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BIOPHYSICAL INTERACTIONS
OF
X-RAYS, ANTIBIOTICS,
PLANT PATHOGENS, AND PLANTS

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TABLE OF CONTENTS

Page

Materials and Methods

B. ORGANIC NUTRITION OF X-RAY-INJURED LIMA BEANS

X-ray-Injured Seeds

References

List of Figures

List of Tables

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BIOPHYSICAL INTERACTIONS OF X-RAYS, ANTIBIOTICS, PLANT PATHOGENS, AND PLANTS

INTRODUCTION

Living organisms in nature are continually subjected to many physical and chemical agents. In the laboratory they can be acted upon by many others. Often these agents have profound effects upon the organisms and the chemicals produced by them. The interactions of a few such agents on each other and on organisms is the subject of this dissertation.

Physical Agents

Temperature, moisture, pH, light, and radiation such as x-rays have considerable influence on the growth and development of organisms. In this study most of these are considered merely from the standpoint of recognizing them as variables and then setting arbitrary limits on them to establish standard conditions.

Temperature. Every organism has a range of temperatures within which it can successfully grow. Within this range there is usually some particular temperature where growth is best. This is known as the "optimum growth temperature", or O.G.T. In designing experiments one sometimes seeks to adjust temperature to near O.G.T. of some organism he wishes to have grow. On the other hand, by shifting temperature outside the growth range it is possible to entirely eliminate life and so remove undesirable organisms from the environment.

In this study the plants were generally grown at room temperature. This fell within the range 70-80°F. and gave satisfactory growth. For germination of seeds a 30°C. cabinet was used. This made possible a more rapid germination than at room temperature. Special temperature conditions for microorganisms used are noted later.

For eliminating organisms entirely an autoclave was used. Experimenters have found that an object maintained for 15 to 20 minutes under a steam pressure of 15 pounds per square inch above atmospheric pressure is rendered completely sterile.

Moisture. Different organisms require different moisture conditions for survival and growth. In the case of plants there are two moisture conditions to consider, that of the stem and leaves and that of the roots. In this study the plants were grown with their roots in water. No attempt was made to aerate this water,as satisfactory growth was obtained without so doing. Room humidity was not controlled but was of the order of 30 per cent. Special environments for the plants to make them more suitable hosts for infectious organisms are described in section III.

Light. Intensity, frequency, and duration of light and dark periods are all important variables in the development of plants. In this study white fluorescent lamps were used. The on and off periods of these lights were controlled by time switches which could be adjusted to give any desired ratio of light period to dark period during a 24 hr. cycle. Description of the lights and the cabinet of which they were a unit is given in section II.

X-Rays. Many authors have studied effects of x-radiation on living cells and on chemicals. These effects can be very great. Work in these two fields is considered in sections I and V.

Chemical Agents

Chemicals, too, affect organisms. In this study consideration is given to effects of chemicals added to the nutrient solution of plants grown in water culture and of certain substances on plant pathogens.

Mineral nutrients and pH. In the culture solution of the plants it is necessary to have salts of a number of the elements. These are principally potassium, phosphorous, nitrogen, sulfur, calcium, iron, and magnesium. Without these and a number of others required in minute amounts, the plants cannot grow properly. There seems to be considerable leaway as to the exact amounts of these which will allow good growth. The particular mixtures used are noted later. It has been indicated that lima beans do best in a pH range of five to six (Turner and Henry, 1939). This was the range used in this work.

Organic nutrients. Amino acids, sugars, and vitamins are not ordinarily used in nutrition studies of higher plants since the plants are normally capable of making these substances for themselves. However, work has been done with organic nutrients and it will be considered in section I. A big factor in such a study is technique of handling the plants and the medium. Since such a nutrient is excellent for bacterial and fungal growth, a sterile technique is required. This, too, is considered in section I.

Antibiotics. These are substances which are produced by an organism and which act to inhibit the growth of another organism. The two organisms involved are usually microorganisms. However, there are antibiotics produced by higher plants. Antibiotic materials are produced by many kinds of molds, fungi, and bacteria. They are organic compounds, usually of relatively high molecular weight. Because of the complexity of such molecules the complete structure of only a few has been determined. The foregoing may suggest that there would be considerable difficulty in determining the amount of such a substance present in a solution or a mixture of other materials. Such is the case. The principal methods of analysis make use of the activity of these substances against bacteria. An analysis of this kind is known as a "bioassay".

Aureomycin is one of these antibiotics. It is produced by the actinomycete Streptomyces aureofaciens. Some of its physical and chemical properties are given in a paper by Broschard et al. (1949). The molecular weight is about 500. Entrance of this substance through the roots of a plant is demonstrated in section II; its action once inside the plant against a plant pathogen is shown in section III; and some of the effects of this substance and two other antibiotics on the plant are considered in section IV. The deterioration of aureomycin under soft x-rays is described in section V.

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I. ASEPTIC CULTURE AND ORGANIC NUTRITION OF X-RAY INJURED LIMA BEANS

The idea behind this study was that the injury caused by x-rays to dry seeds (Long and Kersten, 1937; Smith and Kersten, 1941) might possibly be overcome nutritionally. The object was thus to attempt to supply the plant with building blocks it could no longer make for itself. What the actual mechanism of x-ray injury is has not been clearly established, but it is known that there is a considerable disturbance to the cells. Among others Duggar (1936) and Lea (1947) have written on this.

In order to try to supply the injured plant with a metabolite which it could no longer produce by itself, it became necessary to use an organic nutrient. This required a special technique, for even when plants are grown in plain mineral culture solution there often appear growths of algae and fungi in the solution. When organic materials are added to the nutrient, biological contamination becomes a very serious problem. There are countless bacteria and molds which can grow beautifully on such a medium, robbing the plant of the nutrients and producing substances toxic to the plant. It was therefore necessary to develop a technique for handling a plant in such an organic medium.

A. TECHNIQUE FOR GROWING PLANTS WITH ROOTS IN A STERILE MEDIUM

The use of organic nutrients necessitates control of possible microbiological contaminants. A first attempt to handle the organic nutrient was by a dynamic method. The idea here was to change the nutrient so often that contamination would be kept low enough to prevent its interference with plant growth. This approach was unsuccessful; residual contamination was sufficient to heavily reinfect the nutrient solution.

A second approach was to try to suppress any growth of microorganisms with aureomycin. This failed to give adequate control of contamination but did show interesting stunting and chlorosis of the plants. These effects on the plants are considered in section IV.

Finally, a completely sterile technique was sought. Algae, fungi, and bacteria are handled this way as standard procedure. Moreover, a number of workers have described methods for aseptic culture of higher plants. Several of these are listed under <u>Aseptic Culture</u> in the references on page 29.

Most of these require a container large enough to hold the entire plant. This is a serious limitation. It has inspired the development of various schemes for allowing the top to be free in the air while maintaining the root in a sterile medium (Combes, 1912; Gerretson, 1935; Knudson and Smith, 1919). The first and last methods required a transfer of the seedling plants from their germination container to another container for further growth. The method developed here avoids this complication by disinfecting the seed and planting it aseptically into the top of a specially designed jar unit. Upon germination the root passes down into the jar and continues its development in the sterile medium found there. A procedure similar to this was worked out by Gerretson (1935).

Materials and Methods

Seed disinfection. Lima bean seeds were disinfected with a bleach solution made with HTH (high test hypochlorite) according to the method described by Wilson (1915) or with Clorox (Clorox Chemical Co., 5.25 per cent by weight sodium hypochlorite). For the HTH the procedure was to take 10 g. of the powder, slurry it in 140 ml. of water, and let the mixture stand for 10 minutes. The clear liquid was then decanted and poured over the beans. This was a stronger solution than Wilson used since commercial chloride of lime would have about 28 per cent available chlorine whereas the HTH had about 48 per cent.

To test the maximum treatment time which would permit good germination, seeds which had soaked in the HTH solution for varying lengths of time were given several water rinses and were then planted in a mixture of sand, sawdust, and vermiculite in Petri dishes. This experiment showed that 30 minutes would be a good treatment time. Later experiments showed that a 1:1 dilution of Clorox could be substituted for the HTH solution.

Construction of jar units. The jars themselves were of glass and were 9 cm. tall, 9 cm. in diameter, and had 9 cm. mouths. A hole 3.5 cm. in diameter was punched in the center of each metal, screw-type lid. Into it was inserted a 3.5 cm. long cylinder of thin-walled aluminum tubing, the lower end of which had been turned down on a lathe to leave a shoulder. The thin edge was then flanged down, clamping the lid between it and the shoulder. A disc of thin aluminum sheet was cut to fit the inside of each lid and punched at the center with a hole 3.5 cm. in diameter.

The entire unit was assembled as follows: A piece of cotton gauze (28 x 24 mesh) was laid over the hole of an inverted lid. On top of this an aluminum disc was placed. The lid was screwed into place on a jar containing any desired nutrient solution. Next, the cylinder was half-filled with Terra-Lite (Zonolite Co., vermiculite) and a gauze-wrapped cotton plug was inserted in its top. This entire unit was autoclaved 45 minutes at 15 lb. steam pressure. After cooling, it was ready for the planting operation.

Planting procedure. After the beans had soaked 30 minutes in the bleach solution previously described, they were given five rinses with sterile, distilled water. Into the vermiculite in each aluminum cylinder one of these beans was transferred aseptically. They were then watered with sterile, distilled water. The top of the cylinder was flamed and then reclosed with the plug. In about four days the beans had germinated sufficiently so that the root had penetrated downward through the vermiculite, the supporting gauze, and the two cm. air space into the nutrient solution (fig. 1). Soon after this the plumule emerged from the top of the cylinder, pushing out the plug. The stem was then wrapped with a pad of cotton-filled gauze; this was pushed down into the top of the cylinder to help support the plant.

Results

As a test of technique a nutrient medium consisting of 2 per cent sucrose, 0.01 per cent Difco yeast extract, and 0.01 per cent casein hydrolysate (General Biochemicals, Inc., "Vitamin Free") in Shive's R5S2 (0.0180M. KH_2PO_4, 0.0052M.$Ca(NO_3)2$, 0.0150M. $MgSO_4$, .0044g$FePO_4$ per liter) mineral nutrient solution (Miller, 1938; Loomis, 1937) was used. Plants grown in this way developed to heights of 20 to 30 cm. Contamination generally occurred in less

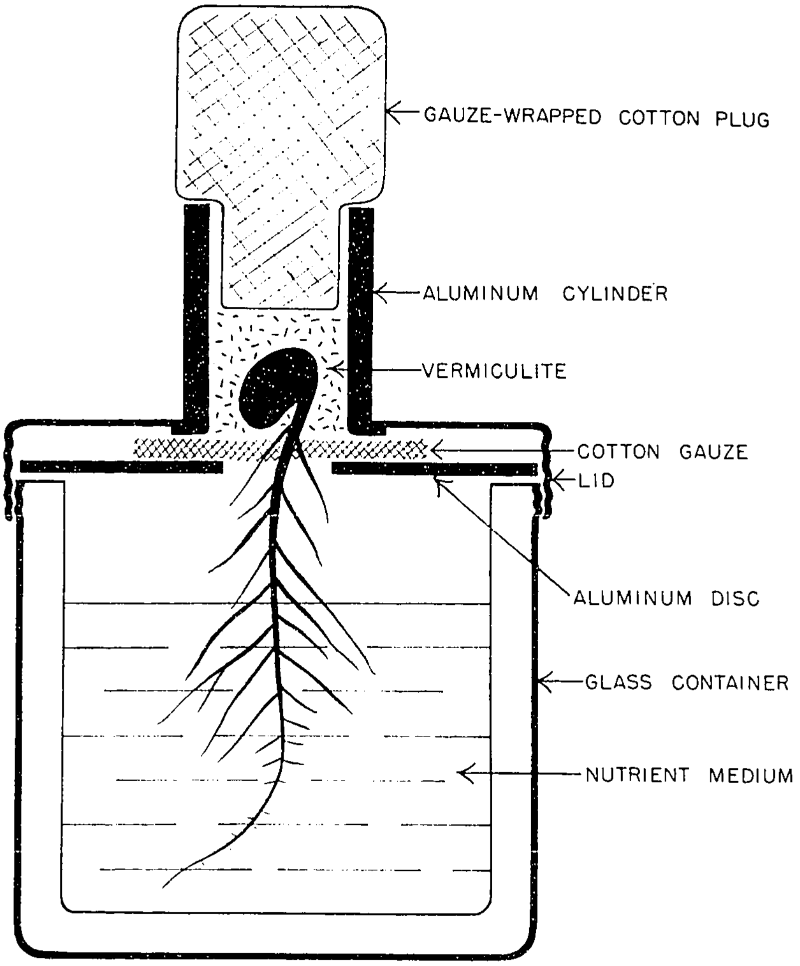

FIG. 1. Cross-sectional diagram of jar unit.

than one jar in 20.

Summary

A simplified technique for growing plants with their roots in a sterile medium has been developed. Lima beans (*Phaseolus lunatus*) were treated with a bleach solution, than planted aseptically into the top of a specially designed jar unit. The germinating bean forced its root down into the sterile medium in the jar, while the top of the plant pushed itself out into the air. This procedure avoided transfer of the young seedling from a germination container to a growth container. The method may be used for experiments in which it is desired to test the effects of various substances in the nutrient solution which would be conducive to bacterial or fungal contamination.

