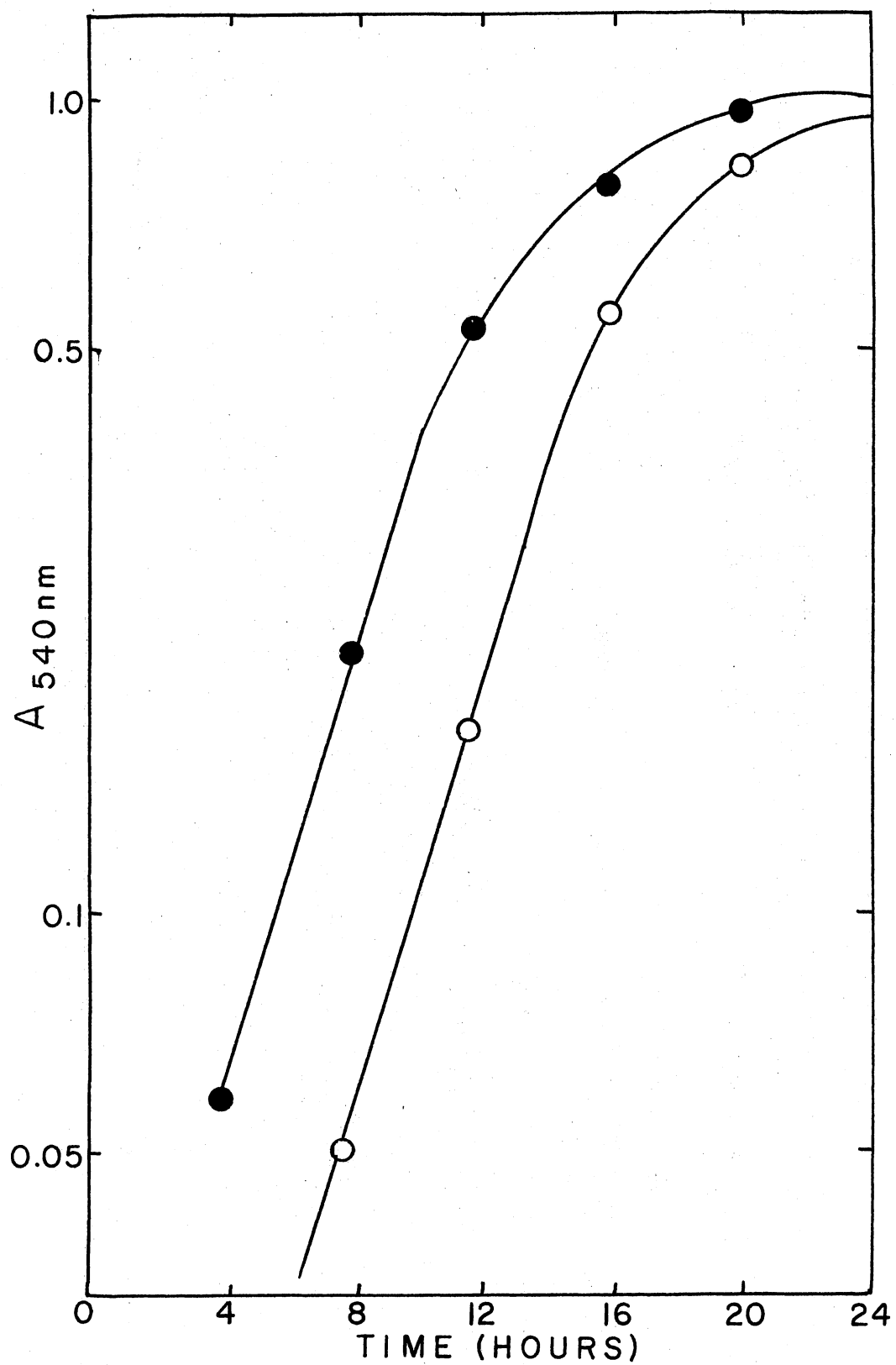
TABLE VII

COMPARISON OF LAG PHASE, EXPONENTIAL GROWTH RATE, AND
TOTAL MASS OF A. TUMEFACIENS WITH ADDITION
OF PLANT HORMONES

Hormone*	Length of** Lag Phase	Log Phase*** Rate	Total Mass[x]
None	4	0.250	0.6
IAA	6	0.175	0.5
None	2	0.250	0.6
Zeatin	2	0.225	0.6
None	2	0.317	0.6
GA_3	0	0.117	0.6
None	5	0.200	0.5
ABA	5	0.180	0.5
None	2	0.325	0.6
All Four	-	-	0.0

*All hormones were at a final concentration of 20 μg/ml.

**Length of lag phase given in hrs.

***Calculated by: rate - (x_2-x_1) - (y_2-y_1) from hr to hr.

[x]Total mass of A. tumefaciens cells at 24 hrs of growth assay m
measured by comparison to Erwinia species standard growth curve.
Given in mg per ml.

