THE DEVELOPMENT OF A POTENT TUBERCULOSIS VACCINE AND THE EFFECT OF SUBTILIN ON THE TUBERCULOUS GUINEA PIGS

By

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TABLE OF CONTENTS

I INTRODUCTION

The ineffectiveness of streptomycin for the treatment of human tuberculosis arises from the streptomycin-resistant strains of Mycobacterium tuberculosis and the development of resistance among the streptomycin-sensitive organisms during prolonged administration of the drug. The neurotoxic action of this antibiotic has been partly overcome by use of the reduced form, dihydro-streptomycin. Still with the use of this drug we are eager to find a more effective therapeutic agent which could combat this most dreaded, unconquered infectious disease without any toxic effects whatsoever.

Among the most promising of the new chemotherapeutic agents for human tuberculosis are aureomycin (Duggar, 1948), various forms of streptothricin, neomycin (Waksman and Lechevalier, 1949) and subtilin (Salle and Jann, 1945; 1946a; Anderson and Wong, 1946; Wong, Hambly and Anderson, 1947; and Anderson, 1947). The latter antibiotic named has shown extreme bacteriostatic and bactericidal action to a few gram negative and many gram positive organisms including acid-fast mycobacteria in vivo and in vitro. However, the data of the effectiveness of subtilin on experimental tuberculosis is still limited and inconclusive. Therefore, it seems highly desirable to carry out strict quantitative experiments in order to acquire data which will yield reliable information

on the effectiveness of subtilin on experimental tuberculosis in guinea pigs.

Many attempts have been made at the development of a suitable vaccine for the prevention of tuberculosis. None of these vaccines developed have been accepted for use with the exception of B. C. G. variety (Calmette, 1936; Corrado, 1938). The use of this vaccine is limited to certain countries in the Eastern Hemisphere. In the United States the practicability of B. C. G. is still doubtful and its use is limited almost entirely to experimentation.

Exposures to ultraviolet rays tend to inactivate many organisms by denaturing their protein molecules. If organisms are not over-irradiated some of their physical, chemical and serological properties may be retained (Stanley, 1944). It has been established that more potent vaccines can be prepared if the organisms are treated in this way instead of by asphyxiation (Potter, 1943), heat (Freund and Casals, 1939; Opie et al, 1939; Alcott, 1939; Wells et al, 1944), and chemicals (Burger, 1928; Spahlinger, 1932; Stanley, 1936, 1944; Hodes, Lavin and Webster, 1937, 1940; Smithburn, 1939; Salk, Lavin and Francis, 1940; Morgan and Lavin, 1941; Webster and Casals, 1942; Levinson et al, 1944; McKinstry and Reading, 1944; Milzer, Oppenheimer, and Levinson, 1944).

However, attempts to prepare tuberculosis vaccine by this method have not proven to be successful, although from the work of Smithburn and Lavin (1939), there are some hopeful possibilities revealed. It is thought that by

improving on the methods of the aforementioned authors and performing the experiments under more rigorous conditions a more potent tuberculosis vaccine can be prepared by this method.

In the experimentation which is reported here on the use of subtilin against experimental tuberculosis the following are recorded: macroscopic and microscopic appearance of tissues of animals treated and untreated with subtilin; the presence or absence of the toxic effect of the drug; the severity, progressiveness or retrogressiveness of the tubercules, differential count of the leucocytes, the change of the body weight, tuberculin sensitivity, life duration, and the mortality rate.

In the evaluation of the effects of vaccination the same observations were performed as mentioned above with the subtilin treated animals, with the addition of a study of the entire blood picture of the animals.

II CRITICAL REVIEW OF LITERATURE

A. Ultraviolet Radiation.

Long before the dawn of modern experimental science the ancient peoples, as Herodotus and Hypocrates mention, by their "trial and error" methods knew the value of sunlight for healing.

However, not until the experimental work of Ward (1892), Strehel (1901), and Barnard and Morgan (1903) was it known that the bactericidal action of sunlight was due to the wave-lengths of the ultraviolet range. Their observations were confirmed by Browning and Russ (1917), Newcomer (1917), Bayne-Jones and Van der Lingen (1923), Coblertz and Fulton (1924), Eidenow (1927), and many others.

Arons is given credit for being the first to discover in 1892 that a container with mercury vapor, if electrified, emitted ultraviolet rays. The effect of mercury vapor lamps on the virulence of the tubercle bacilli was first studied by Mayer and Aworski (1924). These authors found that the Alpine quartz-mercury-vapor lamp, whose burner was about 300 hours old, killed the mycobacteria after three minutes' exposure at a distance of five inches. They tested the viability of the exposed organisms by the guinea pig inoculation test. They also found out that when a saline suspension of mycobacteria was thoroughly mixed with an equal volume of aqueous solution of quinine, a fluorescent substance, twenty-five minutes exposure was required to render them avirulent to guinea pigs. However, in their

first experiment the exposure time was from three to twenty-five minutes, so that the actual time for inactivating the tubercle bacilli may be exactly at three minutes' exposure or only a fraction of it. Furthermore, no specific wave-lengths or energy output of the lamp were mentioned by these authors.

Howze (1926) worked along this line using a very pathogenic variety of tubercle bacilli secured from the sputum of persons having advanced pulmonary tuberculosis. He found out that ultraviolet radiation killed the tubercle bacilli after an exposure of five minutes at a distance of ten inches from the mercury lamp. This author did not standardize the concentration of bacterial suspension; therefore, his results cannot be considered quantitative or conclusive. Moreover, no specific wave-lengths and energy output of the lamp used were given.

Eidenow (1927), by screening with a different vitaglass filter, employed a quartz-mercury-vapor lamp which emitted various ranges of wave-lengths. He found out that one mg./cubic centimeter saline suspension of tubercle bacilli exposed to the radiation of 7,620-2,300 Å at a distance of eight inches were destroyed after ten minutes' exposure. Those organisms exposed to radiations of 5,720-2,300 Å were destroyed or attenuated after thirty minutes exposure. When exposed to the radiations of 5,720-3,300 Å, no lethal effect was manifested. The author concluded that wave-lengths shorter than 3,300 Å are the most bactericidal

on the tubercle bacillus and that the longer wave-lengths from 4,000 to 3,300 Å were without effect. He also pointed out that when the tubercle bacilli were mixed with blood or serum and exposed in a very thin film, they were not killed. However, the energy output of the lamps was not mentioned.

In 1932, Mayer and Dworski employed a water cooled quartz-mercury-vapor arc, operating at 110 volts, 5 amperes, A. C. (Kronmyer lamps) and used oxalic acid and uranyl sulfate method (Anderson and Robinson, 1925) to measure the energy output of the lamp. They found out that a suspension of a moderately virulent tubercle bacilli, strain H-60, grown on glycerol broth for fifteen days and containing 2,750,000 organisms per cc. was rendered avirulent to guinea pigs within four minutes' exposure by absorption of 1.42×10^{9} energy ergs per square centimeter derived from the complete ultraviolet band. They concluded: "A like amount of energy destroyed dried tubercle bacilli on the same suspension within four minutes." It should be pointed out that in their experiments one of the two guinea pigs inoculated with the dried organisms after being exposed for two minutes showed neither a tuberculin sensitive reaction nor tuberculous lesions upon necropsy.

Burger (1928) was the first to point out that bacteria inactivated with ultraviolet irradiation made superior vaccines. Stanley (1936) also mentioned that tobacco-mosaic virus protein could be rendered avirulent by ultraviolet radiation and still retain its immunological

activity. Hodes, Lavin and Webster (1937) reported the infectivity of the rabies virus could be eliminated with ultraviolet radiation and the antigenicity of the same virus could be retained if properly exposed. Kidd (1938) also reported that the infectivity of Shope papilloma virus could be abolished by means of ultraviolet radiation without losing the complement fixing properties of the virus. Stimulated by these findings, Smithburn and Lavin (1939) were the first to test the influence of ultraviolet radiation on the antigenicity of Mycobacterium tuberculosis. These authors found that the concentration of bacterial suspension influenced the length of time necessary to kill the organisms by ultraviolet radiation. They also found that a reduction in virulence was shown after less irradiation than is required to kill the organisms, and that the irradiated viable organisms possessed the property of inducing demonstrable immunity. The authors failed to induce measurable immunity with organisms killed by irradiation. However, they pointed out that "a weak suspension might be used in which very short exposure to radiation would cause death of the bacteria, conceivably without denaturing or rendering ineffective the immunizing antigen."

In recent years many articles have revealed that organisms inactivated by ultraviolet radiation retain more antigenic properties of the organisms than by any other methods. Salk et al (1940) worked on influenza virus; Hodes and Webster (1940), rabies virus; Morgan and Lavin (1941), eastern equine encephalomyelitis virus; Webster and Casals

(1942), rabies virus; McKinstry and Reading (1944), poliomyelitis virus; and Milzer, Oppenheimer, and Levinson (1944), poliomyelitis virus. All of the works reported in these articles showed that potent vaccines could be prepared for the above listed microorganisms by proper exposure to ultraviolet radiation.

Levinson et al (1944) were able to produce potent vaccines of many organisms which could be duplicated with consistent results by standardizing the intensity of the ultraviolet light, concentration and film thickness of organism suspension, time of exposure, and distance from light source. They found that suspensions containing approximately one billion organisms per ml. of the following organisms: (1) E. coli; (2) E. typhosa; (3) S. enteritidis; (4) S. aureus; and (5) rabies 4% uncentrifuged brain tissue suspension were completely killed in 0.17 to 0.33 seconds exposure to ultraviolet radiation. A 4% centrifuged suspension of St. Louis encephalitis virus required twice the exposure time. A new ultraviolet lamp was employed for this work. It emitted wave-lengths below 2,000 Å and it was found more efficient than other types such as Westinghouse steri-lamp, Hanovia cold quartz lamp, etc.

B. Chemotherapy with Subtilin

As pointed out by Freedlander and French (1947), the history of chemotherapy may be conveniently divided into five periods: 1. Pre-Ehrlich period (1870-1900) - In this period many strong germicidal chemicals such as

phenol, mercuric chloride, etc. were employed to treat infectious diseases without paying attention to the toxic effects of these drugs to hosts. 2. Ehrlich period (1900-1920). Specific attention was directed in a search for synthetic compounds which might show higher toxicity to microorganisms than to hosts. 3. Empirical trial period (1920-1935). In this period many heavy metals such as gold, copper, mercury, etc., and many kinds of dyes have been tried without satisfactory results. 4. Sulfonamide era (1935-1945). Because of the low toxicity of sulfa drugs to hosts and their inhibitory effect on many microorganisms which lack the capacity to synthesize and require para-amino benzoic acid as an essential nutritional element, the application of sulfa drugs to many bacterial infection marked the beginning of modern rational chemotherapy. 5. Antibiotic period (1945-). Since the discovery of penicillin (Fleming, 1929, 1932, 1936, 1942a, 1942b, 1943, 1944, 1945 and 1946; Fleming and Queen 1946; Chain, et al, 1940; Abraham et al, 1941, 1942; Florey and Florey 1943; Florey and Cairns, 1943; Berger, 1944; Dala, 1944; Florey, 1944; Hunter and Randall, 1944; Kieway, 1944; McKeen, 1944; Regna, 1944; Chain and Duthie, 1945; the Committee on Med. Res., O.S.R.D., Washington, and the Med. Res. Council, London, 1945; Flosdore, 1945; Murtaugh and Levy, 1945; Pratt and Dufrenoy, 1945; Scudi, 1946), a new antibiotic which is active against many gram positive organisms and has very low toxicity to the host, a great field for cooperative research was opened to the bacteriologists, pathologists, and chemists.

The discovery of streptomycin (Waksman and Schatz, 1945; Waksman, Schatz and Reilly, 1946; Kuehl et al 1945; Brink et al, 1945; Peck et al, 1945a, 1945b; Kuehl et al, 1946), and the establishment of its therapeutic effect against many gram negative, gram positive, especially acid-fast pathogens, (Foldman, Hinshaw and Mann, 1945) gave new courage to the searching for new antibiotics of therapeutic value.

The antagonistic action of B. subtilis has been reported by Mechnikoff (1897) and Nicolle (1907). The lytic effect of B. subtilis against certain bacteria has also been pointed out by Rosenthal (1925) and his co-workers (Rosenthal and Ilitch, 1926; Rosenthal and Duran-Reynals, 1926). The antagonistic activities of B. subtilis against M. tuberculosis (Van Canneyt, 1926), virus (Rakietan, 1936), and fungi (Bitter, 1941; Katznelson, 1942) were also reported.

In 1943, Humfeld and Fenstel found out that by use of asparagus juice to cultivate B. subtilis strain No. 231* of the culture collection of N. R. Smith of the Bureau of Plant Industry, soils, and Agricultural Engineering, U. S. Department of Agriculture, Washington, D. C., a substance was produced, which inhibited the growth of M. conglomeratus,

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* Through personal communication with J. C. Lewis of Bioligical Utilization Section, Fruit and Vegetable By-products Division, Western Regional Research Laboratory to Dr. D. E. Shay, it is known that this strain is now deposited in the American Type Culture Collection as number 6633 and in the culture collection of the Northern Regional Research Laboratory as B-543.

S. aureus, *L. casei*, *P. michiganensis*, *E. amylovora*, and *P. juglandis*.

The nutritional study of the same strain of *B. subtilis* for assuring the yield of the antibiotic was carried out by Jansen and Hirschmann (1944). They found that the addition of manganese in the proportion of 1 ppm. to the media containing sucrose, mineral salts, and several organic nitrogen sources proved essential for high antibiotic activity. The name, "subtilin," was first given by these investigators to the antibacterial substance produced. This antibiotic was reported to be water soluble, highly diffusible, thermolabile under alkaline conditions, relatively stable at pH 2.5, inactivated by light and formaldehyde, and precipitated by 95% alcohol. They found that this antibiotic was also active against *St. viridans*, but not against *E. typhosa*.

Further studies to increase the maximal yield of this active substance were undertaken by Lewis *et al* (1947), Stubbs *et al* (1947) and Feeney *et al* (1947a and 1947b). They found that the highest yields of subtilin in shallow-layer cultures were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor; that requirements for potassium, magnesium, manganese, iron, and zinc were demonstrated; that Cadmium, the only element capable of substitution for zinc, was only partially effective; and that calcium had a detrimental effect on subtilin production, while citrate generally had a beneficial effect.

The purification and chemical nature of subtilin were studied by Dimick et al (1947a, 1947b), Lewis and Jansen (1947), Stanley and Ananenko (1947), and Lineweaver, Klose, and Alderton (1948). It was generally admitted that subtilin is a kind of polypeptide of low molecular weight (7,000-10,000); soluble in acidic water, acetic acid, diluted ethanol and methanol but not in dry ethanol, butanol, pentanol, acetone, ether, or chloroform; precipitated at low concentrations of sodium chloride solution, laevorotary, $(\alpha)\ D^{23} = -30^{\circ}$; surface tension depressor, inactivated by pepsin (pH 2.2-4.8), trypsin (pH 4.4-7.5), pancreatin (pH 4.4-7.5), and erepsin (pH 6.1-7.8); and dialyzable through collodion. It consists of 15.8% total nitrogen (Kjeldahl method), 1.8% amino nitrogen, 2.2% amide nitrogen, and 4.8% sulfur. NO - SH, -SS-, or $-SCH_3$ groups were indicated.

Lewis and Jansen (1947) reported that the bacteriostatic activity of subtilin against a number of organisms was increased about two to five times by methylation, and that the methylated product contained 2.0% $-OCH_3$ by Zeisel as compared with a negligible test (0.15%) in the original sample of unmodified subtilin.

The biological activity of subtilin was further studied by Salle and Jann (1945, 1946a, 1946b, 1946c, 1946d, 1947), Anderson et al (1946), Anderson and Wong (1946), Anderson and Chin (1947), Wong et al (1947), Chin (1947, 1948), and Goodman and Henry (1947). Partially purified subtilin

was found to be active against many gram positive organisms, acid-fast mycobacteria, and certain pathogenic fungi. It was inactive against many gram negative organisms tested except N. catarrhalis and N. gonnorheae. Salle and Jann reported that its effect on M. tuberculosis was bacteriostatic at 1:50,000 dilution and bactericidal at 1:10,000 dilution when Long's liquid medium was employed for the growth of this organism.

While Wong et al reported that by use of modified Dubos and Davis medium for demonstration of antibiotic activity of partially purified subtilin against M. tuberculosis it showed bacteriostatic at 1:400,000 dilution and bactericidal at 1:20,000 or lower dilution. The difference of value of subtilin against M. tuberculosis between these workers might be due to three factors, viz., the purity degree of subtilin used, the susceptibility of different strains of M. tuberculosis, and the different media employed.

For the purpose of uniformity in reports about the biological activity of subtilin, it seems advisable to adopt a standard unit as proposed by Salle and Jann (1946a) for measuring the amount of partially purified subtilin and expressing its value. A unit of subtilin was defined by these authors as the amount contained in 1 ml. of the highest dilution capable of killing S. aureus in 10 minutes at 37°C (F.D.A. Phenol Coefficient Method).

The toxicity of partially purified subtilin to living embryonic chicken heart tissue was found to be one-twentieth of that to S. aureus (Salle and Jann 1946a).

In vivo, it showed remarkable therapeutic value without toxic effect to hosts on type III pneumococcus in mice (Salle and Jann, 1946b), experimental anthrax infections in guinea pigs (Salle and Jann, 1946c), and *St. pyogenes* infections in mice (Salle and Jann, 1946d).

The therapeutic effect on experimental tuberculosis has been studied by Anderson and Wong (1946) with disappointing results. These investigators reported that topical application of subtilin was ineffective in treating experimentally induced tuberculosis infections of the cornea of rabbits. Daily subcutaneous injections of 6 mg. of subtilin continued over a period of 6 weeks failed to affect the course of experimental tuberculosis in Syrian hamsters.

A microbiological assay procedure for subtilin was developed by Lewis *et al* (1947). These workers suggested a turbidimetric method which depended on growth inhibition of *M. conglomeratus*, *St. fecalis*, or *S. aureus*.

The further pharmacological properties of subtilin were studied by Wilson, Lewis and Humphreys (1948). It was found that by subcutaneous injection a dosage of 3 gm./kg. of purified subtilin does not kill mice, that by parenteral administration of subtilin it is precipitated at the site of injection, that amounts up to 100 mg./kg. injected into rabbits intramuscularly no more than 2ppm. of subtilin was indicated in whole blood by cup plate assay method, that a heavy deposit of subtilin was revealed at the injection site twenty-four hours after injection, and that a trace

of subtilin was still able to be detected one month later by blood assay. They also found that by intravenous administration of 10 mg./kg. of subtilin to rabbits through ear vein it gave rise to a blood concentration of about 100-200 ppm. in five minutes, but dropped to 10-30 ppm. in two hours, and dropped to zero in twenty-four hours. The intravenous LD_{50} for mice was determined to be 100 mg./kg. An intravenous infusion at a dose of 20 mg./kg./hr. was given a rabbit for four hours without producing visible symptoms and a final blood level of subtilin after four hours' perfusion was found to be approximately 750 ppm.

III MATERIALS AND METHODS

A. Ultraviolet Radiation.

A 30-watt germicidal mercury lamp, manufactured by the General Electric Company, Cleveland, Ohio, was used as the source of ultraviolet light for this experiment. The lamp radiates most of its energy at the 2537 line. The designation of the lamp is as follows: approximate amperes of lamp, .34; approximate volts of lamp, 103; and approximate energy output of 2537 Å, 7.2 watts at 100 hours' radiation.

The T-8 bulb was cleaned with alcohol just before use. To assure uniformity in energy output, the lamp was turned on half an hour before it was used.

The first step in the development of a safe vaccine disregarding antigenic potency was to determine the minimum time for devitalizing organisms. Two virulent strains of Mycobacterium tuberculosis var. hominis, strain H 37 and strain RL 8207, obtained from American Type Culture Collection, were used. They were first grown on Petragnani medium and then on glycerated egg-yolk medium. Three-week cultures at 37°C were harvested and evenly suspended in sterile distilled water by grinding thoroughly in a sterile mortar and pestle. The concentration of the organisms was one milligram per ml. One milliliter of the uniform suspension was distributed into sterile petri dishes as a thin

film. While being constantly shaken, the petri dishes were exposed to the mercury lamp at a distance of two inches for a definite length of time. Two-tenths ml. of the irradiated suspension was seeded into duplicate tubes of Dubos liquid medium (Dubos and Davis 1946), enriched with Difco TB Medium Serum Experimental, and into duplicate tubes of the same medium enriched with TB Medium Albumin Experimental. The presence or absence of growth was recorded every week for six weeks.

B. Immunization Test.

Sixty apparently healthy guinea pigs with no tuberculin reaction and weighing between 450 to 500 grams, were used for this purpose. They were divided into the control and experimental groups. The controls were sub-divided into three groups which were:

(1) Animals which were injected with non-irradiated organisms.

(2) Animals which were injected with under-irradiated organisms.

(3) Animals which were injected with over-irradiated organisms.

In the experimental group, there were two sub-groups which were:

(1) Animals injected with organisms which had been irradiated for five seconds, which is the minimum time for devitalizing organisms with the mercury vapor lamp used.

(2) Animals which were injected with organisms irradiated for 10 seconds, the maximum time for completely devitalizing organisms with this lamp.

The animals were numbered by either a special numbering system of marking the hair or ear tag. The different sexes were caged separately in a number from three to six except in a particular group where nine animals of the same sex were put in a cage in order to see the influence of crowding and partial starvation on the immunity and pathogenesis of tuberculosis. They were fed with ordinary commercial rabbit chow pellets supplemented with fresh, uncooked vegetables such as cabbage, celery, lettuce, etc., in order to prevent ascorbic acid shortage. Unlimited feeds and fresh water were added twice a day. Ventilation was controlled by a mild electric suction fan without causing a draft. The room temperature was maintained between approximately 20°-25°C. The body weights were taken once a week.

The mycobacterium used for preparing these inoculated suspensions was M. tuberculosis strain H 37. Its virulence had been previously tested on two normal guinea pigs. One guinea pig was inoculated intraperitoneally with one ml. of a four-week old culture grown in Modified Dubos Albumin Experimental liquid medium. The other guinea pig was inoculated subcutaneously with one-half ml. of the same culture. The former died twenty-eight days after inoculation, while the latter died in forty-one

days. The dosage for the immunization test was as shown in Table I.

On the seventh day following the final inoculation, a challenge dose of two mg. of the tubercle bacilli strain H 37 of three-week old glycerolated egg-yolk culture suspended in one milliliter of sterile distilled water was given to all of the animals intraperitoneally except animals Nos. 71, 72 and 73.

C. Subtilin Treatment Tests.

The sample of subtilin used was a pale yellow non-crystalline powder which readily dissolved in distilled water. At a concentration of 1% in water subtilin gave a pale yellow homogenous viscous solution. When added to modified Dubos liquid medium at a concentration of 0.1%, immediate precipitation resulted. One milligram of this powder contained 20 units when determined by F. D. A. phenol coefficient method.

In view of the fact (Wilson, Lewis and Humphreys, 1948) that the solubility of purified subtilin in physiological saline or serum does not exceed 0.05-0.06 gm. per 100 ml., that no more than 2 ppm. of subtilin is contained in whole blood even a large dose is injected parenterally, that a trace of subtilin can still be detected in the animal's blood one month after intramuscular injection with 100 mg./kg. of subtilin, and that its toxicity is exceedingly low; therefore, the writer arbitrarily tried the dosage as shown in Table II for the first experiment.

TABLE I

Vaccination Schedule

Date of Vaccination	1. Non-irradiated		2. Under-irradiated	3. Over-irradiated
	Nos. 501-507	Nos. 71-73	Nos. 401-412	Nos. 101-112
Feb. 23	0.4 mg.	71-1. 0mg. 72.2. 0mg.	0.4 mg.	0.4 mg.
24	"	72.3. 0mg.	"	"
28	0.5 mg.	Intraperitoneally	0.5 mg.	0.5 mg.
Mar. 1	"		"	"
2	"		"	"
3	"		"	"

Experimental

Date of Vaccination	1. Irradiated for 5 seconds Nos. 301-312	2. Irradiated for 10 seconds Nos. 201-212
Feb. 23	0.4 mg.	0.4 mg.
24	"	"
28	0.5 mg.	0.5 mg.
March 1	"	"
2	"	"
3	"	"

Remark: All the injections were made subcutaneously at the left and right thighs alternately except otherwise mentioned.

TABLE II

Time and Dosage Schedule for Subtilin Treatment
(First Experiment)

Date Inj.	Amount of Subtilin (units) Group I (Nos. 501* & 502) 200 units/1 ml. (10 mg.)	intraperitoneally	Group II (Nos. 503 & 504) 200 units/0.5 ml. (5 mg.)	subcutaneously
March				
16	"	"	"	"
17	"	"	"	"
18	"	"	"	"
21	"	"	"	"
24	"	"	"	"
28	"	"	"	"
31	"	"	"	intraperitoneally
April				
4	"	"	"	"
8	"	"	"	"
12	"	"	"	"
14	Subtilin exhausted			
28-30	Necropsied			

Group III - Controls - (Nos. 505, 6 and 7)

* No. 501 was sacrificed on April 11 for preparing histological sections of liver and spleen which showed a few conglomerate tubercles and of some other organs which showed no macroscopic abnormalities.

For the first experiment four emaciated control guinea pigs (Nos. 501, 502, 503 and 504) which had been previously infected with non-irradiated M. tuberculosis strain H 37 as shown in Table I were chosen for subtilin treatment.