B. ORGANIC NUTRITION OF X-RAY-INJURED LIMA BEANS

Normal Seeds

As a preliminary to the work with x-ray injured seeds, measurements of the effects of several organic materials on untreated plants were made. Over a period of years there has been a considerable amount of such organic nutrition work done. A list of references to some of these studies is given in the section on Organic Nutrients in the references on pages 30 to 32.

Materials and Methods. Sterile technique was employed as given in Section I-A. The organic materials and the mineral nutrient used were those given there (see "results" p.10), but they were here employed in the following combinations: Sugar, yeast-sugar, casein hydrolysate-sugar, yeast-casein-hydrolysate-sugar. An additional nutrient made of 100 g. of well rotted leaf mold ground into 1 l. of mineral nutrient in a Waring blendor was used.

Results. The results of these experiments are best given in the form of the graphs, figures 2 to 6. Here the average height of each group of treated plants is plotted as the ordinate with the number of days after planting as abscissa.

In general the organic materials seemed to inhibit rather than help these plants. A possible exception is that of the non-sterile leaf mold, and this is not at all certain because of the small number of plants in the experiment (nine). In this case there was fermentation in the solution as evidenced by gas evolution.

It is interesting to note that a change in dosage of tenfold made no appreciable difference in the inhibiting effect of the casein hydrolysate.

Conclusions. There is apparently little to be gained by this combination of organic nutrients in the case of normal plants. They are able to grow in such

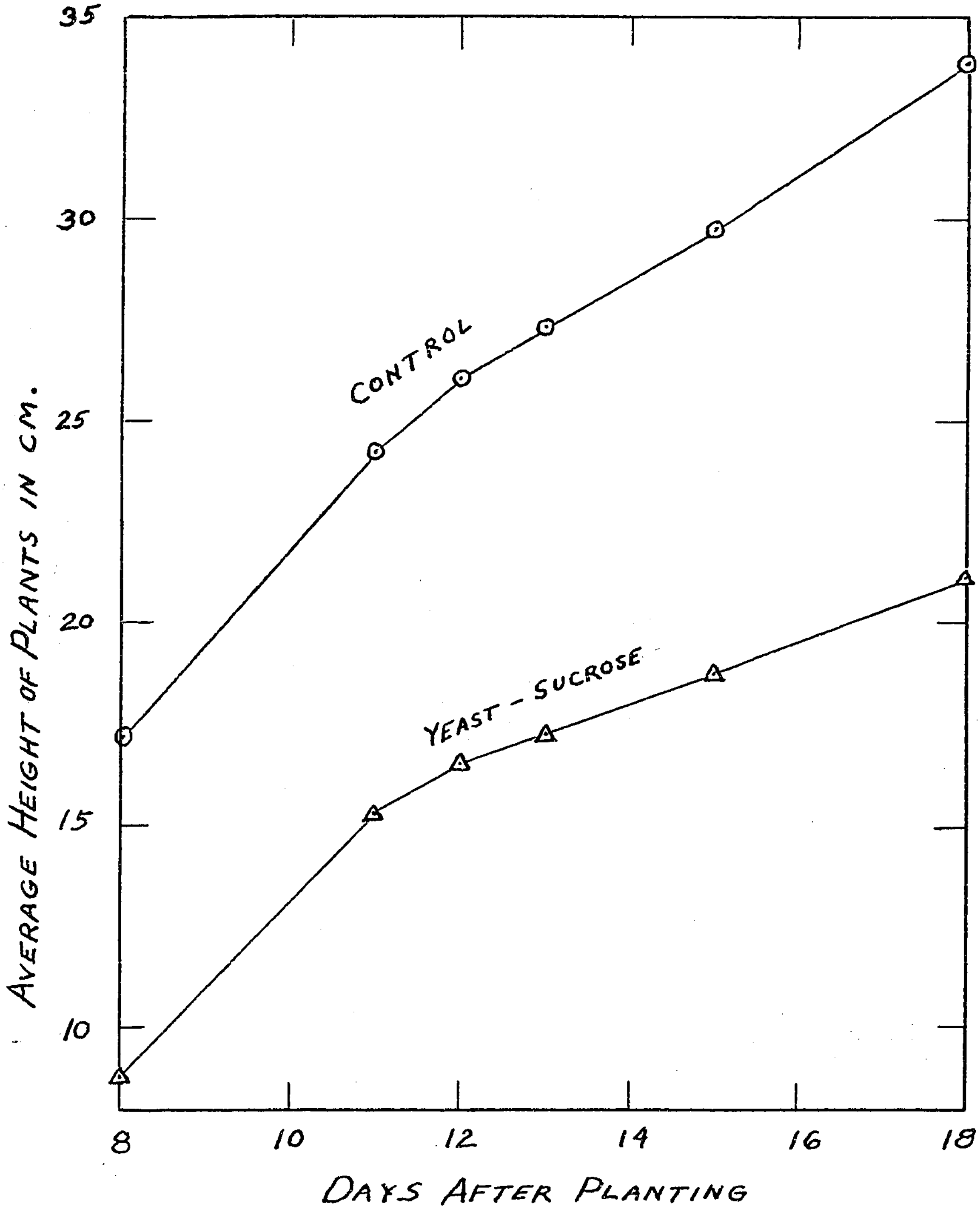

FIGURE 2, GROWTH CURVE, LIMA BEANS IN YEAST EXTRACT-SUCROSE SOLUTION.

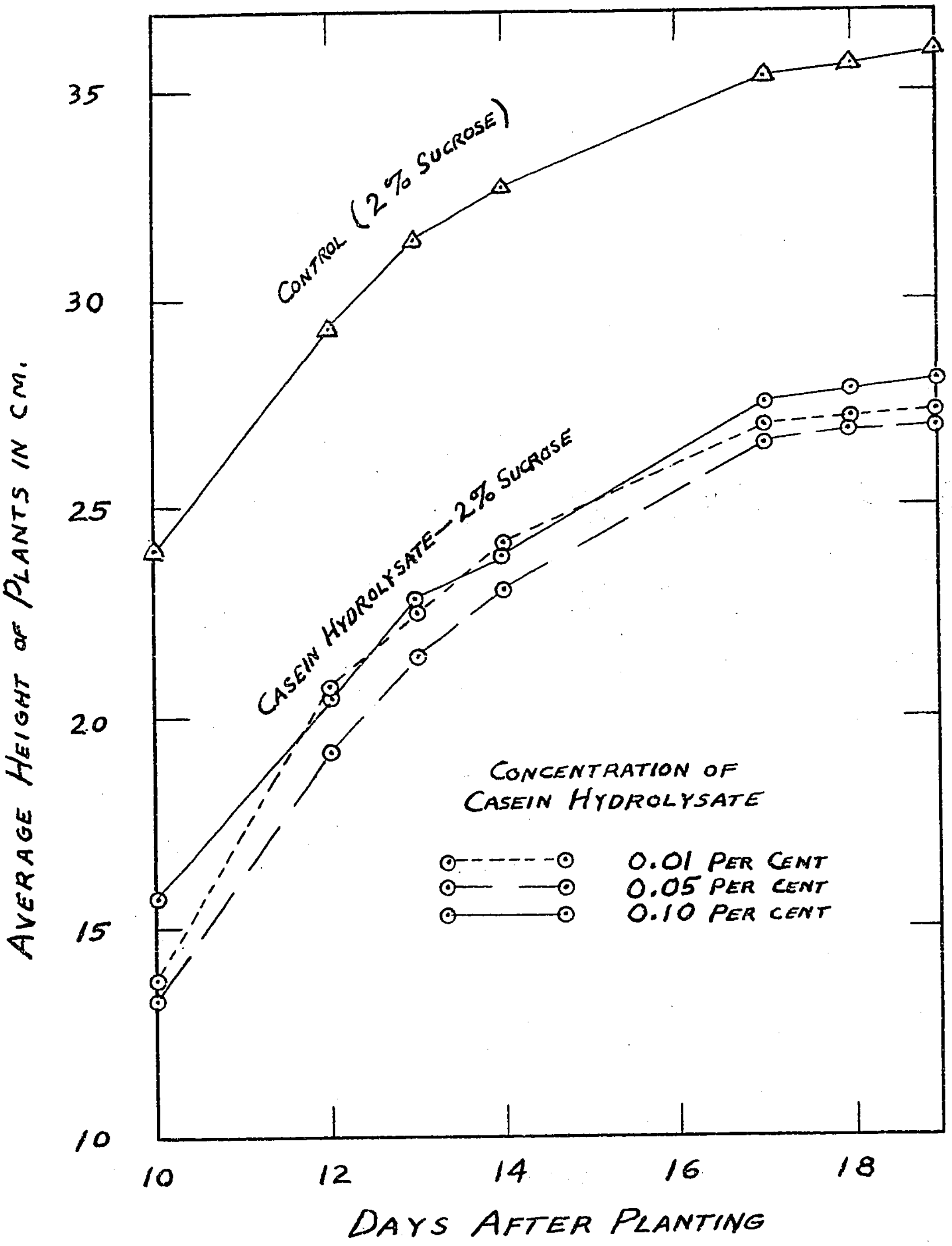

FIGURE 3. GROWTH CURVE, LIMA BEANS IN CASEIN HYDROLYSATE—SUCROSE SOLUTION.

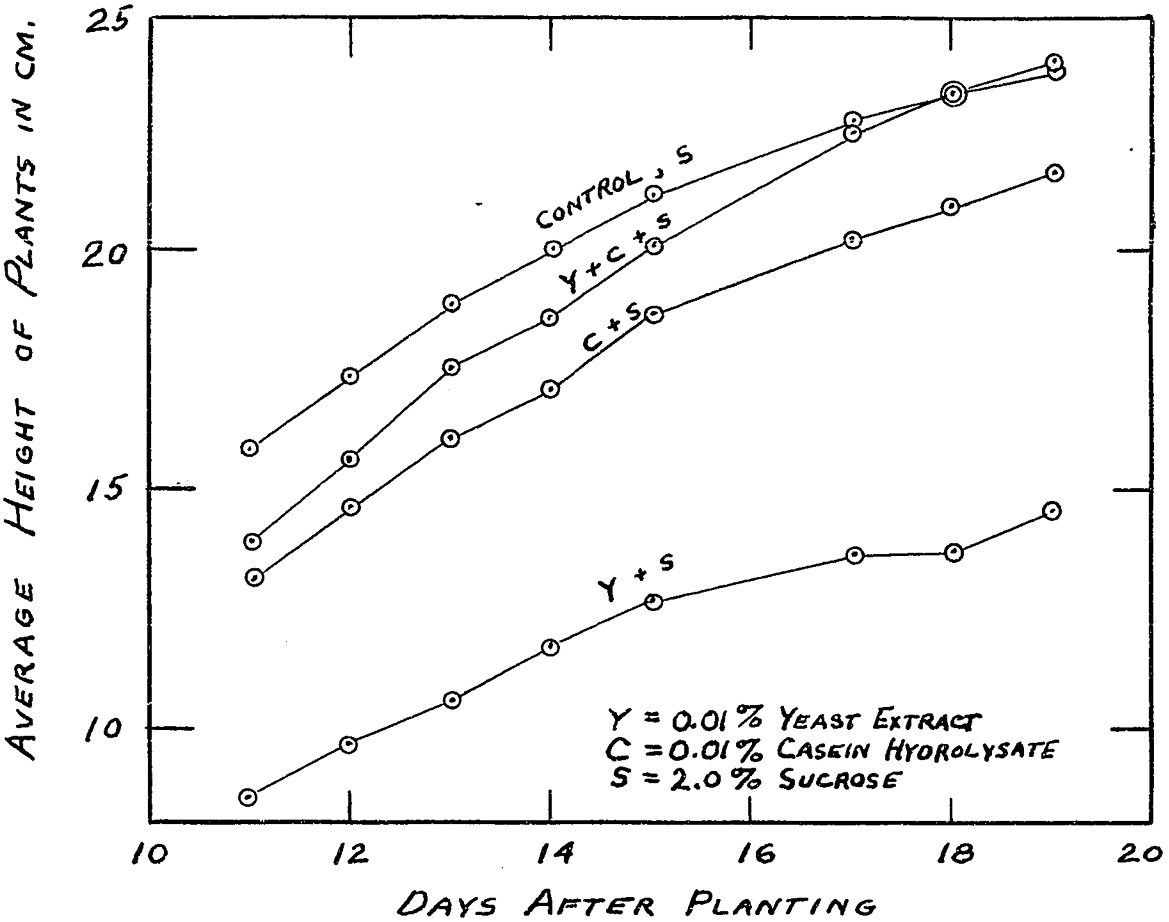

FIGURE 4. GROWTH CURVE, LIMA BEANS IN YEAST EXTRACT – CASEIN HYDROLYSATE – SUCROSE SOLUTION.