Figure 5. Effect of Zeatin at Different Concentrations on Growth of
A. tumefaciens in mannitol-yeast broth at 25 C.
● , control; △ , 0.1, 0.2, 1, 2, 6, 10, and 16 µg/ml
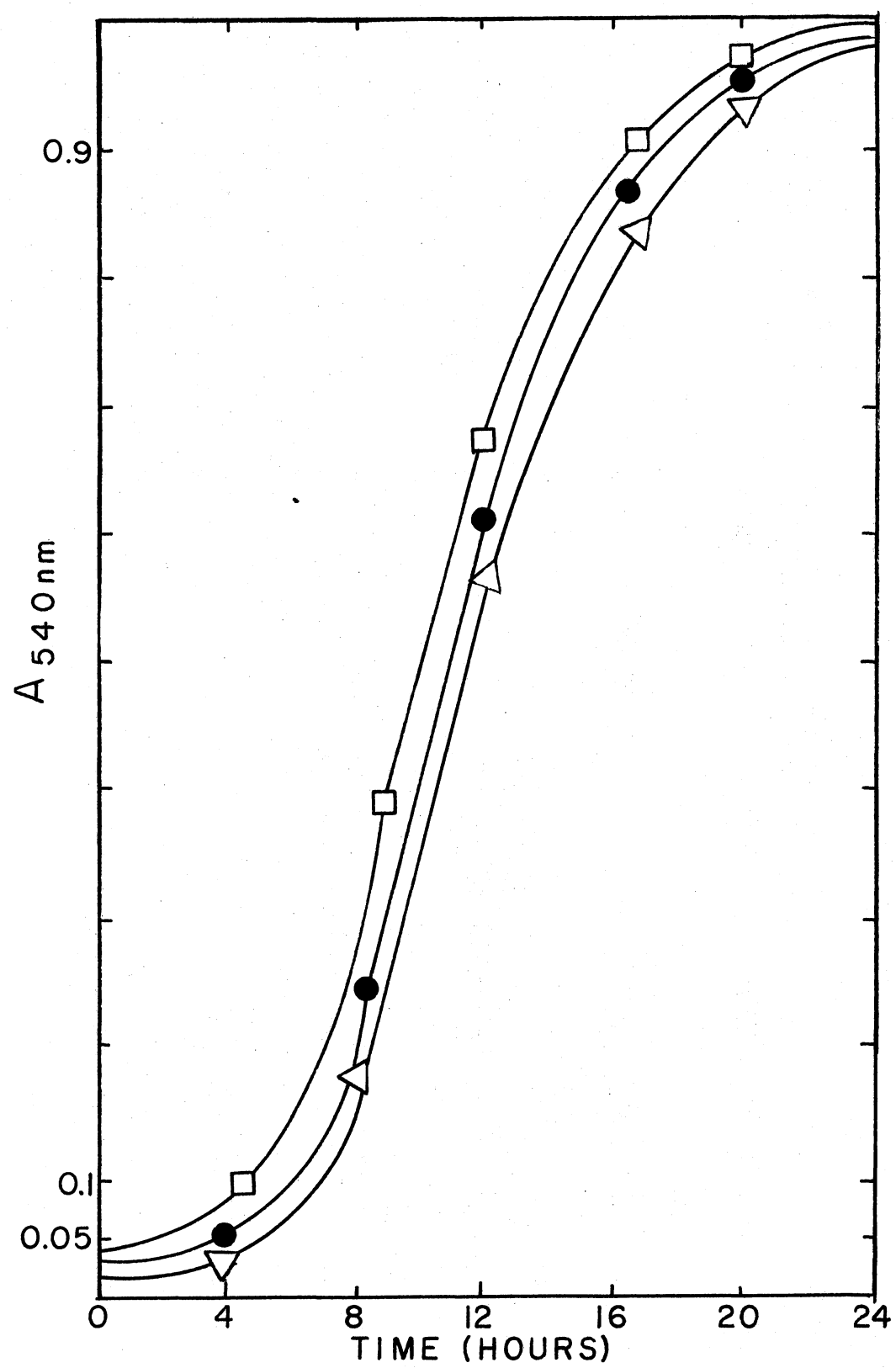Zeatin; ◇ , 20 µg/ml Zeatin.

Figure 6. Effect of Zeatin on Growth of A. tumefaciens in mannitol-
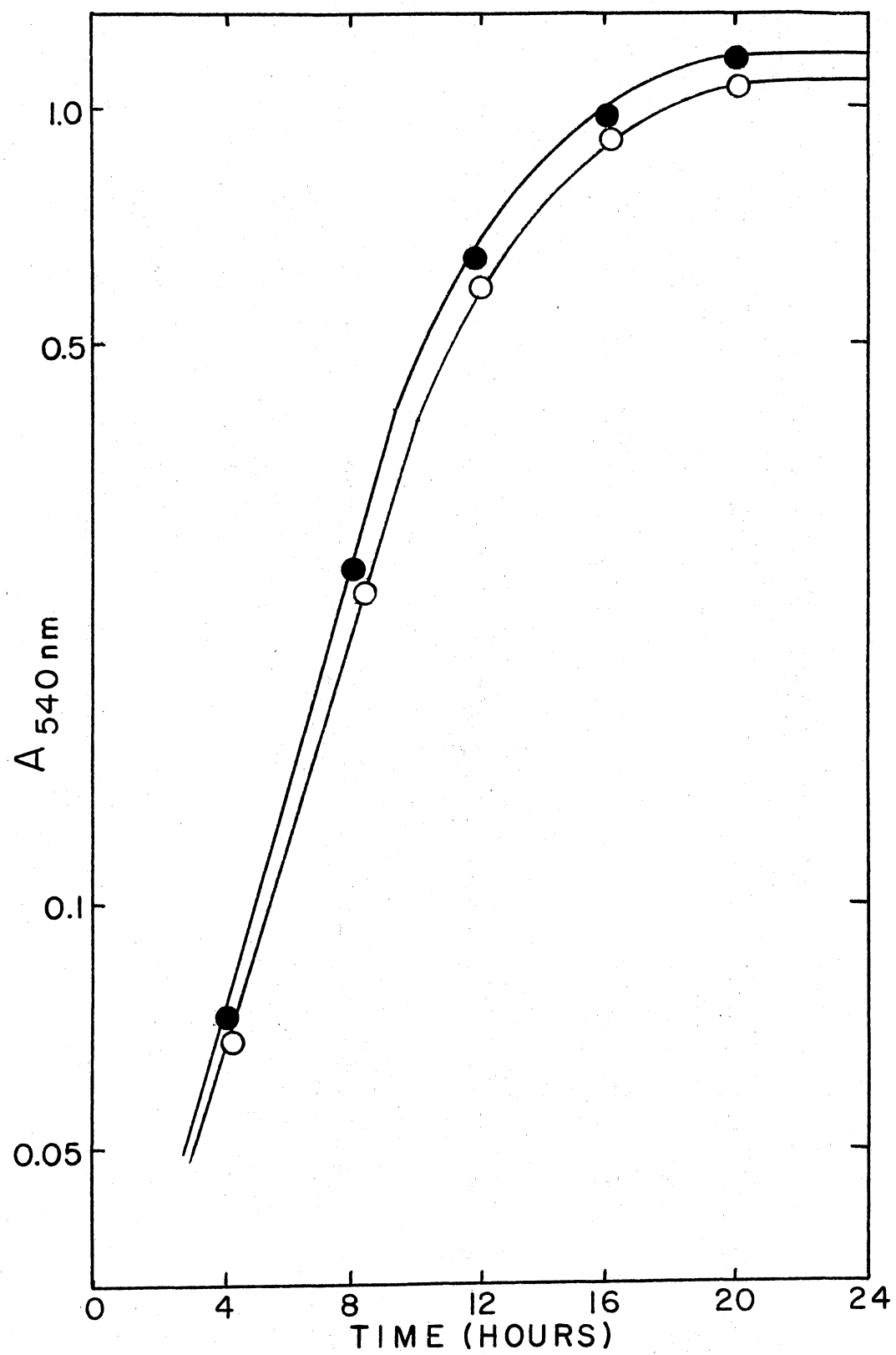yeast broth at 25 C. ⬤ , control; ◯ , 20 µg/ml Zeatin.