After several days treatment the animals showed improved appetite and appearance. It was thought worthwhile to carry out a second experiment, using more animals to eliminate errors due to individual differences of the animals. Another sample of subtilin was requested. In this experiment, twenty apparently healthy male guinea pigs with no tuberculin reaction and weighing between 400 and 500

grams were used. They were divided into four groups with five guinea pigs apiece. Inoculation was done intraperitoneally with 2 mg./1 ml. of a three-week old culture of M. tuberculosis strain H 37. These animals had the same feeding and care as mentioned under the immunization test. The dosage of subtilin was arbitrarily set up as follows: The first group was given 10 mg./ml (200 units), the second group, 20 mg./2 ml. (400 units), the third group 30 mg./3 ml. (600 units), and the fourth group nothing. Injections were started seven days after infection with the organism. They were injected intraperitoneally twice a week for five times, and then interrupted due to the exhaustion of subtilin. All of the surviving animals were necropsied on April 28-30 (forty-three to forty-five days after the first medicament for the animals of first experiment and thirty-one to thirty-three days after the first medicament for the animals of second experiment) to terminate the experiments when the second sample of subtilin was still not received yet.

D. Tuberculin Sensitivity Test.

The method of Mantoux intracutaneous tuberculin test was used for this test. A quantity of 0.1 to 0.2 ml. of 1:1,000 dilution of old tuberculin was injected intradermally on the left thigh. Results were recorded one to three days after inoculation. A high concentration of old tuberculin was used in order to provoke a specific focal reaction in tuberculous guinea pigs with characteristic

change of blood picture and to decrease the possibility of false negative results.

E. Blood Picture Study.

Blood samples were taken by cardiac puncture one to five days after tuberculin injection. The Levy Hemacytometer (Improved Neubauer Single Ruling) counting chamber (manufactured by Arthur H. Thomas Co., Philadelphia, Pennsylvania) was used to count the number of erythocytes and leucocytes. In the preparation of blood smears for leucocyte differential count, Wright's stain was used. In the estimation of hemoglobin value Hallige's haemometer was used throughout the study.

F. The Evaluation of Infection Index.

Since the animals might have died of diseases other than tuberculosis during the observation period and since at autopsy or necropsy the carcasses exhibited various degrees of tuberculosis foci, a modification of Feldman's method for schematic and numerical recording of tuberculous lesions was adopted as shown in figure 1 and Table III.

Fig. 1 Key of Symbols for Recording Tuberculous Lesions.

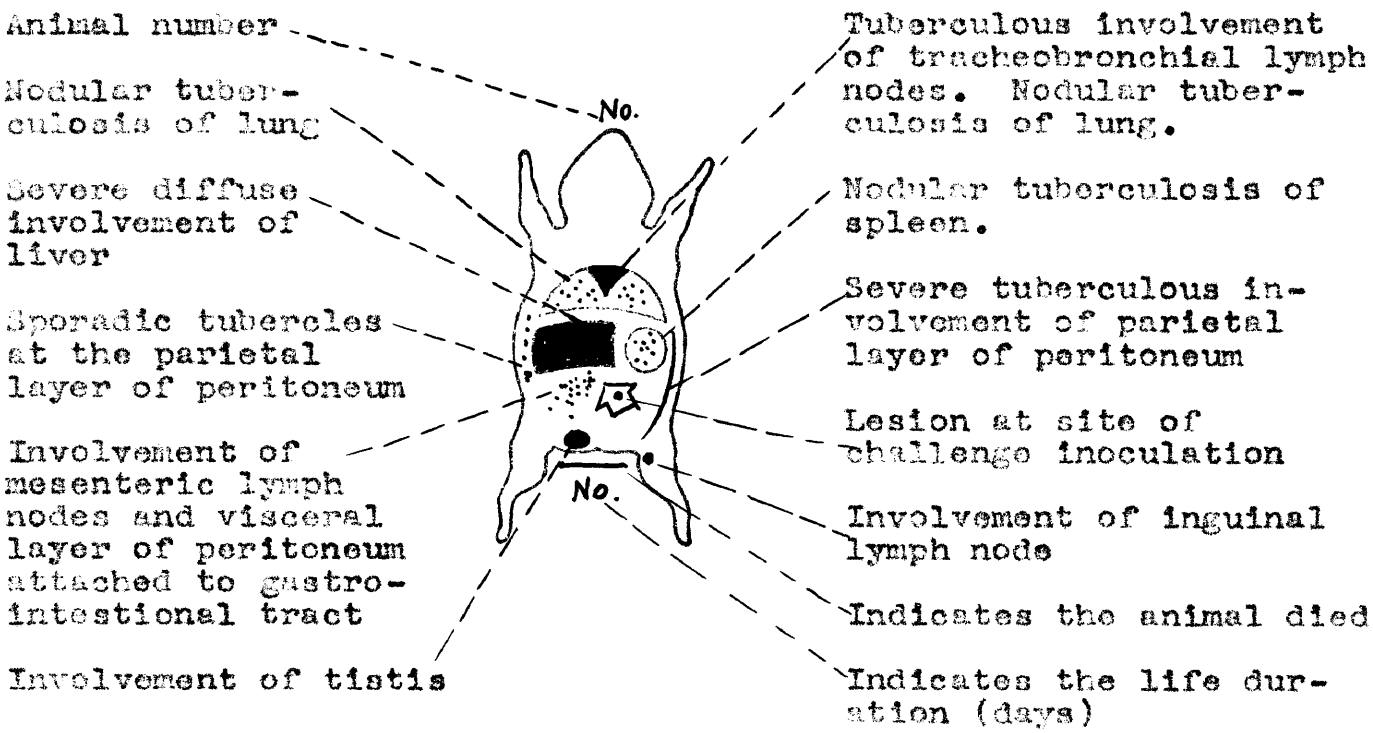


TABLE III

Key for Evaluating Infection Index of Tuberculous Guinea Pigs by Macroscopical and Microscopical Method (A Modification of Feldman's Table)

Extent & Character of Lesions	Liver	Spleen	Peritoneum & Contagious Lymph Nodes	Testis (or ovary) Lungs & Tracheo-bronchial lymph nodes	Total value of Infection Index
Progressive					
Extensive	30	20	30	10	100
Moderate	20	20	20	7	67
Slight	10	10	10	3	33
Retrogressive					
Fibrotic	3	3	3	1	10
Fibrotic & Calcified	2	2	2	0.7	6.7
Only Calcified	0.3	0.3	0.3	0.1	1

During necropsy or autopsy smears from liver and spleen were made to see if acid-fast mycobacteria were present when no macroscopic tuberculous lesions were found. In some cases emulsified liver and spleen suspensions were transferred into duplicate tubes of modified Dubos serum experimental liquid medium and incubated at 37°C for three weeks in order to check negative results in smears. However, the animal inoculation test with the above-mentioned suspensions to detect the tubercle bacilli which might not constitute an inoculum big enough to initiate growth in the previously mentioned medium was not carried out because of the limitation of time.

IV Results and Discussion

A. Effect of Ultraviolet Radiation On The Viability Of Mycobacterium Tuberculosis Var. Homonis.

The irradiation time for the initial test of the effect of ultraviolet radiation on the viability of Mycobacterium tuberculosis var. homonis strains H37 and R1 8207 was arbitrarily set up from five seconds to three hundred seconds as shown in the first column of Table IV. None of the tubes seeded with the organisms irradiated for over ten seconds showed visible growth after six weeks incubation at 37°C. However, in the case of five seconds irradiation the results were variable. One of the four tubes seeded with strain H37 showed visible growth after two weeks incubation at 37°C; while two of the four tubes seeded with strain R1. 8207 showed heavy growth after two weeks incubation under the same conditions.

TABLE IV

Preliminary test of the effect of ultraviolet radiation on the viability of Mycobacterium tuberculosis var. homonis

Irradiation period (sec.)		Strain H37 June 21	June 28	July 5	July 12	July 19	July 20
Control	0	4/4	4/4	4/4	4/4	4/4	4/4
Experimental	5	0/4	0/4	1/4	1/4	1/4	1/4
	10	0/4	0/4	1/4	1/4	1/4	1/4
		Strain R1 8207					
	0	4/4	4/4	4/4	4/4	4/4	4/4
	5	0/4	2/4	2/4	2/4	2/4	2/4
	10	0/4	0/4	0/4	0/4	0/4	0/4

Remark: Numerator = number of tubes showing growth
Denominator = number of tubes inoculated
Irradiation over 10 seconds no growth

The inconsistent results in the case of five seconds irradiation clearly indicated that the tubercle bacilli like other living beings have different susceptibility and resistance to ultraviolet radiation. This difference is indicated not only between different strains but also among the individuals of the same strain. Furthermore, the same organism might have a variable degree of resistance and susceptibility at different stage in its life cycle. An explanation for the variable results, may be attributed to the possibility that by chance the more resistent individuals of the organisms were seeded together into a tube and constituted an inoculum big enough to initiate growth in Modified Bucos liquid medium (as mentioned in the leaflet of Difco Experimental media for the rapid growth of Mycobacterium tuberculosis, a small inoculum of 10^{-7} mg. initiated growth within fifteen days in this medium). On the other hand, the tubes which showed no visible growth after six weeks incubation at 37°C might have consisted of some viable resistant individuals of the organisms in the inocula but not numerous enought to initiate visible growth within six weeks at the given condition.

It should also be pointed out that the seeding inoculum in the negative tubes might have consisted of a great number of viable individuals deprived only of their reproductive capacity and subsequently died naturally.

It is the writer's hypothesis that the pathogenicity of the organisms is generally more delicate than its reproductivity;

reproductivity, more delicate than viability; and viability, more delicate than antigenicity. It is based on this principle that many attenuated organisms may lose their virulence without losing their capacity of reproduction. Many organisms may lose their capacity of reproduction and cytoplasmic viability without losing their antigenicity. It is also based on this same idea that the writer did not use a guinea pig inoculation method to test the viability of the irradiated organisms. Guinea pigs are so extremely susceptible to the virulent tubercle bacilli that a small number of the organism which might not be able to initiate growth in the ordinary glycerolated Lb. media would cause the death of the guinea pigs in fatal tuberculosis. But with the same organisms viable and attenuated it was assumed that no tuberculosis could be developed in the same animals and that the attenuated, viable organisms might be gradually phagocytized by the animal defensive mechanism. On the other hand, such attenuated organisms might grow well in the modified Dubos Lb. medium provided that the inoculum is over 10^{-7} mg. of the organisms.

A second experiment was carried out as recorded in Table V with the time range from one to ten seconds. It showed complete deprivation of reproductivity and viability as shown in those organisms which were irradiated over ten seconds. Partial destruction of the vitality of the organisms was demonstrated in 3, 4, 5, 7, and 9 seconds exposure. The fact that no visible growth was observed in the tubes seeded with the organisms and irradiated for 6 and 8 seconds might

TABLE V

Effect of ultraviolet radiation on the viability of Mycobacterium tuberculosis var. hominis strain H37

Irrad. period (sec.)	Aug. 1'	4"	8	15	22	29	Oct. 5
Control	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Experimental 1	4/4	4/4	4/4	4/4	4/4	4/4	4/4
2	4/4	4/4	4/4	4/4	4/4	4/4	4/4
3	0/4	2/4	3/4	3/4	3/4	3/4	3/4
4	0/4	2/4	2/4	2/4	2/4	2/4	2/4
5	0/4	0/4	1/4	1/4	1/4	1/4	1/4
6	0/4	0/4	0/4	0/4	0/4	0/4	0/4
7	0/4	0/4	0/4	1/4	1/4	1/4	1/4
8	0/4	0/4	0/4	0/4	0/4	0/4	0/4
9	0/4	0/4	0/4	1/4	1/4	1/4	1/4
10	0/4	0/4	0/4	0/4	0/4	0/4	0/4
11	0/4	0/4	0/4	0/4	0/4	0/4	0/4
12	0/4	0/4	0/4	0/4	0/4	0/4	0/4
13	0/4	0/4	0/4	0/4	0/4	0/4	0/4
14	0/4	0/4	0/4	0/4	0/4	0/4	0/4
15	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Remark: Numerator = number of tubes showing growth
Denominator = number of tubes seeded with organism suspension

A confirmation test on the above data was carried out with the time range from two to ten seconds. The results were listed in Table VI.

TABLE VI

Confirmation test of ultraviolet radiation on the viability of Mycobacterium tuberculosis var. homonis strain H37

Irrd. period (sec.)		Oct. 3' 48	10	17	24	31	Nov. 7
Control 0		4/4	4/4	4/4	4/4	4/4	4/4
Experimental	2	4/4	4/4	4/4	4/4	4/4	4/4
	3	0/4	1/4	3/4	3/4	4/4	4/4
	4	0/4	0/4	3/4	3/4	3/4	3/4
	5	0/4	0/4	0/4	0/4	0/4	0/4
	6	0/4	0/4	2/4	2/4	2/4	2/4
	7	0/4	0/4	1/4	1/4	1/4	1/4
	8	0/4	0/4	1/4	1/4	1/4	1/4
	9	0/4	0/4	1/4	1/4	1/4	1/4
	10	0/4	0/4	0/4	0/4	0/4	0/4

Remark: Numerator = number of tubes showing growth
Denominator = number of tubes seeded with organism suspension

be due to the even distribution of the resistant organisms into each of the four tubes of medium.

The results recorded in Table VI, generally confirm the previous experiment that partial destruction of the organisms was manifested at 5 seconds irradiation and no visible growth was detected after 10 seconds exposure at the given conditions. However, it should be pointed out that a few especially resistant organisms might still have been alive for a certain length of time but could not initiate growth within six weeks at the given conditions.

Two of the 4 tubes seeded with organisms irradiated for six seconds and one of each of the 4 tubes seeded with organisms irradiated for seven, eight, and nine seconds respectively showed visible growth after 2 weeks incubation at the given conditions. This implied the possibility that some viable individuals of the organisms might exist in the inocula irradiated for five seconds and that on the other hand the number of the resistant, visble ones was not big enough to initiate visible growth at the given conditions.

The devitalizing time of the organisms of the same strain by ultraviolet radiation was much shorter than that reported by previous investigators. This difference was probably due to the film thickness of the organism suspension. In the previous investigators' experiments their film thickness was of a greater magnitude. As a result, the ultraviolet wave-lengths might not have been able to penetrate the depth of their organism suspension. The distance of the exposure surface to the source of the ultraviolet light (energy out-put

0.02 milliwatts of 2537Å per second) has been standardized at two inches. No attempt has been made to measure the energy absorption of the wavelength per square centimeter. It should be pointed out that by using artificial ultraviolet lamps emitting shorter wavelengths such as the one used by Levinson et al (1944) and making thinner film-thickness of the organism suspensions, shorter periods than 10 seconds irradiation would be expected to be enough to devitalize the organisms.

B. Immunization Experiments

It was assumed that the range between the pathogenicity and the antigenicity of M. tuberculosis var. hominis would be sufficiently wide to permit the use of ultraviolet wavelengths to slightly denature tuberculoproteins, tuberculolipides, and tuberculocarbohydrates. Some organisms tend to lose their virulence and/or their reproductivity and/or their viability without losing their antigenic capacity of the tuberculoproteins (Koch, 1891, Pinner, 1928 Lines, 1929; Seibert, 1932; Heidelberger and Menzel, 1934), phosphatide fractions of the tuberculolipides (Meyer, 1912, Bouet and Negre, 1923) or the tuberculocarbohydrates. The tuberculocarbohydrate fraction alone is not antigenic. Judging from their capacity to give a precipitin reaction in vitro with tuberculous serum at a dilution as high as 1:6,400,000 (Laidlow and Dudley, 1925; Heidelberger and Menzel, 1932), it is obvious that they play an important role in the process of antibody-stimulation during the development of acquired resistance of the host to the tubercle bacilli (Rich, 1944).

It was speculated that by exposing the tubercle bacilli at the conditions as mentioned in the section of "Materials and Methods" for ten seconds, the linkage of the tuberculocarbohydrates with some unknown protein fractions would not be broken. It was also hoped that the same bacilli might lose their pathogenic properties after five seconds irradiation while they were still viable. Therefore, two kinds of experimental tuberculosis vaccine were prepared by exposing to ultraviolet radiation for five and ten seconds respectively. The immunization effective against virulent human tubercle bacilli in guinea pigs with these two kinds of vaccine is shown in the following data:

1. The change in body weight: The data in Table VII showed the gradual increase in body weight of the experimental animals vaccinated with the tubercle bacilli irradiated for five seconds with the exception of the first week after vaccination and during the third week following the challenging dose.

It was assumed that crowding would have a harmful effect on the general health of the experimental animals but would not influence the immunization response to an appreciable extent. The data in Tables VIII, XII, and Figure 8 indicated that this was the case.

The data collected in Tables IX and X showed a general tendency of decreasing body weight in the group of control animals except Nos. 505, 506, and 507. The infection index and blood picture of these three controls as appeared in Tables XIV, XV, and Figure 13 showed severe miliary tuberculosis. It clearly indicates that body weight change can not be relied upon as the sole criterion in evaluating the potency of preventive

or therapeutic agents. The body weight is mainly controlled by the hormones secreted by the hypophysis, thyroid, adrenal, and Langerhan Island cells, provided of course, that feeding conditions and digestive system of the animal are normal. The fever caused by some fractions of the tuberculoproteins would, of course, consume the body weight of the patient. But there are other complicating factors compensating and regulating the mechanism of metabolic rate, subsequently influencing the body weight of the animals.

The data collected in Table X showed the gradual declining of the body weights of the control guinea pigs inoculated with organism under-irradiated. While the surviving male, No.402 and female No. 409 showed a slight increase in their body weight, but they still exhibited numerous progressive tuberculous lesions.

Table XI showed the increase of body weight in the male animals and the decrease of body weights of the females which had been inoculated with over-irradiated organisms (20 seconds). In view of the lower infection index and lower mortality rate for the male than the female in this control group it was assumed that the over-irradiated organisms induced a higher degree of partial immunity to the male than to the female. The reason for this difference between the sexes remained unexplained.

Figures 2, 3, 4, 5, and 6 show the body weight change of each of the vaccinated and control groups respectively. Figure 7 shows the comparison of body weight change among the various groups as a whole. Figure 8 shows the comparison of the final gain or loss of body weight among the various groups.

TABLE VII

Change of body weight (gm.) of guinea pigs for immunization test

(Organisms irradiated for 5 seconds)

Guinea Pig No.		Feb. 23	28	March 7	14	21	28	April 4	11	18	25	Final Difference
Male	301	467	432	465	404	468	520	567	560	560	569	+ 102
	302	487	465	492	Died							+ 5
	303	493	468	479	468	490	536	541	574	578	580	+ 87
	304	468	454	468	415	481	512	521	529	542	575	+ 107
	305	486	460	478	421	475	523	542	560	584	590	+ 104
	306	490	445	461	413	498	513	552	549	542	536	+ 46
Average Weight		481	454	474	424	482	521	546	554	561	570	+ 75
Female	307	468	440	456	401	434	468	498	479	463	498	+ 30
	308	483	466	472	416	484	512	566	649	620	659	+ 176
	309	496	452	469	403	495	561	592	685	668	686	+ 190
	310	450	416	454	408	483	564	598	605	586	589	+ 139
	311	453	408	442	413	492	572	571	591	632	650	+ 197
	312	479	414	456	422	480	491	482	521	532	546	+ 67
Average Weight		472	433	458	411	478	528	551	588	550	605	+ 133
Total Average Wght.		476	443	466	417	480	525	548	571	555	589	+ 104

Remark: + = increase of body weight
- = decrease of body weight

TABLE VIII

Change of body weight (gm.) of guinea pigs for immunization test

(Organisms irradiated for 10 seconds)

Guinea Pig No.		Feb. 23	28	March 7	14	21	28	April 4	11	18	21	25	Final Difference
Male	201	466	454	452	390	416	423	475	455	436	420	468	+ 2
	202	479	462	458	387	414	425	434	432	469	502	541	+ 62
	203	481	480	472	396	426	486	569	545	581	659	675	+ 194
	204	454	424	414	394	417	386	347	359	367	321	Died	- 103
	205	462	434	430	384	423	448	455	416	420	470	498	+ 64
	206	460	422	418	379	464	453	443	412	468	519	543	+ 121
	207	471	467	464	408	478	469	464	432	491	539	596	+ 129
	208	485	458	449	414	465	478	499	485	524	565	614	+ 156
	209	458	446	480	Died								+ 22
Average Weight		468	450	449	394	438	446	461	442	470	499	562	+ 72
Female	210	453	441	456	434	485	568	669	676	726	770	780	+ 327
	211	483	456	464	442	496	590	659	675	706	722	768	+ 285
	212	495	480	498	477	528	533	547	556	500	521	546	+ 51
Average Weight		477	459	473	451	564	564	625	636	644	671	698	+ 211
Total Average Wght.		471	452	455	410	472	478	506	495	518	546	603	+ 109

Remark: + = increase of body weight
- = decrease of body weight

TABLE IX

Change of body weight (gm.) of guinea pigs for immunization test

Control - organisms nonirradiated

Guinea Pig No.		Feb. 23	28	March 7	14	21	28	April 4	11	18	25	28	Final Difference
Female	505	498	482	490	466	483	498	534	596	602	614	616	+ 118
	506	485	475	483	360	442	458	464	501	540	556	564	+ 79
	507	476	463	452	398	459	483	502	548	Died			+ 72
Average Weight		486	473	475	408	461	480	500	548	571	585	590	+ 90
(Only one inoculation intraperitoneally)													
Female	71	496	416	422	432	446	456	462	425	384	Died		- 112
	72	487	402	412	406	368	325	Died					- 162
	73	495	428	416	420	451	462	435	465	412	389		- 106
Average Weight		499	415	417	419	422	414	449	445	398	389	___	- 127
Total Average Wght.		493	444	446	414	442	447	479	507	485	520	590	- 37

Remark: + = increase of body weight
- = decrease of body weight

TABLE X

Change of body weight (gm.) of guinea pigs for immunization test

Control - organisms underirradiated (2 seconds)

Guinea Pig No.		Feb. 23	28	March 7	14	21	28	April 4	11	18	25	Final Difference
Male	401	483	472	470	408	412	405	398	329	Died		- 154
	402	468	456	463	442	460	458	469	482	496	530	+ 62
	403	472	450	448	403	408	415	413	365	Died		- 107
	404	495	460	468	405	412	424	392	385	333	Died	- 110
	405	462	454	443	386	406	412	406	348	Died		- 114
	406	483	473	459	406	392	483	358	379	Died		- 104
	407	496	486	463	418	386	392	408	341	Died		- 155
Average Weight		480	464	460	410	411	427	406	376	415	530	- 97
Female	408	439	470	459	398	382	375	336	Died			- 153
	409	466	452	494	419	406	403	398	412	425	472	+ 6
	410	486	469	432	420	408	391	356	319	Died		- 167
	411	498	454	413	396	385	378	370	280	Died		- 218
	412	473	461	452	419	364	342	326	351	Died		- 122
Average Weight		482	461	450	410	389	378	357	341	425	472	- 131
Total Average Wght.		481	463	456	410	402	407	385	363	418	501	- 111

Remark: + = increase of body weight
- = decrease of body weight

TABLE XI

Change of body weight (gm.) of guinea pigs for immunisation test

Control - organisms overirradiated (20 seconds)

Guinea Pig No.		Feb. 23	28	March 7	14	21	28	April 4	11	18	25	Final Difference
Male	101	458	446	469	431	485	499	531	590	623	655	+ 197
	102	452	450	472	421	491	487	485	479	492	568	+ 116
	103	492	436	489	462	484	469	484	555	583	645	+ 153
	104	483	468	492	454	469	470	473	562	576	590	+ 107
	105	463	432	484	464	487	466	503	560	596	625	+ 162
Average Weight		470	446	481	446	483	478	495	549	574	617	+ 147
Female	107	458	413	445	403	408	404	417	427	401	383	- 75
	108	452	422	462	372	380	364	337	394	390	397	- 55
	109	454	431	432	383	391	382	399	359	Died		- 95
	110	464	424	454	396	384	363	379	376	368	Died	- 96
	111	492	468	478	362	359	364	354	432	447	458	- 34
	112	470	442	468	389	364	380	363	387	330	Died	- 140
Average Weight		465	433	457	384	381	376	375	396	387	413	- 52
Total Average Wght.		468	403	409	378	392	387	430	427	437	432	+ 95

Remark: + = increase of body weight
- = decrease of body weight

Figure 2

Curve of body weight changes in experimental guinea pigs
Organisms irradiated for 5 seconds