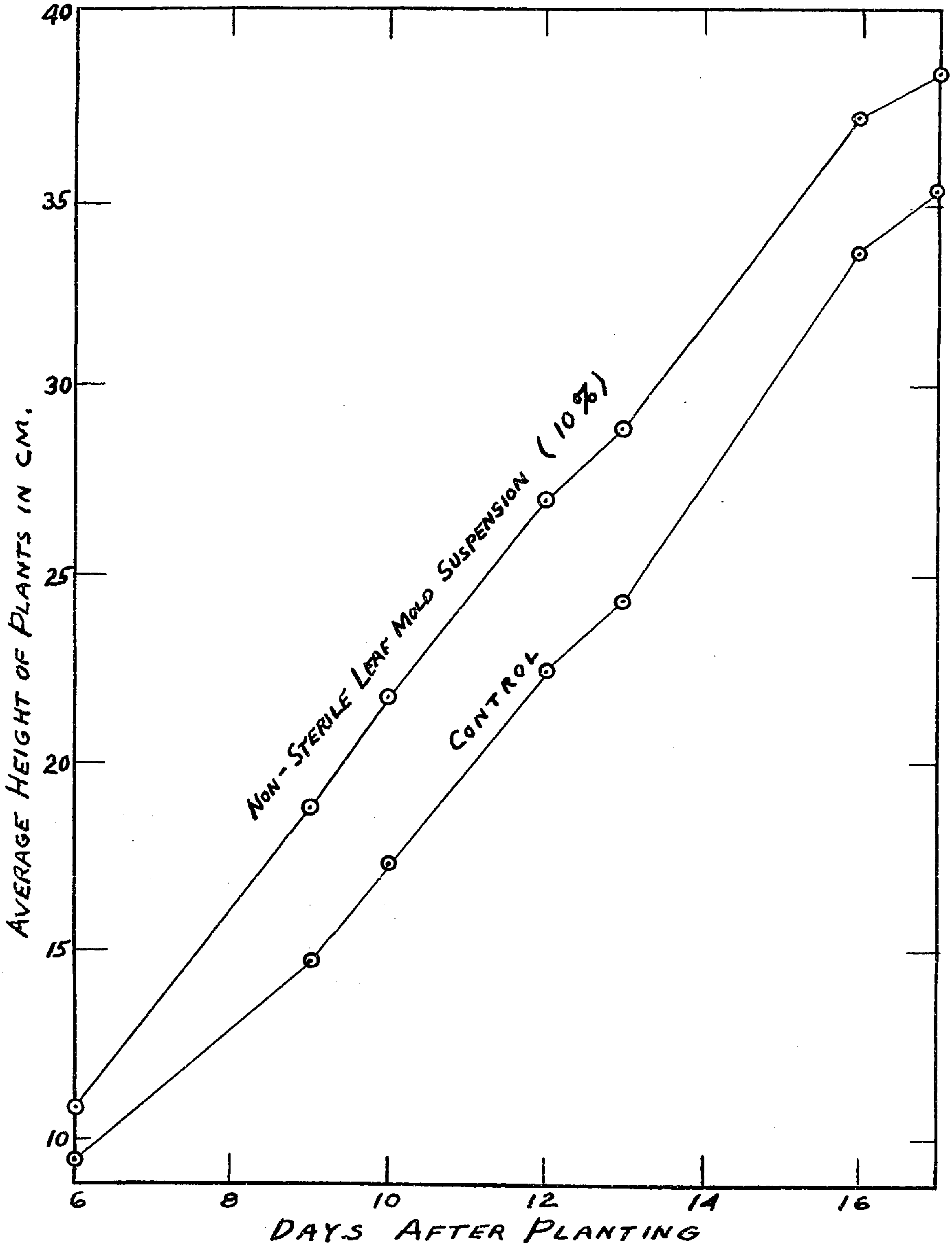

FIGURE 5. Growth curve, lima beans in non-sterile leaf mold suspension.

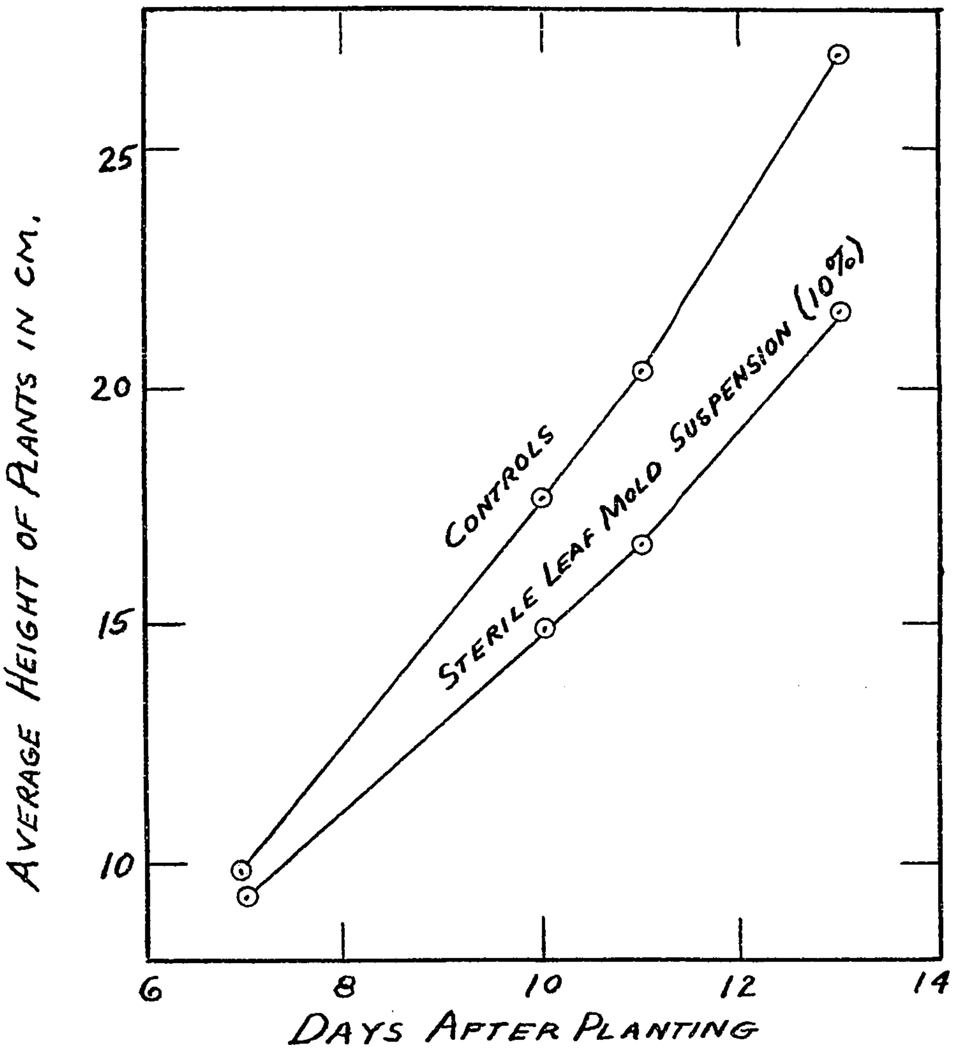

FIGURE 6. GROWTH CURVE, LIMA BEANS IN STERILE LEAF MOLD SUSPENSION.

nutrients but not as well as in plain mineral solutions.

X-ray-Injured Seeds

Materials and Methods. The x-rays were produced by a Machlett AEG-50-T Beryllium window tube (Machlett Laboratories, Inc., Springdale, Conn. U.S.A.). This was operated at about 35 kv and 10ma in this work. The range of wavelengths was from about 0.4 Angstroms to about 4.4 Angstroms with a peak intensity at about 1.7 Angstroms. Intensity of the radiation was of the order of 12,000 roentgens per minute as estimated from data on this tube in a paper by T.H. Rogers (1947).

The bean seeds were placed on edge, hilum side up, in sand in a Petri dish (no cover). This dish was placed under the window of the x-ray tube at a distance of 5 cm. for the preliminary trial and 6 cm. for all later work. Dosages were varied by changing the time of exposure.

In the preliminary trial a series of exposure times ranging from 1 to 300 seconds was used. The seeds so treated were than planted to sterile mixtures of sand, sawdust, and vermiculite in Petri dishes (2 beans to a dish). Details of this planting procedure are given in section II. After 3 days the lengths of the roots were measured. (Table 1). This experiment indicated that a dosage of 60 seconds or slightly more should be about

TABLE 1: X-ray lethal dose experiment on beans[a]

	Root lengths in centimeters Three day growth in Petri dishes at 30°C.						
x-ray time in seconds	Dish number 1		2		3		number satisfactory[b]
1	6.5*	8*	8.5*	8*	3.5	6.5*	5
5	8 *	6*	4.5*	7.5*	1	3	4
15	7.5*	1	3	10.5*	-	8*	3
30	7 *	-	4*	1	5*	-	3
60	6.5*	6*	2	3	1	4*	3
300	3	0.5	4*	2.5	1	3	1

a. Irradiations at 5 cm.from x-ray tube window

b. Those 2 cm. or longer could probably have crossed the air gap in the sterile jars.

* Those fulfilling requirement of note a. :

right to show effects on the plants but leave sufficient vitality for the root to cross the 2 cm. air gap into the nutrient of the sterile jars.

Handling of the seeds after treatment and of the controls for all subsequent experiments was by the sterile technique previously described. The organic nutrient used was the same as that given in the results of Section I-A, but with some indole-acetic acid (IAA), a plant hormone, added to attempt to stimulate root and stem growth.

Results. A first experiment using 0.6 x 10^{-4} M.IAA was made with 60 seconds of x-radiation. When this IAA concentration was chosen it was thought to be at a level for optimum growth stimulation as indicated by Muir et al. (1949). However, when the roots entered the solution considerable masses of tissue developed in clumps on them where they touched the solution. This suggested a huge overdose of IAA. This is confirmed by a figure in Meyer and Anderson, p. 580, (1939). A sketch of this is given in figure 7 . From this it was seen that although 10^{-4}M. is good for stems (the work of Muir et al. was with stem tissue culture), 10^{-9} or 10^{-10}M. would be a better choice for the entire plant. The growth of these plants in 27 days is given in table 2.

With the IAA dropped to 10^{-10}M. but with the x-ray

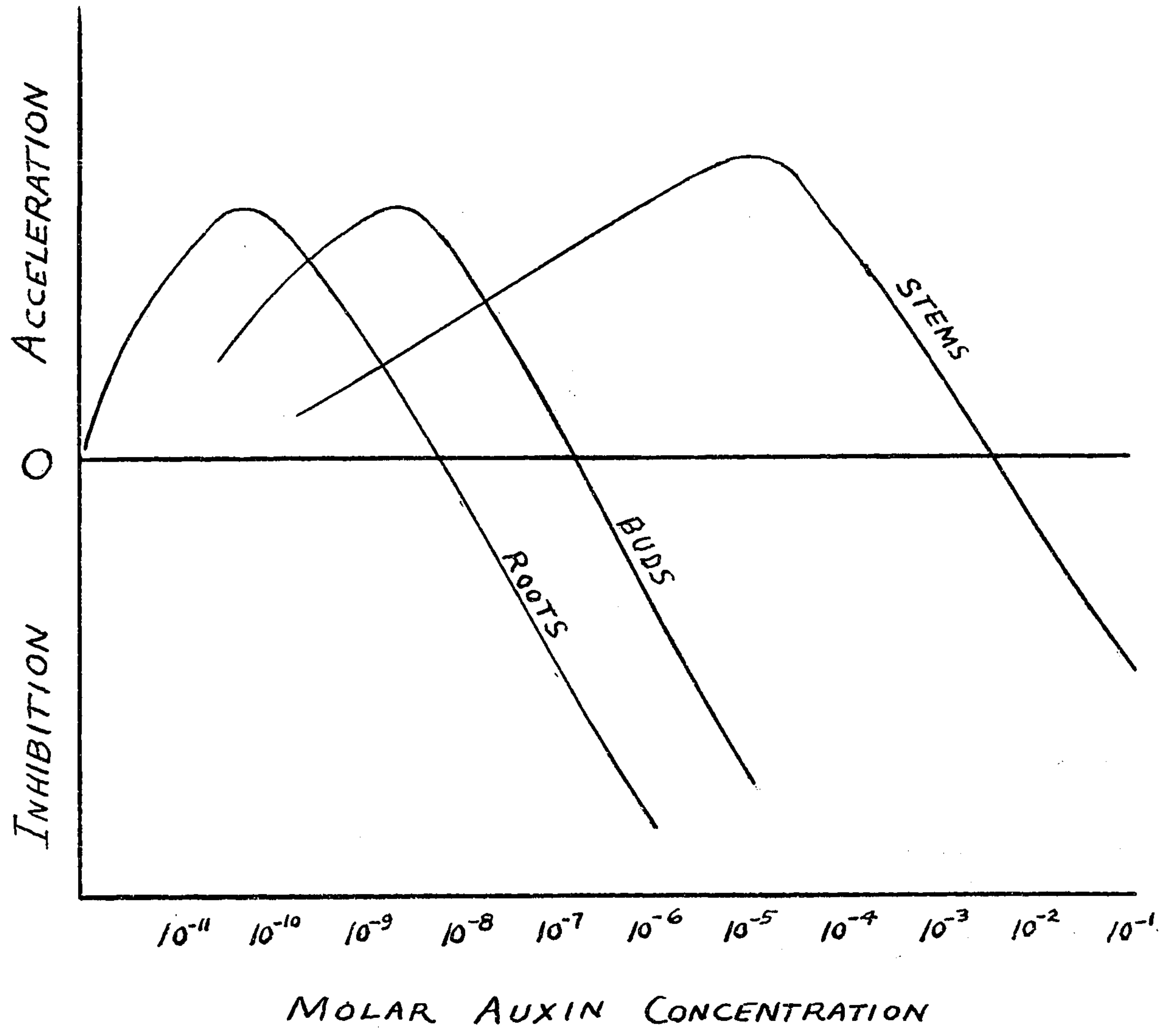

FIGURE 7. RELATION OF AUXIN CONCENTRATION TO ELONGATION OF ROOTS, BUDS AND STEMS ACCORDING TO THIMANN (1937). TAKEN FROM MEYER AND ANDERSON (1939).