Figure 7. Effect of GA$_3$ at Different Concentrations on Growth of
A. tumefaciens in mannitol-yeast broth at 25 C.
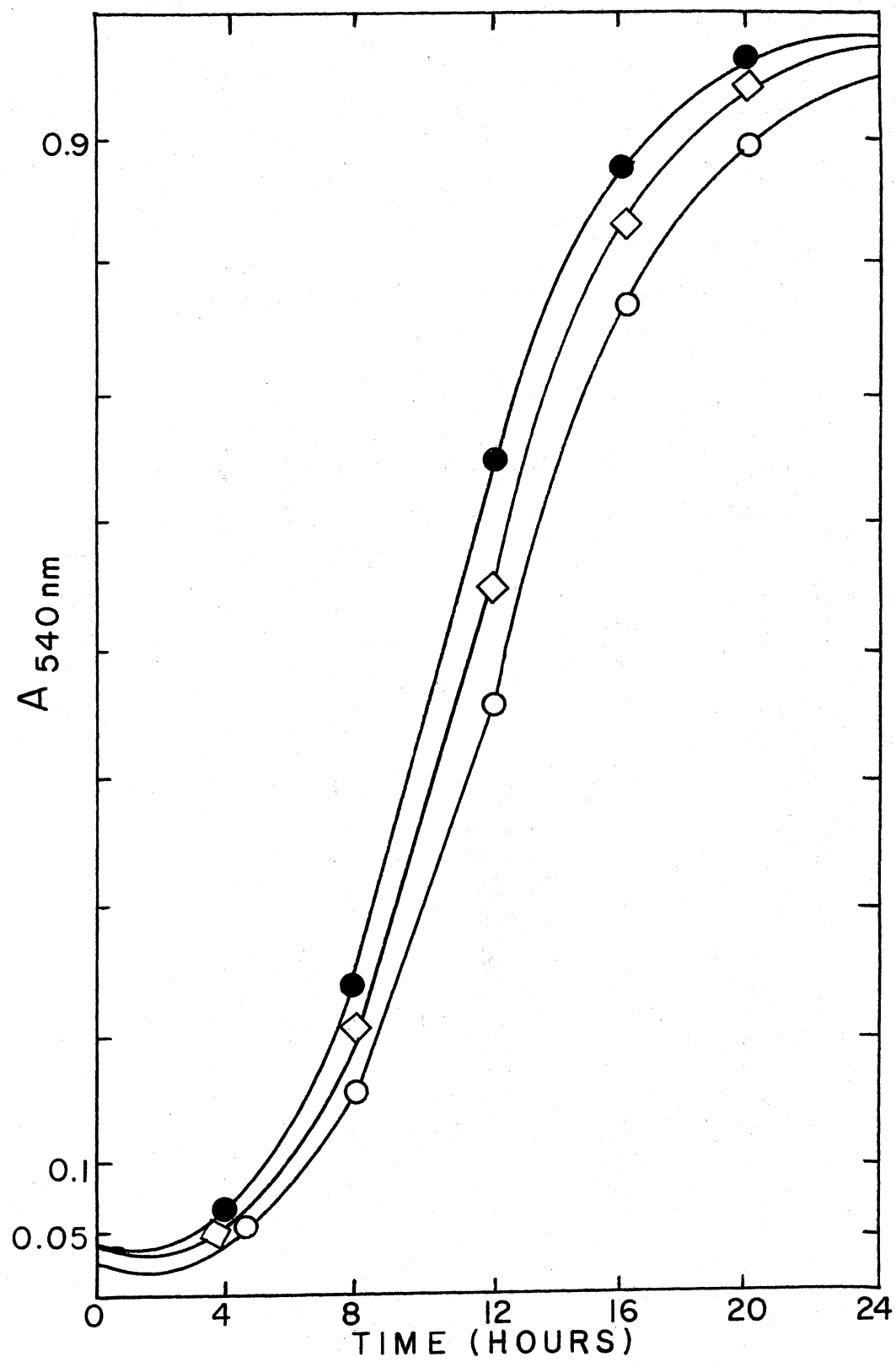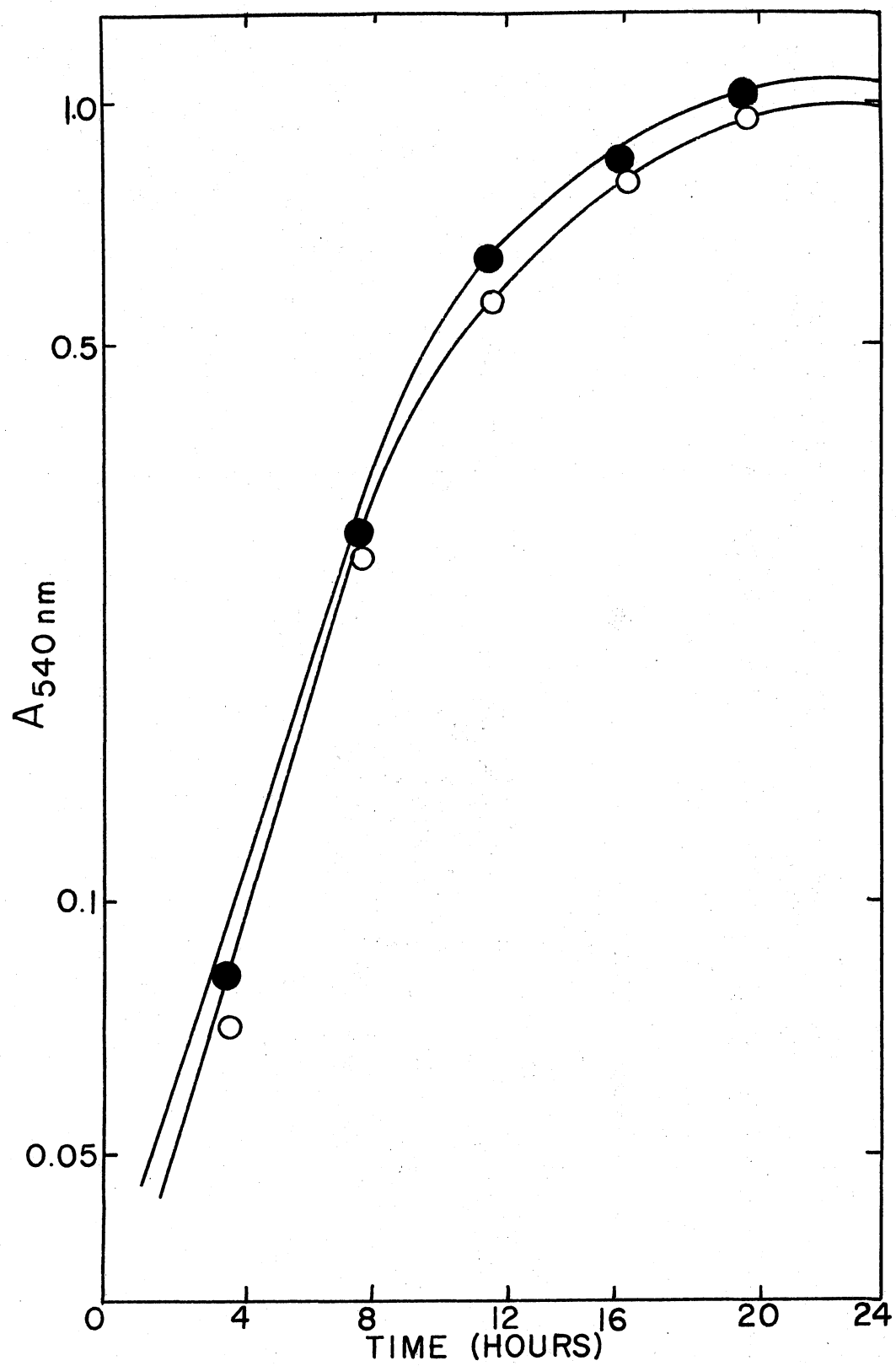● , control; ◇ , 0.1, 0.2, 1, 2, 6, 16, and 20
μg/ml GA$_3$; ○ , 10 μg/ml GA$_3$.

