TOXICITY STUDIES OF AQUATIC ACTINOMYCETES

APPROVED:

D. K. S. Silvey

Major Professor

R. K. Maguire Jr.

Minor Professor

B. B. Koonce

Director of the Department of Biology

Jack Johnson

Dean of the Graduate School

TOXICITY STUDIES OF AQUATIC ACTINOMYCETES

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Helena Juengermann Fair, B. S.

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INTRODUCTION AND HISTORY

The toxic effects of certain members of the Order Actinomycetales have long been recognized both from the pathogenic effects of the plants themselves on the experimental animal and from toxic reactions produced histologically and physiologically by extracts from the plants.

The first reported isolation of an Actinomycete was made by R. Foerster from the concretions of the lacrymal duct and was designated as Streptothrix Foerster by Ferdinand Cohn (1875). Harz (1877) was the first to use the term Actinomyces, applying it to an infective organism from cattle which was discovered by Bollinger (1877).

Nocardia as a type genus of the Actinomycetes was first described in 1889. However, it was not until 1905 that Wright distinguished between nocardiosis and actinomycosis. This was the initial recognition of a pathogenic difference in genera of the Actinomycetales. Since that time extensive work has been done in describing the pathology of these infections together with effective treatments by means of penicillin, sulfonamides thymol, and the Streptomyces series of antibiotics (Colebrook, 1921; Cope, 1938;

Lyons, Owens and Ayers, 1943; Dobson and Cutling, 1941 and 1945).

Interest in the toxic effects of extracts and by-products arose parallel to the development of antibiotics from the Actinomycetales. Gratia and Dath (1924) were the first to recognize the antibacterial activity of a metabolic product of a certain strain of Actinomycetes (Frieden, 1945). This product had been designated as actinomycetin, but work on its toxicity was not continued. This antibacterial substance was probably similar if not identical to other antibiotics developed later.

In 1939, Waksman and his co-workers began an extensive program on the isolation of actino-types to serve as potential antibiotic producers. By 1942, a total of 244 types had been isolated from a wide variety of natural habitats. Of the total examined, only nine were obtained from lake mud; the others were isolated from various soil types, compost and manure. Only one of the nine aquatic organisms, namely Micromonospora sp., Strain 1, revealed any antagonistic properties (Waksman, Horning, Wilson, and Woodruff, 1942). Actinomycin (Waksman and Woodruff, 1940 and 1941) was the first antibiotic substance isolated for study from the survey begun in 1939. This compound from Streptomyces antibioticus (Waksman and Woodruff) proved to be one of the most toxic substances isolated from any

Streptomyces. Regardless of whether the injection was made into the blood, muscle or peritoneal cavity, 10 micrograms of the substance per 20 grams of body weight were sufficient to kill a mouse, while 0.5 to 1.0 micrograms killed fowls. A lysozyme from Actinomycis reported in this same year by the Russians (Kriss, 1940) showed little antibacterial promise.

Welsch (1941) at the University of Liege isolated Actinomycetin from Streptomyces albus (Rossi Doria emend. Krainsky). No information is available concerning its toxic qualities.

Micromonospora sp., Strain 1, as stated above, was studied as a potential antibiotic producing strain of Actinomycetes. Micromonosporin was recovered from cultures of this organism but its antibacterial spectrum was limited so study was discontinued.

Professor Gardner of Oxford in 1942 described a substance, isolated from Nocardia (Proactinomyces) gardneri (Waksman); to which he consigned the name proactinomycin. Sequential studies have revealed also A, B and C types of proactinomycin. All varieties have exhibited chronic toxicity with repeated sublethal doses accompanied by possible liver damage. In a concentration of 1 part proactinomycin to 1,000-3,000 human leucocytes, the blood cells were killed in 30 minutes to one hour while an intravenous injection of 2.0 milligrams per kilogram body weight was lethal to mice (Baron, 1950).

The first antibiotic of any promise, encountered by Waksman, Woodruff and Horning (1941), was produced by Streptomyces (Actinomyces) lavendulae (Waksman and Curtis) and was termed streptothricin. Subsequent research (Metzger, 1942; Robinson, 1944; Heilman, 1945; Smith, 1945) indicated a residual toxic effect inherent in the compound which was not lost by purification. Using streptothricin of 5 to 300 E. coli units per milligram, the intravenous minimal lethal dose for mice has been established at 500,000 to 750,000 units per kilogram body weight. This may be accompanied by intestinal gangrene, myocarditis, and lesions of the kidney, liver or nervous system (Baron, 1950).

In September of 1943, a compound was isolated from Streptomyces griseus (Krainsky) characterized by low toxicity to animals. It was rapidly excreted from body tissues and owed its toxic reactions primarily to impurities incurred in the production process. Toxic effects were initial, not residual, as in the case of streptothricin. This substance was named Streptomycin (Schatz, Bugie and Waksman, 1944). Further observations have revealed new sources and types of streptomycin. The crude concentrates of streptomycin will also yield streptomycin B in addition to streptomycin. Commercially, dihydrostreptomycin, mannosidostreptomycin or streptomycin B and dihydromannosidostreptomycin have been developed by further laboratory

purification. The later commercial compounds have proved less toxic than the original streptomycin compounds.

Since 1943 some thirty to forty new compounds have been reported from different species of the Actinomycetales, exhibiting varied antibiotic activities and eliciting diverse toxic responses from the animal body. Among these new actinomycete antibiotics may be included actidione, actinorubin, aureomycin, borrelidine, chloromycetin, grisein, lavendulin, litmocidin, mycomycin, neomycin, nocardin, streptolin, sulfactin, terramycin and xanthomycin (Ehrlich, 1947; Smadel, 1947; Gottlieb, 1948; Irving and Herrick, 1949; Baron, 1950). Characteristic toxicity of each mentioned above has proven so conclusive that it has been suggested as a basis of classification by Waksman (1950).

Two of the newer antibiotics, aureomycin and chloromycetin, have exhibited extremely low toxicity accompanied by moderate gastrointestinal irritation. The main toxic response depends both on the idiosyncrasy of patients under treatment and the purity of the drugs (Harvey, 1949; Maxwell, 1949; Woodward, 1949).

The major interest in actinomycete research has centered primarily on soil forms with little attention devoted to the toxicity of Actinomycetes existing either in spore forms or as mycelial masses in water. The production of tastes and odors in lakes, rivers and

reservoirs has only recently focused attention on this group of aquatic organisms (Egorva, 1942; Issatchenko, 1944; Silvey, 1950). As far back as 1936, however, Thaysen had reported the isolation of an odoriferous substance from a strain of actinomyces growing on reeds in the Thames River in England. Experimentally, in the laboratory, a characteristic earthy odor was imparted to the flesh of fish identical to the taint observed in animals recovered from the stream. No toxic effect on the fish was noted; no toxic reactions from human consumption of tainted flesh were noted.

STATEMENT OF PROBLEM

Since Actinomycetes have been isolated from finished public drinking water, it is believed that the organisms are unaffected by the chlorination and flocculation of water treatment plants and pass as spores through the filters into the general distribution system. For this reason it was deemed imperative to study the toxic effects of these organisms.

ISOLATION AND CULTURE OF ACTINOMYCETES

The Actinomycetes used in this study were first plated from raw, untreated water samples obtained at Lakes Hefner and Overholser, Oklahoma City, Oklahoma, in February, 1951. The samples were shaken, and, under sterile conditions, 0.1 ml. of the water was pipetted into a sterile petri dish containing approximately 15 ml. of sterile, single-strength nutrient agar. After the plates were revolved to encourage distribution of the water film, they were inverted and incubated at room temperature for fourteen to twenty-one days. Two or, if necessary, three successive transfers were made until bacterial and fungal contaminations were no longer evident.

On September 8, 1951, fifteen uncontaminated actinomycete types were arranged in a series and consigned a number. The location, from which the original sample had been obtained was recorded together with other available data, such as, the date of initial isolation, colony color and water depth in meters at which the sample was taken. The colony types were then transferred to prepared nutrient agar slants for use as experimental stock cultures.

The following types were selected from the stock culture collection to be used in the toxicity study:

8-isolated July 25, 1951, from a Lake Hefner water sample collected at 16 meters; gray colony type.

9-isolated July 25, 1951, from a Lake Hefner water sample collected at 14 meters; gray and white colony type.

10-isolated July 25, 1951, from a Lake Hefner water sample collected at 14 meters; gray type.