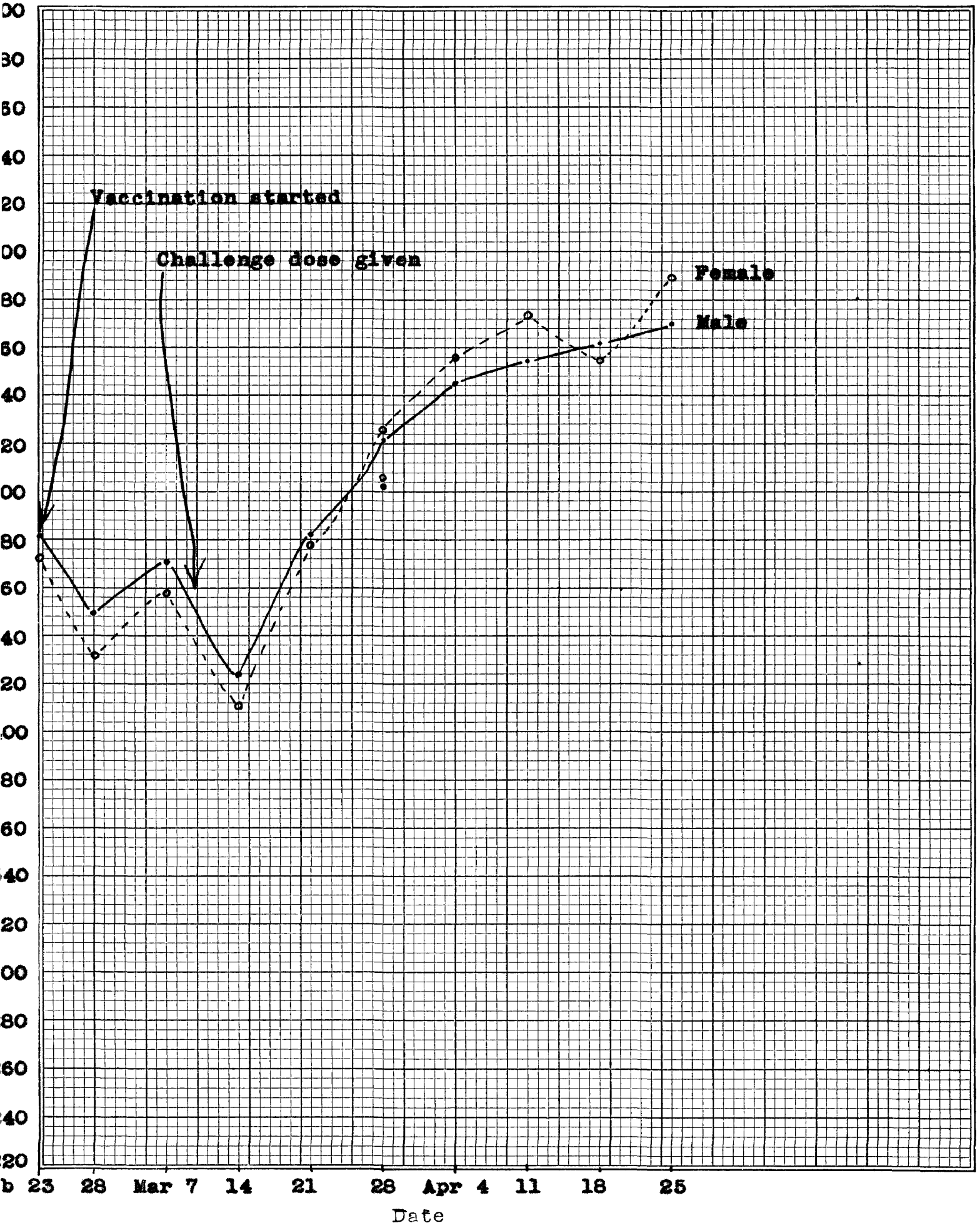


Figure 3

Curve of body weight changes in experimental guinea pigs Organisms Irradiated for 10 seconds

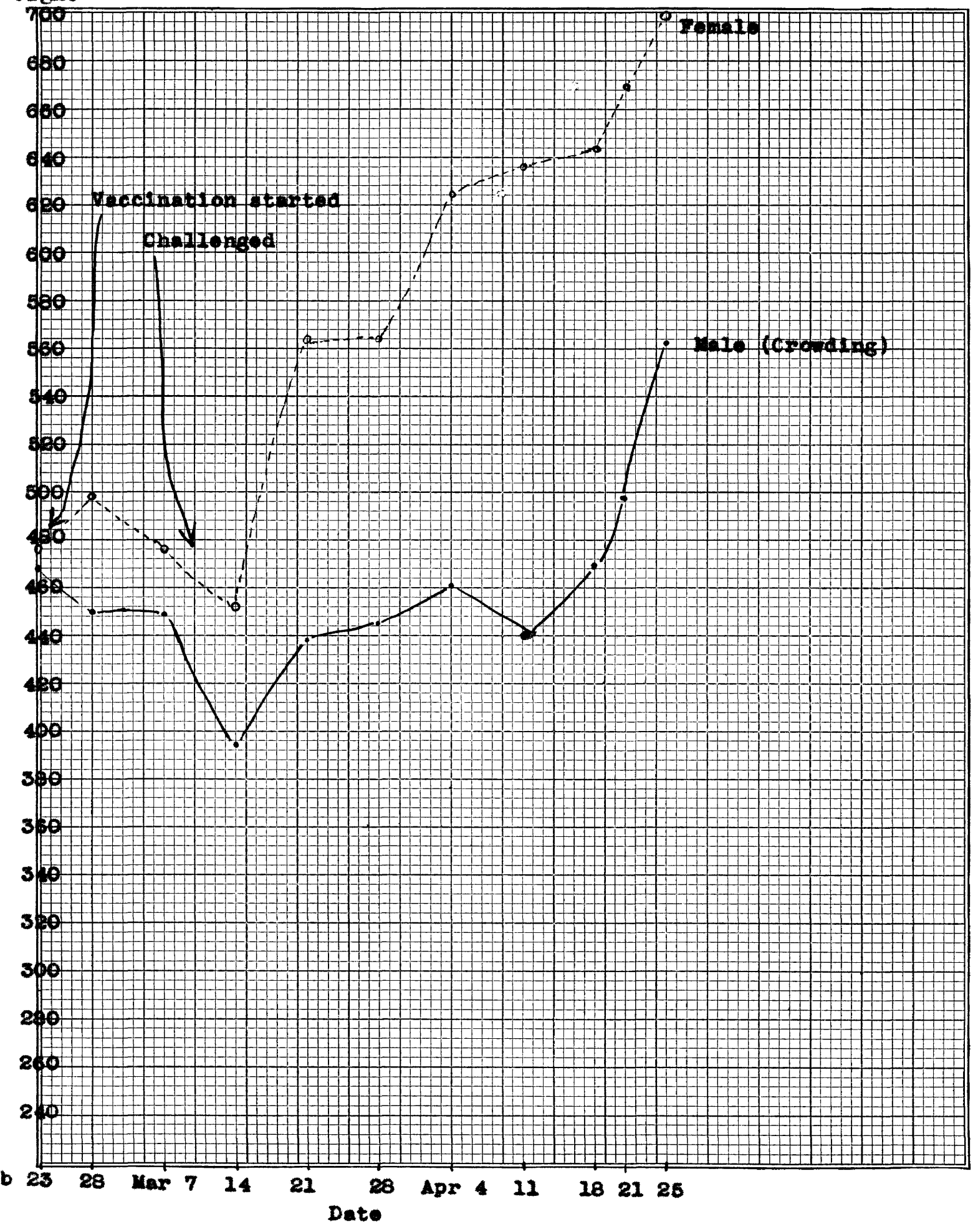


Figure 4
Curve of body change in control guinea pigs
(Organisms non-irradiated)

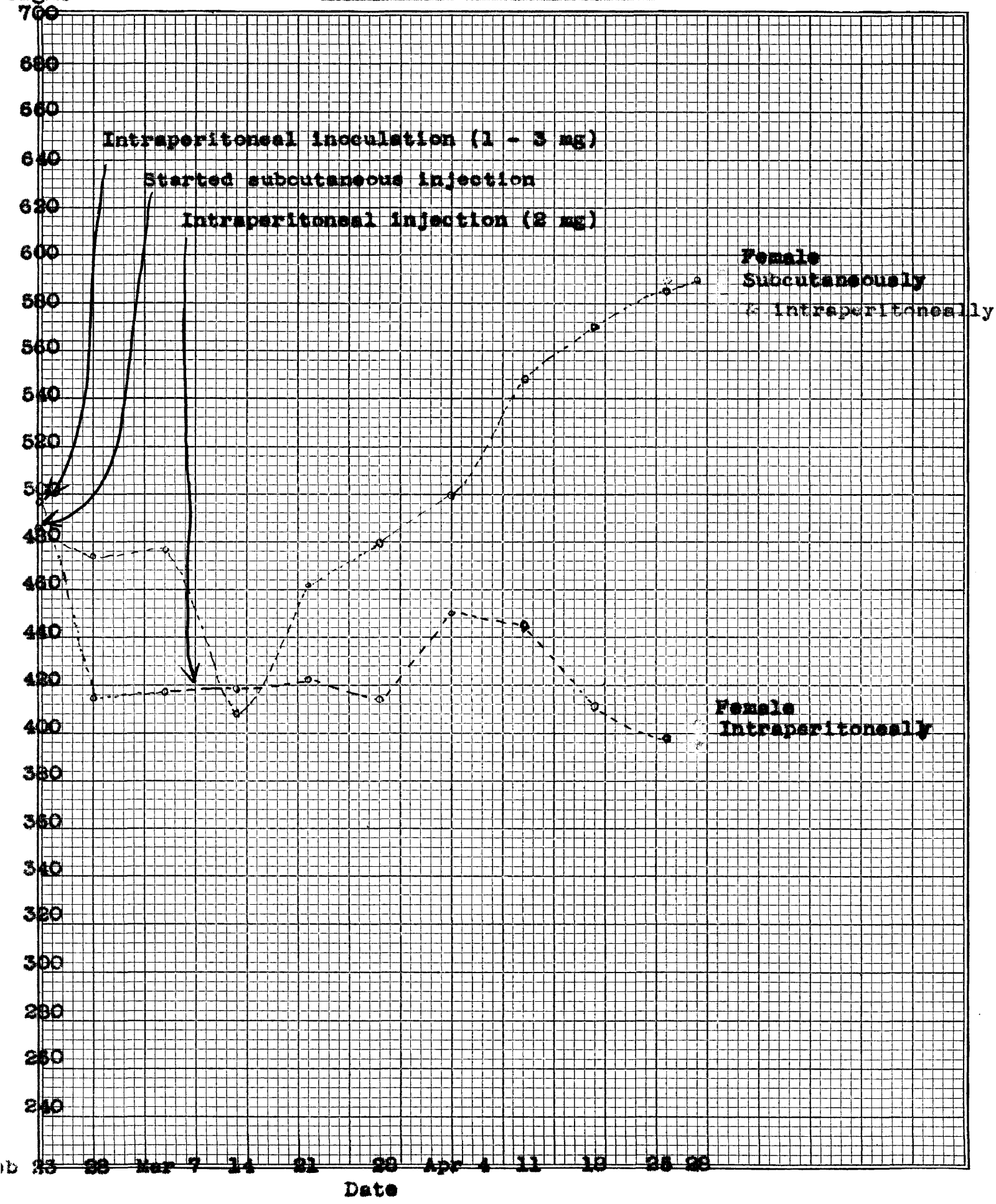


Figure 5

Curve of body weight changes in control guinea pigs
Organisms under-irradiated (2 seconds)

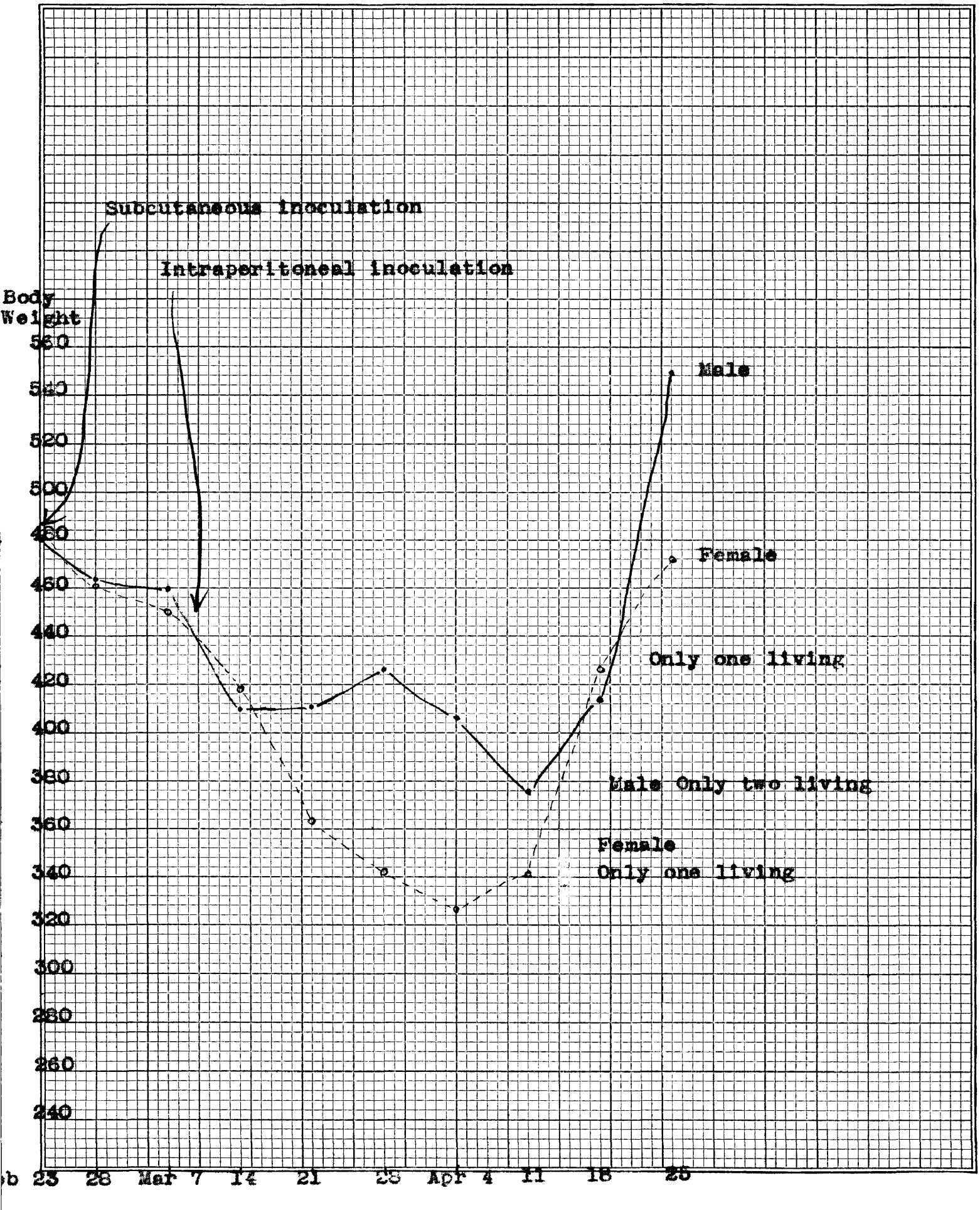


Figure 6
Curve of body change in control guinea pigs
(Organisms over-irradiated)

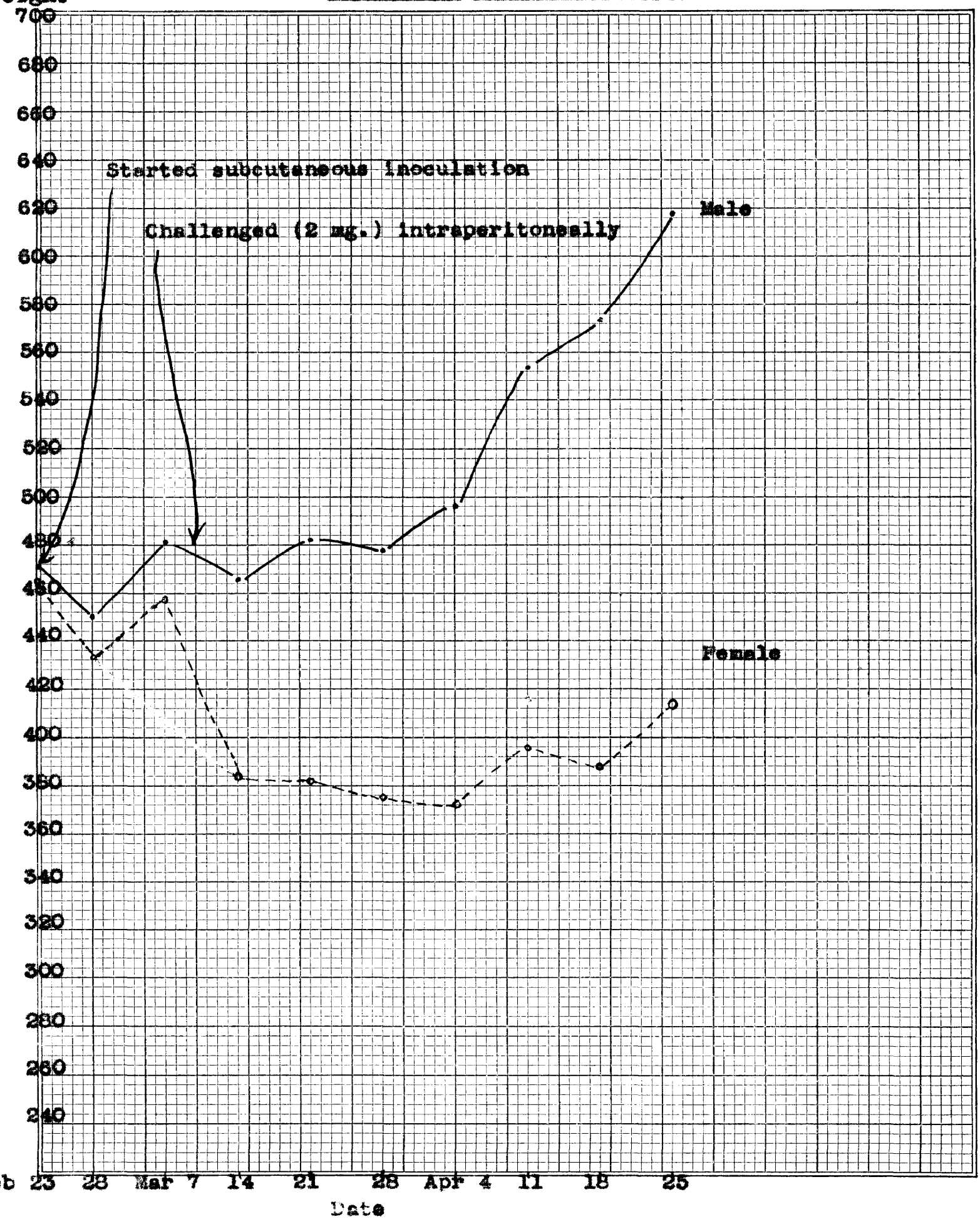


Figure 7

Comparison of total average body weight changes among various groups

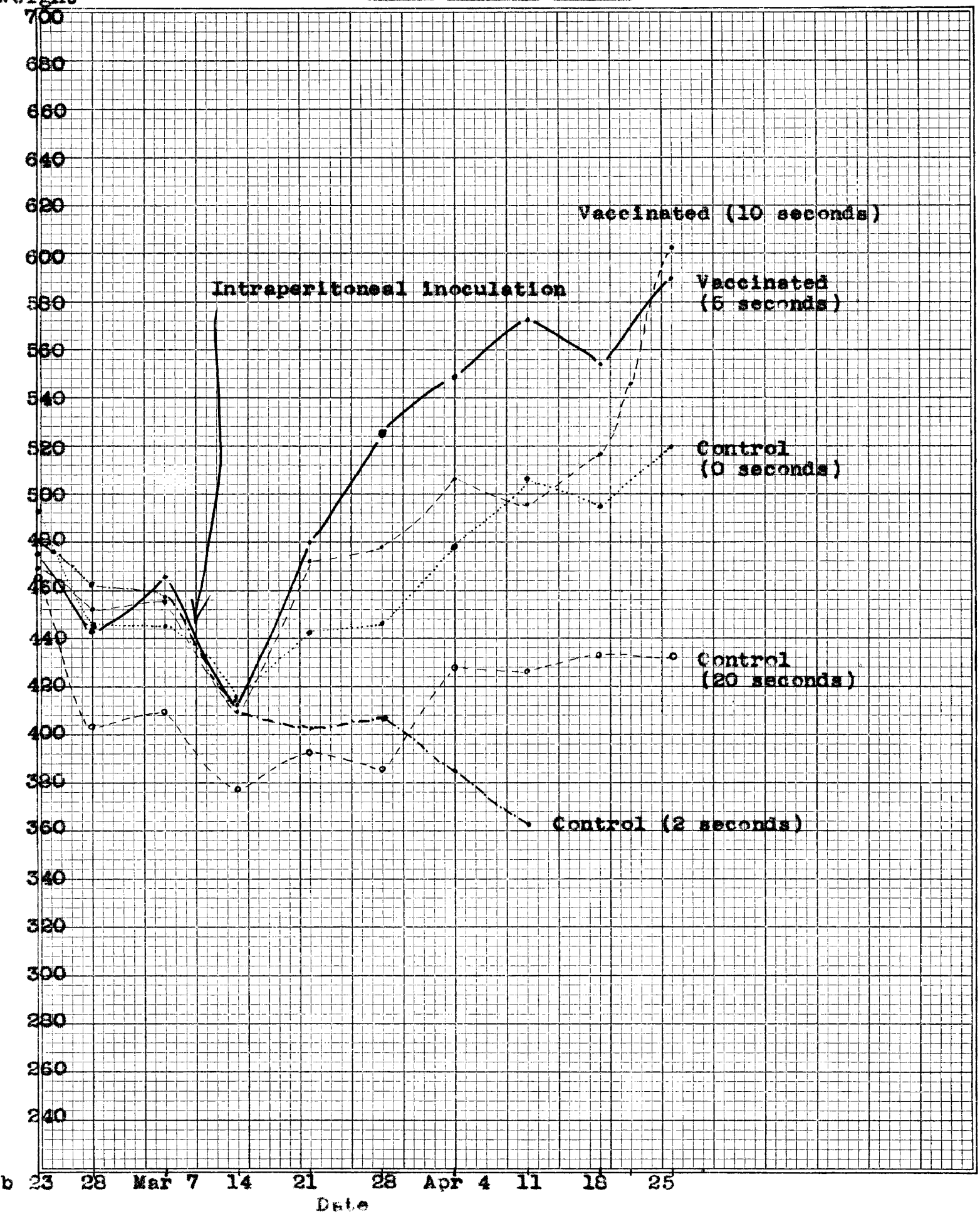
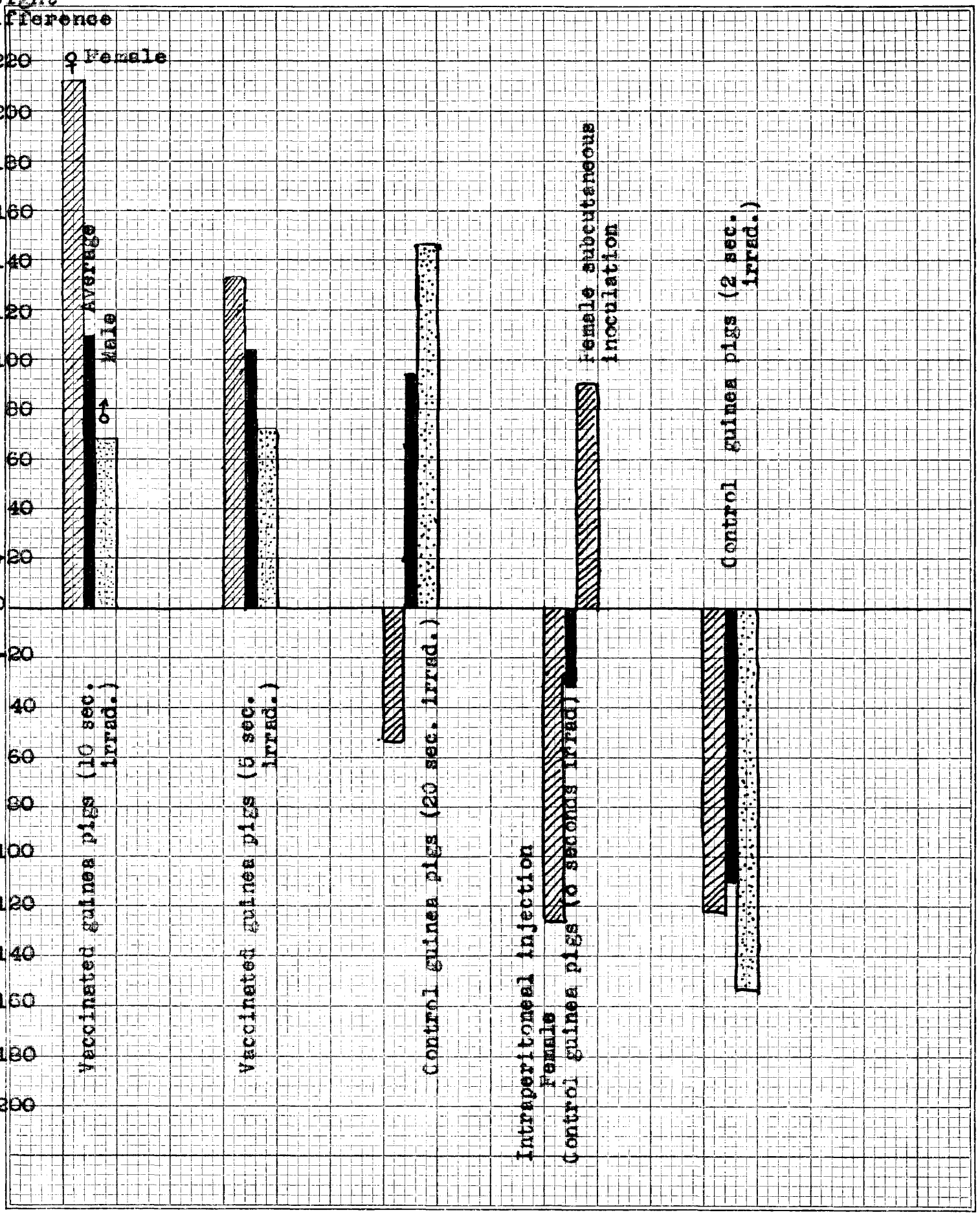


Figure 8
Final difference of average body weight among various groups



2. Tuberculin Sensitivity

As indicated in Table XII, three guinea pigs in each of the experimental groups showed tuberculin sensitivity reaction. Of the control guinea pigs which survived, two of them in the over-irradiated group and one of them in each of the non-irradiated (No.73) and under-irradiated (No. 409) groups manifested no tuberculin sensitivity reaction. However, the tuberculin-negative guinea pigs Nos. 73 and 409 were emaciated and feeble by the time of tuberculin test and exhibited severe tuberculous lesions at necropsy. The blood picture of these animals showed characteristic features of fatal tuberculosis.