TABLE 2: Twenty seven day growth of x-ray injured beans in yeast extract - casein - hydrolysate - sucrose + 0.6 x 10^{-4} M. IAA.*

Plant No.	Height of plants in centimeters			
	M	MX	C	OX
1	40.5	47	7	7.5
2	44	48	24	12
3	48.5	49	27.5	13.5
4	49	51.5	29	24
5	54.5	60	30.5	25.5
6	55	64	32	31
7	56	-	34.5	35
8	57	-	40	38.5
9	60	-	40	43
10	66.5	-	44	-
average	53.1	53.2	30.9	26.7

-	failed to germinate
*	large clumps of tissue developed on the root tips; apparently too much IAA.
M	mineral nutrient - normal seed
MX	mineral nutrient - seed x-rayed 60 seconds
C	organic nutrient - normal seed
OX	organic nutrient - seed x-rayed 60 seconds

dosage raised to 90 seconds, another experiment was made. Results of this are presented in table 3. Apparently the increase in x-ray dosage was too great.

A final experiment was set up with 60 seconds x-ray treatment and 10^{-10} M. IAA. The effect on length of roots one week after planting is given in table 4. This indicates that the x-ray treatment did injure the plants, but that there was little difference in root growth between mineral and organic nutrients. The average heights of the x-rayed plants at 26 days were: mineral nutrient - 44 cm., organic nutrient-29 cm. Averages for the controls were: mineral nutrient - 48 cm., organic nutrient 48 cm. The appearance of these plants in their growth cabinet is shown in figure 8. Those in the organic nutrient tended to become somewhat chlorotic.

Conclusions. This particular combination of organic materials (2 per cent sucrose, 0.01 per cent yeast extract, 0.01 per cent casein hydrolysate, and 10^{-10} M. indoleacetic acid) does not seem to be able to counteract the x-ray injury which the plants received. It even has a tendency to cause chlorosis and inhibition. The inhibition is especially marked on plants that are already weakened by x-ray injury (in contrast with the controls).

It is doubtful whether any other medium would be more successful although the possibility is not ruled out.

TABLE 3: Twenty-three day growth of x-ray injured beans in yeast extract - casein -hydrolysate-sucrose + 10^{-10}M. IAA.

Plant No.	Height of plants in centimeters			
	M	MX	O	OX
1	57	27.5	46	12.5*
2	48	6.	44.5*	8.5
3	42.5	+	39	+
4	42	-	38.5	+
5	36.5	-	38	-
6	35	-	30.5	-
7	34.5	-	28**	-
8	34	-	27.5**	-
9	33.5	-	26**	-
10	33.5	-	-	-
average	39.7	16.8	30.9	10.5

+	root dipping into solution but plug not ejected from top
*	contamination
**	heavy contamination
-	failed to germinate
M	mineral nutrient - normal seeds
MX	mineral nutrient - seed x-rayed 90 seconds
O	organic nutrient - normal seeds
OX	organic nutrient - seed x-rayed 90 seconds

TABLE 4. Seven day root growth of x-ray-injured lima beans in yeast extract-casein hydrolysate-sucrose ≠ 10^{-10}M. indole acetic acid.

Plant no.	Root lengths in centimeters			
	M	MX	O	OX
1	8*	2	5*	4*
2	8*	4*	5*	2
3	8*	4*	5*	5*
4	-	-	3	3*
5	8*	4*	6*	3*
6	3*	2	6*	3*
7	2	4*	8*	5*
8	9*≠	1	7*	1
9	3*	-	7*	3*
10	9*	2	8*≠≠	2
average (all)	6	3	6	3
average (*only)	7	4	6	4

*	root in solution
≠	top out
≠≠	jar cracked, contamination
-	failed to germinate
M	mineral nutrient - normal seeds
MX	mineral nutrient - seed x-rayed 60 seconds
O	organic nutrient - normal seeds
OX	organic nutrient - seed x-rayed 60 seconds

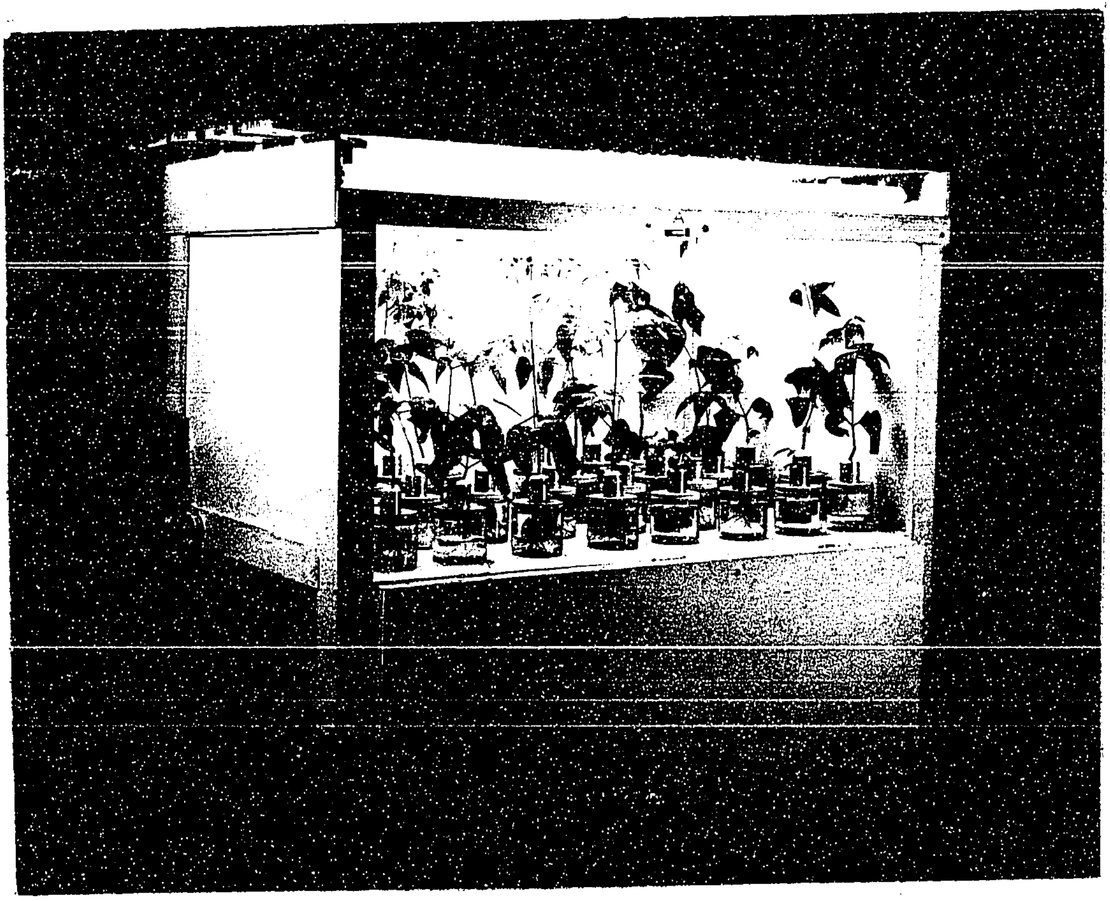

FIGURE 8. Lima beans, with roots in sterile media, growing in fluorescent light cabinet.

Four rows to right were x-rayed 1 min. at 12,000 r/min.
Four rows to left were controls.

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Two rows on each end were grown in mineral nutrient.
Four rows in middle were grown in organic nutrient.

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II. UPTAKE OF AUREOMYCIN THROUGH THE ROOTS OF LIMA BEANS

During the preliminary search for a method for aseptic culture of plants, it was noticed that aureomycin in the culture solution seemed to have effects on the roots and the tops of the plants. The latter suggested that some of the drug might have entered the upper parts of the plant.

This study was designed to determine whether any of the aureomycin penetrated the roots and was translocated to the stems and leaves. The procedure was to grow the plants in aureomycin solutions and then run assays for it on extracts prepared from the plant parts. Positive assays for streptomycin have been obtained by Anderson (1947) in extracts prepared from soybean plants grown in solutions of that antibiotic.

Materials and Methods

Germination technique. Nine centimeter Petri dishes, filled with a mixture of sand, sawdust, and vermiculite (approximately equal portions of each by volume) were wrapped in brown paper and autoclaved at 15 lb. steam pressure for 15 minutes.

One hundred and fifty lima bean seeds (Phaseolus lunatus) were treated by shaking for 1 minute in Arasan (DuPont, 50 per cent tetramethyl thiuramdisulfide, 50 per cent inert materials), after which they were shaken free of excess dust and inserted three to a Petri dish. They were placed close together, away from the center, and with their hila toward the center of the dish. After 20 ml. of distilled water had been added to each dish, the dishes were closed and stacked on edge (beans uppermost) in a germination cabinet.

This was a box 2x2x2 feet kept at 30°C. by a Fenwal thermostat-controlled heater. The heater consisted of a small blower which pulled room air past a 100-watt light bulb and forced it into the cabinet. Both the bulb and the blower were actuated by the thermostat.

Growth technique. Three days after the seeds had been placed in the Petri dishes, the seedlings were removed, rinsed, and sorted to remove defective plants. The remaining plants were then placed with their roots in 1.2 cm. holes in the screw lids of short wide-mouth jars (9 cm. diameter, 9 cm. mouth, 9 cm. height). They were held in place in the lid by a pad of cotton-filled gauze wrapped around the stem. The jars were filled about two-thirds full (250 ml.) of mineral culture solution; the lid with its seedling was then screwed into place on the jar. The culture solution used was basically Formula I of Shive and Robbins (0.0023M KH_2PO_4, 0.0045 M. $Ca(NO_3)_2 \cdot 4H_2O$, 0.0023 M. $MgSO_4 \cdot 7H_2O$, 0.0007M $(NH_4)_2SO_4$) (1948) supplemented with an "A-Z" solution as suggested by Hoagland and Snyder (1933) (their solution "A".

The jars, with the seedlings as described above, were then placed in a cabinet 4 ft. by 2.5 ft. by 2 ft. high. Its walls were constructed of wallboard and were painted white. Above a top of sheet glass was a bank of ten 40-watt white fluorescent lights operating on a 10 hr. light-14 hr. dark cycle. Illumination at the jar lids was about 700 f.c. No attempt was made to aerate the solutions. The temperature inside the cabinet ranged from 75°-80°F. A picture of the cabinet is given in figure 8.

Selection and treatment of plants. After 5 days in the light cabinet, the jars were sorted to give a line of descending heights of plants. Extremely tall, stunted, or damaged plants were removed; from the remainder, the sixty most uniform plants were chosen (fifty for experiment #2. These were sorted into six groups (five for experiment #2) of ten plants each. This was done according to a systematized Latin square to provide an equivalent range of tall to short plants in each group. Use of the Latin square method for selection of samples is described by Vickery, et al. (1949).

As controls, the plants in three of the groups (two for experiment #2) were than transferred to jars containing freshly prepared culture solution. Three other groups were transferred to similar solutions to which had been added 10, 100, and 1000 μg. aureomycin/ml., respectively. All solutions were adjusted to pH range 4.6-4.9.

Aureomycin analysis. Seven days later, the plants were removed from their containers, rinsed in distilled water, and divided into roots, stems, and leaves. All the roots of each group were placed together in a beaker and dried overnight at 80°C. These dried samples were than weighed to give total dry weight for the ten roots of each group. The stems and leaves were treated similarly.

Each of these samples was then pulverized in a mortar, placed in a small glass bottle, and suspended in 25 ml. of distilled water. An hour later they were frozen until further work could be done on them. After several days they were thawed, centrifuged, and the clear supernatant fluid refrozen until needed.

The final analysis was a serial dilution assay using *Bacillus cereus* #5 (Dornbush and Pelcak, 1948). Briefly the method is to add equal quantities of an overnight, broth culture of *Bacillus cereus* to a series of increasingly dilute solutions of the sample being considered. A standard solution of aureomycin of known strength is treated in the same way. After incubation at

37°C. for 4 hrs., these solutions are examined. Any turbidity indicates bacterial growth. A clear solution indicates inhibition of bacterial growth. The most dilute unknown solution remaining clear is then considered to have the same aureomycin concentration as the most dilute, clear, standard solution. The results have been expressed in micrograms of aureomycin activity per gram of total dry weight of the plant part for each group.

Results and Discussion

The results of the analyses are presented in table 5. They would seem to point very strongly to the fact that aureomycin can be absorbed by the plant and is translocated to stems and leaves. The actual amount of aureomycin entering the plant is quite small, although the amount adsorbed to or absorbed into the root is relatively much larger. The uptake is apparently influenced by a great many factors, and although efforts were made to keep the experiments uniform, it was impossible to obtain quantitatively reproducible results.

To check on deterioration of the aureomycin in the media, a composite sample was taken of the culture solution of each treated group and of the controls at the end of the experiment. These samples were kept frozen until the time of assay. The assay showed that although there was decrease in aureomycin activity in the culture solutions considerable activity remained at the conclusion of the

TABLE 5: Uptake of aureomycin through the roots of *Phaseolus lunatus*.