12-isolated August 12, 1951, from a Lake Overholser algae sample; white colony type.

13-isolated August 12, 1951, from a Lake Overholser algae sample; gray colony type.

14-isolated August 12, 1951, from a Lake Overholser algae sample; white colony type.

In addition to these six types, a seventh Actinomycete, designated as Oklahoma City II, was chosen from a series of three types isolated by Mr. John McReynolds, Filtration Bacteriologist, from the filter of the Lake Hefner Purification Plant and sent to North Texas in January, 1951.

If large growths of mycelia were required, a transfer was made with an innoculating needle from a stock culture tube into a flask containing 50 ml. of sterile nutrient broth. The flasks were incubated at room temperature for one to three months. Then, 5 ml. of the broth culture was pipetted into one-gallon Duraglass Coca-Cola jugs, containing two liters of sterile modified Czapek's media. The media was composed of the following ingredients:

600 grams brown sugar
40 grams $NaNO_3$
20 grams KH_2PO_4
20 grams KCL or NACl
10 grams $MgSO_4$

0.2 grams $FeSO_4$
0.2 grams $ZnSO_4$
10 or 20 grams Tryptose, Peptone or Tryptose Broth
1.0 grams Tyrosine
20,000 ml. distilled water
20 grams Bacto Agar or Nutrient Agar dissolved in 1,000 ml. distilled water.

All of the above ingredients except the agar were dissolved in the distilled water, and the pH checked for a range between 6 and 8, the recommended pH being 7. Adjustments were made with sodium hydroxide or phosphoric acid. Two liters of the solution were added to each of ten containers together with 100 ml. of the melted agar and autoclaved at 17 lbs. pressure for 20 minutes. After cooling, these jugs were seeded as stated previously and incubated at approximately 30° to 40° C. Also the jugs were shaken daily to encourage oxygen distribution and to prevent formation of the pellicle only on the surface. Growth was allowed to continue for 30 to 60 days, depending on mycelial development.

At the end of the growth period, only one of the ten jugs, showing the best mycelial development, was selected, and its contents were poured through a clean, unsterile flour-sack or surgical cap which retained the mycelial particles and agar. After repeated washings with tapwater to eliminate the agar, the cap was squeezed and allowed to dry at room temperature for 20 to 30 minutes. The cloth was turned, and the mycelia scraped onto paper toweling and dried for three to four more hours. The dried mycelia

was cut into approximately 2 mm. flakes, and refrigerated in a screw cap jar for later use.

TOXICITY PROCEDURES

Actinomycetes were recovered on nutrient agar plates from finished drinking water from the mains of Waco, Texas, from a drinking fountain at Galveston, Texas, and from finished tapwater at the Lake Hefner Water Purification Plant. Thus the organisms must have been unaffected by the purification process and passed, probably in the spore form, into the distribution system. Therefore, the toxicity study was conducted with these organisms in the spore form or as a mycelial growth rather than concentrating strictly on the extracts of these plants as has been the approach in clinical research.

Fourteen mice were given a single direct exposure to Actinomycetes as a first study. A type isolated from a Lake Hefner algae sample was fed to a total of seven mice in two cages. (This type was not included in the numbered stock culture series and has been lost.) Mycelia and spores were scraped from a nutrient agar plate and added to the regular mixture of oats, Pablum and powdered milk. Carrot slices were dragged, individually, across the plate until they were coated with a powdery layer of gray spores. No other food was given the experimental animals. Simultaneously, by the

same procedure, seven mice were fed Actinomycetes from Lake Overholser (a composite of number 12, 13 and 14 in the series), and fourteen control mice were fed regular untreated rations. The weight of each animal was recorded daily before feeding. Animals were observed for any unusual reactions.

Following the single exposure to Actinomycetes, eight mice were given a continued exposure of fourteen feedings. The first seven feedings were Oklahoma City II Actinomycete and the last seven were from a composite of numbers 12, 13 and 14. Actinomycete particles were mixed in the regular ration. (Preparation of the Actinomycete flakes is found in the section on Isolation and Culture.) Four mice in Cage III received 0.5 grams of the particles with each feeding, while six mice were selected as control animals.

The records of the daily weighings of the first two studies have been arranged graphically in Figures 1 through 8.

A third study was devised to determine whether a sterile water suspension of actinomycetes would be toxic, if injected into the tissue of a mouse, to determine the most effective method of injection and to determine the effects of various quantities injected.

Three vaccine vials were stoppered with rubber nipples and evacuated by means of a 20 ml. syringe. The vials were autoclaved at 17 lbs. pressure for 45 minutes, and then

allowed to cool before they were filled. Three nutrient agar slants of Actinomycetes 8, 9 and 10 were selected. Between 12 and 14 ml. of sterile, deeminized water were added to each slant with sterile syringes. The tubes were shaken and the cotton stoppers discarded. The water suspension was drawn into sterile syringes and added to each of the vials. The filled vials were stored at room temperature until injections were initiated 24 days later.

Three cages, each containing four mice, were designated I, II and III to correspond in sequence with the vials of Actinomycete suspensions numbered 8, 9 and 10. Three of the mice in Cage I were injected subcutaneously in the region of the back; the three in Cage II were injected intramuscularly in the region of the thigh on the hind legs, alternating dorsal and ventral surfaces; and the three in Cage III were injected peritoneally in the lower half of the abdomen.

The experimental animals within a cage were differentiated by means of three organic dyes, green, purple and red. The fourth unmarked mouse in each cage remained as a control animal. Each day the green mouse received 0.2 ml. of the vial suspension; the purple mouse received 0.6 ml. and the red mouse received 1.0 ml. Daily, for a period of five days, the mice were weighed, injected and fed.

The injections were given with 22 and 24 gauge needles attached to 0.5 ml. and 1.0 ml. sterile syringes, one being

used for each mouse. Vials, syringes and the work table were exposed to ultraviolet irradiation for 20 minutes before each experimental series was injected. Each mouse was treated in the region of intended inoculation by sponging with 70 per cent ethyl alcohol, followed by a 5 per cent solution of iodine.

After the final injections had been given, 0.2 ml. of the water suspension was removed by a sterile syringe from each vial, and added to two test tubes, each containing 5 ml. of nutrient broth. After incubation at room temperature for twenty-four hours, all six tubes showed growth of the actinomycete with no contamination.

Weights of the animals are recorded in Table 9, together with the date of killing or death.

All of the red mice in the third study were autopsied in order to observe possible tissue damage and to attempt recovery of the actinomycete colonies from the animals.

The technique employed in these autopsies was as follows: Each mouse was chloroformed and pinned, ventral side up, with sterile insect pins fixed to a sterile board. They were examined externally in the region of injection for any visible lesions or swellings. After a sponging with 70 per cent ethanol, an incision was made through the dermal layer, extending from the base of the head to the tail. After examination of the internal surface of the skin and external surface of the muscles, another incision was made

through the muscular layer. The examination was conducted either from the dorsal or ventral surface, depending on the site of injection.

During each examination parts were removed and placed in sterile petri dishes. The entire tissue collection from each mouse was transferred to a sterile mortar. Sterile banding sand was added, and the organs and tissue were pulverized. Sterile deeminized water was added and mixed. One-half milliliter of this suspension was removed with a pipette and added to a prepared nutrient agar plate. Three plates per mouse were prepared. One-half milliliter of the tissue-water suspension was also added to 5 ml. of nutrient broth. Plates and broth tubes were incubated at room temperature for 72 hours. Tabulation of the observations of plates and tubes have been recorded in Table 3. Any actinomycete colonies recovered by plating were transferred to nutrient agar slants and added, unnumbered, to the general stock culture collection.

The green mouse in Cage II was found dead four days after the final injection. This animal had received 0.2 ml. daily by intramuscular injection from Vial 9. Autopsy was performed immediately. Tissues removed at this time were kept separate and from these, three nutrient agar plates were innoculated as follows:

Plate I-Bloody streak on the underside of the dermis extending about one-half inch down the dorsal side of each hind leg.

Plate II-Haematomas from the muscles of the calf of each hind leg and the inguinal lymphatics from one side.

Plate III-The vagina, inguinal lymphatics from the other side and a bloody band covering the first six ribs and extending under both scapulae.

The plates were incubated at room temperature for three days. Observation of the plates is recorded in Table 3.

The purple mice in Cage II and Cage III also died. No autopsies were performed on these animals, since death had occurred several hours previous to the discovery of the bodies.