In all of the tuberculin-positive guinea pigs in the experimental groups, a few epithelioid and lymphocyte infiltration tubercles could be found in some of the organs, such as the liver, spleen, peritoneum, and testicles. While in the control groups, with a few exceptions in the group inoculated with over-irradiated organisms, almost all of the tuberculin-positive guinea pigs manifested severe miliary tuberculosis.

Neither visible tuberculous lesions nor tuberculosis symptoms could be found in any of the tuberculin-negative guinea pigs with the exception of Nos.73 and 409 as mentioned above.

3. Blood Picture

As shown in Table XII the average hemoglobin value and blood cells count of the experimental groups were 13.8 gms/100 ml. blood for the group inoculated with the organisms irradiated for five seconds and 13.2 gms/100 ml. blood for the other group.

The erythrocyte count was higher in the former group than the latter. Some of the experimental guinea pigs showed a slight increase in their leucocyte count. In the same group, the hemoglobin value and erythrocyte counts of the male were generally higher than that of the female. The leucocyte differential count was within the normal range for most of the experimental guinea pigs with the exception of the seven shown in Table XIII. The abnormally high lymphocyte count and low monocytic-lymphocytic index indicated regressive tuberculosis. At necropsy, a few hard tubercles were found in one or two of the predilected organs of the guinea pigs with the exception of No. 201 in which case no tubercle was noted. The higher count of segmented neutrophils and stabs in No.201 guinea pig when compared with the rest (except No.210) indicated that its blood picture was shifted toward the normal status.

The control groups inoculated with non-irradiated and under-irradiated organisms generally showed a decrease of hemoglobin value and blood cells count with the exception of No.409 where an increase of leucocyte count was recorded. In the remainder of the control group inoculated with over-irradiated organisms, the hemoglobin value and blood cells count were about normal. (See Table XIV). However, the leucocyte differential count in all of the surviving guinea pigs showed a marked increase in young lymphocytes as shown in Tavle XV. No increase was noted in No. 73 or 409. This phenomenon indicated the retrogressive tendency of the chronic tuberculosis with rather optimistic prognosis.

Nos. 73 and 409 showed a marked "shift to left" of lymphocytes and an increase of monocytes with the monocytic-lymphocytic index 1.23 and 8.8 respectively.

As mentioned previously, these two guinea pigs were particularly emaciated and feeble by the time of necropsy.

4. Tuberculous Lesions and Infection Index

To terminate the experiment all of the surviving guinea pigs were killed. This sacrificing of animals was done 62-66 days following the first inoculation of the organisms. The animals were killed by removing 10 to 21 mls. of blood by cardiac puncture. Any visible tubercles observed were recorded in a schematic picture. Among the twenty-four experimental guinea pigs, six of them exhibited a few tubercles in either one or two of the predilection organs. The guinea pigs Nos. 209, 302, and 204 died on the fifteenth, nineteenth, and fifty-eighth day respectively after inoculation with the organism. No visible tuberculous lesions were found. Acid-fast bacilli were found in the smears from the liver of No. 209 but not that of Nos. 302 and 204. However, acid-fast bacilli were recovered in the Modified Lubos liquid medium by seeding the suspensions of macerated liver and spleen of No. 302.

At the necropsy of guinea pig No. 207, no visible pathological signs were manifested macroscopically. However, some small areas of lymphocytic infiltration were detected by histological examination (Figure 9). The few tubercles seen in the liver and spleen were found, later on, to be of epitheloid type with many Langhans giant cells (Figures 10 and 11). Severe hemorrhagic areas and cloudy degeneration of the spermatogenic cells were observed in

one of the tuberculous testicles (Figure 12).

The data of the infection index calculated and recorded in Tables XII and XIII was based on macroscopic and microscopic findings. These results are graphically presented in Figure 17.

At time of autopsy or necropsy, all of the control guinea pigs inoculated with non-irradiated and under-irradiated organisms exhibited soft, conglomerate tubercles in the liver, spleen, peritoneum, lungs, their adjacent lumph nodes and testicles with the exception or No.402. In this animal, no visible tubercles were found in the peritoneum, testes, liver or lungs. (Figure 13,13a, and 13b). However, a few small epithelioid tubercles were found in the macroscopically clean lungs and liver (Figures 14 and 15)

In the control group inoculated with over-irradiated organisms, guinea pigs No. 102 and 108 exhibited no visible tubercles. The infection index (Table XIV) was much lower than that of the other control groups (See Figure17). This fact would seem to indicate that the overirradiated organisms retained some remnants of the antigenicity which gave complete protection to these two guinea pigs and partial protection to most of the males.

TABLE XII

Tuberculin reaction, hemoglobin value, blood cells count, and infection index of vaccinated guinea pigs. Organisms irradiated for 5 and 10 seconds

Animal No. Irrad. 5 sec.	Tuberculin Test	Hb. value (gms/100 ml. blood	Erythrocyte count (per c.mm.)*	Leucocyte count (per c.mm)	Infection index
Male 301	pos.	14.9	6,120	11,850	15
302	-	-	-	-	0
303	neg.	13.0	5,240	9,700	0
304	neg.	15.1	6,320	9,950	0
305	pos.	13.6	5,690	9,200	1
306	neg.	13.9	5,800	12,100	0
Average		14.1	5,834	10,560	2.5
Female					
307	neg.	13.8	5,380	9,850	0
308	pos.	13.2	4,910	13,100	10
309	neg.	13.9	5,640	10,250	0
310	neg.	13.4	5,440	8,600	0
311	neg.	13.0	5,050	9,150	0
312	neg.	13.8	5,630	9,400	0
Average		13.8	5,338	10,058	1.7
Irrad. 10 sec.					
Male 201	neg.	12.9	4,920	8,950	0
202	neg.	13.6	4,960	13,200	0
203	neg.	13.9	5,680	12,450	0
204	-	-	-	-	0
205	neg.	14.0	5,640	9,300	0
206	pos.	12.9	4,980	8,750	11
207	pos.	13.6	4,790	13,600	1
208	neg.	14.1	5,700	9,500	0
209	-	-	-	-	0
Average		13.4	5,238	10,821	1.2
Female					
210	pos.	12.5	4,620	9,850	20
211	neg.	13.2	5,180	9,400	0
212	neg.	12.3	4,590	9,150	0
Average		12.7	4,796	9,467	6.7
Total Average		13.2	5,106	10,415	2.7

* unit = in thousands

TABLE XIII

Leucocyte differential count of vaccinated guinea pigs
organisms irritated for 5 and 10 seconds

Animal No. Irrtd. & sec.		Bas.	Eos.	Myel.	Juv.	Stabs.	Seg.	Lymph	Mono.	Mono. Lymph. Index
Male	301	0	0	0	0	2	9	87	2	0.02
	305	0	0	0	0	4	8	88	0	0.07
Female	308	0	0	0	1	5	13	79	4	0.05
Irrtd. 10 sec.										
Male	201	0	1	0	0	4	16	76	3	0.04
	206	0	0	0	0	0	1	98	1	0.01
	207	0	0	0	1	2	12	84	1	0.12
Female	210	0	0	0	0	4	23	57	16	0.38

TABLE XIV

Tuberculin reaction, hemoglobin value, blood cells count, and infection index of control guinea pigs organisms non-irradiated, under-irradiated, and over-irradiated

Animal No.		Tuberculin Test	Hb. value (gms/100 ml. blood)	Erythrocyte count (per c.mm.)*	Leucocyte count (per c.mm.)	Infection index
Non-irrad	505	pos.	11.8	4,310	6,800	100
	506	pos.	11.3	4,130	6,450	100
	507		-	-	-	100
	71		-	-	-	100
	72		-	-	-	100
	73	neg.	11.2	4,070	6,100	100
			11.4	4,166	6,417	100
Average						100
Under-irrad.						100
Male	401		-	-	-	100
	402	pos.	11.6	4,510	7,200	43
	403		-	-	-	100
	404		-	-	-	100
	405		-	-	-	100
	406		-	-	-	100
	407		-	-	-	100
Average			11.6			91.9
Female	408		-	-	-	97
	409	neg.	10.8	3,680	12,150	100
	410		-	-	-	100
	411		-	-	-	100
	412		-	-	-	100
Average			10.8			99.4
Total Average			11.2	4,095	9,675	99
Over-irrad.						
Male	101	pos.	14.2	5,940	7,650	3
	102	neg.	13.4	5,380	8,400	0
	103	pos.	13.6	5,640	8,950	23
	104	pos.	13.7	5,790	7,250	43
	105	pos.	13.4	5,650	6,850	30
Average			13.7	5,680	7,820	19.8
Female	107	pos.	11.9	4,630	6,500	100
	108	neg.	13.4	5,410	7,350	0
	109		-	-	-	98
	110		-	-	-	70
	111	pos.	13.8	5,820	9,250	33
	112		-	-	-	46
Average			13.0	5,280	7,700	57.8
Total Average			13.4	5,532	7,775	40.5

Table IV

Leucocyte differential count (%) of control guinea pigs
animals non-irradiated, under-irradiated, and
over-irradiated

Animal No.		Bas.	Eos.	Myel.	Juv.	Stabs	Seg.	Lymph	Monoc	Monoc. Lymph. Index
Non-irrad.										
Female	505	0	0	2	0	3	8	73	4	0.05
	506	0	0	1	0	4	11	72	12	0.17
	75	0	0	2	0	2	9	39	48	1.23
Average		0	0	[illegible]	0	3	9	61	21	0.48
Under-irrad.										
Male	402	0	0	0	0	2	4	84	10	0.12
Female	409	0	0	0	0	1	1	10	88	8.8
Average		0	0	0	0	2	3	47	49	4.46
Over-irrad.										
Male	101	0	0	0	0	4	18	75	3	0.04
	102	0	0	0	0	0	1	99	0	
	103	0	1	0	0	6	3	78	12	0.15
	104	0	0	0	0	0	2	84	14	0.17
	105	1	0	0	0	0	2	92	5	0.05
Average		0	0	0	0	2	5	81	7	0.08
Female	107	0	0	0	0	4	21	62	13	0.21
	108	2	6	0	0	3	22	57	12	0.21
	111	3	0	0	0	0	10	82	5	0.06
Average		2	2	0	0	2	18	67	10	0.16
Male and female total average		1	1	0	0	2	10	79	8	0.1

a

Figure 8
Schematic presentation of gross tuberculous lesions
at necropsy or autopsy
Vaccinated guinea pigs
1.Organisms irradiated for 5 seconds

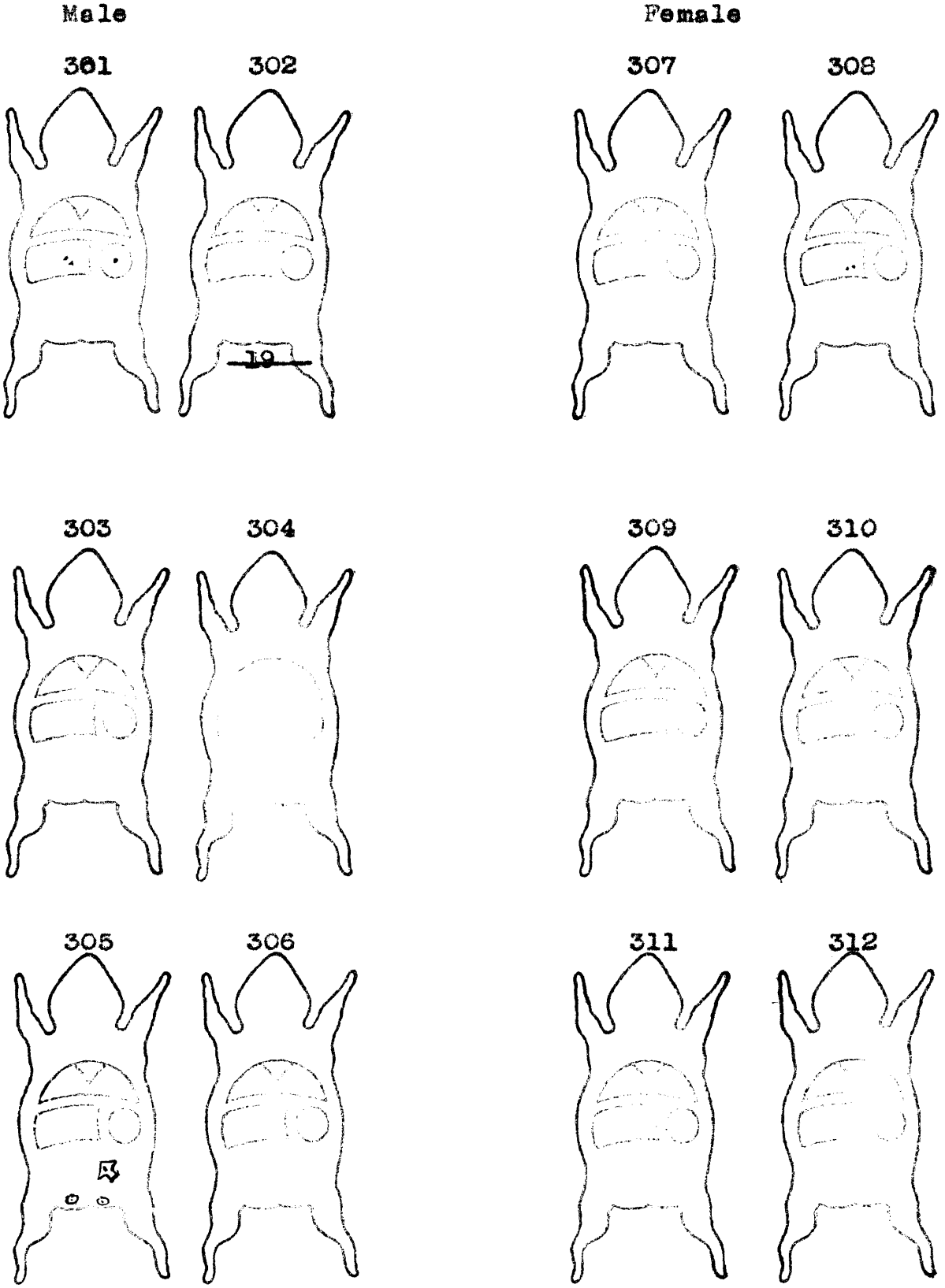


Figure 8a(Continued)
Schematic presentation of gross tuberculous lesions
at necropsy or autopsy
Vaccinated guinea pigs
2.Organisms irradiated for 5 seconds

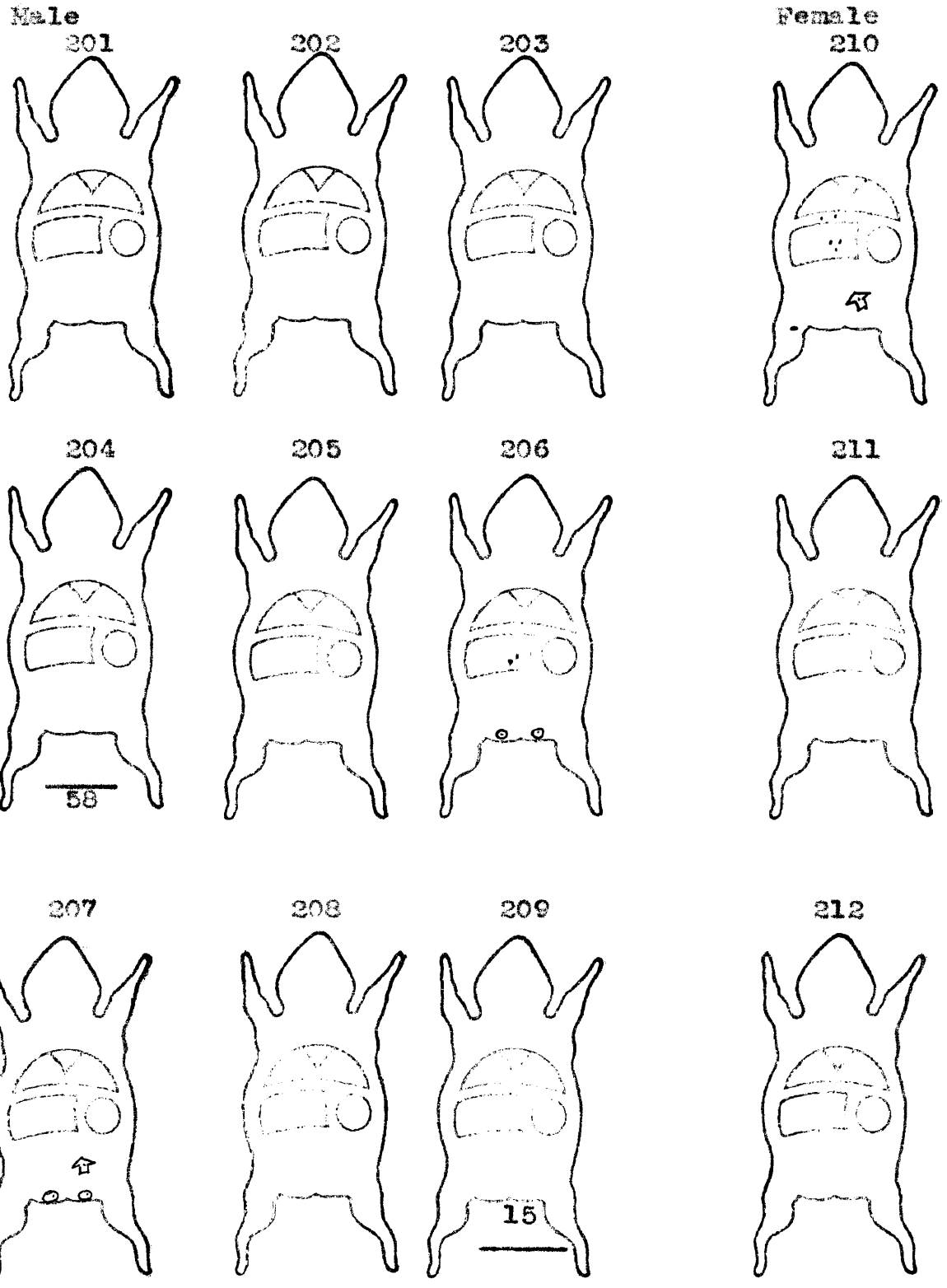
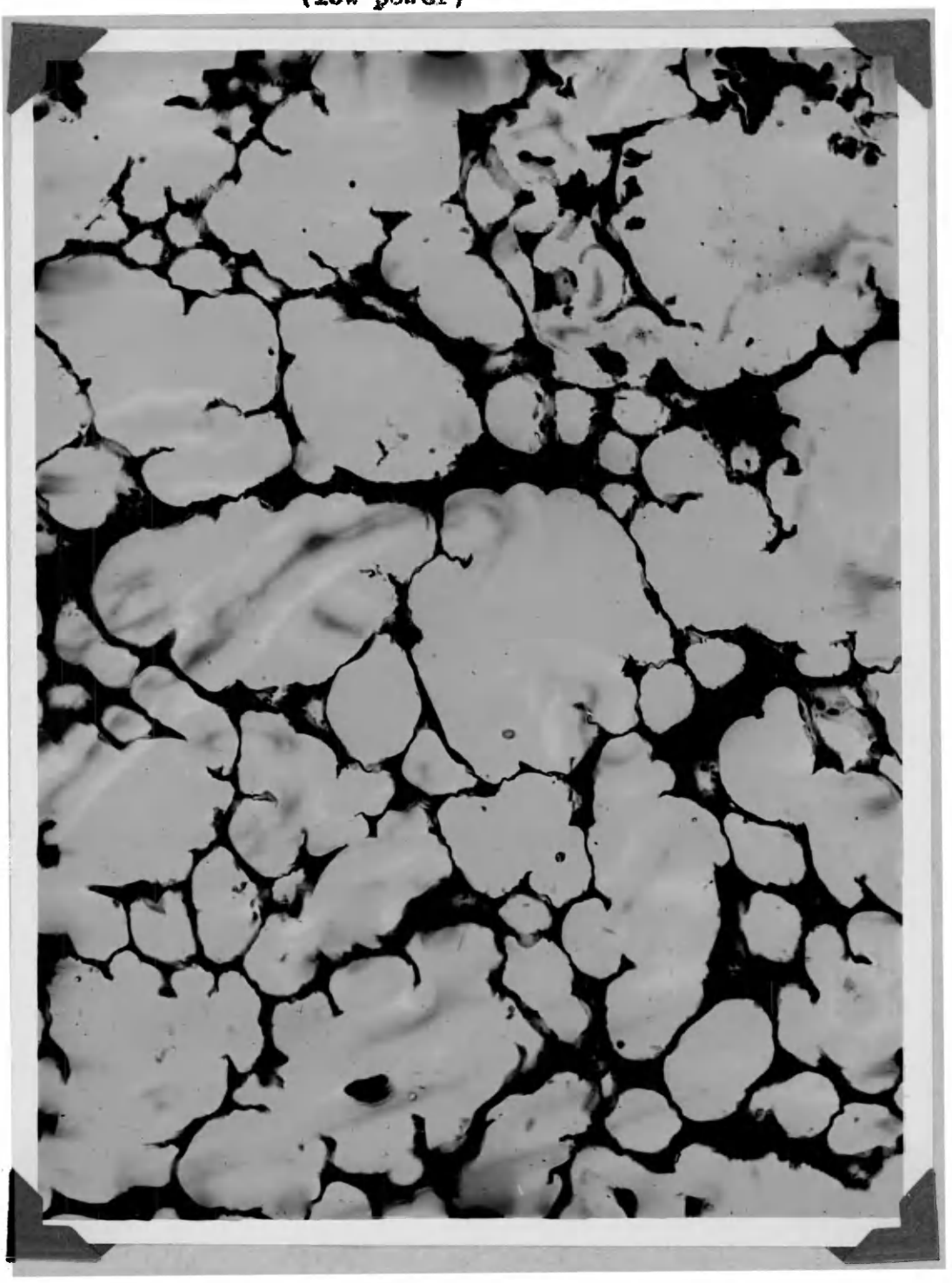


Figure 9
Lung (guinea pig No. 207) Photomicrograph
(low power)