Figure 8. Effect of GA$_3$ on Growth of <u>A. tumefaciens</u> in mannitol-
yeast broth at 25 C.   ● , control; ○ , 20 µg/ml GA$_3$.

lag period is significant and it may be similar to the effect GA_3 has

on plant cells. There were no morphological differences in the cells

with GA_3 when examined microscopically. Although GA_3 seemed to acti-

vate cell division in the lag phase, it did not do so during the

exponential growth phase. If it indeed exerted any effect on A.

tumefaciens cells, it decreased their growth rate. The control cells

grew at a rate of 0.317 while they grew at a rate of only 0.117 in the

presence of 20 µg/ml GA_3 (decrease of 0.2). The total mass of A.

tumefaciens cells at the end of 24 hrs of growth was the same as the

total mass of cells plus GA_3, 0.6 mg/ml. No morphological differences

in the cells were observed during any phase of growth.

Abscisic acid, a natural plant growth inhibitor hormone, which

increases plant cell membrane permeability and inhibits DNA and RNA

synthesis, also seemed to inhibit growth of A. tumefaciens cells to a

small degree during the exponential growth phase (Figures 9, 10;

Table VII). The length of the lag phase was 5 hrs for both control

cells and for cells containing ABA (20 µg/ml). The total mass at the

end of the 24 hrs of growth was 0.5 mg/ml for the control cells and

0.5 mg/ml for the cells plus ABA. Microscopic examination of the

cells at 24 hrs revealed no morphological changes.

The combination of naturally occurring plant growth hormones: IAA,

zeatin, and GA_3 and the naturally occurring growth inhibitor, ABA, had

a profound effect on growing A. tumefaciens cells at concentrations of

16 and 20 µg/ml. Growth was stopped immediately and never resumed

(Figures 11, 12; Table VII). At the lesser concentrations of 10 and

6 µg/ml, only an inhibitory effect was observed. The lag period for the

control cells lasted 2 hrs while the cells containing all of the plant

Figure 9. Effect of ABA at Different Concentrations on Growth of
          A. tumefaciens in mannitol-yeast broth at 25 C.
          ● , control; ▲ , 0.1, 0.2, 1, 2, 6, 10, 16, and
          20 µg/ml ABA.
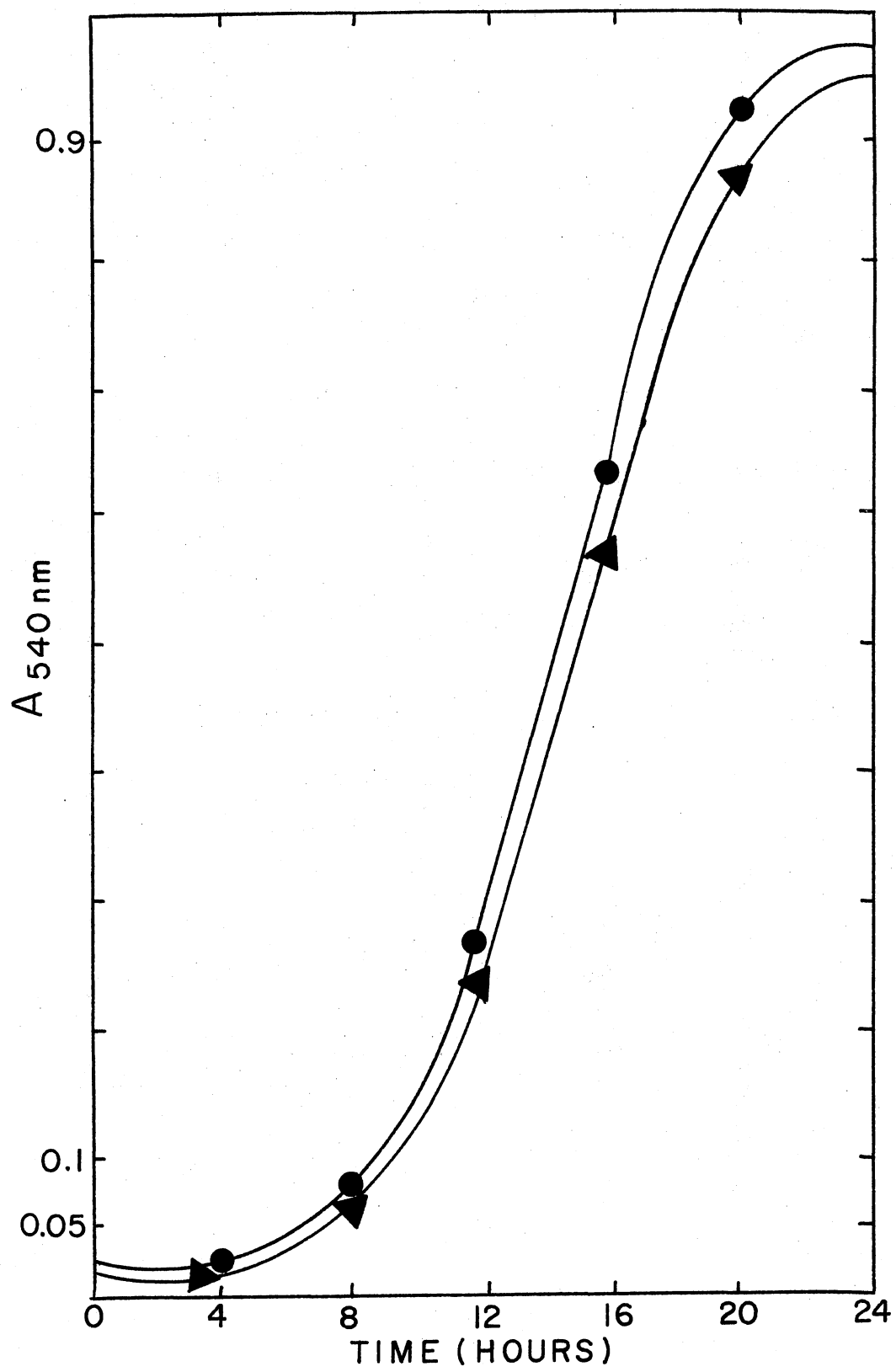
Figure 10. Effect of ABA on Growth of A. tumefaciens in mannitol-
        yeast broth at 25 C. ● , control; ○ , 20 μg/ml.