Aureomycin added to culture solution (μg/ml)	Aureomycin in medium after experiment (μg/ml)	Dry weight per group (g.)			Aureomycin in extracts (μg/g. dry weight)		
		Roots	Stems	Leaves	Roots	Stems	Leaves
0	< 0.05	0.88	3.52	4.28	< 1.3	< 0.4	< 0.3
0	< 0.05	0.88	3.87	4.50	< 1.3	< 0.3	< 0.3
0	< 0.05	0.78	3.30	4.05	< 1.3	< 0.4	< 0.3
10	< 2.5	0.73	3.55	4.08	44.0	1.4	0.6
100	100.	0.68	3.61	3.50	> 47.0	> 11.1	1.4
1000	1000.	0.75	4.16	2.58	> 42.5	> 9.6	7.7
. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
0	< 0.05	0.95	3.35	3.68	< 1.3	< 0.7	< 0.4
0	< 0.05	0.87	3.18	3.92	< 1.4	< 0.6	< 0.4
10	1.	0.65	3.69	3.92	76.9	< 0.5	0.4
100	50.	0.76	3.72	2.95	1316.	1.9	6.1
1000	500.	0.83	4.32	1.94	> 193.	3.2	3.1

Note 1: Where more than one value is given for μg/g., each figure represents a separate assay on the extract.

Note 2: Wherever the symbol < appears, the sample tested had an aureomycin activity, if any, less than the lower limit of the microbiological assay (o.05μg/ml.)

Note 3: Each row represents data for a group of ten plants.

TABLE 5: Uptake of aureomycin through the roots of *Phaseolus lunatus* (cont.).

Aureomycin added to culture solution (μg/ml.)	Aureomycin in medium after experiment (μg/ml.)	Dry weight per group (g.)			Aureomycin in extracts (μg/g. dry weight)		
		Roots	Stems	Leaves	Roots	Stems	Leaves
0	< 0.05	0.94	3.30	3.54	<1.3	<0.5	<0.3
0	< 0.05	1.00	3.60	3.80	<1.0	<0.3	<0.3
0	< 0.05	0.87	3.22	3.54	< 1.1	<0.4	<0.3
10	2.	0.82	3.35	3.29	48.7	<0.4	<0.3
					24.4		
					< 60.9		
					48.7		
100	50.	0.87	3.71	2.93	115.	<0.3	<0.3
					115.	0.7	<1.0
					230.		0.7
1000	500.	1.03	3.61	1.99	1550.	1.4	1.0
					1940.	1.4	2.0
					3890.	2.8	
					5820.	2.8	

growth period. As a check on aureomycin deterioration apart from the influence of the plants, dummy jars for each treatment solution were prepared and closed with a lid and a cotton plug. At the end of the experiment, samples of each of these were taken and frozen until needed. This assay showed that there had been about the same amount of deterioration as for the solutions which had had plants growing in them.

Summary

Lima bean plants which had been grown for 5 days under controlled conditions were used in experiments designed to investigate whether aureomycin could enter the plants through their root systems. Various concentrations of aureomycin were added to the mineral culture solution supplied to the roots. Assays for aureomycin were made on the roots, stems, and leaves 1 week later. The results leave little doubt that aureomycin penetrated through the roots and was translocated to the stems and leaves of the plants.

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III. AUREOMYCIN CHEMOTHERAPY OF CROWN GALL IN TOMATOES

Plant chemotherapy, the introduction of some chemical agent to the internal system of a plant to act against bacterial, fungal, or virus infections, has been explored by a number of workers. An excellent review of the field through 1948 is given by Stoddard and Dimond (1949). An attempt to use an antibiotic for this purpose was reported by Anderson and Nienow (1947). They found that soybeans containing 4 to 5 units of streptomycin per ml. of expressed sap developed typical symptoms of infection when inoculated with Xanthomonas phaseoli,var. sojense.

The presence of aureomycin in a plant when it is grown with its roots in a solution of this antibiotic, as shown in section II, suggested the use of this drug for phytochemotherapy. Preliminary work seemed to indicate that both aureomycin treated and untreated lima beans were equally susceptible to Xanthomonas phaseoli (Erw. Smith) Dowson, (American Type Culture Collection No. 9563), the organism causing bean blight. Other work with the organism of crown gall, Agrobacterium tumefaciens (Smith and Towsend) Conn[1], on tomatoes indicated a lower incidence and degree of infection in aureomycin treated plants than

[1] Culture supplied by Dr. B.M. Dugger of Lederle Laboratories

in controls. Chemical treatments of plants against crown gall have been reported previously, among these: Ark (1941), Brown and Boyle (1944, 1945, 1945), Hampton (1948), and Verona (1947).

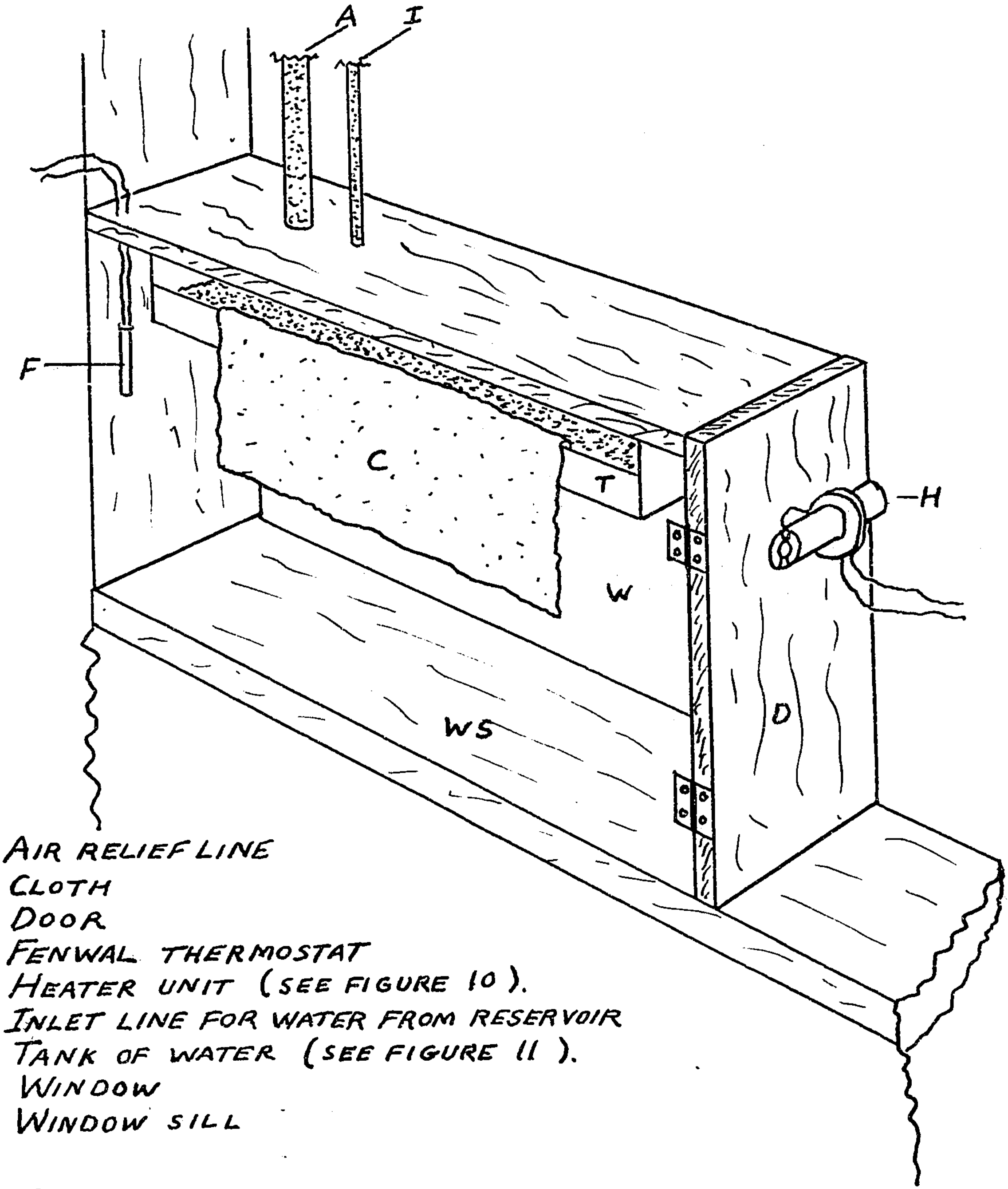
The purpose of the study reported here was to see if aureomycin could exert chemotherapeutic action on crown gall of tomatoes.

Materials and Methods

Mineral culture. Tomato plants (Bonnie Best) were grown in mineral culture solution in 400 ml. wide mouth glass jars. Shive and Robbins' (1948) mineral nutrient, Formula I, (see page 34) with added supplementary solution "A" of Hoagland and Snyder (1933) was used. Each plant was held in place in a 0,5 in. hole in a metal jar lid with a pad of cotton-filled gauze wrapped around the stem. To transfer a plant from one solution to another, it was only necessary to unscrew the lid and screw it in place over another jar.

"Moist" cabinet. The plants were grown in two different cabinets, a "light" cabinet and a "moist" cabinet. The former is described in Section II. Its bank of ten 40-watt, white fluorescent lamps was operated on a cycle of 14 hours of light and 10 hours of darkness. The "moist" cabinet was a box built up around a recess in the laboratory window utilizing a portion of the window for one wall (fig. 9). During the winter this tended to bring the temperature in the cabinet to 10-15°C. However, the air was warmed and held to 18-22°C by a thermostatically controlled blower which pulled air from the room past an electrical resistance heater element and forced it into the cabinet (fig.10). Humidity was raised to about 75 per cent by evaporation of water from a wet cloth which was hung in the cabinet. The cloth was kept wet by placing it with one edge dipping into a tank of water. When the water level in this tank dropped about half a centimeter, air entered the air relief line of an adjoining reservoir permitting a siphon to operate. This refilled the tank to a level such that the water again covered the inlet to the **air relief** line (fig.11). The entire cabinet was therefore automatic in operation. The temperature and moisture conditions for this cabinet were chosen to lie within a range conducive to tumor initiation (Riker, 1926).

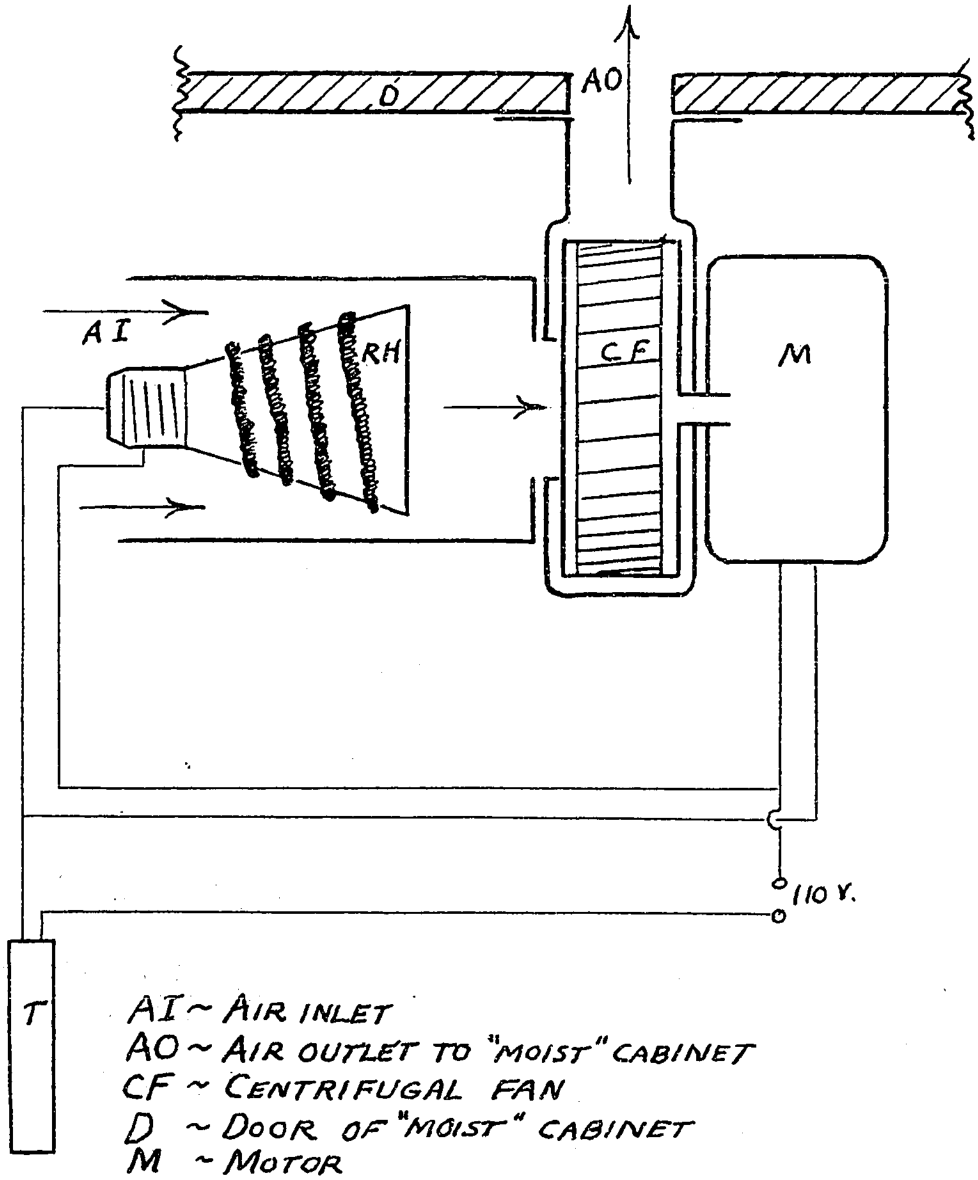
Inoculations. The inoculations were made by puncturing the stem with a needle dipped in a 4-day culture of Agrobacterium tumefaciens, which had grown at 24° C in A.C. Broth (Difco). Two or more inoculations were made in each stem, with the lowest one a centimeter or two from the roots, and the others spaced about a centimeter apart along the stem.