Note the dark area of lymphocytic infiltration

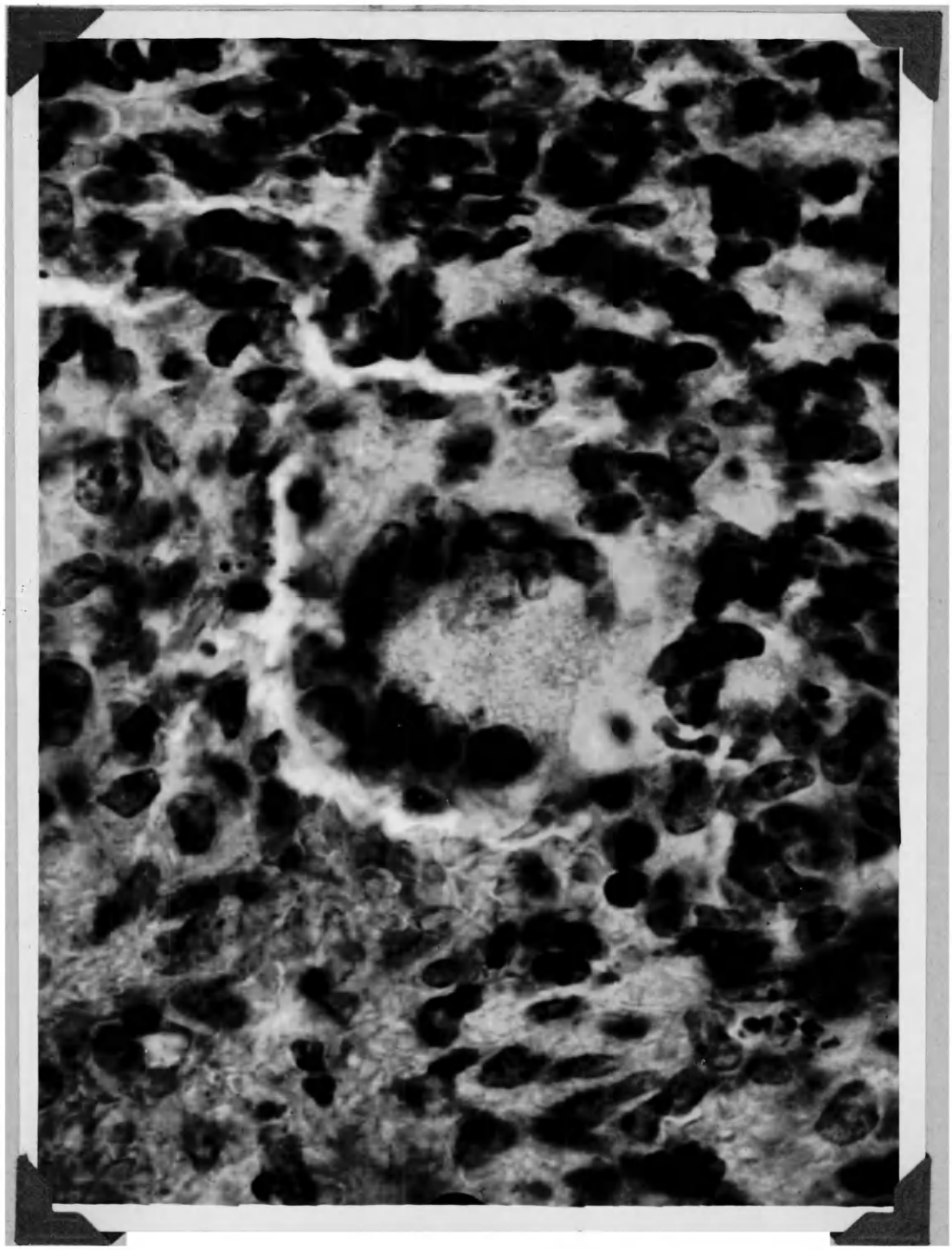
Figure 10

Liver(guinea pig No.210) Photomicrograph
High power

Note the epithelioid tubercle replacing the hepatic parenchyma

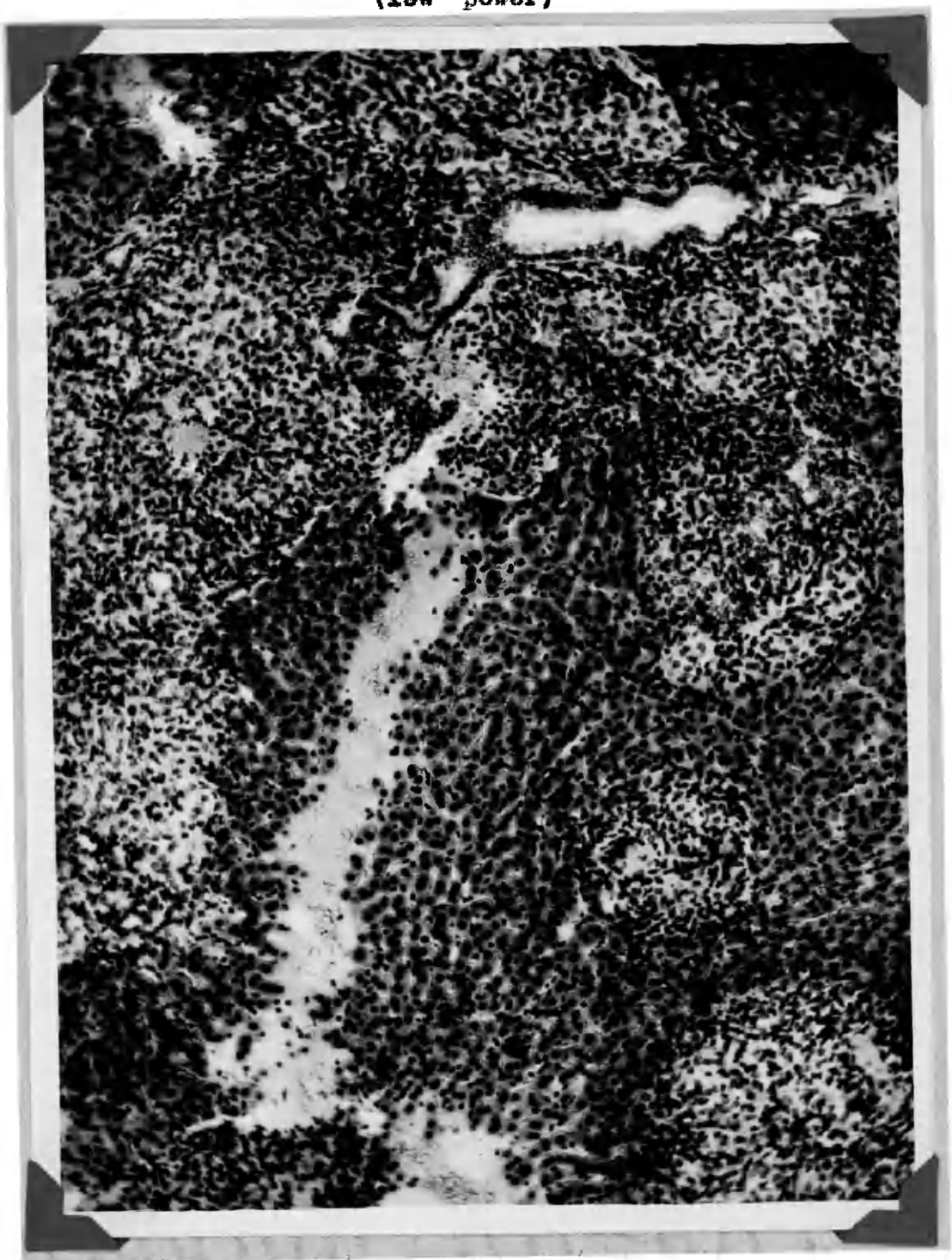
Figure 11

Spleen (guinea pig No. 301) Photomicrograph
High power



Note the Langhans giant cells

Figure 12
Testis(guinea pig No.306) Photomicrograph
(low power)

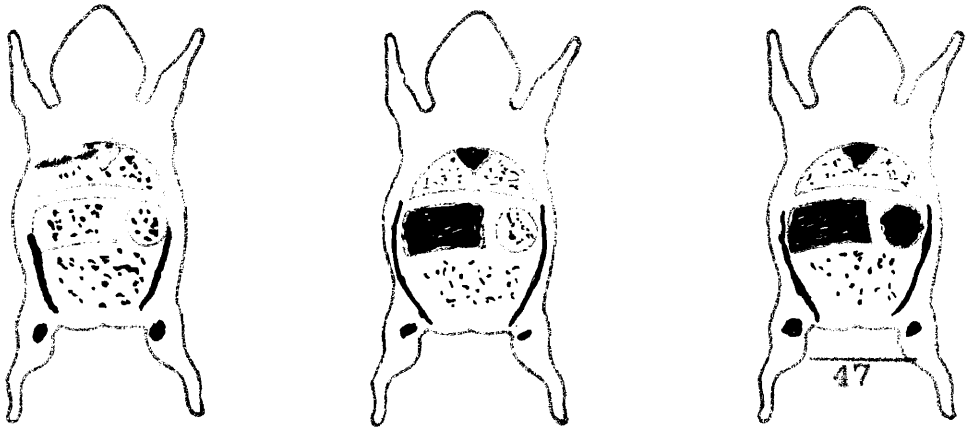


Note the hemorrhagic area,shrunken convoluted seminiferous tubules,and the cloudy degeneration of the spermatogenic cells

Figure 13
Schematic presentation of gross tuberculous lesions
at necropsy or autopsy
Control animals
1. Organisms non-irradiated

Female

505 506 507



Female

71 72 73

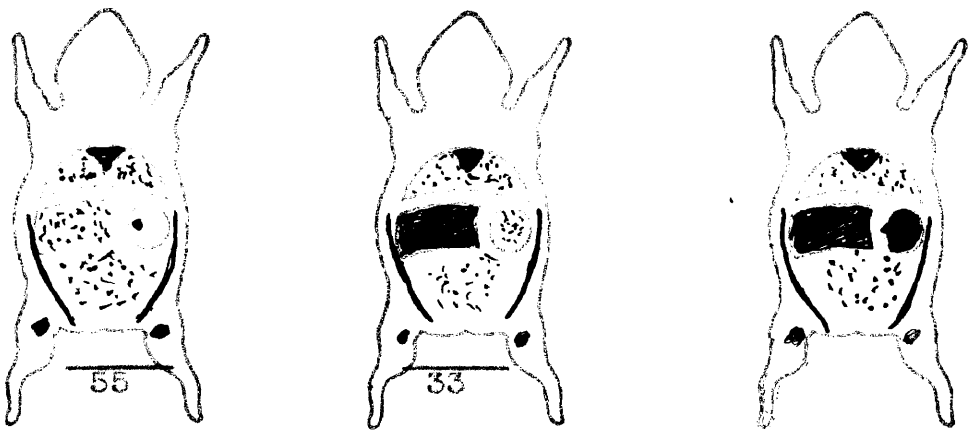
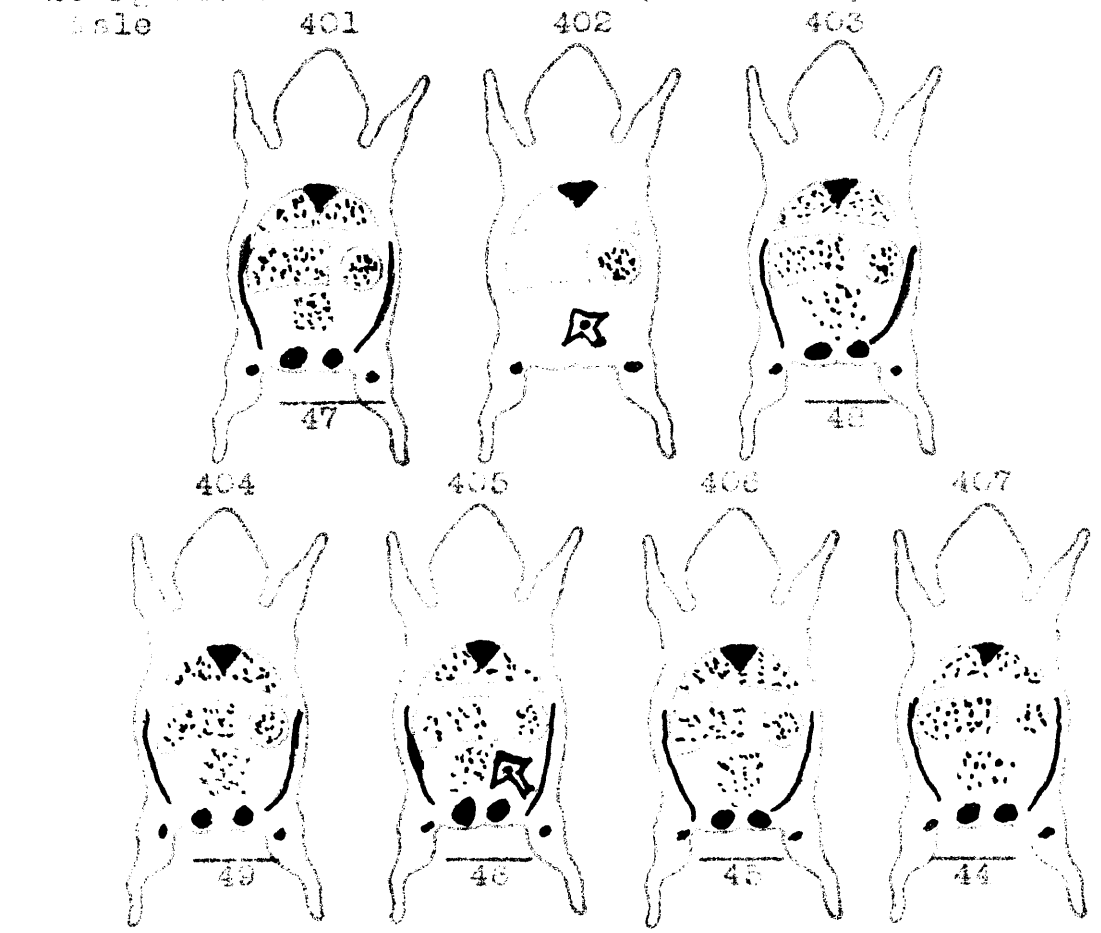


Figure 13(continued)
Schematic presentation of gross tuberculous lesions
at necropsy or autopsy
Control animals
2. Organisms under-irradiated(2 seconds)

Male



Female

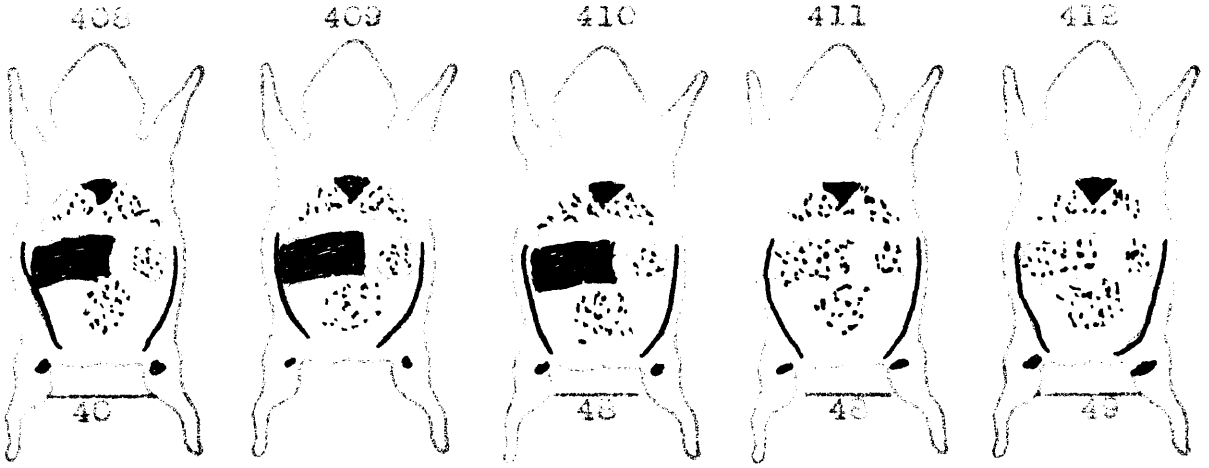


Figure 13a
Control guinea pig died of tuberculosis(Photograph)

Note the tubercles on lungs,liver,spleen,and intestine(Part of the liver and spleen has been removed to make tissue sections and suspensions).

Figure 13b
Liver and spleen of control guinea pig died of tuberculosis (photograph)

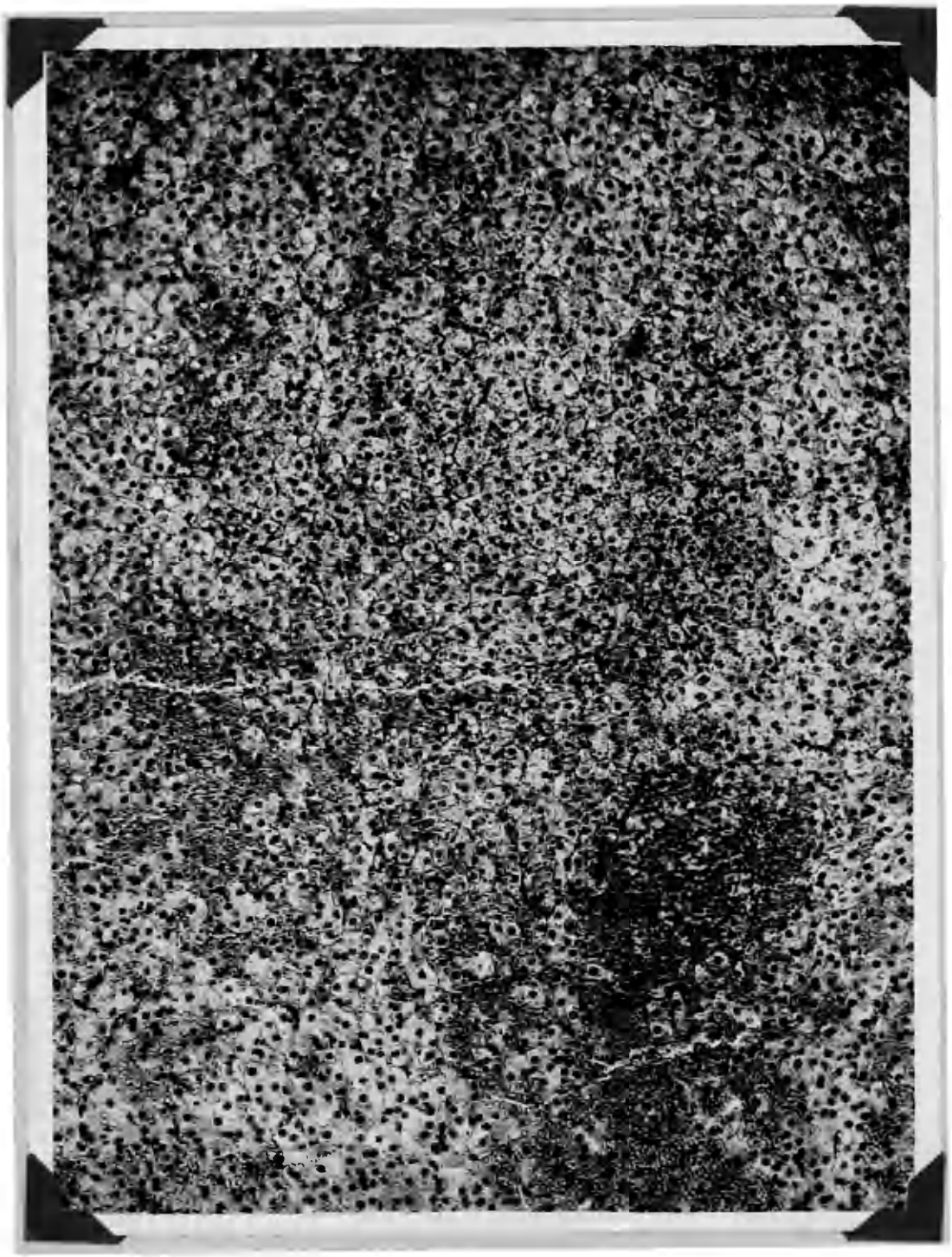
Note the conglomerate caseous tubercles(part of the liver and spleen has been removed for preparing tissue sections and suspensions)

Figure 14

Lung(guinea pig No.402) Photomicrograph
(low power)

Note the progressive epithelioid tubercules and the macrophages in the air sacs

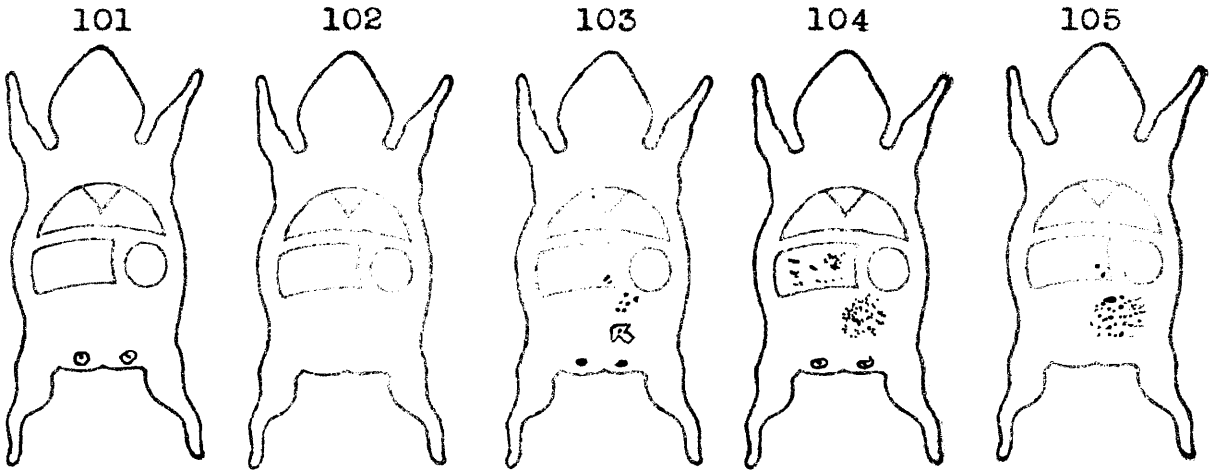
Figure 15
Liver (guinea pig No.402) Photomicrograph
(low power)



Note the epithelioid tubercles replacing the hepatic parenchyma

Figure 16
Schematic presentation of gross tuberculous lesions at necropsy or autopsy
Control animals
3.Organisms over-irradiated (20 seconds)

Male



Female

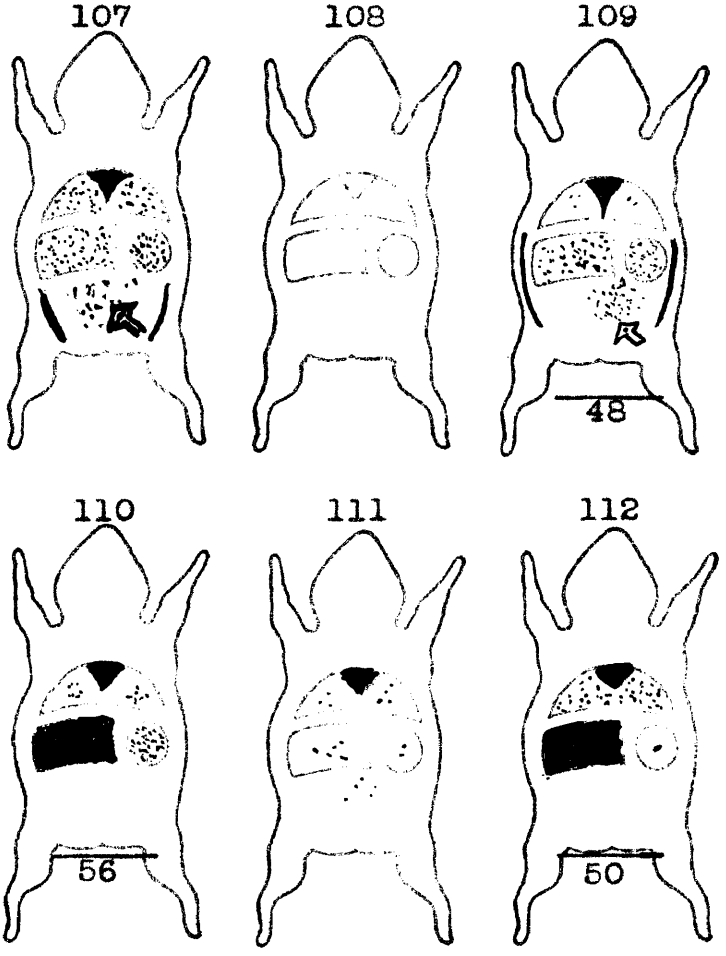
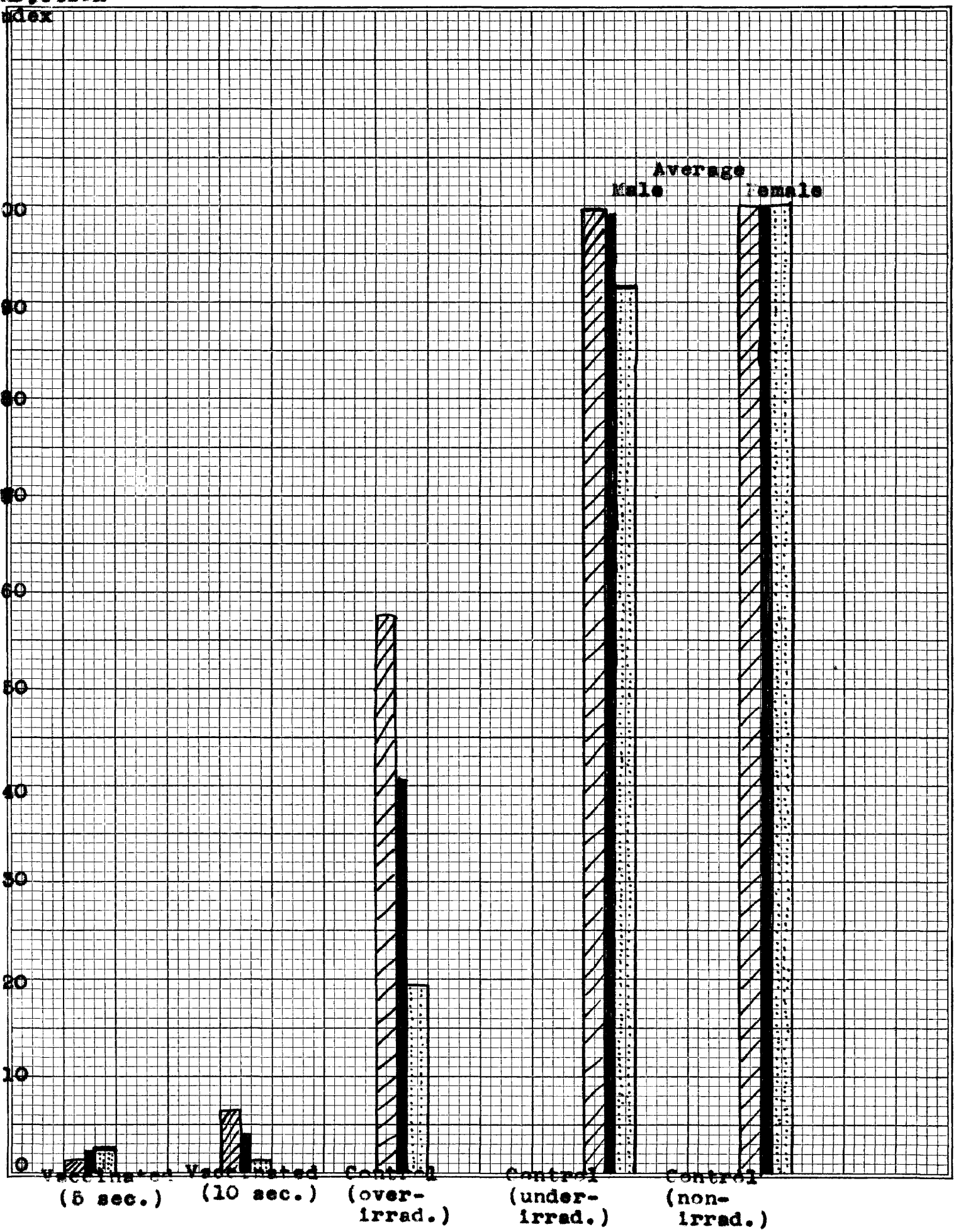


Figure 17
Infection Index



5. Mortality rate

The higher mortality rate in the control group inoculated with under-irradiated organisms than that with non-irradiated organisms might be due to the fact that the underirradiated organisms, after being stimulated by the ultraviolet radiation without being harmfully affected, multiplied more rapidly than the non-irradiated organisms. Consequently, they killed the guinea pigs quicker than the non-irradiated ones did (Table XVI).

The difference of the mortality rate among the various groups and between the different sexes is graphically presented in Figure 18.