Figure 11. Effect of Four Plant Hormones (IAA, GA_3, Zeatin, and ABA)
at Different Concentrations on Growth of A. tumefaciens
in mannitol-yeast broth at 25 C. ● , control; ⬡ , 0.1,
0.2, 1, and 2 μg/ml of All Four Hormones; ▲ , 6 μg/ml
of All Four Hormones; ☐ , 10 μg/ml of All Four Hormones;
◆ , 16 μg/ml of All Four Hormones; ◯ , 20 μg/ml of All
Four Hormones.
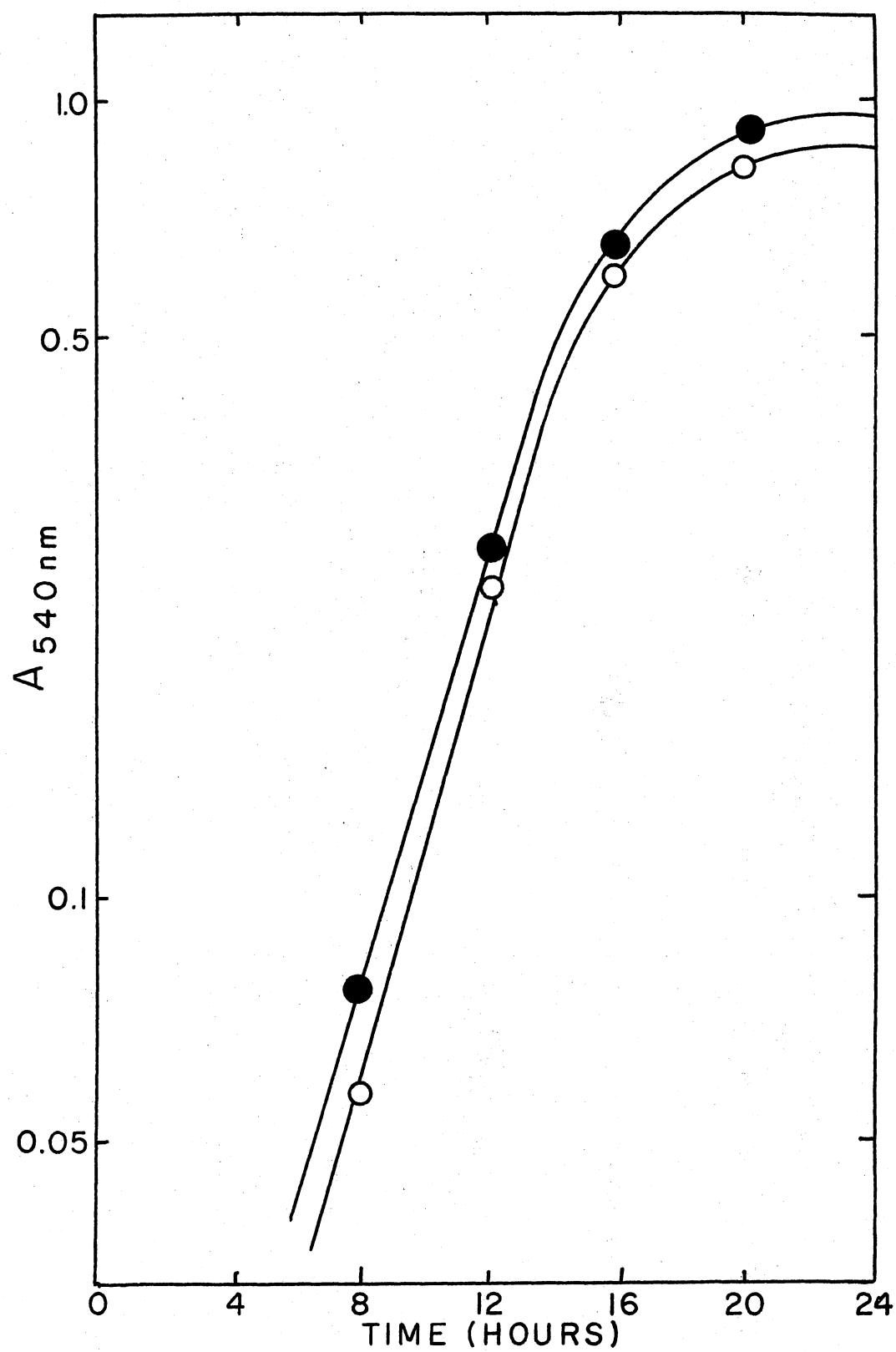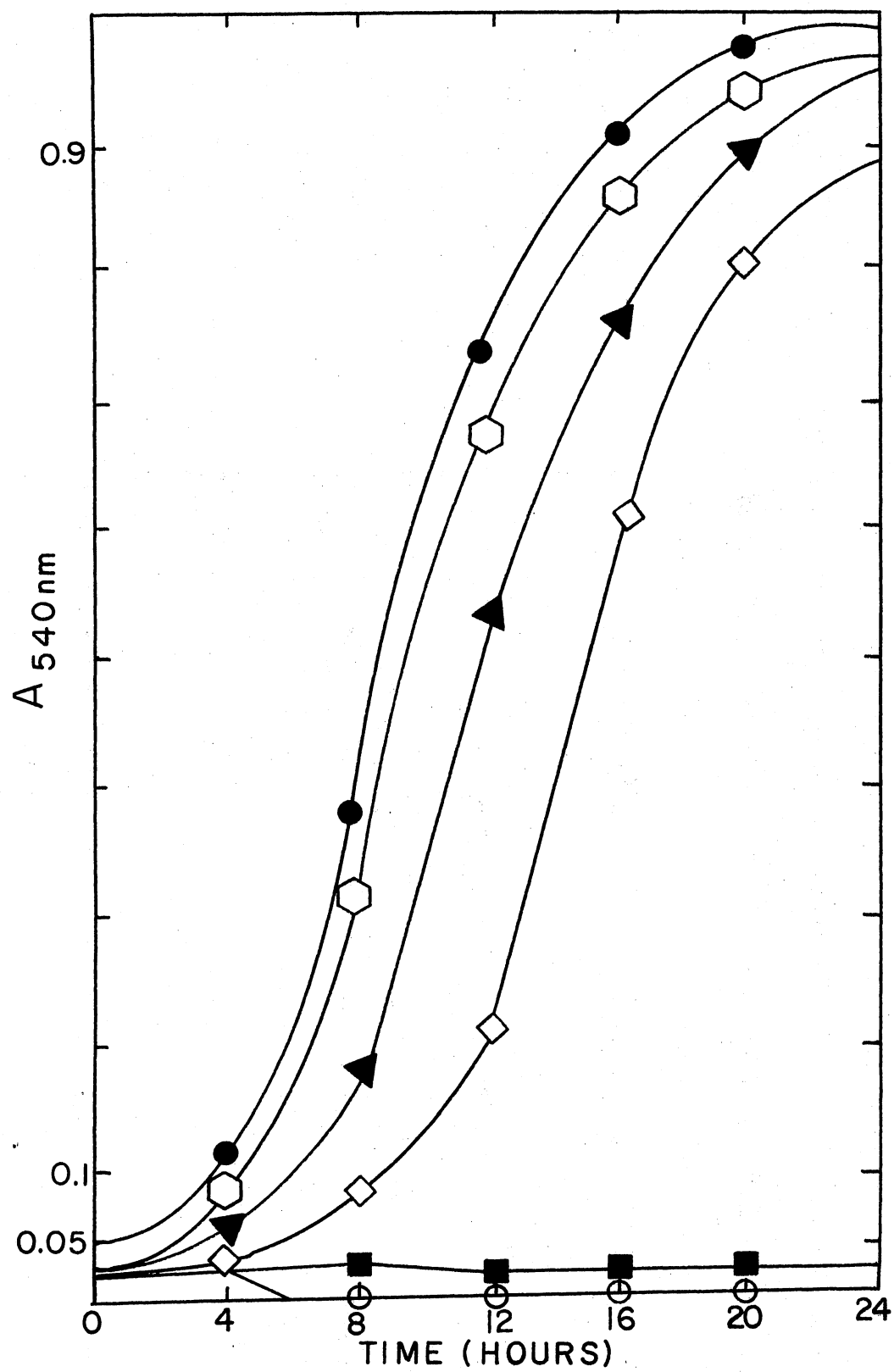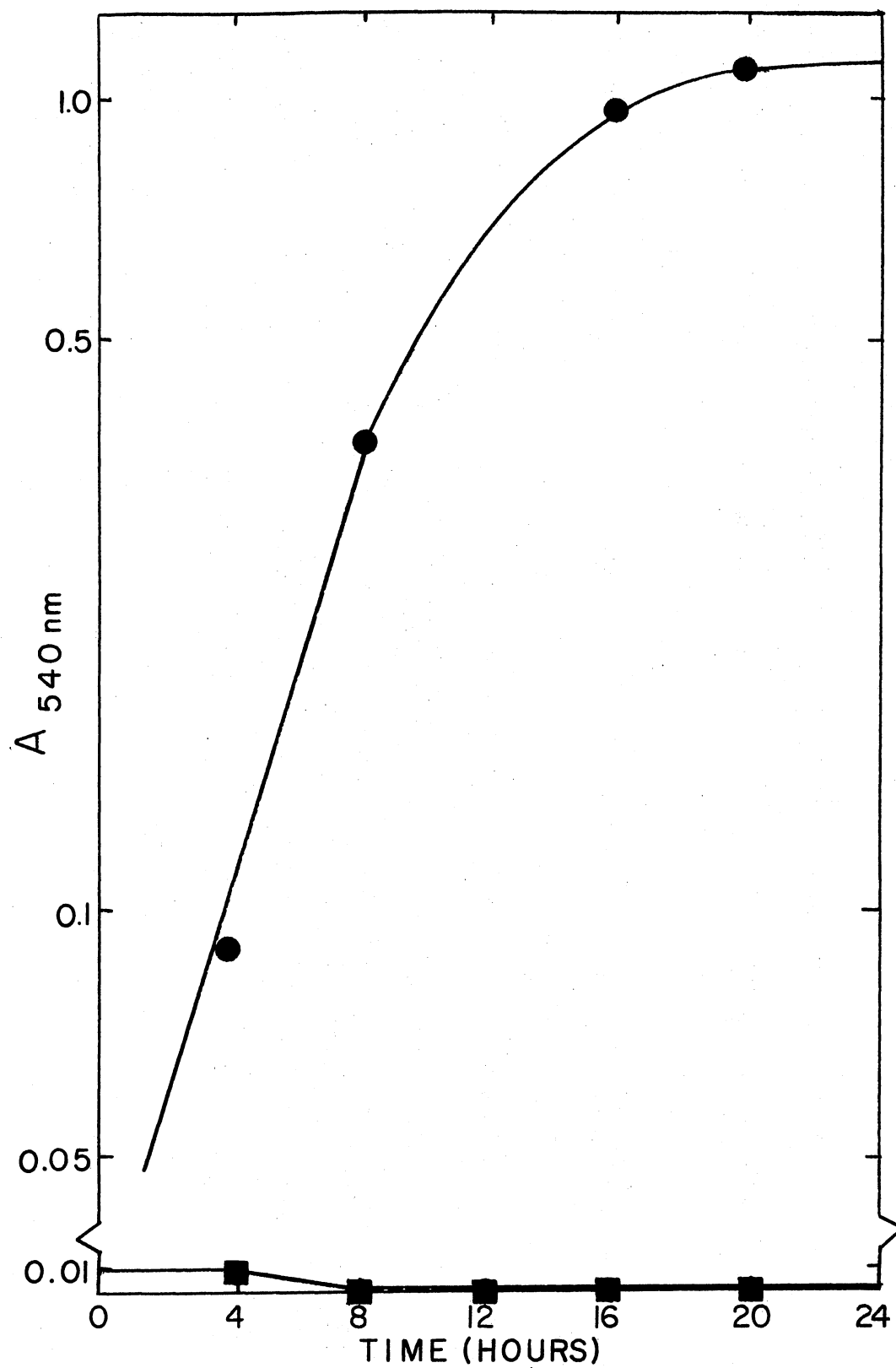
Figure 12. Effect of Four Plant Hormones (IAA, GA_3, Zeatin, and ABA)
on Growth of A. tumefaciens in mannitol-yeast broth at
25 C.   ● , control; ■ , 20 μg/ml of All Four Hormones.