The previous study indicated intramuscular injection of Actinomycete 9 as the most promising from the viewpoint of toxicity. An attempt was made to confirm these findings. Since the plants had been recovered by plating from mouse tissue injected with from 0.2 ml. to 1.0 ml. of suspension per day, 0.5 ml. was selected as an average dosage for all injections.

Two sterile vaccine vials were filled with deeminized water suspensions of actinomycetes by the technique outlined in the preceding study. One of the vials (Vial A) was filled with a suspension of Actinomycete 9. The other vial (Vial B) was filled from a transfer of Actinomycete 9,

re-isolated from pulverized mouse tissue (Plate III) from red mouse in Cage II), plated in the third study.

A cage containing four mice was selected for use with each vial. Three mice in each cage were used experimentally while the fourth remained as a control. Weights were recorded each day, beginning just before the first injection. A total of eight injections were given intramuscularly. Weighings continued through the twenty-second of the month and are recorded in Table 4

As tissue was removed for plating, similar sections were removed for histological study. Each tissue was dipped in Bouin's Fluid Fixative, wrapped in gauze, tagged with a number and replaced immediately in Bouin's Solution. Those tissues removed for fixing were:

1 - left inguinal lymph node

2 - lymphatic tissue from left leg

3 - section of muscle from leison on left hind leg.

After twenty-four hours the tissue was removed from the fixative and dehydrated with formalin and alcohol. Each tissue mass was imbedded in paraffin, sectioned and fixed on slides in serial sections. Staining was done with eosin.

Actinomycete 9 has also been studied in tissue culture. A water suspension was made from an agar slant. The embryonic extract (EE_1) was added to the water suspension. One drop of the EE_1 was placed on a cover slip together

with a section of eight-day chick heart. A clot was formed by the addition and mixing of one drop of fowl plasma. The cover slip was inverted over a hanging drop slide and sealed with melted paraffin. Slides were either photomicrographed with phase contrast movies at the rate of 100 feet in 30 hours or stained with Giemsa's stain or May-Greenwald stain for microscopic observation.

RESULTS

A comparison of Figures 1 through 8, consisting of daily weighings of mice used in the first two studies, shows that all animals had a same general pattern of weight increase. The mice in control Cages V and VI show average weight gains of 3.6 grams and 4.3 grams. The fourteen mice receiving a single feeding of Actinomycetes showed average weight increases as follows: four mice in Cage I, 3.75 grams; three mice in Cage II, 3.5 grams; four mice in Cage VII, 5.0 grams; and three mice in Cage VIII, 1.5 grams. The eight animals fed mycelia continuously for fourteen feedings showed average gains of 6.5 grams for the four mice in Cage III and 5.75 grams for the four mice in Cage IV.

Weight gains indicate that direct ingestion of these particular Actinomycete types, rather than being toxic, had been beneficial to the experimental animals. Largest weight gains appear among the mice exposed for the greatest length of time. No deaths occurred. No unusual reactions were observed.

A single feeding of mycelia in no way interfered with reproduction. A mouse in Cage II and a mouse in Cage VIII each delivered normal offspring. Continued exposure may have had some bearing on reproductive processes although

the data collected were not extensive enough to be conclusive. Over a month after the last of the fourteen feedings, the animals in Cages III and IV had not produced young, although animals from control Cages V and VI had done so.

After the first five continual feedings, the animals refused the mycelial particles of Oklahoma City II Actinomycete, selecting only the oats and Pablum in the feeding mixture. When the actinomycete type was changed, the animals resumed consumption of the dried mycelium. The second type, a composite of numbers 12, 13 and 14, was not refused even after seven feedings.

No attempt was made to recover actinomycete colonies from the digestive tract. However, the color of the feces was observed to change from the usual dark brown to a golden color on extended feeding.

The day after conclusion of the fourteen feedings, mice in Cages III and IV showed a decided weight drop. This was not construed as significant since all of the animals showed this same drop.

In the second study, a total of 7.0 grams of mycelia was fed to four mice in Cage III over a period of fourteen feedings. The average amount per mouse totaled 1.75 grams or 0.125 grams daily. Each animal in Cage IV received 0.25 grams daily or a total of 3.5 grams. The total cage ration was 14.0 grams for fourteen feedings.

Figures 1-8 follow immediately on the next pages.

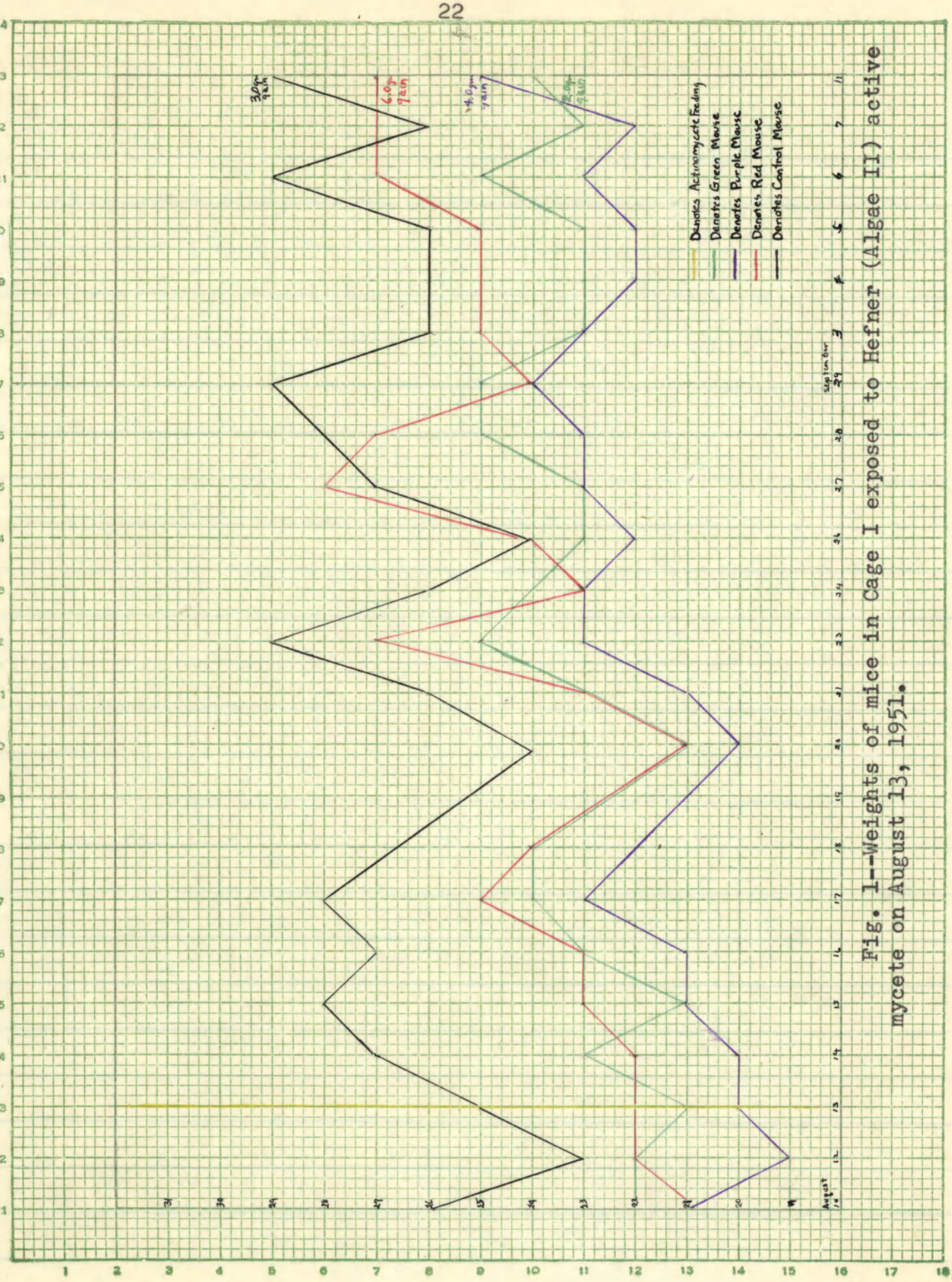

Fig. 1--Weights of mice in Cage I exposed to Hefner (Algae II) active mycete on August 13, 1951.