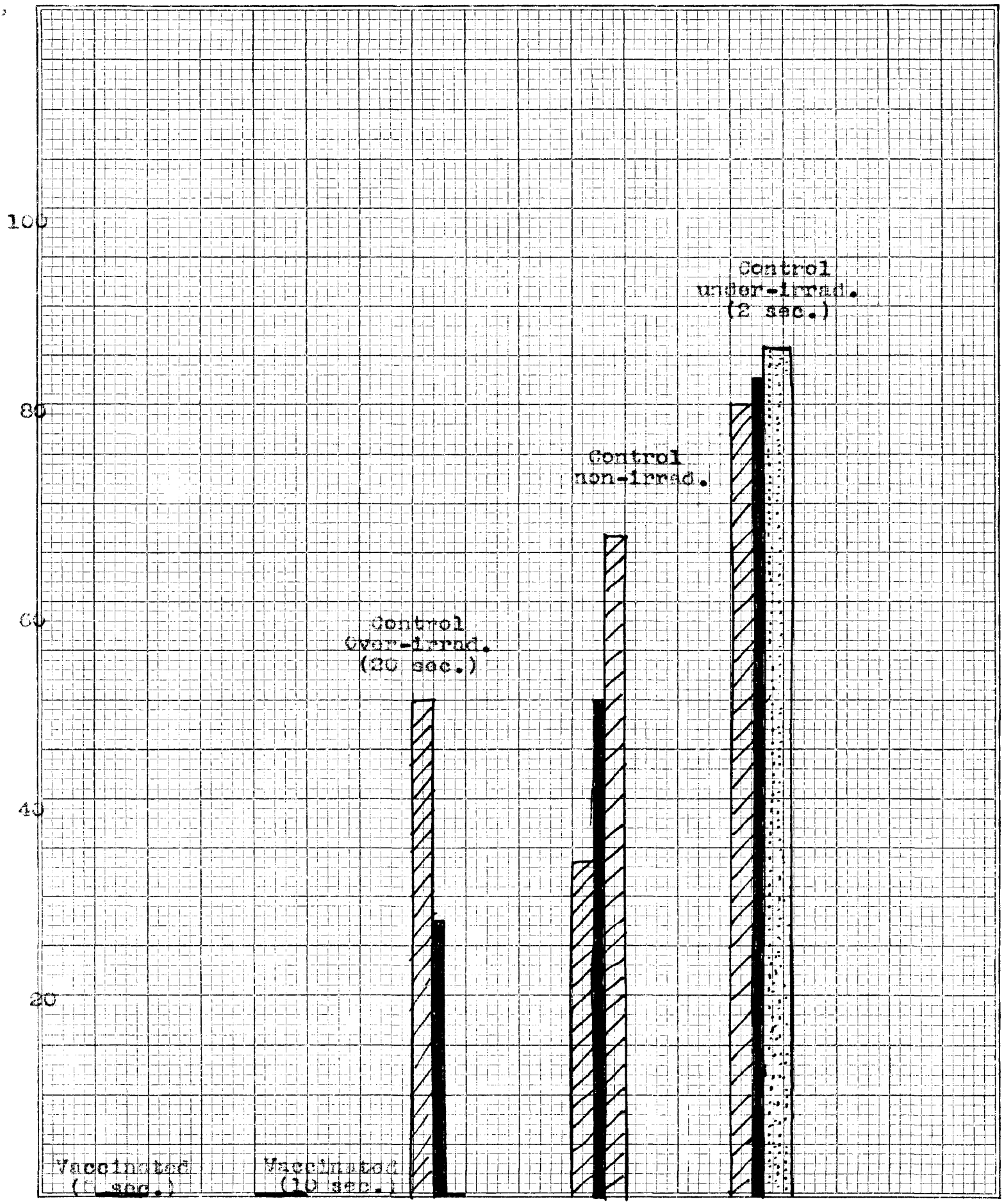
TABLE XVI

Mortality

Experimental		No. of g. pigs died of T.B. / No. of g. pigs inoculated	Mortality rate (%)
I. Vac. with organisms irrad. for 5 sec.	Male	0/6	0
	Female	0/6	0
II. Vac. with organisms irrad. for 10 sec.	Male	0/6	0
	Female	0/6	0
	Total	0/12	0
Control			
I. Organisms non-irrad.	Subcutan.	1/3	33.3
	Intraperit.	2/3	66.7
	Total	3/6	50.0
II. Organisms under-irrad. (2 sec.)	Male	6/7	85.7
	Female	4/5	80.0
	Total	10/12	82.8
III. Organisms over-irrad. (20 sec.)	Male	0/5	0
	Female	3/6	50
	Total	3/11	27.3

Remark: The guinea pigs died of diseases other than tuberculosis were excluded.

Figure 18
Mortality Rate



C. Subtilin Treatment

1. The body weight change:

The data for body weight change in the first experiment are recorded in Table XVII and graphically presented in Figure 19. They show the gradual increase in body weight following the administration of subtilin with the total average final gaining of twenty-nine grams for group I and seventy-eight grams for group II.

Table XVII

CHANGE OF BODY WEIGHT (gm.) OF TUBERCULOUS GUINEA PIGS TREATED WITH SUBTILIN

FIRST EXPERIMENT

Guinea Pig No.	Feb.23	28	Mar.7	14	16	23	30	Apr.6	13	20	27	Final difference
Group I												
501	455	431	385	324	307	354	437	465	necropsy on April 13th.			10
502	468	448	406	361	308	348	404	459	468	482	516	48
Average Weight	462	440	396	342	308	351	421	462	468	482	516	29
Group II												
503	473	463	419	370	328	383	435	451	473	496	528	55
504	492	485	470	406	349	421	491	542	556	584	592	100
Average Weight	483	474	445	388	339	402	463	497	515	540	560	78
Group III (control)												
505	see table IX											
506	" " "											
507	" " "											

The data of body weight changes in the second experiment (Table XVlll,Figure 20) showed a general tendency for weight loss with the exception of group 1 where a slight increase in weight was indicated.

A review of the average final difference in the body weight among the various groups of guinea pigs (Figure 21) revealed the fact that the dosage of 30 mg. (or 600 units) and 20 mg. (or 400 units) of the partially purified subtilin administered through the interperitoneal route twice a week was slightly toxic to the experimental guinea pigs employed.

2. Tuberculin test:

Tuberculin tests on the surviving guinea pigs treated with subtilin two to five days before necropsy manifested positive sensitivity reaction with the exception in animal No.34 where no reaction was observed. Guinea pig No.34 exhibited a feeble and emaciated appearance.

3. Leucocytes Differential Count:

As shown in Table XlX, the monocytic-lymphocytic index of all the survived guinea pigs was much higher than the normal figure with the exception of Nos. 501,502,22,and42. The high figure of the monocytic-lymphocytic index generally indicated a grave prognosis.

TABLE XVIII

Change of Body Weight (Gm.) of Tuberculous Guinea Pigs Treated with Subtilin Second Experiment

Guinea Pig No. / Weekly dose (20 mg.) / Group I		March 21	28	April 4	11	18	25	Final Diff.
Number	11	466	413	424	477	419	496	↗ 30
	12	423	402	440	465	471	485	↗ 62
	13	425	396	502	434	447	465	↗ 30
	14	495	407	494	485	496	498	↗ 3
	15	408	359	472	419	417	406	- 2
Average Wt.		443	395	466	456	462	470	↗ 27
Weekly dose (40 mg.) Group II								
Number	21	496	413	489	479	484	502	↗ 6
	22	434	392	432	477	479	493	↗ 59
	23	409	398	457	430	-	-	↗ 21
	24	463	421	459	408	358	-	↗105
	25	425	334	343	368	359	-	- 66
Average Wt.		445	392	436	432	420	498	- 17
Group III No.	31	458	423	460	354	331	-	-127
	32	479	437	461	-	-	-	- 18
	33	483	425	436	420	398	343	- 40
	34	491	463	504	492	423	374	- 17
	35	402	338	351	421	360	-	- 42
Average Wt.		463	417	442	422	378	358	- 48
Group IV No.	41	450	408	440	451	467	454	↗ 4
	42	474	431	478	505	494	463	- 11
	43	493	420	-	-	-	-	- 73
	44	482	421	446	-	-	-	- 36
	45	431	419	481	504	460	424	- 7
Average Wt.		466	420	461	487	474	447	- 25

Table XIX

LEUCOCYTE DIFFERENTIAL COUNT OF THE SURVIVING GUINEA PIGS FOR SUBTILIN TREATMENT TEST

Animal	No.	Bas.	Eos.	Myel.	Juv.	Stabs	Seg.	Lymph.	Monoc.	Monocytic-lymphocytic index
1st exp.	501	-	-	-	-	-	-	-	-	-
	502	0	0	0	1	1	21	67	10	0.15
	503	0	0	0	0	0	1	89	10	0.11
	504	0	0	0	0	1	4	36	59	1.64
Average		0	0	0	0	1	9	64	26	0.63
2nd exp.										
Weekly dose	: 20 mg. (or 400 units)									
	11	0	0	0	0	1	3	11	85	7.73
	12	0	0	0	0	2	7	54	37	0.69
	13	-	-	-	-	-	-	-	-	-
	14	0	0	0	1	1	12	16	70	4.38
	15	0	0	1	0	15	13	55	16	0.29
Average		0	0	0	0	5	9	34	52	3.27
Weekly dose	: 40 mg.(or 800 units)									
	21	0	0	0	1	12	18	24	45	1.88
	22	0	0	0	2	9	12	66	11	0.17
Average		0	0	0	2	11	15	45	28	1.03
Weekly dose	: 60 mg.(or 1,200 units)									
	33	0	1	0	1	2	12	35	14	0.40
	34	0	3	0	0	2	36	8	51	6.38
Average		0	2	0	1	2	24	22	33	3.39
Control										
	41	0	0	0	0	29	6	41	24	0.59
	42	0	0	0	1	5	27	57	10	0.18
	45	0	0	0	0	3	5	29	63	2.17
Average		0	0	0	0	13	13	42	32	0.98

Figure 19

Average body weight change of tuberculous guinea pigs treated with subtilin. First experiment

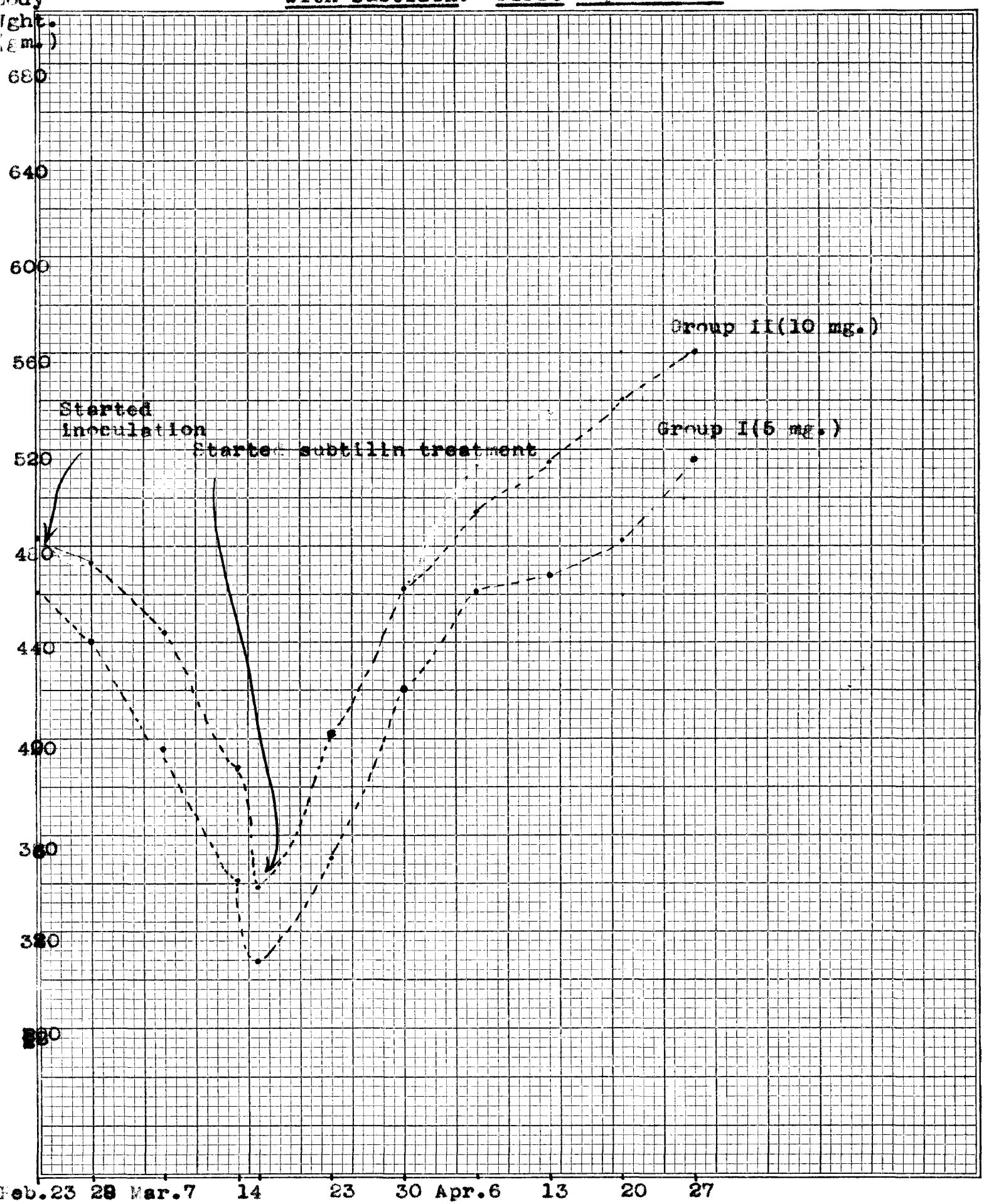


Figure 25

Curve of average body weight change of tuberculous guinea pigs
Second experiment of subtilin treatment

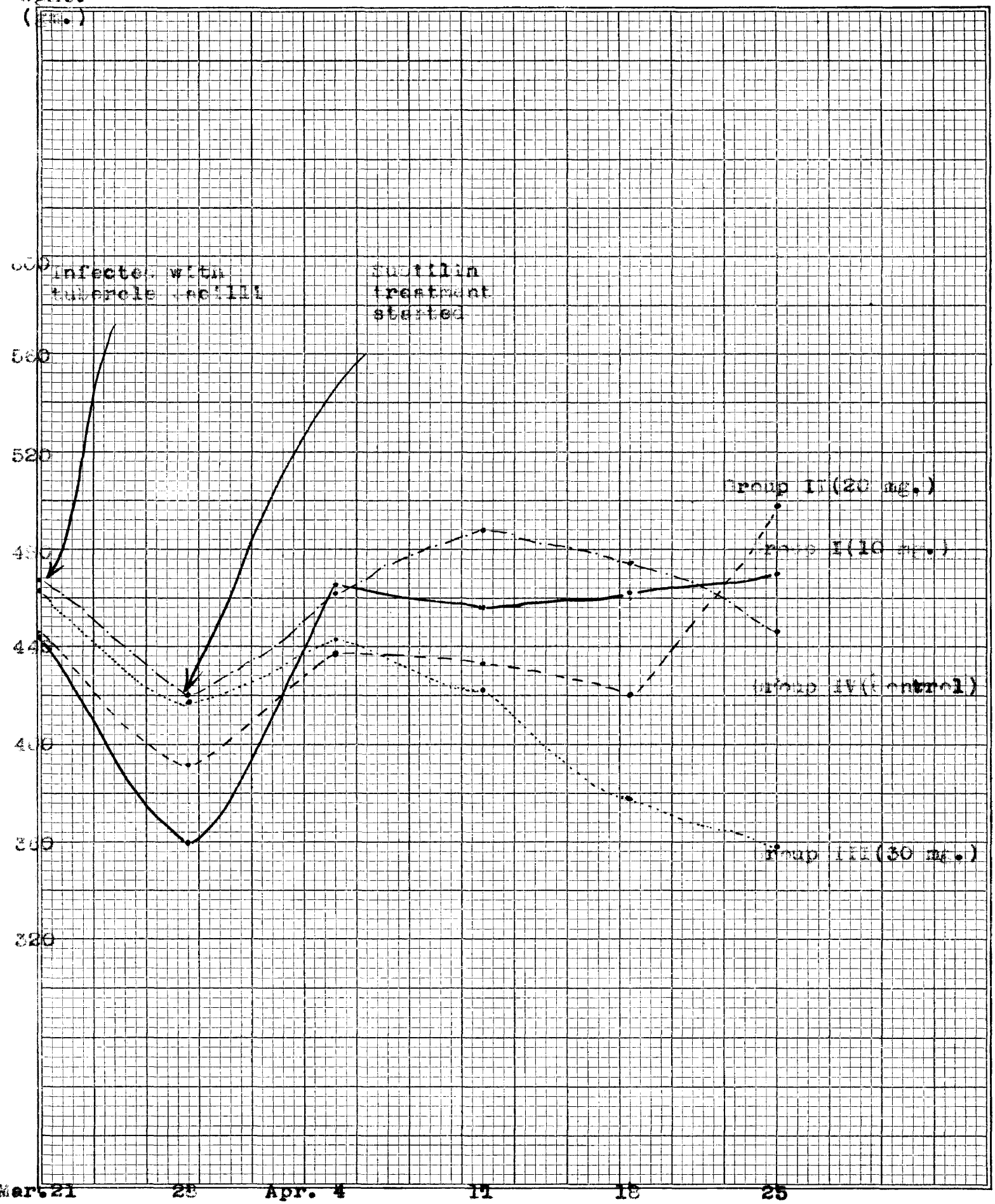
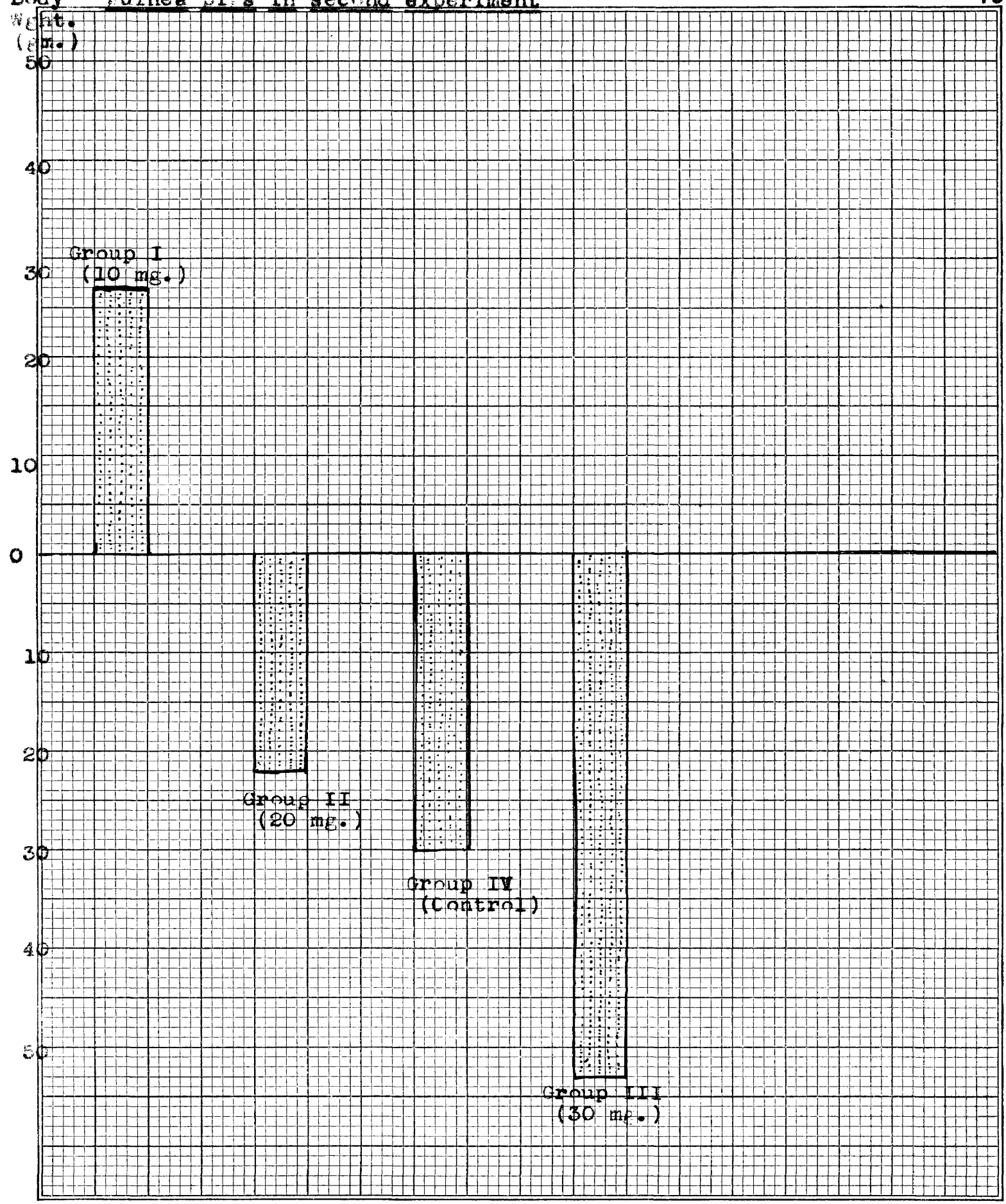


Figure 21

The average final gain or loss of weight among various groups of guinea pigs in second experiment

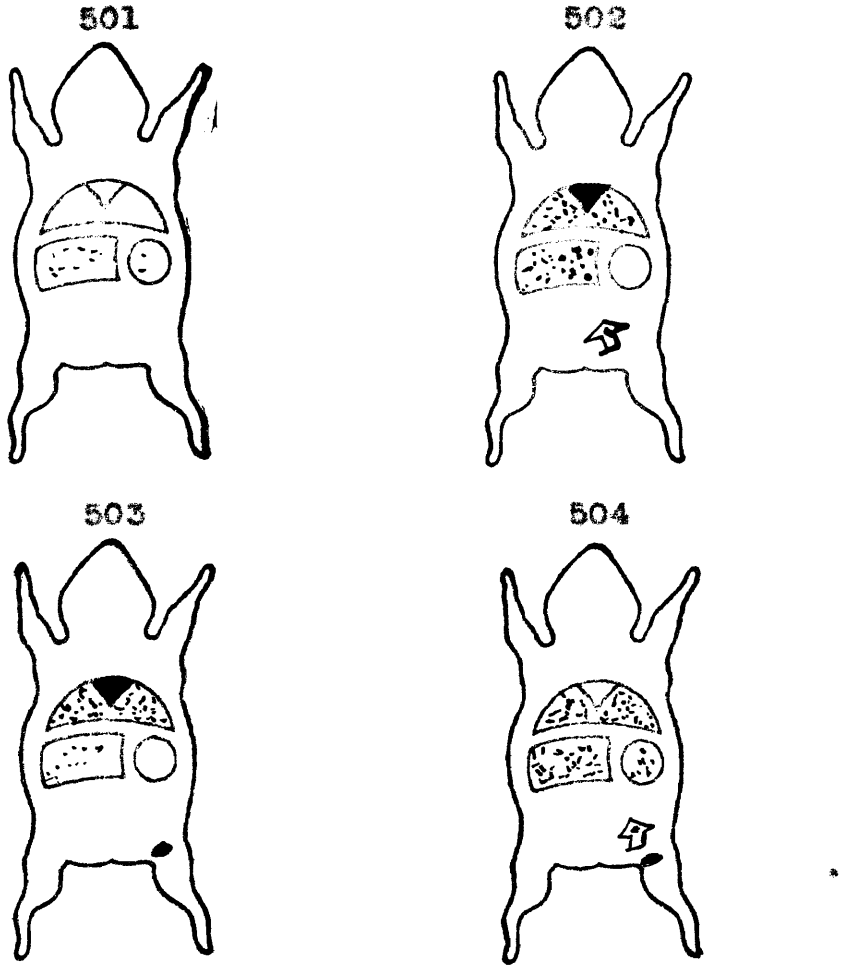


4. Tuberculous Lesions and Infection Index

The visible tubercles observed at autopsy or necropsy in the guinea pigs were schematically recorded in Figures 22 and 23. In the control guinea pigs numerous conglomerate caseous tubercles were unmistakingly found in peritoneum, liver, spleen, lungs, testicles and their adjacent lymph nodes, (see Figure 24) without exception.

In the experimental guinea pigs the tuberculous lesions were generally less extensive than those of the control ones. It was especially so in the abdominal organs. No peritoneal tubercles were observed in any of the experimental guinea pigs for the first experiment. However, in the second experiment there was one guinea pig in Group 1, three in Group 11, and four in Group 111 developed peritoneal tubercles. The higher percentage of guinea pigs in Group 111 developing peritoneal tuberculous lesions might be attributed to the toxic effect of the over-dosage, which invalidated the defense mechanism of the host and subsequently counteracted the value of subtilin.