hormones (20 µg/ml) never grew, therefore, their lag period could not

be measured. Microscopic examination (24 hrs) showed only a small

amount of cells per field with no notable morphological changes.

All plant hormones used in the growth assays including the combi-

nation were utilized as possible chemical antagonists to tumor formation.

Each in a final concentration of 20 µg/ml was added to A. tumefaciens

and injected into susceptible host plants. Also just the hormones alone

(20 µg/ml) were injected into plants.

All hormones or hormone-A. tumefaciens combinations produced

tumors; therefore, none behaved entirely antagonistically toward

ability of A. tumefaciens to form tumors. In fact, the hormones alone

produced tumor-like overgrowths that looked like crown gall tumors.

Several of these were located on one side of the stem only rather than

on both sides of the wound and injection site as occurs when A.

tumefaciens is the initiating agent. On the average, IAA produced a

growth of 1.0 cm in diameter, GA_3: 1.3 cm, zeatin: 1.0 cm, ABA: 1.1 cm,

and the combination of all four: 0.9 cm (Table VIII). These overgrowths

were excised and isolations for possible bacterial contamination were

carried out. No bacteria were isolated from the overgrowths produced by

the plant hormones. (Tumors produced by A. tumefaciens plus the plant

hormones allowed the usual reisolation of A. tumefaciens).

No hormone was entirely antagonistic to ability of A. tumefaciens

to form tumors. The combination of all four hormones seems to have

behaved somewhat antagonistically toward A. tumefaciens since tumors

were 1.1 cm on the average as compared to tumors of 1.3 cm on the

average formed by A. tumefaciens alone. Indole acetic acid, GA_3, and

zeatin allowed A. tumefaciens to form tumors of 1.5 cm in diameter.

TABLE VIII

EFFECT OF PLANT HORMONES ON TUMOR FORMATION BY
A. TUMEFACIENS IN LYCOPERSICON ESCULENTUM

Plant #*	Hormone[xx]	Site**	Tumor Size***		Stem Size***	
301	None	(1)	2.5		0.7	
301	"	(3)	2.3		0.6	
301	"	(5)	1.7	2.2	0.5	0.6
302	"	(1)	1.9		0.7	
302	"	(3)	2.0		0.7	
302	"	(5)	1.5	1.8	0.6	0.7
303	"	(1)	2.4		0.7	
303	"	(3)	2.2		0.8	
303	"	(5)	1.8	2.1	0.8	0.7
			Corrected Value:			1.3
304	IAA	(1)	1.8		0.6	
304	"	(3)	2.2		0.7	
304	"	(5)	1.8	1.9	0.7	0.6
305	"	(1)	2.1		0.6	
305	"	(3)	2.7		0.6	
305	"	(5)	2.0	2.3	0.6	0.6
			Corrected Value:			1.5
306[x]	"	(1)	1.8		0.6	
306[x]	"	(3)	1.8		0.7	
306[x]	"	(5)	1.4	1.7	0.7	0.7
			Corrected Value:			1.0
			Adjusted Value:			0.5
307	GA$_3$	(1)	2.1		0.6	
307	"	(3)	2.4		0.6	
307	"	(5)	2.1	2.2	0.6	0.6
308	"	(1)	2.1		0.7	
308	"	(3)	2.1		0.7	
308	"	(5)	2.2	2.1	0.7	0.7
			Corrected Value:			1.5
309[x]	"	(1)	2.0		0.7	
309[x]	"	(3)	1.7		0.7	
309[x]	"	(5)	2.2	2.0	0.6	0.7
			Corrected Value:			1.3
			Adjusted Value:			0.2
310	Zeatin	(1)	2.6		0.7	
310	"	(3)	2.1		0.7	
310	"	(5)	2.4	2.4	0.6	0.7

TABLE VIII (CONTINUED)

Plant #*	Hormone^{xx}	Site**	Tumor Size***		Stem Size***	
311	Zeatin	(1)	2.3		1.0	
311	"	(3)	2.4		0.9	
311	"	(5)	2.6@ 2.4		1.1	1.0
			Corrected Value:			1.5
312^x	"	(1)	1.7		0.6	
312^x	"	(3)	1.6		0.7	
312^x	"	(5)	1.6 1.6		0.6	0.6
			Corrected Value:			1.0
			Adjusted Value:			0.5
313	ABA	(1)	2.2		0.7	
313	"	(3)	1.6		0.6	
313	"	(5)	2.0 1.9		0.6	0.6
314	"	(1)	2.2		0.6	
314	"	(3)	1.8		0.6	
314	"	(5)	1.9 2.0		0.6	0.6
			Corrected Value:			1.4
315^x	"	(1)	2.4		0.7	
315^x	"	(3)	1.6		0.7	
315^x	"	(5)	1.4 1.8		0.6	0.7
			Corrected Value:			1.1
			Adjusted Value:			0.3
316	All Four	(1)	1.8		0.7	
316	"	(3)	1.8		0.6	
316	"	(5)	1.7 1.8		0.4	0.6
317	"	(1)	1.3		0.6	
317	"	(3)	1.6		0.7	
317	"	(5)	1.7 1.5		0.5	0.6
			Corrected Value:			1.1
318^x	"	(1)	1.4		0.7	
318^x	"	(3)	1.7		0.7	
318^x	"	(5)	1.6 1.6		0.7	0.7
			Corrected Value:			0.9
			Adjusted Value:			0.2

*All plants received 2 X 10^8 cells of <u>A. tumefaciens</u> unless otherwise noted (x).

**Site: (1), (3), and (5) denoted internode 1, 3, and 5 respectively beginning with the one directly above the cotyledons.

***Tumor, overgrowth, stem, and average sizes given as diameters in cm.

^{x}Plants received only the designated hormone; produced overgrowths.

^{xx}Hormone concentrations were all 20 µg/ml.

@Tumor split stem. Inner tumor diameter: 1.1 cm. Corrected Value: denotes the average tumor or overgrowth size minus the average stem size in cm. Adjusted Value: denotes the corrected tumor size minus the corrected overgrowth size in cm.

However, when one takes into account the size of the overgrowths
formed by each of these hormones and adjusts each situation for tumor
formation by A. tumefaciens, one arrives at different and perhaps more
meaningful results. Indole acetic acid plus A. tumefaciens adjusted for
IAA overgrowth formation formed a tumor of 0.5 cm diameter on the
average (Table VIII). Gibberellic acid and the combination each allowed
for 0.2 cm tumors, ABA allowed for 0.3 cm tumors, and zeatin for 0.5 cm
tumors (all adjusted). These results indicate that each plant hormone
and the combination of all four at a concentration of 20 μg/ml behaved
as chemical antagonists to formation of tumors by A. tumefaciens.
Also, since A. tumefaciens alone forms tumors of 1.3 cm diameter on the
average, the concentration of these plant hormones in the plant is
probably less than 20 μg/ml.

CHAPTER IV

SUMMARY AND CONCLUSIONS

No test organism utilized was able to significantly inhibit formation of crown gall tumor disease by A. tumefaciens. Since each isolate, other than the anaerobic bacteria, was screened for nuclease production, it was possible to determine if any connection exists between crown gall tumor formation by A. tumefaciens and production of DNase, RNase, both DNase and RNase, and neither nuclease. It was concluded that no evidence exists to directly implicate either DNA or RNA in tumor formation.