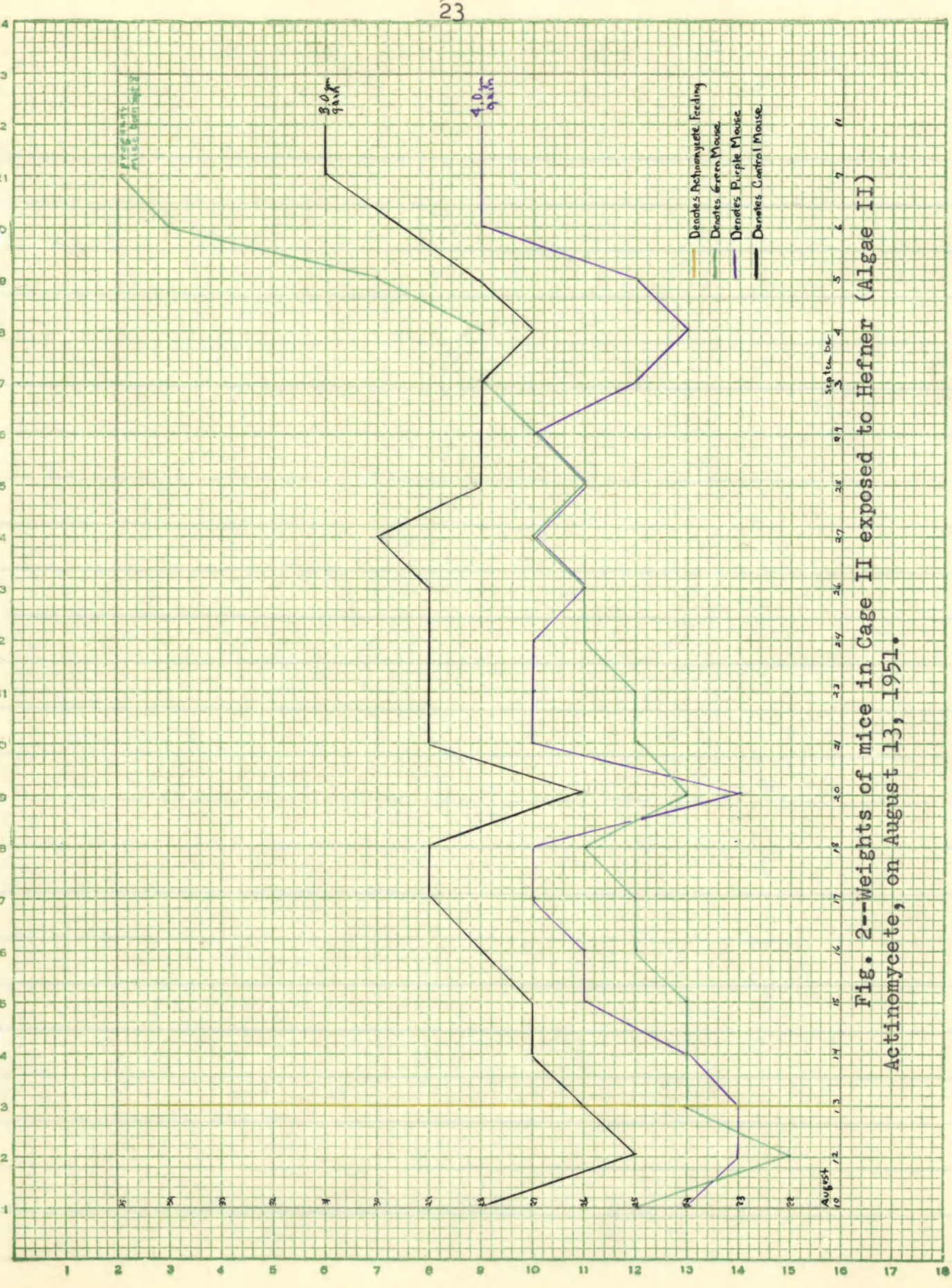

Fig. 2--Weights of mice in Cage II exposed to Hefner (Algae II) Actinomycete, on August 13, 1951.

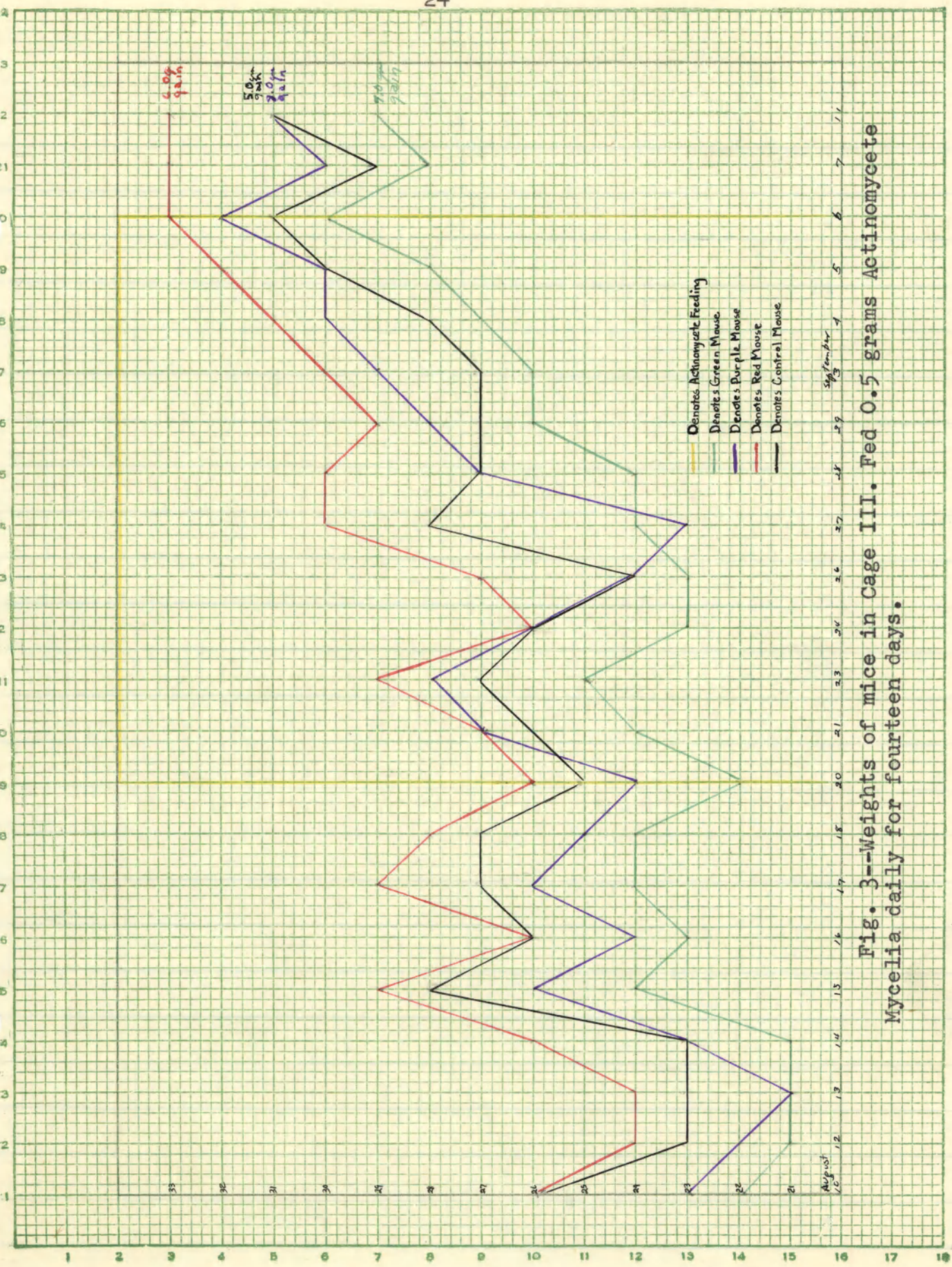

Fig. 3--Weights of mice in Cage III. Fed 0.5 grams Actinomycete Mycelia daily for fourteen days.