A ~ Air relief line
C ~ Cloth
D ~ Door
F ~ Fenwal thermostat
H ~ Heater unit (see figure 10).
I ~ Inlet line for water from reservoir
T ~ Tank of water (see figure 11).
W ~ Window
WS ~ Window sill

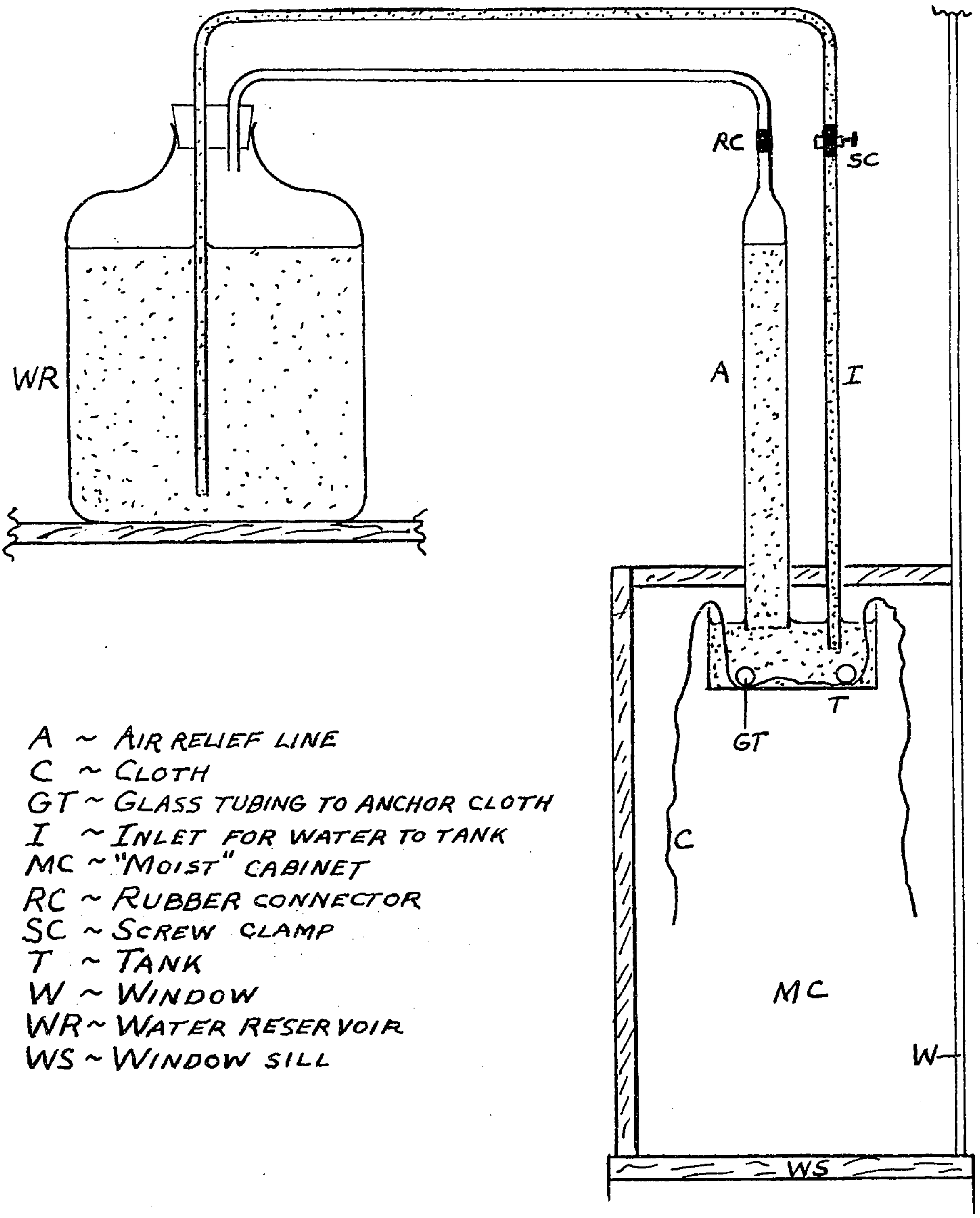
Front removed to show details

FIGURE 9. "Moist" cabinet.



AI ~ Air inlet
AO ~ Air outlet to "moist" cabinet
CF ~ Centrifugal fan
D ~ Door of "moist" cabinet
M ~ Motor
RH ~ Resistance heater element
T ~ Thermostat

FIGURE 10. Diagram of heater unit.



A ~ Air relief line
C ~ Cloth
GT ~ Glass tubing to anchor cloth
I ~ Inlet for water to tank
MC ~ "Moist" cabinet
RC ~ Rubber connector
SC ~ Screw clamp
T ~ Tank
W ~ Window
WR ~ Water reservoir
WS ~ Window sill

FIGURE 11. Automatic humidity system for "moist" cabinet.

Experimental procedure. The procedure for each experiment was as follows: One month old plants, which had been in the "light" cabinet, were inoculated as described above and then arranged in two groups. One group, the control plants, was transferred to jars containing fresh nutrient solution. The other, the treated plants, was transferred to jars of the same solution to which Aureomycin hydrochloride (Lederle)[2] had been added. Concentrations of 5, 10, and 20 μg. of aureomycin per ml. of nutrient solution were used. A preliminary experiment showed that 50 μg. per ml. was the minimum dosage which would cause severe wilting and stunting of the plants. In order to stay well below this range, the upper dosage level of these experiments was set at 20 μg. per ml.

Both groups of plants were placed in the "moist" cabinet. This was done to provide optimum conditions for the development of tumor cells from host cells. They were kept there 4 days, since the conversion seems to take about this length of time (Braun, 1943). After this period, the plants were returned to the "light" cabinet. When the level of the nutrient in the jars became low (about 2 weeks) both treated and control plants were transferred to plain mineral nutrient.

[2] Supplied by Lederle Laboratories Division, American Cyanamid Co.

Gall development. After 5 weeks, a gall of 3 to 20 mm. in diameter had developed at most of the inoculation points on each control plant. In general there were fewer galls on the treated plants. The diameter of each such gall was measured even where there were more than one at a given inoculation point. The galls from each group were sliced off and weighed as a group.

Computations. Three percentage ratios were computed from this data. The first was the ratio of the number of inoculation sites at which galls developed on the treated plants to the number of inoculation sites which showed galls on the control plants. The second was the ratio of the wet weight of galls of treated plants to that of control plants. The third was the ratio of the sum of the cross sectional areas of all galls developed on the treated plants to that of those on the control ones. This calculation was made assuming that the area of each gall is given by $\pi D^2/4$, where D is the measured diameter of the gall. The area ratio was chosen in preference to a diameter ratio in order to make possible a more accurate representation of gall development at inoculation points where more than one gall had developed.

Results

Fewer galls developed on plants treated with aureomycin than on the controls. The galls of the treated plants were of lower average area and weight than those of the controls. The difference in appearance of the gall growth of the two groups is shown in figure 12.

Confirmation of the effectiveness of the treatment is provided by the fact that all the percentage ratios described earlier are considerably less than 100 (table 6). For the dosage of 20 μg. per ml., the very small percentages, the general appearance of the galls, and the gall sizes themselves, as given in table 7, indicate nearly complete control. There was some browning of the roots at this dosage but no noticeable wilting or difference in general appearance of the control and treated plants. Neither was there any measureable difference in heights of the control and treated plants.

Discussion

In connection with previous attempts to control crown gall, it might be noted that Stoddard and Dimond (1949, p. 360) point out that "there appears to be no record of crown gall being completely controlled by the introduction of materials into a plant by root absorption or by injection, the only successful treatments having been by application of chemicals directly into or onto the gall tissue". In the present work with aureomycin, the treatment was by root absorption and nearly complete control was obtained at a dosage of 20 μg. of aureomycin per ml. of nutrient.

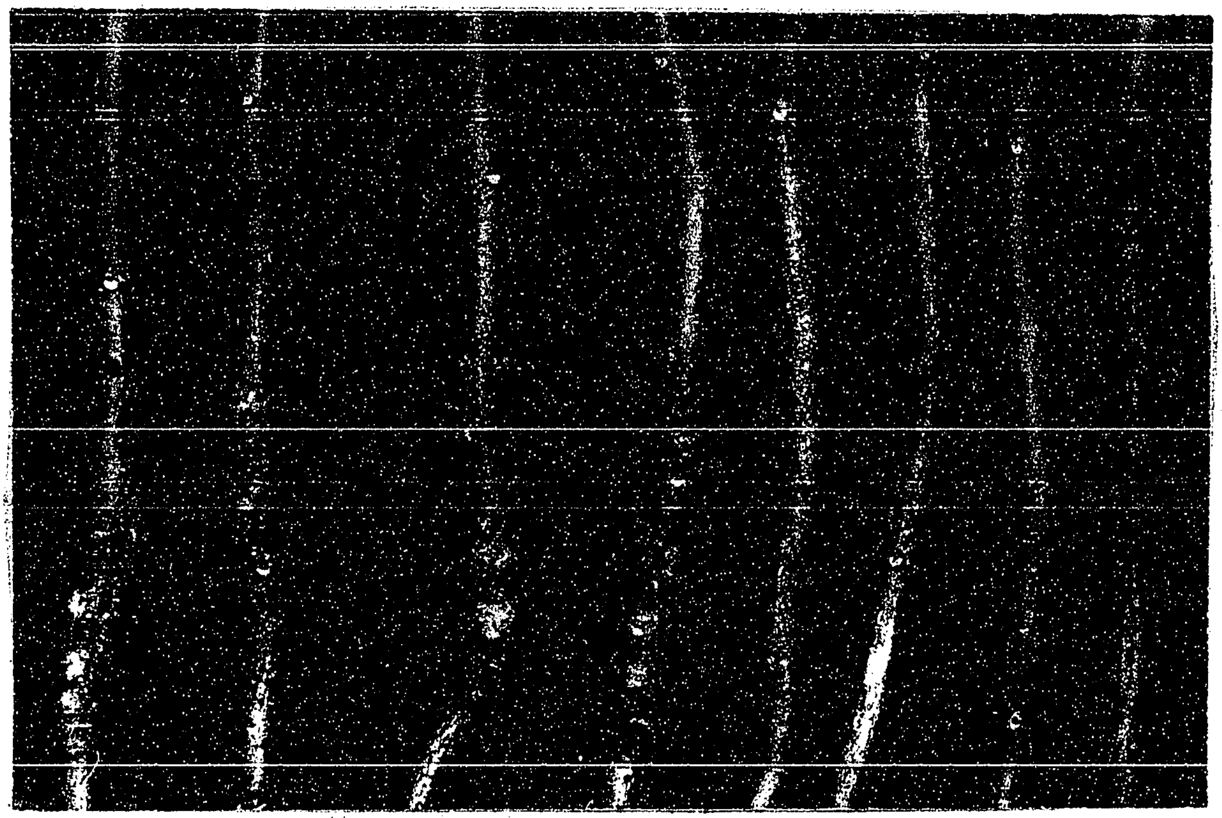


FIGURE 12. Stems of tomato plants inoculated at four points with crown gall. The four on the right were grown in mineral nutrient solution containing 20 μg. of Aureomycin per ml. The four on the left were controls.

TABLE 6 - Number of inoculation sites with galls, and weight and area of galls on treated plants as per cent of those on control plants.

Concentration of Aureomycin in nutrient of treated plants	No. of plants[a]	Inoculations per plant	No. of positive inoculations	Wet weight of galls	Area of galls[b]
μg. per ml.			per cent	per cent	per cent
5	14	2	77	32	42
10	6	4	37	—	8
10	22	4	83	35	43
10	6	6	72	12	14
20	8	4	6	0.2	0.5

a. In each experiment half of the plants were grown in a solution of aureomycin in mineral nutrient, the other half were grown in mineral nutrient alone.

b. Area of cross section from $A=\pi D^2/4$ where D is the measured diameter in mm.

TABLE 7 - Diameter of each gall developed on tomato plants grown in 20μg. per ml. Aureomycin solution and on controls. Total weights of all galls in each group.

Treatment	Plant No.	Diameter of galls at Inoculation position[a] on stem:				Total Wet Weight of Galls
		1st	2nd	3rd	4th	
20μg. per ml. Aureomycin		mm.	mm.	mm.	mm.	gm.
	1	0	0	0	0	0.0036
	2	3	0	0	0	
	3	0	0	0	0	
	4	0	0	0	0	
Controls						
	1	7	8	10	7	1.946
	2	19	22	4	6	
	3	6	3,8[b]	5,7, 4,4,3	10,7	
	4	3,6	8	6	6	

---

[a] Numbered from root upwards. First position 1 or 2 cm. above root.