Figure 22
Schematic Presentation of gross tuberculous lesions
in guinea pigs at necropsy or autopsy
Subtilin treatment
First experiment



Remark: For control animals, Nos. 505, 506, and 507 see Figure 13

Figure 23
Schematic presentation of gross tuberculous lesions in guinea pigs at necropsy or autopsy
Subtilin treatment
Second experiment

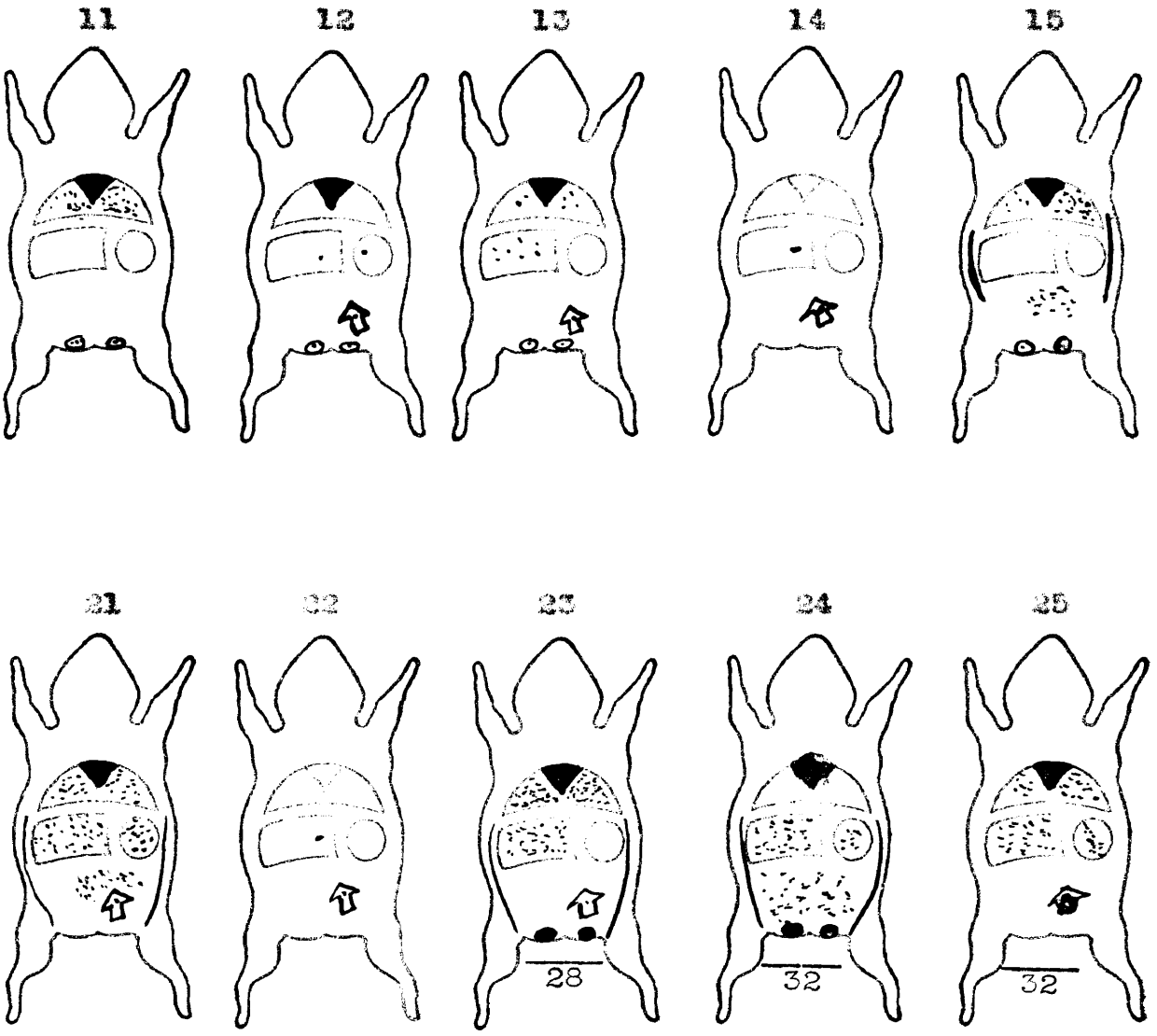


Figure 23 (Continued)
Schematic presentation of gross tuberculous lesions in guinea pigs at necropsy or autopsy
Subtilin treatment
Second experiment

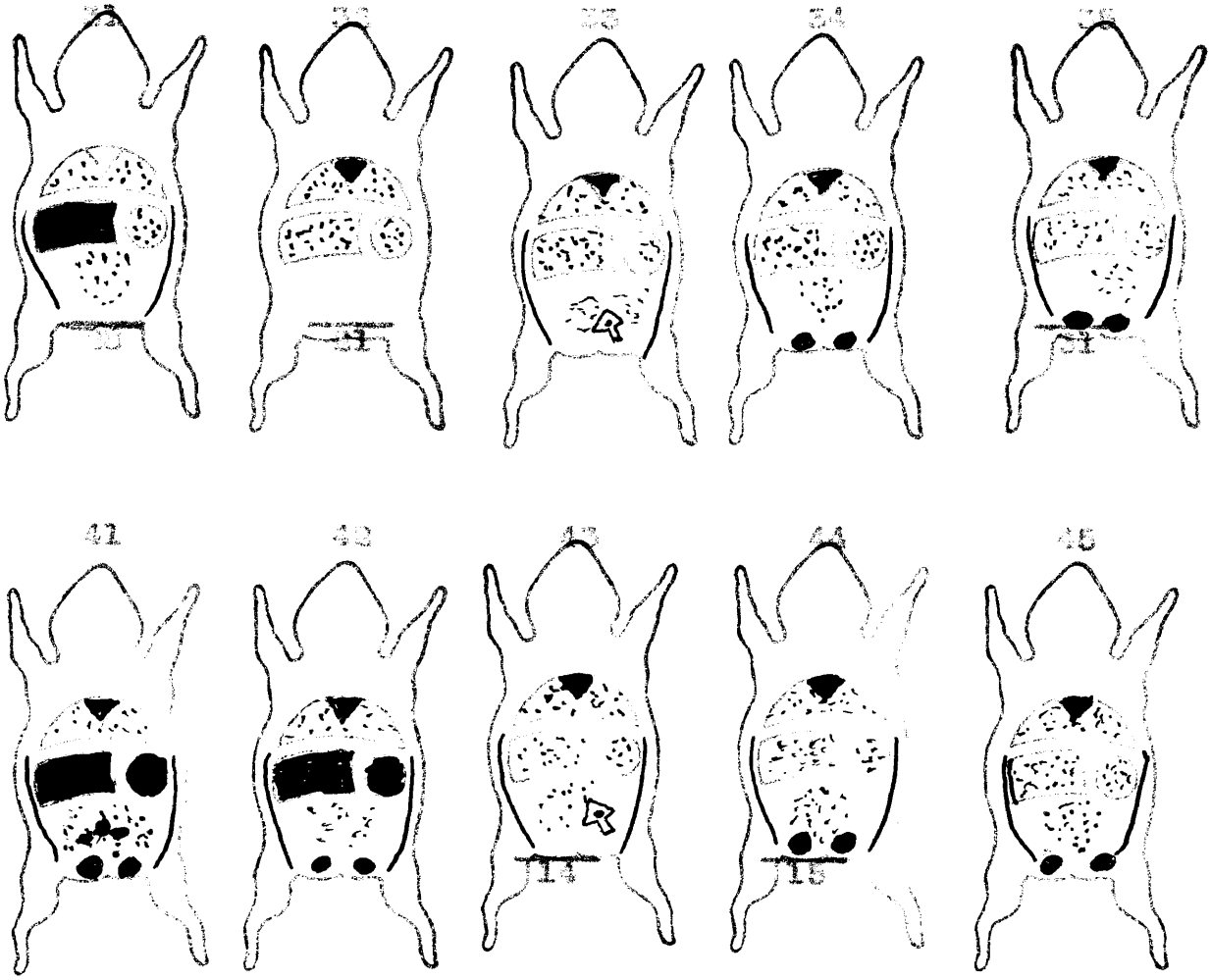


Figure 24

Guinea Pig No. 43

Died of Tuberculosis

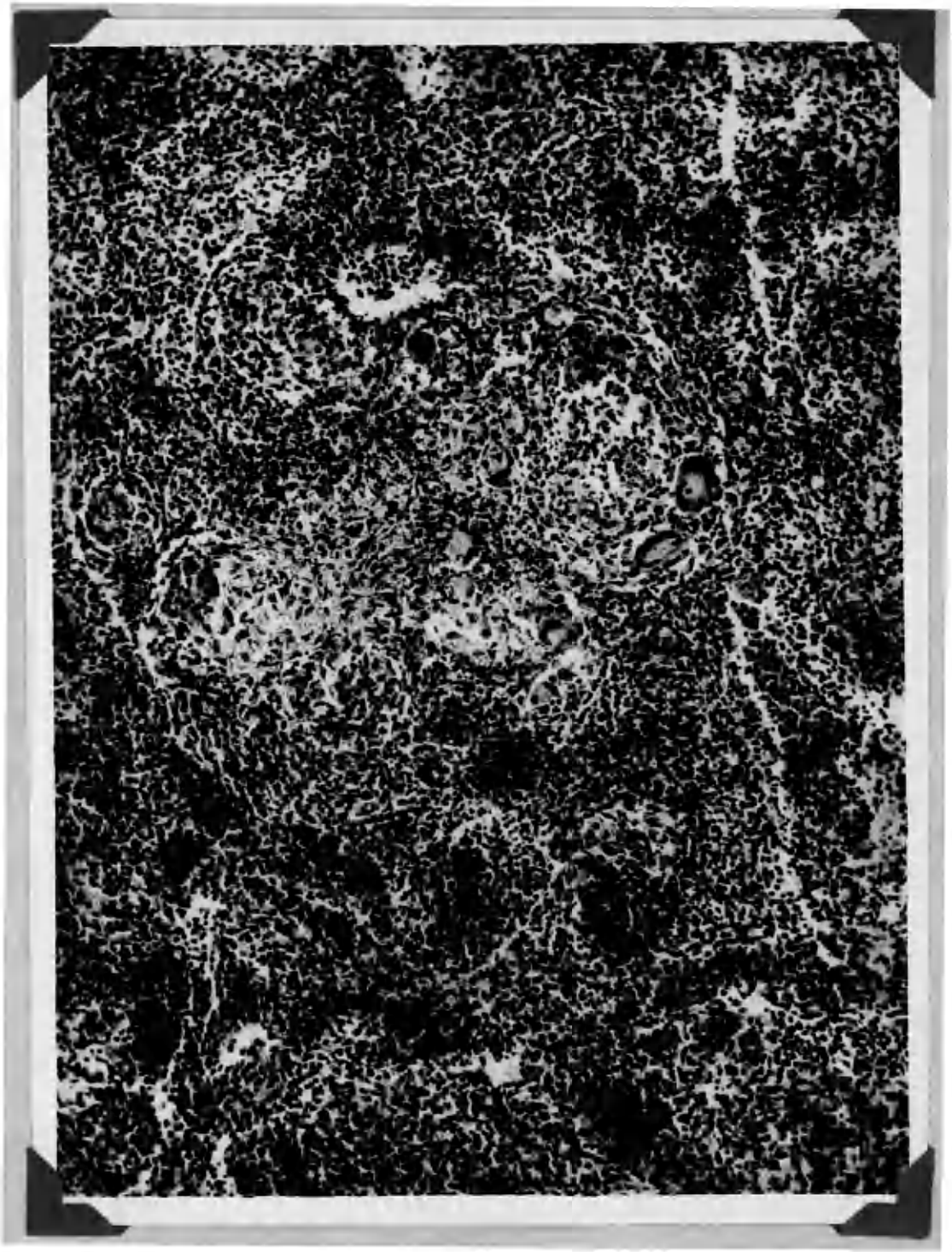
Note particularly the tubercles on the parietal layer of peritoneum under abdominal muscles and on the visceral layer attached to the intestines.

The histological studies showed progressive epitheloid tuberculous foci in all of the organs involved. The liver and spleen were especially damaged (Figure 25). Macroscopically, no visible tubercles were seen in kidney and adrenal. The histological studies (Figures 26,27, and 28) nullified the prevalent conception that the kidneys are rarely affected. However, the kidney of No. 501 guinea pig treated with subtilin, neither tubercles, hemorrhage, nor hyperemia was observed (Figure 29). It indicated at least that the dosage of subtilin given as shown in second column of Table 11 exerted no harmful effect or irritation upon the kidneys of the recipient.

Up to this point, the tuberculous lesions have not as yet been found in the pancreas. In a case of severe miliary tuberculosis, the tubular cells and Langerhan Island cells of the pancreas remained unharmed (Figure 30).

N either calcified nor fibrotic foci was observed in these tissue sections.

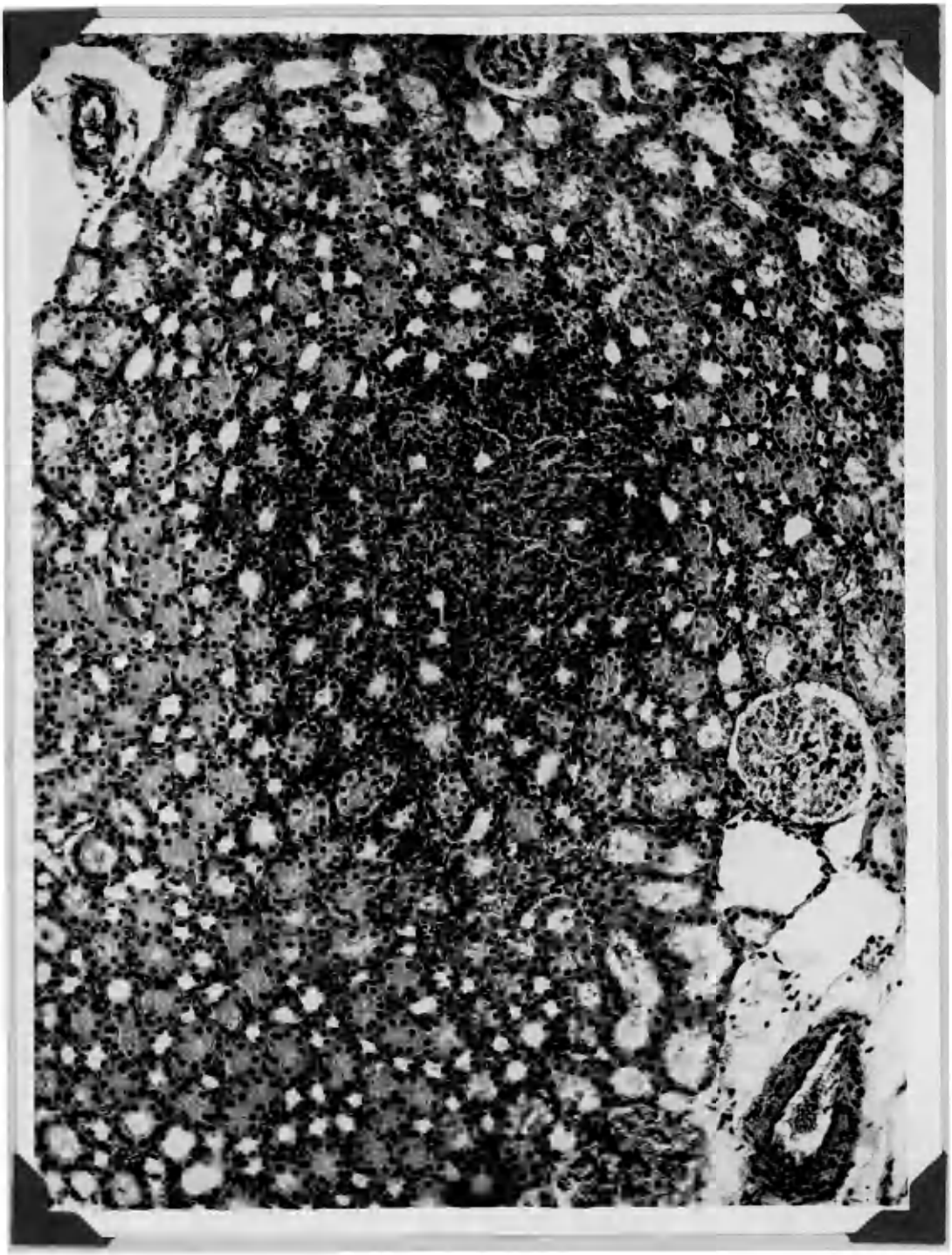
Figure 25
Spleen(guinea pig No.507) Photomicrograph
(low power)



N ote the Lan ghans giant cells

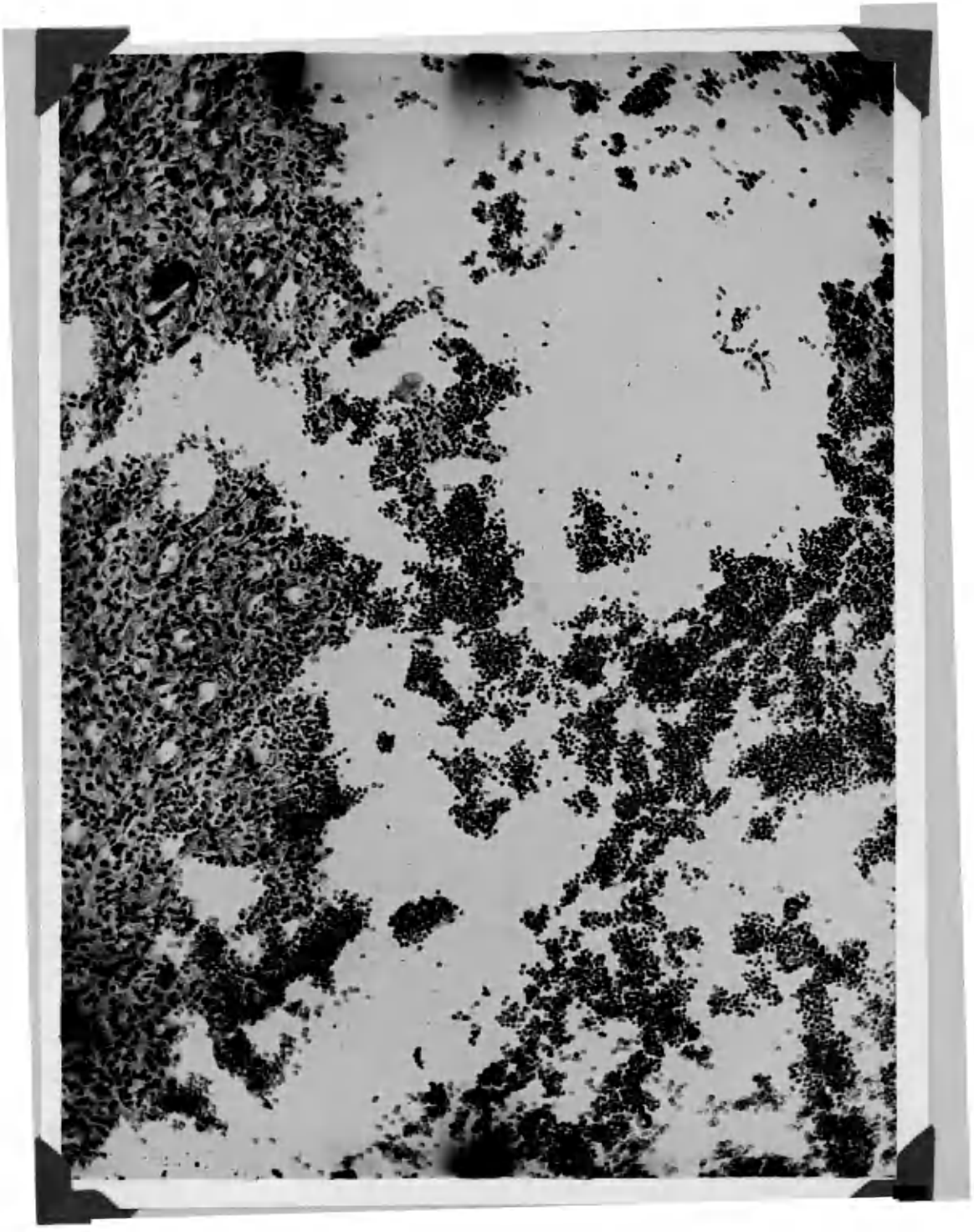
Figure 26

Kidney (Guinea pig No.41) Photomicrograph
(low power)



Note the epithelioid tubercles in the cortex

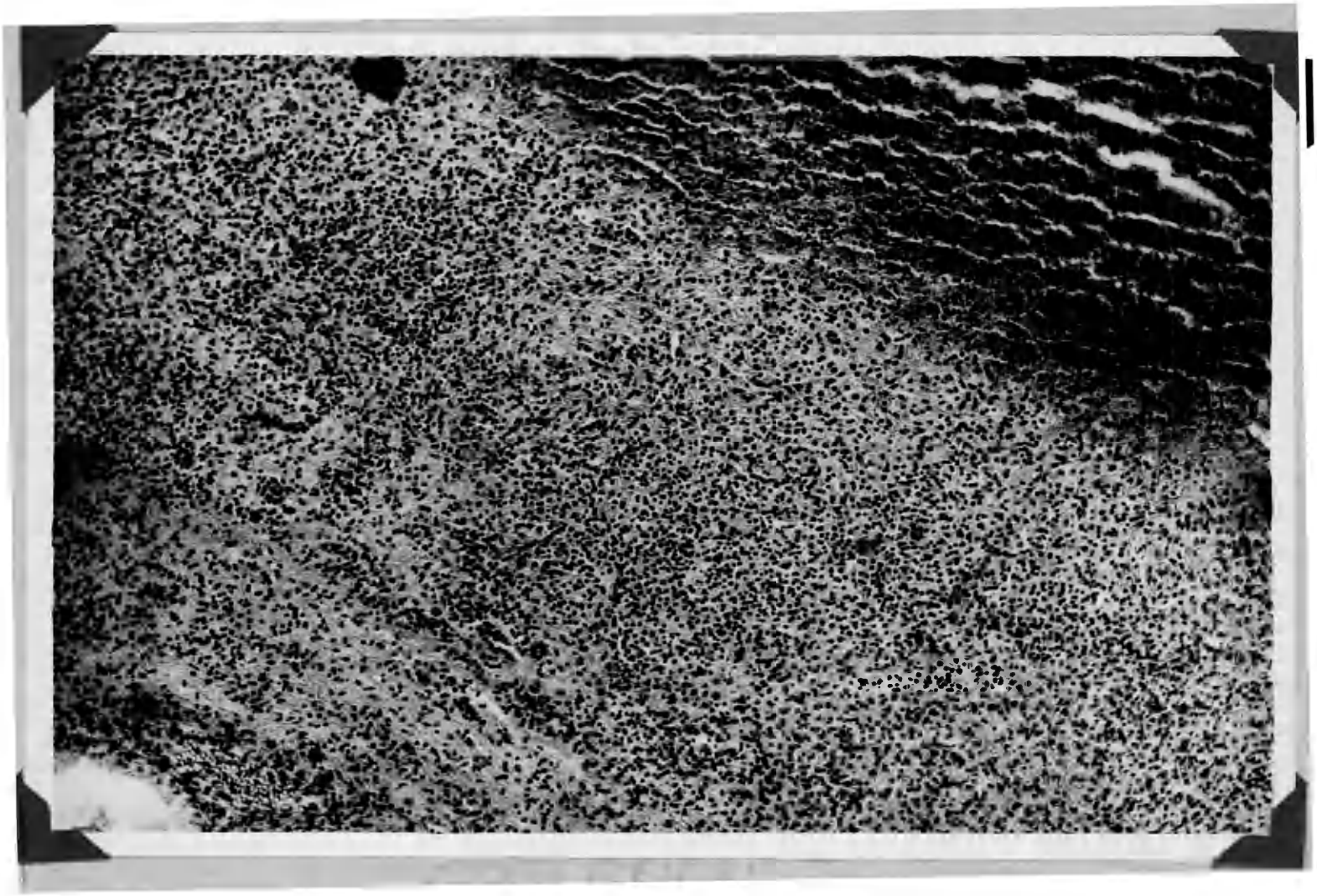
Figure 27
Kidney(guinea pig No.506) Photomicrograph
(Low power)



Note the damage area and extensive hemorrhage

Figure 28

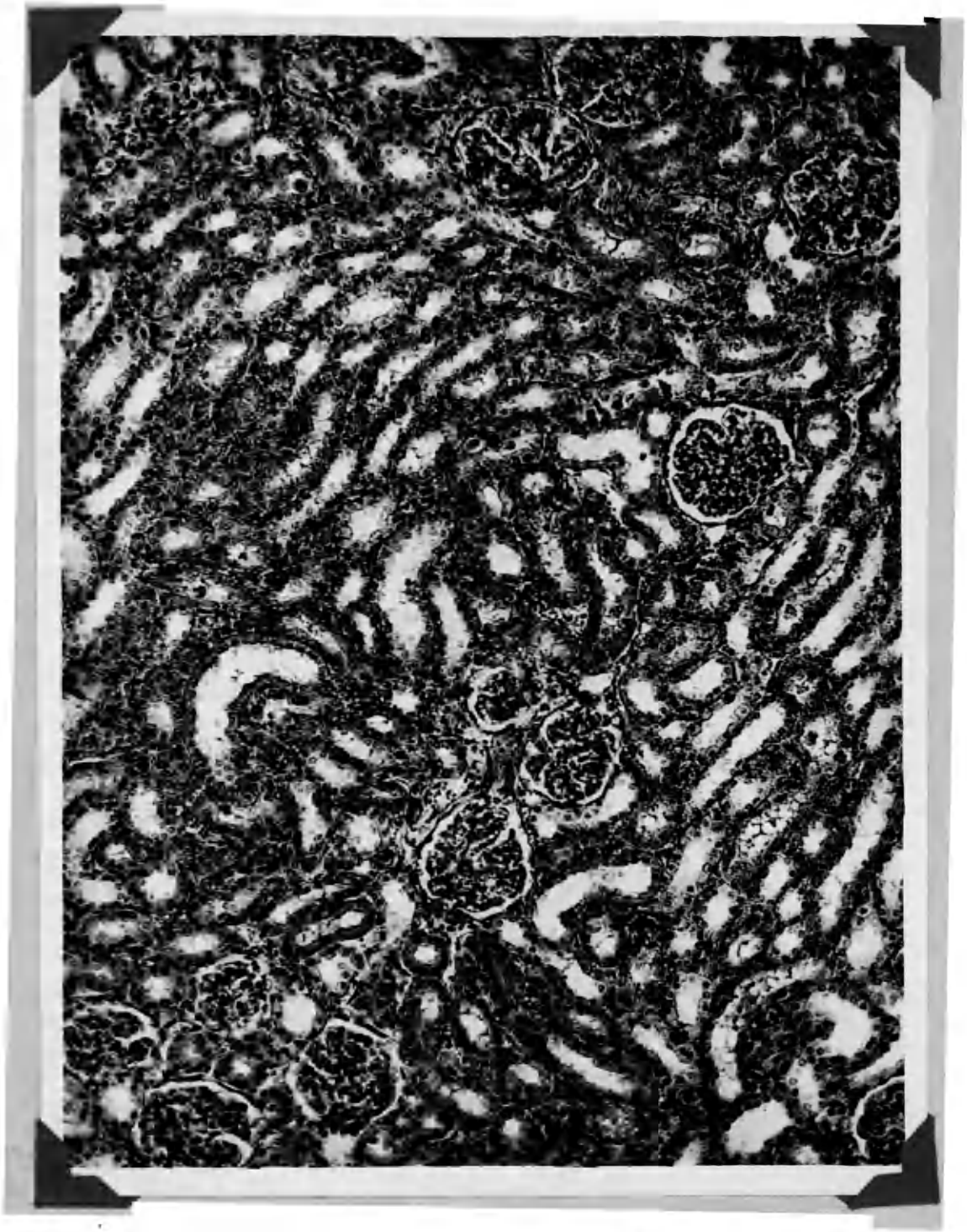
Adrenal(guinea pig No.42) Photomicrograph
(low power)