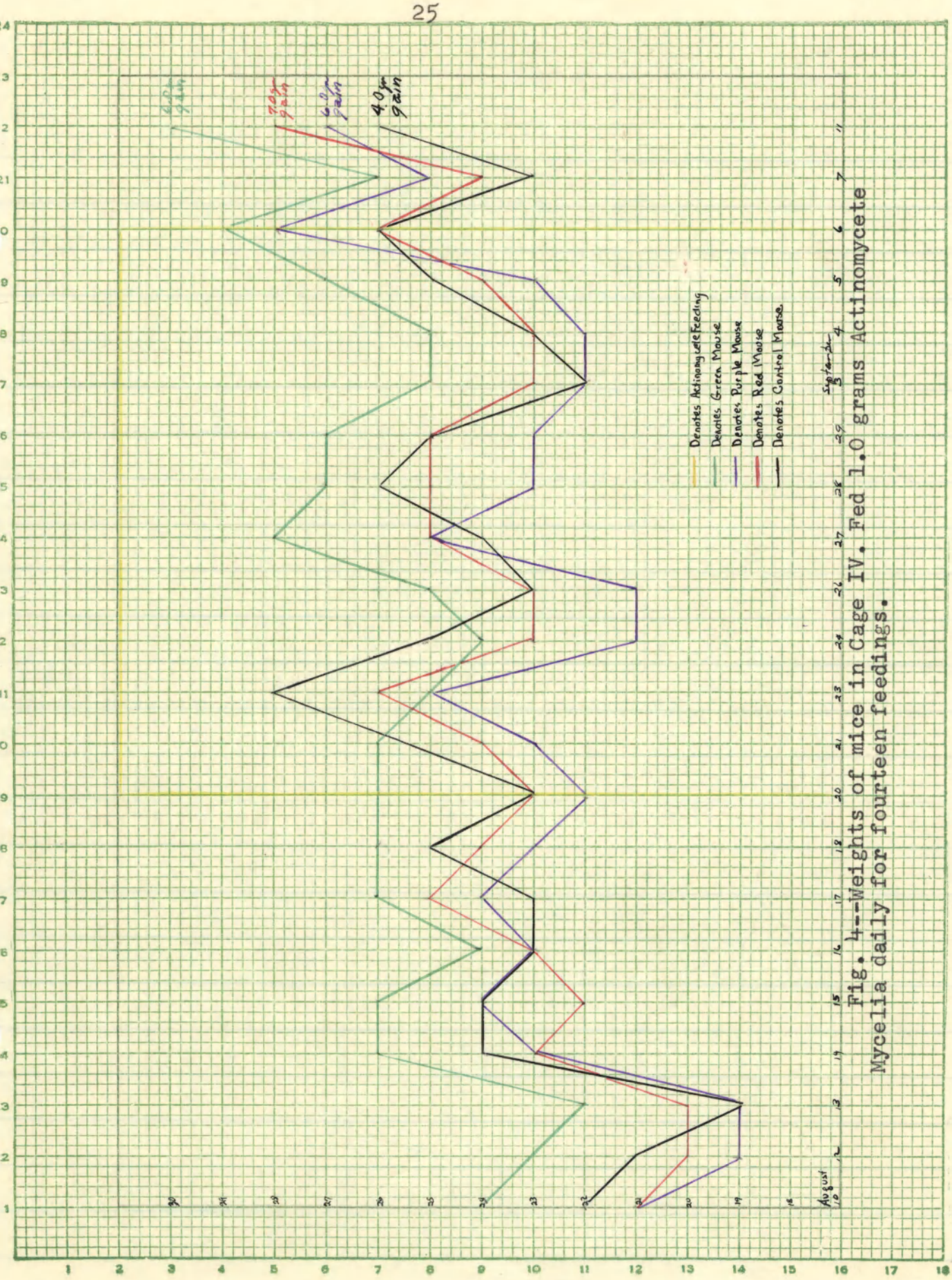

Fig. 4--Weights of mice in Cage IV. Fed 1.0 grams Actinomycete Mycelia daily for fourteen feedings.

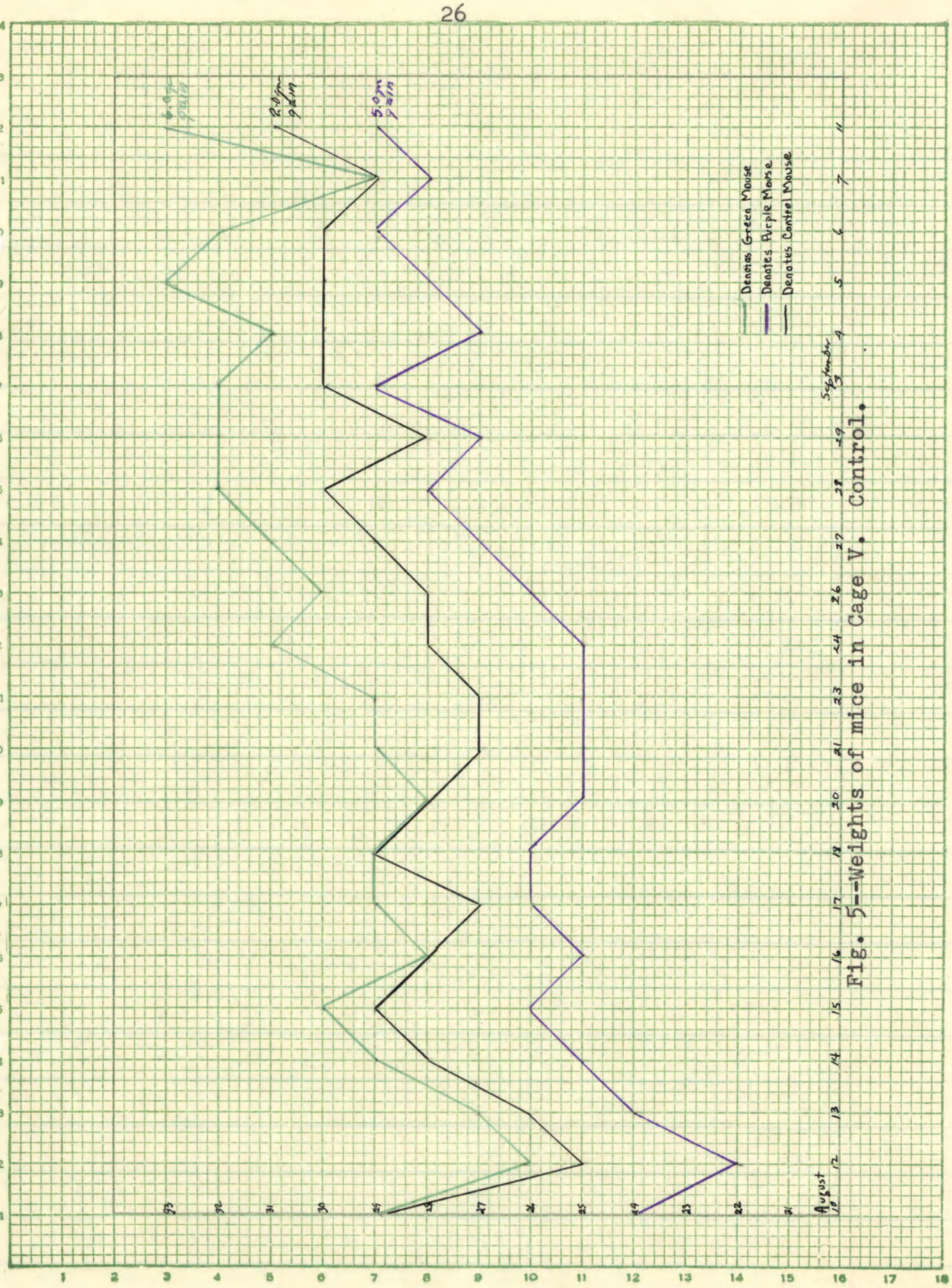

Fig. 5--Weights of mice in Cage V. Control.