[b] Entry of two or more different diameters indicates there were that number of more or less separate swellings at the given inoculation position.

Throughout this work it was assumed that the principal action of the aureomycin was on the bacteria rather than on the tumor cells. Tissue culture experiments of de Ropp (1948, 1949) have indicated that this is true when streptomycin is used against crown gall. In view of this, inoculations and treatments were performed at the same time in the present study. This was to allow the aureomycin a chance to act on the bacteria before they had caused the completion of the conversion of host cells to tumor cells. It has been indicated that once this change takes place the tumor may continue to grow without the bacteria (White and Braun, 1941).

Summary

Fewer and smaller galls developed on tomato plants grown in a solution of Aureomycin hydrochloride (Lederle) in mineral nutrient following needle inoculation with <u>Agrobacterium</u> <u>tumefaciens</u> than on similar plants grown in mineral solution alone.

Concentrations of 5 and 10 µg. of aureomycin per ml. of nutrient solution gave a slight reduction in incidence and extent of gall growth. A dosage of 20 µg. per ml. gave practically complete control. Severe wilting, stunting, and death of the plants was caused by doses of 50 µg. per ml. or greater.

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IV. SOME EFFECTS OF ANTIBIOTICS ON PLANTS

In previous sections, the penetration of aureomycin into a plant and its action against a plant pathogen, crown gall, have been considered. In experiments associated with the above studies and in separate studies, it has been found that lima beans grown in a nutrient containing aureomycin, penicillin, or streptomycin showed differences in their appearance and extent of growth as compared with controls in plain mineral nutrient. This section presents data on some of these effects.

Previous work seems to have concerned itself primarily with effects on root growth. Anderson (1947) investigated the effect of streptomycin on soybean, tomato, radish, and wheat seedlings. He found that concentrations beyond 50 units per ml. were toxic to tomato and radish seedlings, while wheat was not injured at 200 units per ml. Soybeans were not killed but showed a marked stunting of lateral roots. Macht has reported studies of the effect of a number of chemicals on the root growth of Lupinus albus. This work seems to have been concerned primarily with using the plants as a test organism for a pharmacological evaluation of the chemicals. Penicillin, streptomycin, and aureomycin have been found to have phytotoxic effects (Macht, 1949; Macht and Farkas, 1949).

Some effects of aureomycin on stems, leaves, and total growth of lima beans as well as on their roots are considered here. A comparison of the stunting action of aureomycin, penicillin, and streptomycin is also made.

Materials and Methods

Growth conditions. Lima beans were grown in Shive and Robbins' mineral solution, Formula I, (Shive and Robbins, 1948), as given on page 34, with added solution "A" of Hoagland and Snyder (1933). They were grown under white fluorescent lights operating on a cycle of 10 hrs. light and 14 hrs. darkness. The jars in which they were grown and the cabinet and lights were the same as described in Section II.

Treatment. Plants of about 15 cm. in height were sorted into two groups. One was transferred to fresh mineral nutrient. The other was transferred to the same solution with an antibiotic added. Two types of experiments were performed. In one the effects of three concentrations of aureomycin were observed. Concentrations of 10, 100, and 1000 μg. per ml. were used. In the other a comparison of the effects of relatively heavy doses (2500 μg. per ml. of aureomycin, penicillin, and streptomycin) was made. For this latter experiment the following quantities of antibiotics were used: (a) 5 g. of Aureomycin hydrochloride (Lederle), (b) approximately 5 g.

(34 bottles) of Buffered Penicillin G. Sodium (Lederle) at 200,000 units per bottle, (c) one 5 g. size bottle of Dihydrostreptomycin Sulfate (Abbott). Each of these was made up to 2000 ml. with mineral nutrient.

Observations and Computations. Measurement of the height of treated and control plants was made at the time of the transfer to antibiotic solutions and again one week later.

The average height of each group and the difference between the initial and final values of this average were computed. The percentage ratio of the average growth of the treated plants to that of the controls was calculated. In a few cases, where this value was near 100 per cent, the "t" and the "Xi^2" tests (Fisher, 1950) were applied to determine whether there was statistical significance in the variation from 100 per cent.

Any difference between the appearance of the roots, stems, or leaves of treated and control plants was noted.

Results and Discussion

Height effect. Aureomycin at a dosage of 1000 µg. per ml. caused severe stunting (table 8). The growth was reduced to 10 to 30 per cent of control growth. At 100 µg. per ml. the stunting was less severe. In this case the growth was reduced to 60 to 80 per cent of that of the controls. The average of the experiments at 10 µg.

TABLE 8. One week growth of lima bean plants with Aureomycin in their nutrient solution. Given as per cent of control growth.

	10 µg. Aureomycin per ml.		100 µg. Aureomycin per ml.		1000 µg. Aureomycin per ml.	
	Per cent of Control Growth	Number of Treated Plants[a]	Per cent of Control Growth	Number of Treated Plants	Per cent of Control Growth	Number of Treated Plants
	111.2	(10)	77.5	(10)	9.1	(5)
	111.1	(25)	59.6	(25)	8.3	(6)
	119.2	(20)	62.3	(10)	27.8	(10)
	117.2	(10)	69.0	(10)	29.8	(10)
	92.9	(10)			34.5	(10)
	112.8	(20)				
	105.4	(50)				
	107.8	(40)				
	103.8	(40)				
	104.8	(40)				
	104.8	(40)				
	85.4	(40)				
avg.	106.3		67.1		21.9	

a. At least as many control plants as treated plants were used in each experiment.

per ml. (106 per cent) suggests a slight stimulation. However, the statistical tests failed to show significance for this.

In the comparison experiment 80 plants were used. Of these 10 were grown in each of the three antibiotics and 50 served as controls. The percentages of control growth were (a) aureomycin 15 per cent, (b) penicillin 58 per cent, (c) streptomycin 87 per cent. Of these, the growth reduction for (c) does not show up as statistically significant. Perhaps it could be established with a larger group of plants.

<u>General appearance.</u> The 10 μg. per ml. aureomycin solution caused a slight browning of the roots and some inhibition of lateral root development. Both of these effects were more pronounced at 100 μg. per ml. At 1000 μg. per ml. the roots became dark brown and stopped growing.

There was no obvious effect on the lower part of the stems at any dosage. However, the upper tip of the stems and the young leaves developed a chlorosis after the plants had been in the aureomycin solution somewhat longer than the week used in the height experiments. This effect was especially marked at 100 and 1000 μg. per ml.

Summary

Lima beans were grown under fluorescent lights in a mineral nutrient (Shive & Robbins') with added antibiotics. In experiments with several dosages of Aureomycin hydrochloride (Lederle), the following effects were observed:

1. At 10 μg. per ml. there was either no effect on height of plants or possibly a slight stimulation.
2. At 100 μg. per ml. the plants were stunted and a chlorosis developed at the stem tips and in the younger leaves.
3. At 1000 μg. per ml. the plants were severely stunted and very chlorotic.

A comparison of the effects of three antibiotics at a dosage of 2500 μg. per ml. showed the following effects:

1. Aureomycin stunted the plants severely.
2. Penicillin G Sodium (Lederle) stunted the plants moderately.
3. Dihydrostreptomycin Sulfate (Abbott) seemed to cause some stunting.

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V. EFFECT OF SOFT X-RAYS ON AUREOMYCIN

The fact that many chemicals can be altered by the action of x-rays is well known (see, for example, Lea, 1947). Dale (1940, 1943, 1943) has reported on their action on enzymes and biologically active compounds. The following study was made to determine whether x-rays would have an effect on aureomycin. This is of particular interest in light of the prominent consideration being given to the use of such antibiotics in connection with defense against radiation injuries from atomic energy and atomic bombs. If serious damage can be caused by radiations to aureomycin, it would be necessary to protect the drug in storage with adequate shielding.

Materials and Methods

Laboratory-dry crystalline aureomycin, 100 μg. per ml. solutions of aureomycin in distilled water, and frozen aureomycin solutions of 100 μg. per ml. were exposed to x-radiation from the tube described in Section IB. The operating conditions were again 35 kv, 10 ma. The samples were contained in Lucite (DuPont) cups 17 mm. in diameter and 6 mm. deep. During treatment these were held within 2 or 3 mm. of the window of the x-ray tube. The x-ray intensity at this distance was about 250,000 r units per minute.

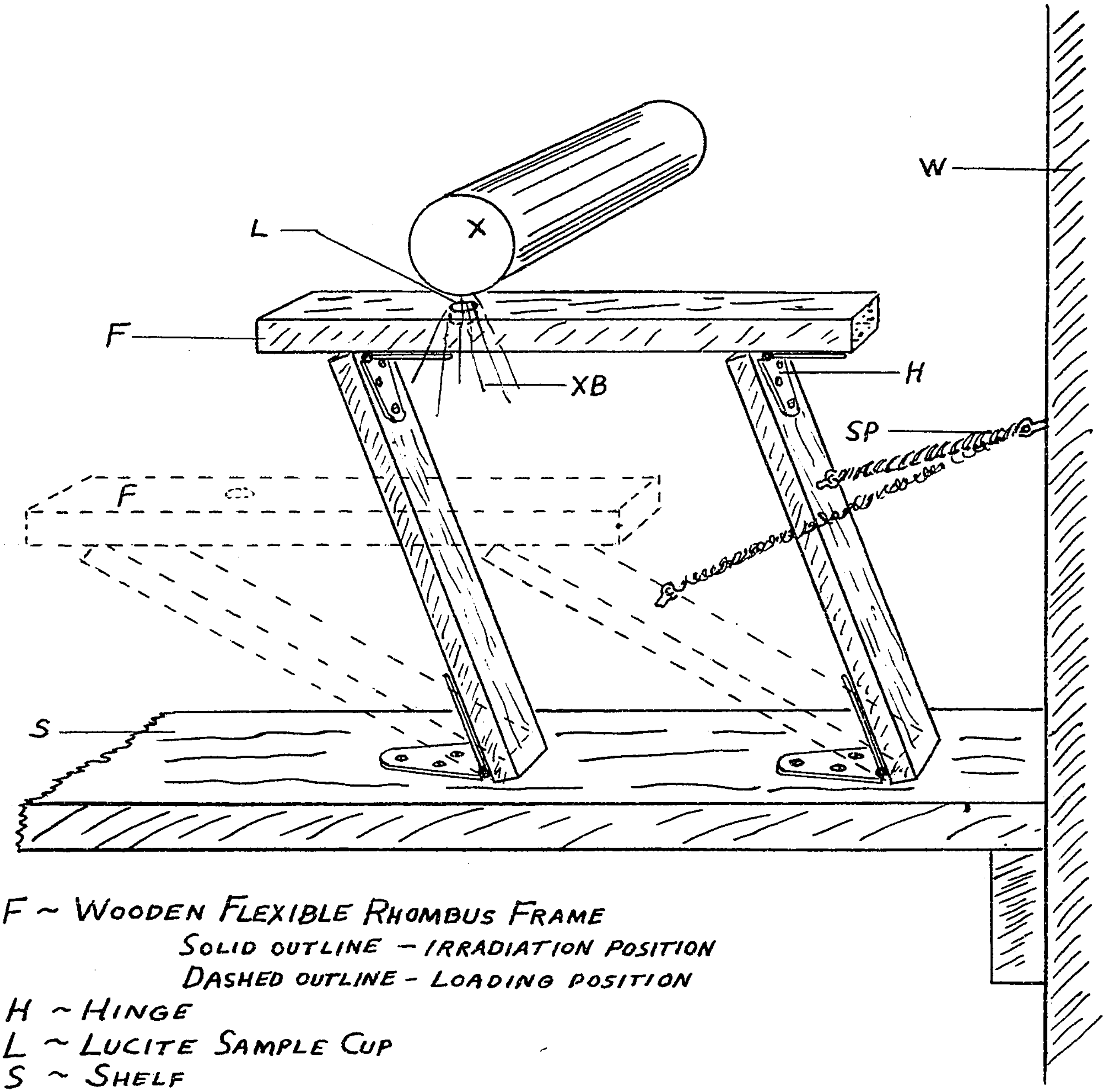
For dry samples and solutions these cups were placed in a flexible rhombus frame of light wood (fig. 13). This made possible a rapid changing of samples with fairly accurate positioning of them under the x-ray tube.

A different arrangement was used for the frozen samples. For this a lead-filled brass tube 28 mm. in diameter, which had a shallow well drilled into one end to receive the Lucite cup, was inserted into a Dewar flask. A mixture of crushed dry ice and acetone was packed around the rod in the flask. A cup with a sample solution was placed in the well and when the solution had frozen, the whole unit was set in position 2 or 3 mm. beneath the window of the x-ray tube (fig. 14). The sample was thus kept solidly frozen during the entire irradiation.

Bioassays were run on the treated samples and on controls by the serial dilution method of Dornbush and Pelcak (1948) as described on page 36.

Results

1. No noticeable change was measured in dry, crystalline aureomycin irradiated up to 20 minutes (approximately 5×10^6 roentgens).
2. After 3 minutes irradiation (approximately 8×10^5 roentgens), the aureomycin solution lost about half its strength (as measured by its action against



F ~ WOODEN FLEXIBLE RHOMBUS FRAME
SOLID OUTLINE – IRRADIATION POSITION
DASHED OUTLINE – LOADING POSITION
H ~ HINGE
L ~ LUCITE SAMPLE CUP
S ~ SHELF
SP ~ SPRING
W ~ WALL
X ~ X-RAY TUBE (SUPPORTS AND DETAIL OMITTED FOR SIMPLICITY)
XB ~ X-RAY BEAM

FIGURE 13. EQUIPMENT USED FOR IRRADIATING DRY SAMPLES AND SOLUTIONS.