The anaerobes which were not tested for nuclease production accentuated tumor formation in some cases; however, as a group, no definite trend could be established.

The agrobacteria other than tumefaciens B_6 and the bacterial isolates from insects showed the greatest degree of antagonism towards A. tumefaciens. Seventy-one percent of the agrobacteria inhibited full expression of tumor formation by A. tumefaciens and 64% of the insect isolates did the same. Again, however, no general correlation to nuclease production is possible since most of the insect isolates produced both nucleases whereas in the agrobacteria, the majority either produced both nucleases or no nuclease. The bacterial isolates producing no nuclease accentuated and inhibited the elaboration of TIP in nearly equal amounts; thereby revealing no new evidence for or

against the direct implication of bacterial nucleic acids in tumor
formation. Recently Eden et al. (1974), Drlica and Kado (1974), and
Chilton et al. (1974), found no convincing evidence for bacterial DNA
in crown gall tumor DNA. Kado and Lurquin (1975) reported that no A.
tumefaciens DNA was replicated in mung bean seedlings under conditions
specified in other published uptake, integration, and replication
reports of bacterial DNA in higher plants. Their results do not agree
with previous reports that at least 30% of the plant genome of the
bacterial DNA treated plants comes from bacterial DNA. Similar results
have been reported by Phillips and Butcher (1975) who found no evidence
for the induction of tumors with A. tumefaciens DNA on carrot root
explants; funflower and tobacco stem segments; callus cultures of sun-
flower; tobacco, carrot, and sunflower stems using similar reported
successful methods.

As reisolation studies of the two organisms inoculated into the
host gave approximately equal amounts of the same two organisms from
well developed (5 week) tumors, the bacteria had the opportunity to
interact. Assuming that the bacterial isolates continued to produce
nuclease (they produced their specific nuclease when reisolated from
tumors), such interaction did not inactivate TIP even though the
nuclease enzymes produced by the test organisms should have inactivated
DNA or RNA. It follows, therefore, that TIP is not A. tumefaciens DNA
nor RNA but some other entity. The possibility exists, however, that
TIP may be a nucleic acid that is somehow sheltered from the attack of
DNase and/or RNase produced by the antagonistic organisms. Perhaps the
recent reports implicating a bacterial plasmid from oncogenic A.
tumefaciens as the carrier of genetic information for tumor inducing

ability of <u>A. tumefaciens</u> are correct (Hamilton and Chopin, 1975; Van Larebeket et al., 1975; Watson et al., 1975). Circular plasmid DNA is covalently closed; although such structuring makes it resistant to denaturation (Helinski and Clewell, 1971; Humphreys, Willshaw, and Anderson, 1975), it is still susceptible to attack by nucleases. However, if this plasmid DNA were sheltered from the nucleases, they would not denature it. Therefore, tumors would still result regardless of the presence or absence of these enzymes. Bogers (1972) reported that cell walls of <u>A. tumefaciens</u> fuse with plant cell walls in the tumor induction process and Schilperoort (1971) suggested that the attachment of bacteria to receptors on cell walls may be a preliminary step to the injection of bacterial DNA into intact plant cells. Since wounding is essential to tumor formation and in the process of wounding, autolytic enzymes are released and can attack and modify the cell wall, bacterial attachment to plant cell walls can occur. Plasmids are similar to the bacterial sex factors (both are extrachromosomal elements of DNA) which according to Hayes (1968) must be attached to the bacterial cell membranes where they produce local surface changes that determine where cellular contact and subsequent connection with another cell occurs. It is through this contact that a connection between the cells is established and the plasmid is transferred to another cell (Stanier, Doudoroff, and Adelberg, 1970). (It is never released into the medium.) Perhaps this is the means of plasmid transfer from <u>A. tumefaciens</u> to the plant cell in the process of crown gall tumor formation.

No concentration of tetracycline was entirely antagonistic towards ability of <u>A. tumefaciens</u> to elaborate TIP, however, all concentrations

did allow for partial inhibition of its elaboration, the size of the

tumors being indirectly proportional to the concentration and directly

proportional to the amount of growth inhibition of A. tumefaciens.

No concentration of rifampicin utilized allowed for entire

antagonism to the elaboration of TIP. However, rifampicin did behave

partially antagonistically toward ability of A. tumefaciens to elaborate

TIP even though no general positive correlation between concentration of

the antibiotic and tumor production is evident.

No general trend can be established with PL and tumor antagonism

as some concentrations allowed partial tumor inhibition while others

allowed tumor accentuation.

None of the plant hormones exerted full inhibition on ability of

A. tumefaciens to elaborate TIP, although these hormones all behaved

partially antagonistically toward ability of A. tumefaciens to elaborate

TIP by forming small tumors. The combination of 4 hormones in concen-

trations of 16 and 20 μg/ml had a significant synergistic effect on

viable A. tumefaciens cells. Perhaps further investigations with

varying concentrations of each hormone in the mixture would yield some

more interesting results. No general positive correlation between

growth assay results and tumor formation by A. tumefaciens can be made.

Since each hormone at 20 μg/ml inhibited A. tumefaciens from elaborating

TIP to the fullest, perhaps these naturally occurring plant hormones

occur at a lesser concentration in the normal plant susceptible to the

crown gall tumor disease.

None of the bacteria nor any of the chemicals utilized inhibited

ability of A. tumefaciens to cause tumor formation. Partial antagonisms

or accentuation to tumor formation were evident with each possible

antagonist. Naked nucleic acid does not appear to be involved in crown

gall tumor formation, however plasmid DNA may be.

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VITA $^\curlyvee$

Rosalie K. Truby

Candidate for the Degree of

Master of Science

Thesis: BACTERIAL AND CHEMICAL ANTAGONISTS TO AGROBACTERIUM
TUMEFACIENS AND THE CROWN GALL TUMOR DISEASE

Major Field: Microbiology

Biographical:

Personal Data: Born in Wilkes Barre, Pennsylvania, July 27, 1941,
the daughter of Anthony V. and Angela B. Kackauskas; married
to Wilbur J. Truby, March 31, 1972.

Education: Graduated from Ashley High School, Ashley,
Pennsylvania, in June, 1959; received Bachelor of Arts
degree in Biology from Wilkes College, Wilkes Barre,
Pennsylvania, 1963; began graduate study at Hofstra
University, Hempstead, New York, 1971; enrolled in University
of Oklahoma, Norman, Oklahoma, 1972; completed the require-
ments for the Master of Science degree at Oklahoma State
University in December, 1975.

Professional Experience: Undergraduate teaching assistant,
Wilkes College, 1961-1963; Research Chemist, Surface
Activation Corporation, Westbury, New York, 1971-1972;
graduate teaching assistant in Microbiology, Oklahoma State
University, 1973-1975.