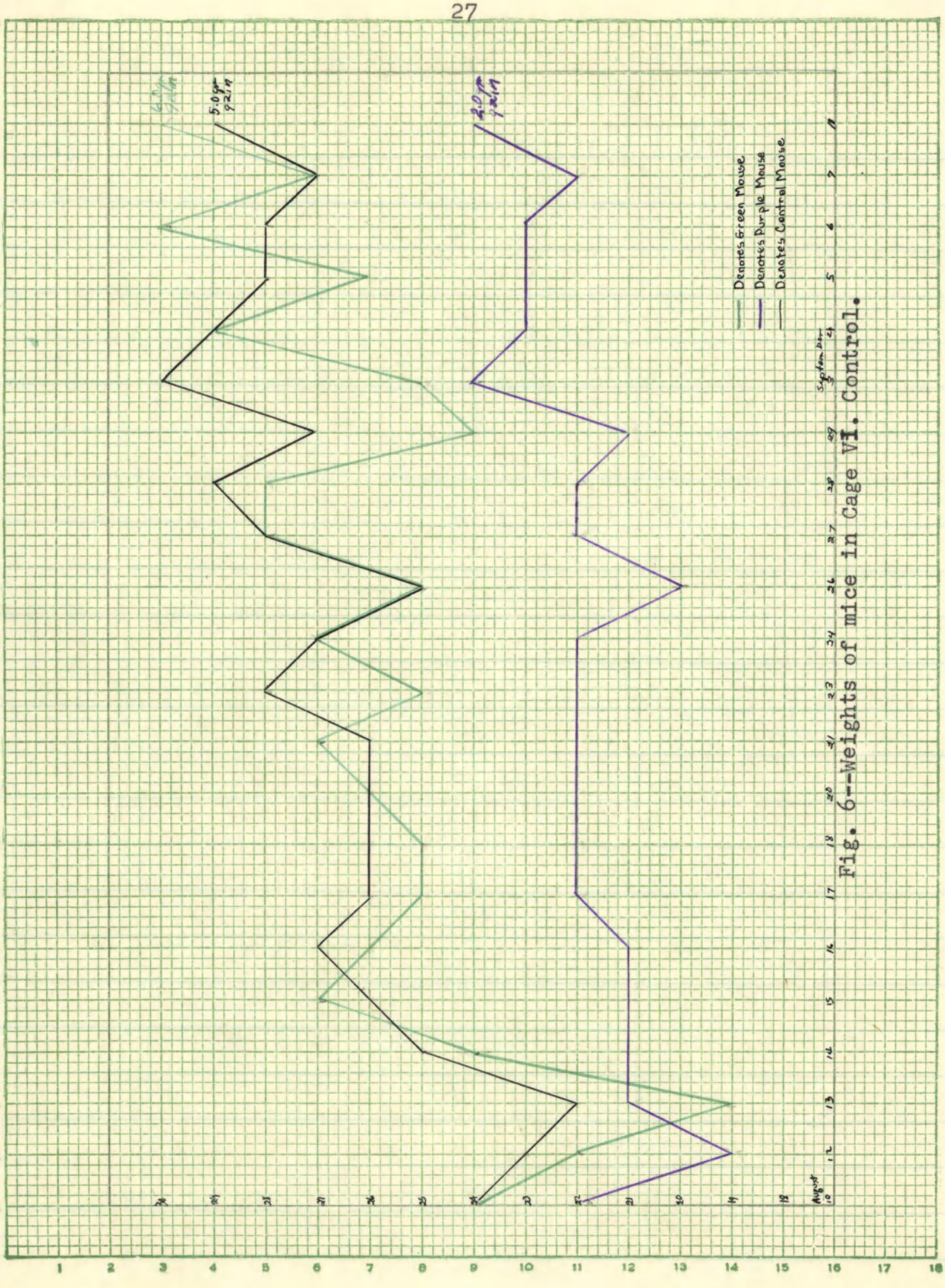

Fig. 6--Weights of mice in Cage VI. Control.

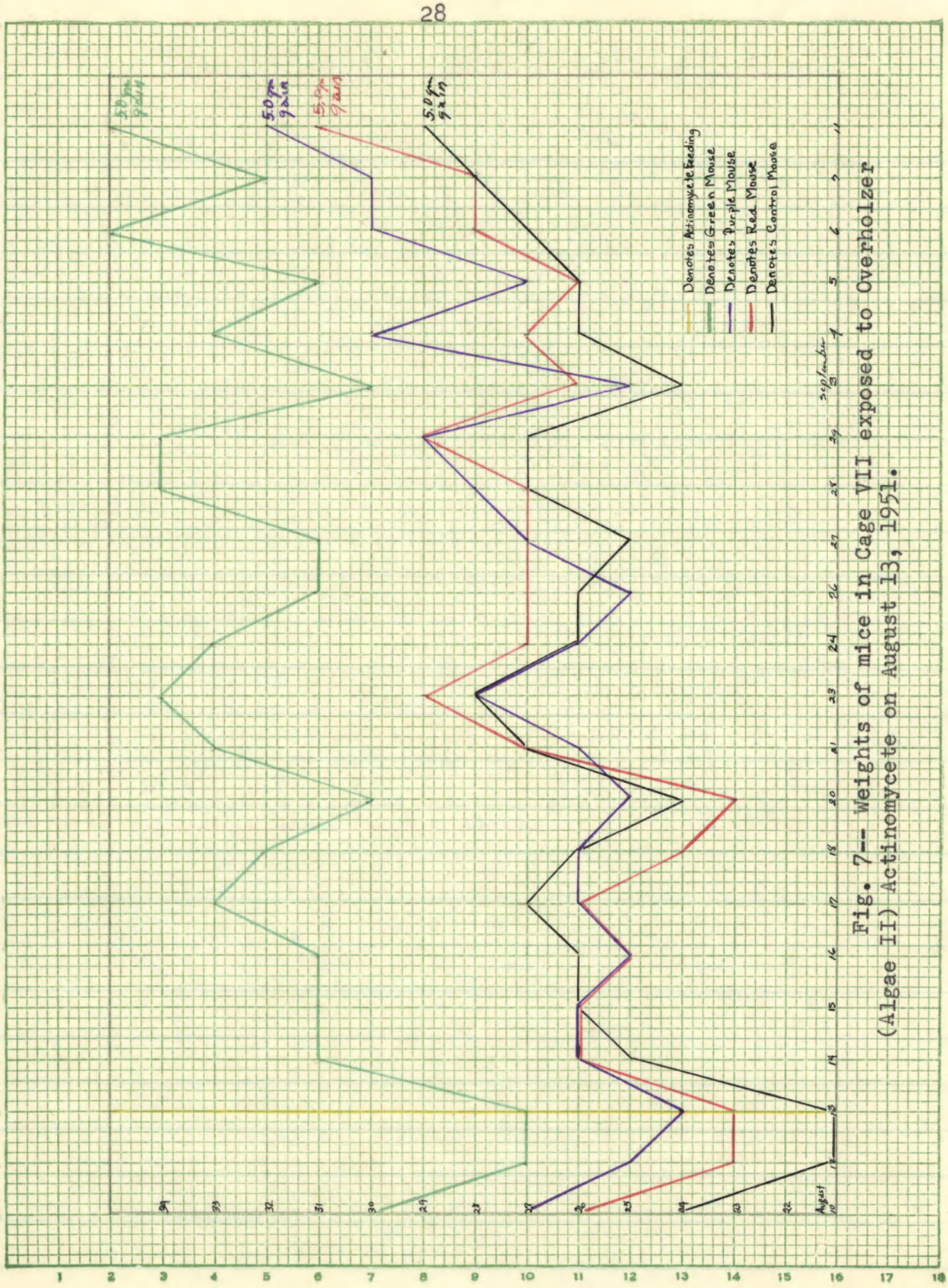

Fig. 7-- Weights of mice in Cage VII exposed to Overholzer (Algae II) Actinomycete on August 13, 1951.