Note the necrotic area and the degenerating glandular cells

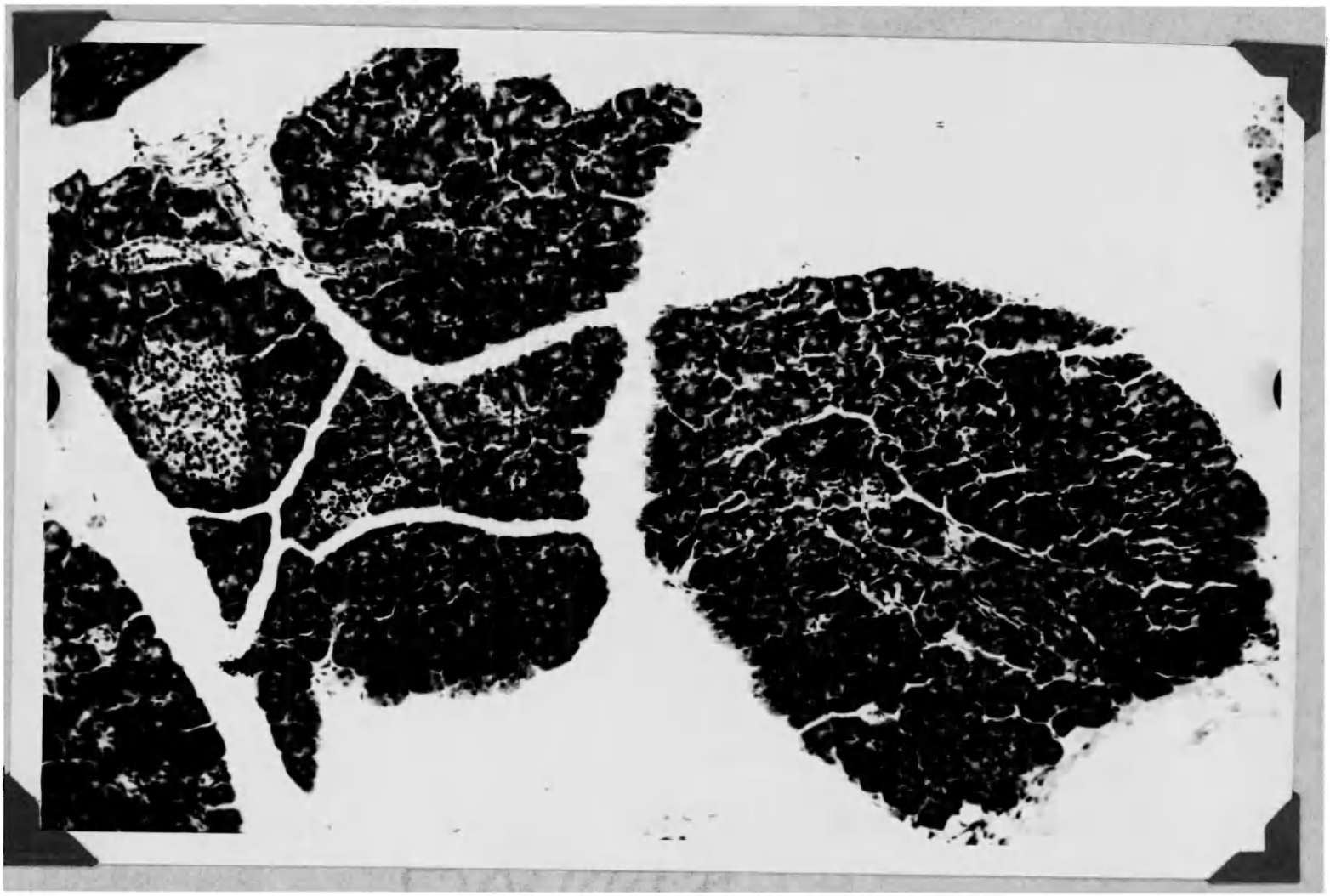
Figure 29

Kidney (guinea pig No.501) Photomicrograph (low power)



No pathological modification was observed.

Figure 30
Pancreas(guinea pig No.507) Photomicrograph



Note the tubular cells and Langerhan Island cells are still intact(This guinea pig died of miliary tuberculosis)

The infection index of the experimentally induced tuberculous guinea pigs for the first experiment (Table XX and Figure31) showed an appreciable decrease. For the second experiment, the data were recorded in Table XXl and graphically presented in Figure 32. The difference in infection indices among the various groups indicated the fact that this new anitbiotic did show partial suppressive effect of the experimentally induced tuberculosis in guinea pigs. The maximum dosage for this partially purified subtilin seemed to be about 10 mg or 200 units under this particular condition. It should be pointed out that with a more purified form of subtilin used in order to avoid the undesirable toxic side-effect of the impurities, a bigger dosage with higher units of subtilin might be administered without causing loss of body weight or toxic effect to the host. Consequently, a higher suppressive value of this new antibiotic against tuberculous guinea pigs could be expected. Moreover, by increasing the frequency of medicament of this antibiotic and by improving the administration route, e.f., through intravenous injection, better results comparable with streptomycin might be expected.

The fact that no calcified or fibrotic foci were found in these subtilin-treated guinea pigs greatly counteracted the value of this new antibiotic to be a chemotherapeutic agent to tuberculous guinea pigs.

TABLE XX

Infection index
First experiment

Guinea Pig No.		Infection Index
Experimental (5 mg.)	501	30
	502	99
Average		65
Experimental (10 mg.)	503	40
	504	75
Average		58
Control	505	100
	506	100
	507	100
Average		100

TABLE XXI

Infection index
Second experiment

Guinea Pig No.		Infection Index
Experimental (weekly dose: 20 mg. or 400 units)	11	10
	12	23
	13	12
	14	10
	15	40
Average		19
Experimental (weekly dose: 40 mg. or 800 units)	21	100
	22	10
	23	70
	24	100
	25	70
Average		70
Experimental (weekly dose: 60 mg. or 1,200 units)	31	83
	32	56
	33	66
	34	90
	35	100
Average		79
Control	41	100
	42	100
	43	100
	44	100
	45	100
Average		100

Figure 31

Infection index of tuberculous guinea pigs treated with subtilin

First experiment

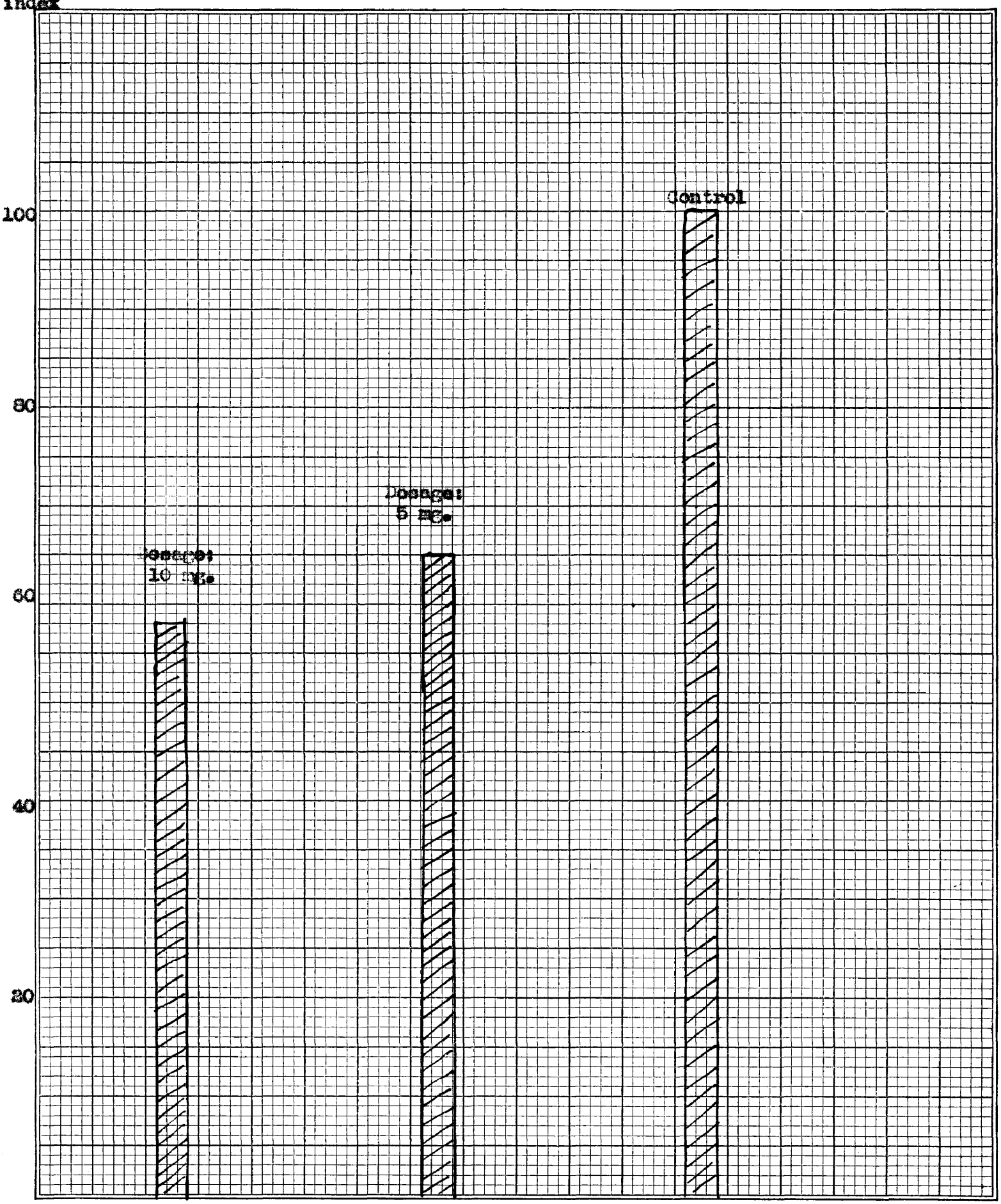
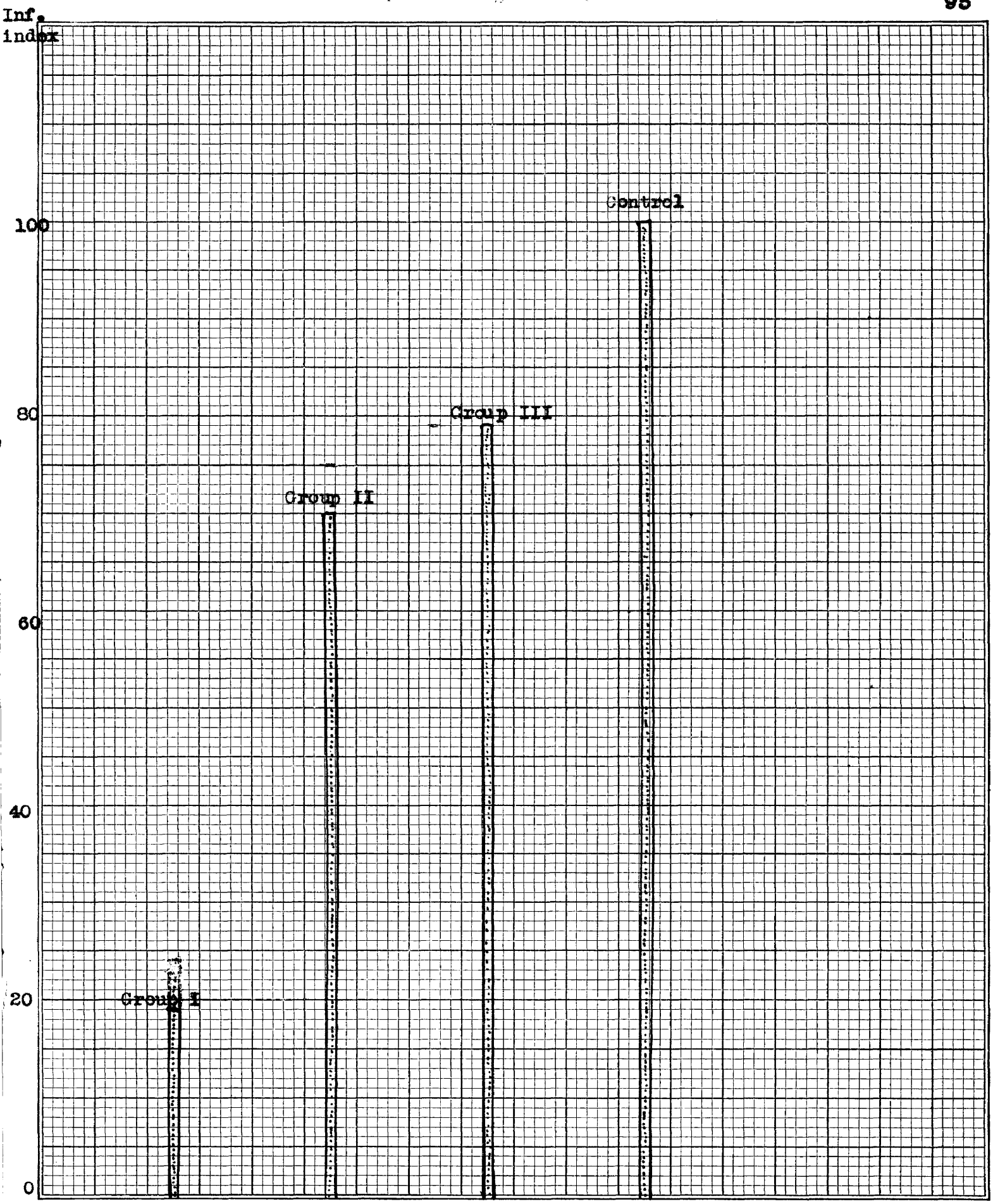


Figure 32

Infection index of tuberculous guinea pigs treated with subtilin
(Second experiment)



5. Longevity of Life Duration and Mortality Rate

The significance of the longevity of life duration among the experimental and control guinea pigs is best seen in Figure 33. The mortality rate for the subtilin-treated and control groups is graphically presented in Figure 34. Since the number of guinea pigs used for each group was not big enough to diminish the errors arising from the individual difference of susceptibility and resistance, the data of mortality rate were of little value.

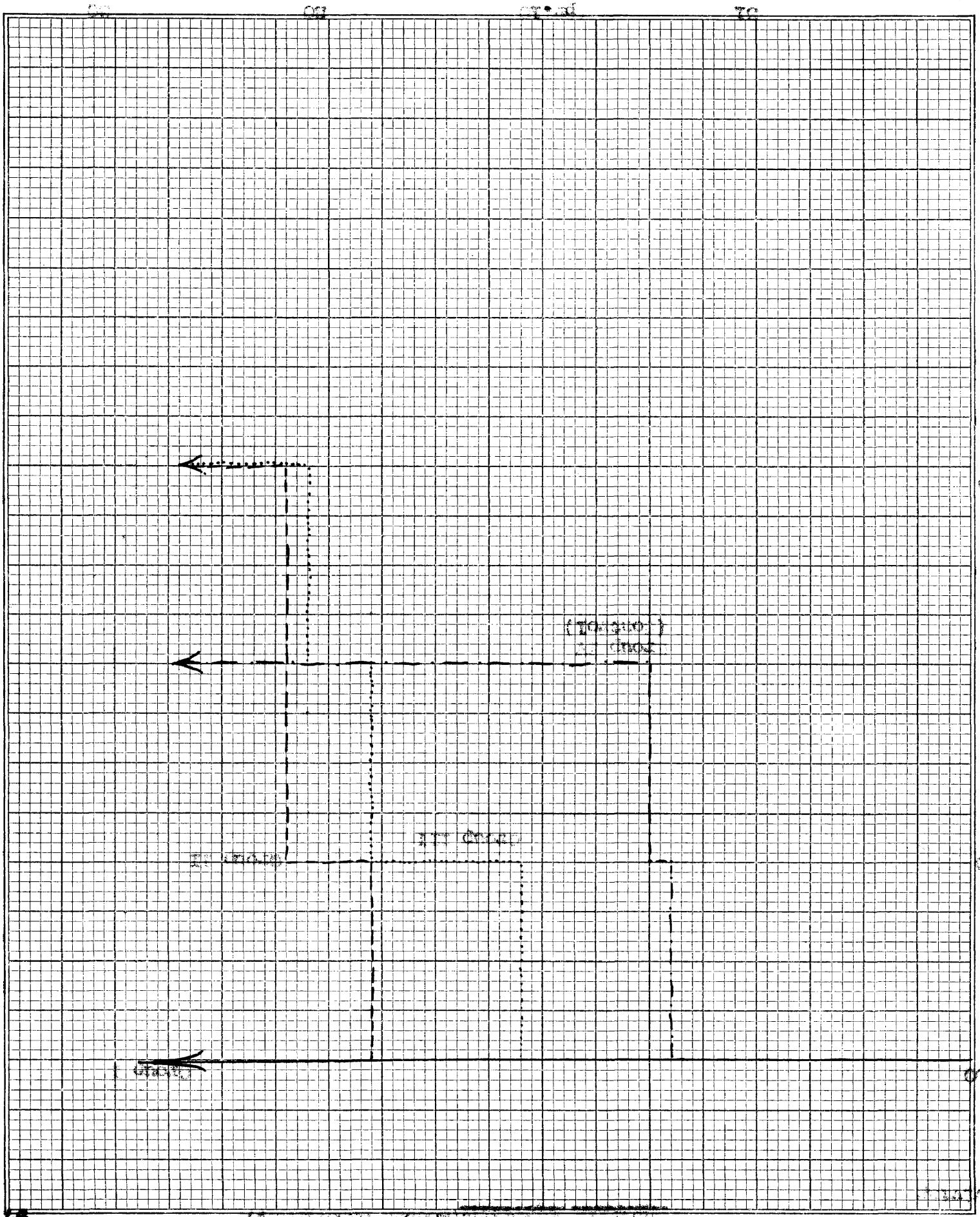
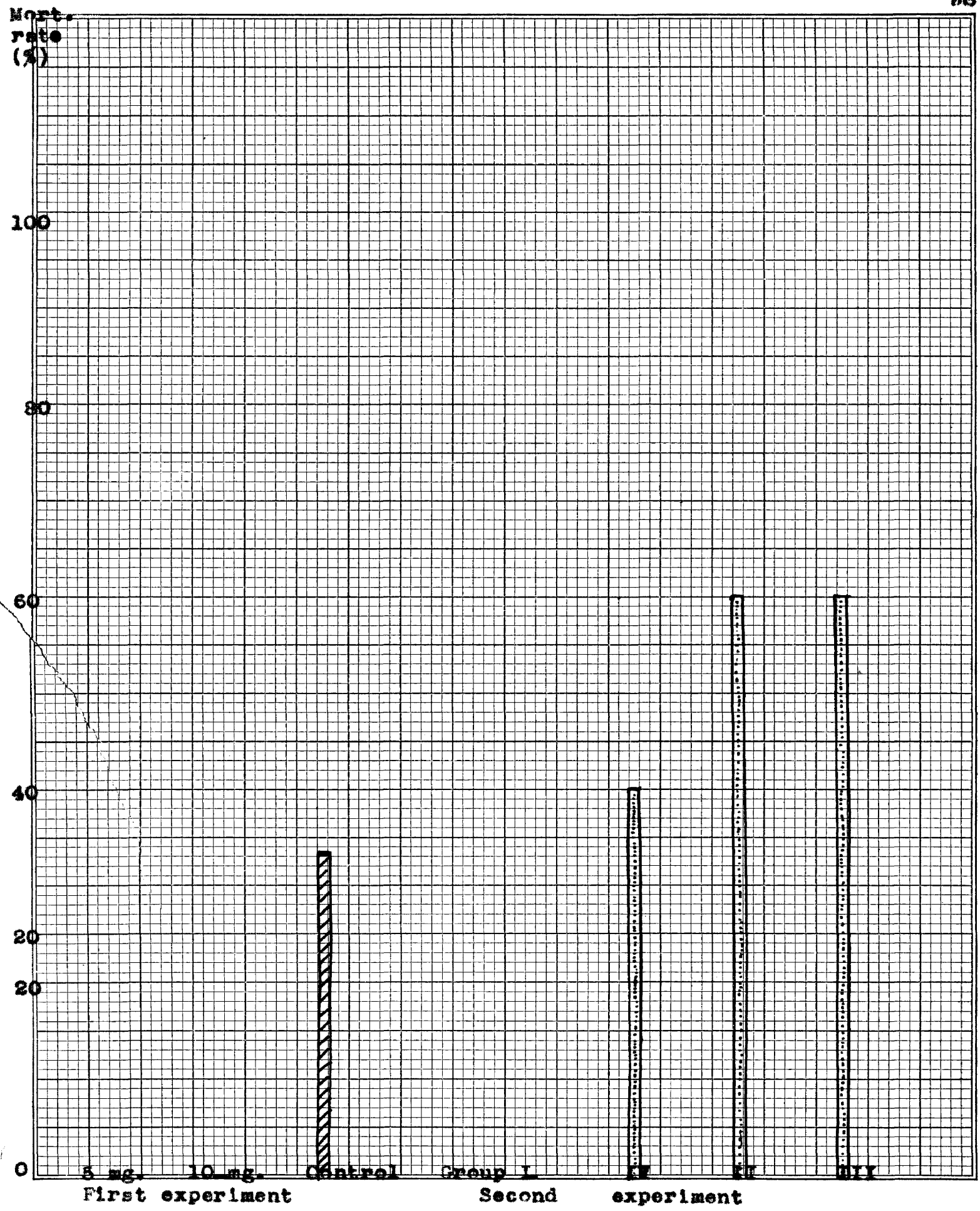


Figure 34
Mortality rate of subtilin-treated and control guinea pigs



V CONCLUSION

Guinea pigs vaccinated with Mycobacterium tuberculosis vas homonis strain H37 which was previously irradiated by ultraviolet light for five and ten seconds survived a challenging dose of two milligrams of virulent organisms when injected intraperitoneally. When autopsied or necropsied, most of the vaccinated guinea pigs exhibited no visible tuberculous lesions. The controls invariably developed a miliary tuberculosis with a short life duration and high mortality. The aforementioned results give evidence of the potency of the vaccine used. The data collected in these studies also pointed out that the pathogenicity of the M. tuberculosis var. homonis strain H 37 is more delicate than its reproductivity and viability; and that its reproductivity and viability, more delicate than its antigenicity. This principle might be generally applied not only to the other strains of tubercle bacilli but also to many other species. It is based on this same principle that many pathogenic organisms might lose their virulence without losing their capacity of reproduction and cytoplasmic metabolism. Their reproductivity and viability might be lost without losing their specific antigenic properties.

It is speculated that by measuring the oxygen consumption and carbon dioxide production, one might prove the principle that the organisms might lose their capacity of reproduction but still retain their capacity of cytoplasmic metabolism.

As pointed out by Mellon (1946), sufficient evidence has accumulated to indicate that B.C.G. variety can not be regarded as a "fixed virus", but is susceptible like all other organisms of undergoing variation in one or more directions. Consequently, its virulence may be regained. On the other hand, it seems possible that its antigenic properties might gradually be lost due to mutation to such an extent that no more significant preventive value can be demonstrated. Theoretically, the pathogens generally have their own specific antigens besides the group antigenic properties among different strains of the same species as well as among species of the same genus. Therefore, it would be the best idea to prevent the disease by a vaccine prepared from that particular strain causing the disease. However, it should be pointed out that before this type of tuberculosis vaccine can be used for human beings, further studies should be made on the standardization of the amount of energy absorption of various wavelengths in ultraviolet region required to destroy the pathogenicity and reproductivity of various virulent strains of human and bovine tubercle bacilli. The difference of immunization response between guinea pigs and human beings should also be determined.

The chemotheropeutic value of the new antibiotic, subtilin, against experimentally induced tuberculosis in guinea pigs is inconclusive. The data collected in these studies indicated partial suppression of no important significance. However, it should be pointed out that by increasing the frequency of medicament, higher units of dosage using the more purified form of the antibiotic,

and improving the administration route, a higher suppressive effect could be expected.

VI SUMMARY

1. A potent tuberculosis vaccine was developed by ultraviolet radiation.

2. Guinea pigs vaccinated with this type of tuberculosis vaccine survived a challenge dose of two mg. of virulent human tubercle bacilli strain H 37. At necropsy, no tuberculous lesions were found in most of the guinea pigs previously vaccinated. The control ones invariably developed miliary tuberculosis with short life duration and high mortality.

3. The human tubercle bacilli H 37 irradiated for two seconds, killed the guinea pigs faster than the non-irradiated bacilli. Those irradiated for twenty seconds under the same conditions gave a partial protection to the guinea pigs against the same challenge dose as mentioned above. Organisms irradiated for five and ten seconds under the same conditions exhibited a tremendous preventive potency.

4. The results of chemotherapeutic value of subtilin against experimentally induced tuberculosis in guinea pigs were inconclusive. The partial suppressive effect of this antibiotic against experimentally induced tuberculosis as appeared in these experiments encourages further studies of this new antibiotic against tuberculosis.

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