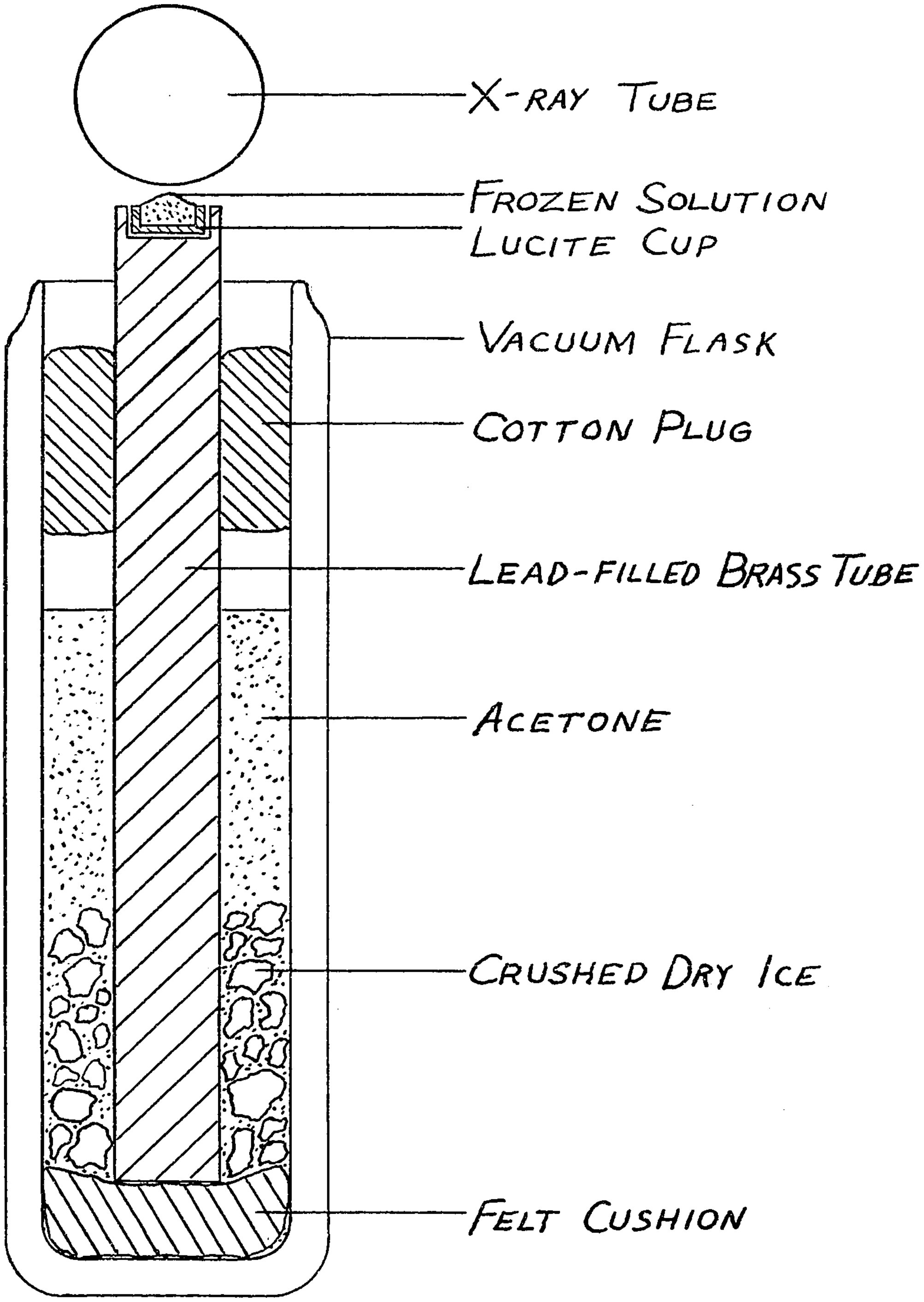


FIGURE 14. EQUIPMENT USED FOR IRRADIATING FROZEN SOLUTIONS.

Bacillus cereus). The complete residual activity curve for irradiation from zero to fifteen minutes is given in figure 15.

3. There was no measurable change in the frozen solutions with up to 20 minutes irradiation (fig. 16).

Summary

Aqueous solutions, frozen aqueous solutions, and dry crystalline Aureomycin hydrochloride (Lederle) were subjected to doses of soft-x-rays at about 250,000 r units per minute. A bioassay was then used to determine whether there had been any change in the inhibiting action of the treated material on *Bacillus cereus* #5. There was no measurable change in the dry or frozen material with dosages as high as 5×10^6 roentgens (20 minutes). A solution ($100 \mu g.$ of aureomycin per ml. initially) assayed only 50 $\mu g.$ per ml. following a dosage of 8×10^5 roentgens.

The gamma ray dosage to be expected 2100 ft. from the explosion of a nominal (20 ton TNT equivalent) atomic bomb is 10^4 roentgens (Glasstone, 1950, fig. 7.42). Thus it would seem that shielding of dry crystalline aureomycin would probably be unnecessary as far as x-rays are concerned. The much higher intensity over a short period, as would be the case for the bomb explosion, might alter this result.

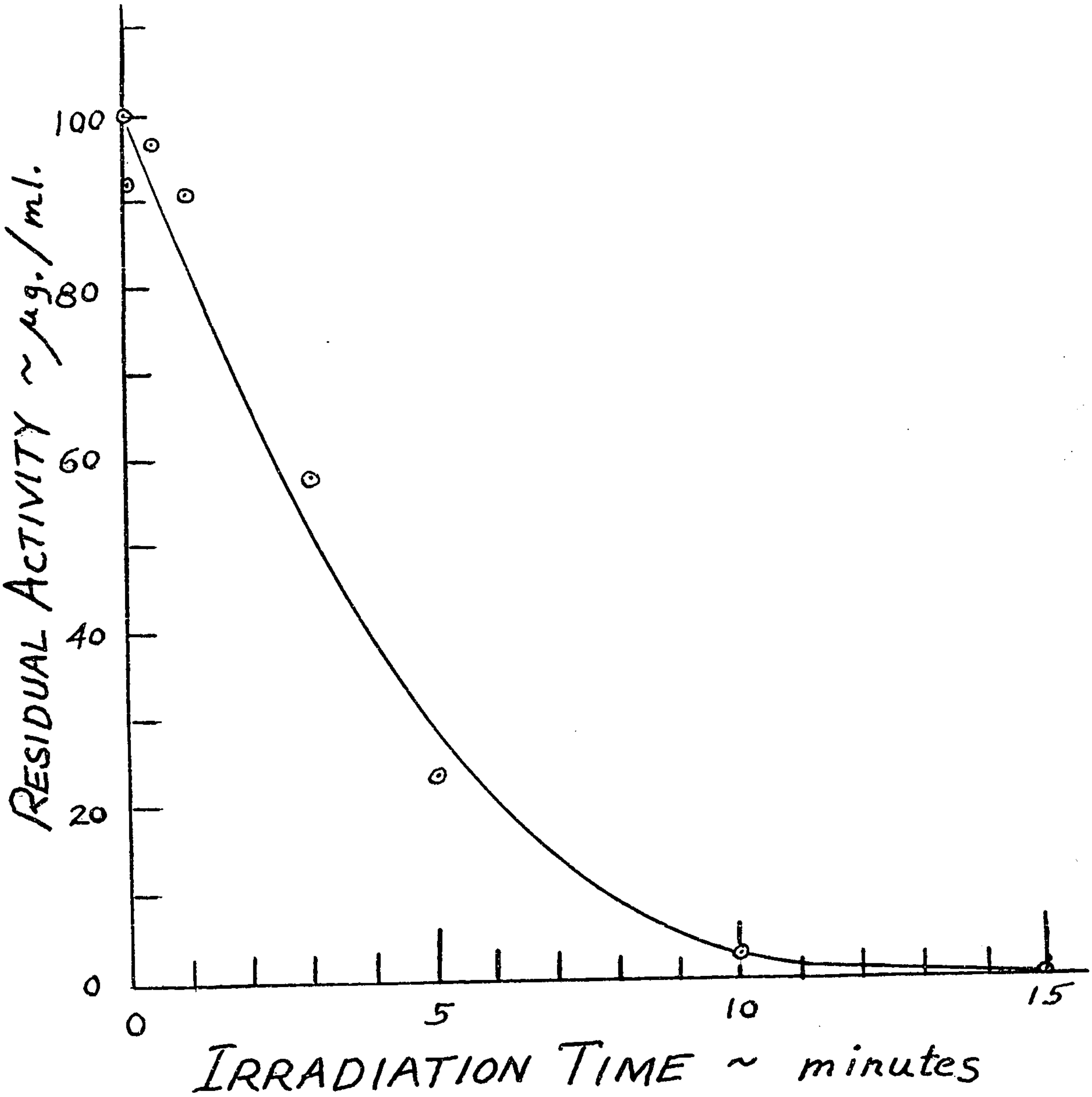


FIGURE 15. BIOLOGICAL ACTIVITY OF AUREOMYCIN SOLUTION IRRADIATED WITH SOFT X-RAYS. DOSAGE: 250,000 r/MIN.

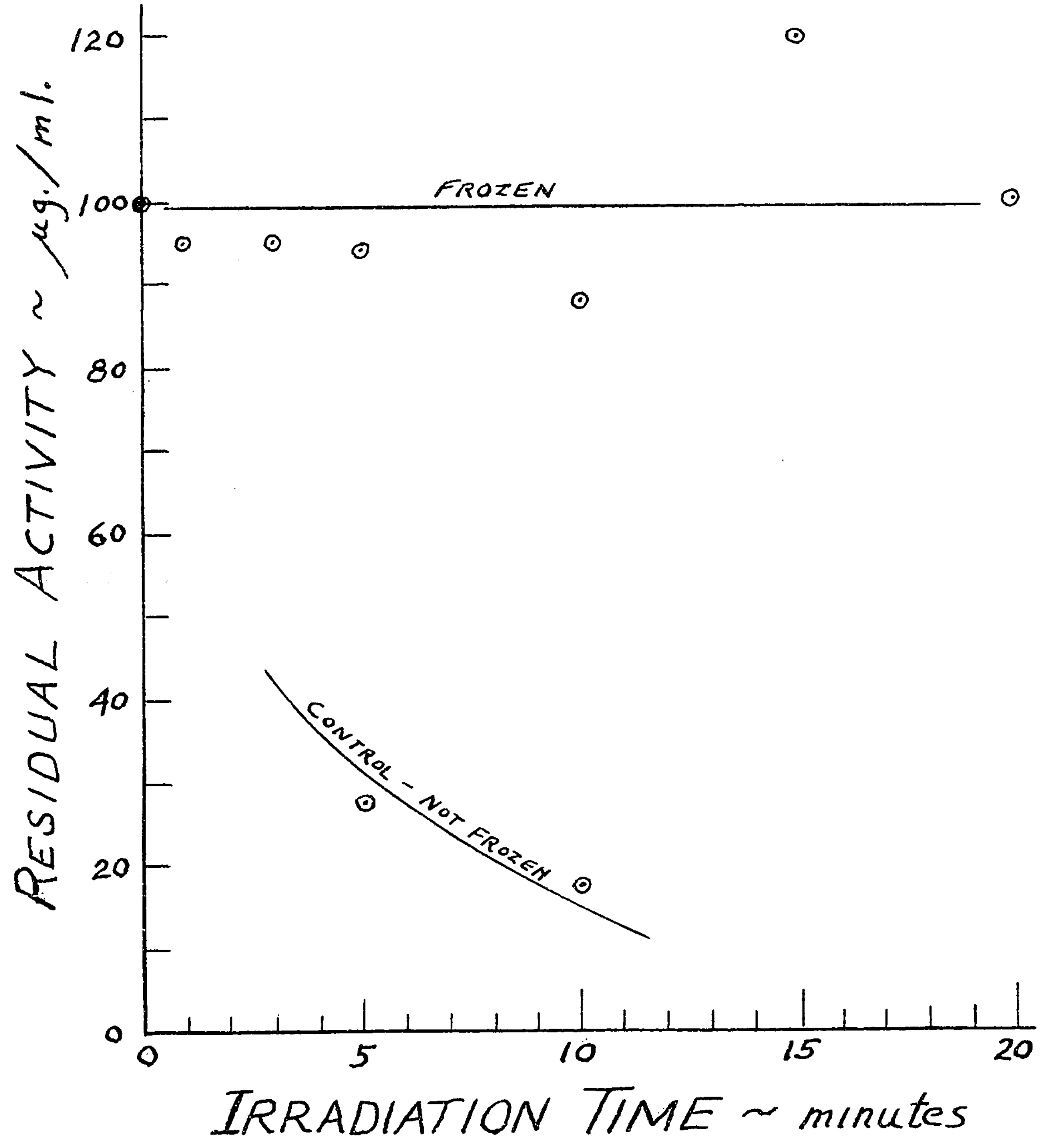


FIGURE 16. BIOLOGICAL ACTIVITY OF FROZEN AUREOMYCIN SOLUTION IRRADIATED WITH SOFT X-RAYS. DOSAGE: 250,000 r/MIN.

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