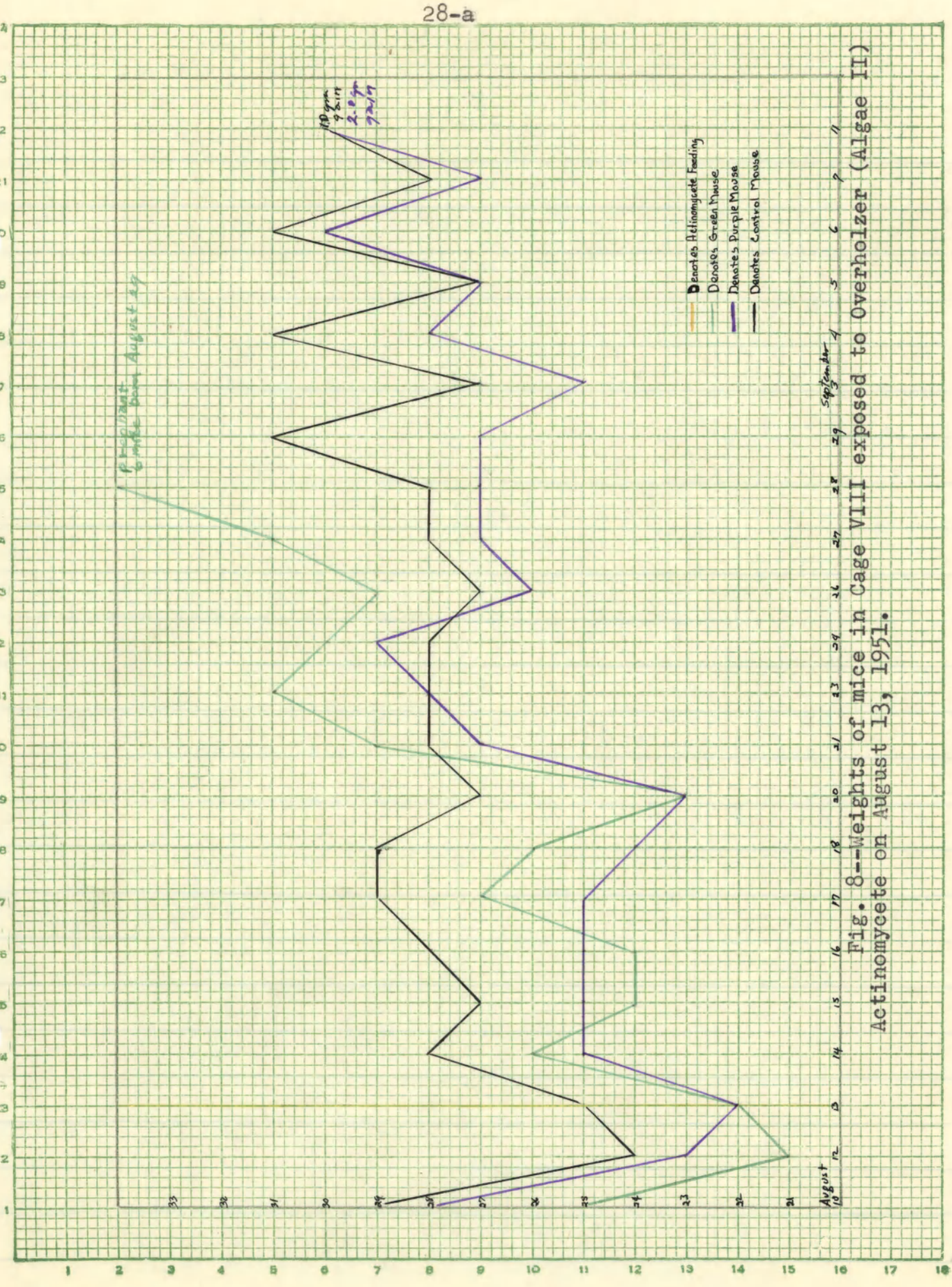

Fig. 8--Weights of mice in Cage VIII exposed to Overholzer (Algae II) Actinomycete on August 13, 1951.

In the third study, concerned with injections of water suspensions of actinomycetes (See Table 1), initial and final weights of both experimental and control animals were essentially the same. Weightings, therefore, offered no clues to effects of the injections or to impending demise of the animals.

All control animals survived the study. Of the nine mice in the experimental group, only three remained alive. Three were found dead, and three were killed for autopsy.

Since no experimental animals lived from Cage II, the intramuscular injection was probably the most successful although the deaths may have been due to a greater toxicity of Actinomycete 9. Toxic dosage could not be established because all of the animals died. Length of time required for death was undetermined since the animal receiving the larger dose survived longer.

Toxic dosage for subcutaneous injection of Actinomycete 8 remains undetermined since all animals, not autopsied, survived.

Table 1, concerning the weight of the mice injected with water suspension of Actinomycetes, Table 2, giving observations on autopsies of mice, and Table 3 showing plates and nutrient broth tubes from autopsies of mice, follow each other in consecutive order.

TABLE 1

WEIGHT (EXPRESSED IN GRAMS) OF MICE INJECTED WITH WATER SUSPENSION OF ACTINOMYCETES

	Dates									
	1-3-52	1-4-52	1-5-52	1-6-52	1-7-52	1-8-52	1-9-52	1-10-52	1-16-52	Survival
Cage I Sub-cutaneous										
Green	31.0	30.0	30.0	28.0	27.0	29.0	31.0	31.0	31.0	---
Purple	32.0	30.0	31.0	29.0	30.0	31.0	31.0	31.0	31.0	---
Red	27.0	26.0	28.0	26.0	26.0	27.0	28.0	29.0	killed 1-11-52	killed 1-11-52
White	26.0	26.0	27.0	25.0	25.0	28.0	27.0	27.0	29.0	---
Cage II Intra-muscular										
Green	26.0	25.0	29.0	25.0	25.0	28.0	27.0	27.0	Died 1-16-52	Died 1-16-52
Purple	32.0	32.0	30.0	31.0	32.0	33.0	32.0	32.0	30.0	Died 1-28-52
Red	32.0	31.0	34.0	31.0	30.0	31.0	32.0	31.0	killed 1-11-52	killed 1-11-52
White	36.0	35.0	35.0	32.0	32.0	35.0	34.0	33.0	30.0	---
Cage III Intra-peritoneal										
Green	32.0	30.0	27.0	28.0	29.0	30.0	30.0	30.0	31.0	---
Purple	26.0	25.0	26.0	28.0	29.0	31.0	31.0	31.0	41.0*	Died 2-20-52
Red	25.0	24.0	23.0	24.0	23.0	25.0	24.0	25.0	killed 1-11-52	Killed 1-11-52
White	29.0	29.0	29.0	30.0	31.0	31.0	31.0	31.0	29.0	---

* Pregnant

Injections ended

TABLE 2

OBSERVATIONS ON AUTOPSIES OF MICE INJECTED IN THIRD STUDY

	Autopsy Results					
	External Dorsal	External Ventral	Sub-cutaneous Dorsal	Sub-cutaneous Ventral	Abdominal Cavity	Removals for Plating
Cage I Red mouse	---	---	Lesion on under side of skin and top of muscle; ¼" diameter; gray center circled with red; located at side of vertebral column.	No irregularities	- - -	1.Section of shoulder muscle 2.Fascia from lesion on muscle 3.Fascia from lesion on skin
Cage II Red mouse	A lesion on each hind leg; appear as clean slit; length, 1/16"	---	Both left & right inguinal lymph nodes enlarged. Muscle necrosis evident on thighs of both legs. Scar tissue formation on skin in right axillary region.	---	All organs in good condition	1.Scar tissue from muscle 2.Inguinal lymph nodes 3.Scar tissue from
Cage III Red mouse	---	Three clean puncture holes in skin and muscle on abdomen	---	Inflamed area in fascia on underside of dermis; located ½" above hind legs on right and left sides; about ¼" diameter	General picture good. Early pregnancy.	1.Section of fascia from inflamed area. 2.Three embryos in right section of uterus. 3.Section of liver.

Case II
Green mouse

Lesion in lumbar region. Not inflamed.	---	Haematomas on muscles of both legs in calf region, appears as bloody streak. Bloody streak ½" long on dermis of both hind legs (possible lymphatic infection). Inguinal lymphatics inflamed but not enlarged. Bloody band with bloody mat underneath, covering first 6 ribs and running under both scapulae.	---	General picture good. Death recent since aorta bled as it was severed.	1. Haematomas from both calf muscles. 2. Both dermal streaks. 3. Left and right inguinal lymphatics. 4. Entire bloody area. 5. Vagina

TABLE 3

PLATES AND NUTRIENT BROTH TUBES FROM AUTOPSIES OF MICE JANUARY, 1952

	Plates			Tubes
	Plate 1	Plate 2	Plate 3	Nutrient Broth Tubes
Cage I Sub-cutaneous 1.0 ml.	Bacteria Mold	Bacteria Mold	Bacteria 2 Actinomycete colonies	Bacterial contamination
Cage II Intra-muscular 1.0 ml.	61 Actinomycete colonies No contamination	18 Actinomycete colonies Bacteria	84 Actinomycete colonies No contamination	Actinomycete growth No contamination
Cage III Intra-peritoneal 1.0 ml.	Bacteria Mold 2 Actinomycete colonies	Bacteria Mold	Bacteria Mold	Bacterial contamination
Cage II Intra-muscular 0.2 ml.	Bacteria	4 Actinomycete colonies	Sterile	---

Intraperitoneal injections of Type 10 appear to be toxic in amounts between 0.2 ml. and 0.6 ml. daily for an undetermined period of time.

The viability and sterility of the three suspensions was demonstrated by the six nutrient broth tubes innoculated after the final injections were given. The ability of the actinomycete to survive for at least six days within mouse tissue was shown by the plates from the autopsy of the green mouse in Cage II. The number of colonies recovered was fewer than those obtained in autopsies four and five days after the final injections.

From observations during the autopsy (See Table 2), it would appear that any lesions present occurred in the region of injection. Possibly, this was due to introduced bacterial contaminations or to trauma produced by the quantities injected. Involvment of the inguinal lymphatics in cases of intramuscular injection may indicate a reaction to the actinomycete. No major organic disturbances were discernible within the abdominal cavity.

One of the three surviving animals, the green mouse from Cage III, produced a litter of seven young a month after the injections were discontinued. All grew to maturity normally.

Tables 4, 5, and 6 follow consecutively.

TABLE 4

WEIGHTS (EXPRESSED IN GRAMS) OF MICE INJECTED INTRAMUSCULARLY WITH 0.5 ML. ACTINOMYCETE SUSPENSION

	Dates During Injection							
	2-11-52	2-12-52	2-13-52	2-14-52	2-15-52	2-16-52	2-17-52	2-18-52
Cage I, Vial A								
Green	34.0	37.0	35.0	34.0	34.0	34.0	33.0	35.0
Purple	28.0	30.0	30.0	20.0	31.0	31.0	30.0	33.0
Red	25.0	26.0	26.0	25.0	23.0	25.0	23.0	25.0
White	27.0	28.0	27.0	27.0	27.0	29.0	27.0	29.0
Cage II, Vial B								
Green	32.0	32.0	33.0	33.0	33.0	35.0	32.0	33.0
Purple	29.0	27.0	28.0	29.0	29.0	29.0	27.0	30.0
Red	33.0	33.0	35.0	36.0	36.0	35.0	33.0	35.0
White	31.0	32.0	34.0	35.0	33.0	32.0	31.0	33.0

	Dates After Injection			
	2-19-52	2-20-52	2-21-52	2-22-52
Cage I, Vial A				
Green	34.0	34.0	35.0	40.0
Purple	33.0	33.0	34.0	38.0
Red	24.0	24.0	26.0	29.0
White	28.0	28.0	28.0	31.0
Cage II, Vial B				
Green	32.0	32.0	34.0	36.0
Purple	30.0	30.0	30.0	34.0
Red	33.0	33.0	34.0	36.0
White	33.0	33.0	33.0	36.0

TABLE 5

OBSERVATIONS OF NUTRIENT AGAR PLATES PREPARED FROM MOUSE TISSUE INJECTED IN THE FOURTH STUDY

Source	A-Plates	B-Plates
Right inguinal lymph node	Bacteria Mold	Sterile
Lymphatic tissue from right leg	Bacteria	Sterile
Muscle section from lesion on left hind leg	Broken	Bacteria

An attempt to repeat the trends indicated in the above study proved unsuccessful. Tissue was removed during autopsy, macerated and plated on nutrient agar. No actinomycete colonies were recovered. Sections removed and stained for histological examination revealed nothing. All mice, except the one killed for autopsy, survived.

Observations on autopsy of the red mouse from Cage I again indicated a possible lymphatic involvment (See Table 6). However, no actinomycetes were recovered from plates of the gland.

Use of the actinomycete type isolated from macerated mouse tissue in study three produced no more toxic effect than the original Type 9.

TABLE 6

OBSERVATIONS ON AUTOPSY OF MOUSE IN FOURTH STUDY

Ventral Aspect	Internal	Dorsal Aspect	Removals for Plating	Removals for Histological Study
Slightly enlarged inguinal	Good general appearance. Early pregnancy.	Lesions through fur and skin, on thigh; clean holes not inflamed. Possible lymphatic enlargement extending from ventral side of hind legs.	1. Inguinal lymph node from right hind leg. 2. Lymphatic tissue from right hind leg. 3. Section of muscle from leison on right hind leg.	1. Inguinal lymph node from left hind leg. 2. Lymphatic tissue from left hind leg. 3. Section of muscle from lesion on left hind leg.

Altogether a total of 4 ml. of water suspension was introduced into the tissue of each animal over a period of eight days, no toxic responses of any type were observed.

In tissue culture Actinomycete 9 exhibited definite proteolytic properties. On the slides proliferating cells of the chick heart appeared to have been destroyed by some enzyme or metabolic waste-product produced by the plant. Phase contrast movies showed that initial growth of the cells gradually lessened as a larger mycelial mass developed.

DISCUSSION

Actinomycetes selected for this study were isolated from raw and finished water and consigned a laboratory series number. The actual identification of the genus and species of the types has not been undertaken. General microscopic observation of these organisms indicates that mixtures of more than one genus probably occurred together in a culture and were assigned a single number. Until actual identification of each organism has been completed, attempts to duplicate any part of this study will be impossible since component types are frequently lost in a series of successive transfers.

The fact that these organisms can withstand the treatments employed in the filtration process for the preparation of drinking water indicates a high degree of resistance to present purification methods and calls attention to the fact that the survival of these plants may contribute to public health problems. However, the present studies have not indicated a great degree of toxicity for the organisms observed. Toxic reactions were noted only when spores or mycelia were brought into direct contact with living cells, either by injection into the tissue of experimental animals or by exposure to growing cells in

tissue culture. Ingestion of the plant masses produced no toxic responses.

This lack of toxic response may be noted in the first two experiments. A single ingestion produced no discernible effect while continued feedings produced marked increases in the average weight gain of each animal and reduced fertility. The increase might have been due to the actual nutritional content of the dried mycelial flakes or to the effects of the plant on the bacterial flora of the digestive tract of the animal. The only explanation of the reduced fertility, other than that of toxic response, is that the mating selection of the experimental animals was poor.

The many variable factors, mode of injection, amount of dosage and type of actinomycete, involved in the third study make impossible the selection of a single fatal factor. Since all of the control animals survived, one, if not all, of these experimental factors must have contributed directly to the deaths of the mice. Viability and sterility of the water suspensions were adequately demonstrated. Contaminations, sufficient to be fatal, were not introduced with the innoculum or demonstrated on plates after autopsy. Enlargements of the lymphatic system alone appeared consistently throughout the findings of the autopsies and probably were responses either to the amount of fluid

injected or to the actinomycete itself. Further studies are indicated to conclude which of these contributing factors actually caused the deaths of the experimental animals.

Study four, an attempt to repeat the previous experiment, failed to elicit any response. Neither the agar plates nor the sections taken for histological study revealed any actinomycete colonies. The transfers may have died after innoculation into sterile water, or the toxic component of the actinomycete type may have been lost in the series of transfers made on nutrient agar slants between the time of the third and fourth studies. The water suspension itself was not tested for viability or sterility. However, the lack of contaminations on the nutrient agar plates of macerated mouse tissue indicated that the techniques employed in autopsy and injection had introduced no contaminations.

Slides and movies of actinomycetes grown in tissue culture revealed only the extreme toxic responses. However, in the general aspects of music health, isolated, individual living cells would probably never come in contact with these plants.

This preliminary study has indicated that these eight actinomycete types produced no toxic response when ingested directly but may damage individual cells if introduced into a lesion.

SUMMARY AND CONCLUSIONS

1. The isolation of actinomycetes from finished public drinking water suggested the possibility that these may exhibit pathogenicity usually attributed to other organisms. Therefore eight actinomycete types were isolated from Lakes Hefner and Overholser, municipal reservoirs of Oklahoma City, Oklahoma.

2. The actinomycetes were cultured in modified Czapek's medium for direct feeding of large quantities of mycelia to mice. Water suspensions of spores were taken from nutrient agar slants of the actinomycetes for injection into experimental animals. No extracts from the plant were employed in this study.

3. Eight mice were given flakes of mycelia in fourteen continuous feedings. All of the animals showed marked increases in weight with a probable reduction of fertility, although the data are insufficient to be conclusive.

4. Sub-cutaneous, intramuscular and intraperitoneal injections of actinomycete-water suspensions, given to nine mice, produced lesions in the region of injection, probable involvment of the lymphatic system and death in three instances.

5. Actinomycete colonies were recovered by plating from the macerated tissue of four mice. The greatest number of colonies was recovered from a mouse injected intramuscularly with Actinomycete 9.

6. A second attempt at intramuscular injection of Actinomycete 9 failed to produce death in any of six mice. No colonies were recovered by plating mascerated tissue, and none were observed in tissues removed for histological study. Colonies, recovered from mascerated tissue in the preceding experiment and re-injected into mice, exhibited no unusual effects. This second study of Actinomycete 9 indicated a complete non-toxicity largely because this type consisted of a mixture and in re-isolation all strains were probably not recovered.

7. Tissue culture of Actinomycete 9 revealed the presence of proteolytic enzymes or by-products of the plant which caused destruction of the proliferating cells of explanted chick heart.

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