TUBERCULOSIS IN A FROG *

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Numerous cases of spontaneous tuberculosis in cold-blooded animals have been reported. Friedmann [1] isolated an acid-fast bacillus from two cases of spontaneous infection in salt-water turtles which is pathogenic for cold-blooded animals and guinea pigs, and can be grown at 37.5°C. Aronson reported tuberculosis in a salt-water fish [2] and in snakes [3]; the organism isolated from the salt-water fishes is pathogenic for cold-blooded animals and for mice. Kuster [4] isolated an acid-fast bacillus from the liver of frogs which is pathogenic for all cold-blooded animals and whose optimum temperature is 25°C. Rupprecht [5] also studied another case of spontaneous tuberculosis in a frog.

In one of the tanks of the Philadelphia Aquarium, Dr. Henry Winsor found a dead frog (*Rana esculenta*) which showed at necropsy numerous tubercles in the internal organs. The spleen measured 25 × 18 mm. in size, and was studded with small subperitoneal, firm, gray-white tubercles; many necrotic patches were noted in this organ. The liver was somewhat larger than normal and studded with numerous, grayish-white, subperitoneal nodules measuring less than 1 to 2 mm. in size. These were most abundant on the anterior surface of the organ. No gross tubercles were observed in the kidneys. In the mediastinal region a grayish-white mass, 10 × 12 mm. in size was found, from which smears were made which showed numerous acid and alcohol-fast, granular, long and thin bacilli, many being in clumps. In the spleen and liver, short coccoid acid-fast and alcohol-fast bacilli were demonstrated.

HISTOPATHOLOGY:

In sections made from the lungs no tubercles were found. The pleural lining was intact; the blood vessels of the parenchyma were moderately congested, and within the alveoli were rather numerous, large, mononuclear cells of

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histiocytic type; these were also present within some of the bronchi. In the liver several tubercles were in evidence, some of which were relatively small and young, and composed exclusively of large cells with pinkish cytoplasm, with poorly defined cell boundaries and large oval-shaped, rather vesicular nuclei; these cells were not frankly epithelioid in appearance. About the periphery was a narrow zone of flattened cells of connective tissue origin. The older tubercles were larger. The greater part of the central portion of these tubercles had undergone necrosis, and the constituent cells presented small contracted pyknotic nuclei. At the very center there was a small amount of nuclear dust and highly acidophilic fibrinoid material. The periphery of these older tubercles was composed of a broad zone of flattened fibroblasts between which was moderately abundant collagenous substance. Small numbers of small round cells of lymphoid type were present in both the central and peripheral portions of all the tubercles. The spleen demonstrated throughout the pulp numerous areas of necrosis with formation of highly acidophilic fibrinoid material and much fragmentation of nuclei. An occasional ill-defined group of epithelioid cells was in evidence, but these groups presented no peripheral zone of fibrosis. A few of the blood vessels were thrombosed. Acid-fast bacilli could not be identified in the tubercles found in the liver, while in the spleen they occurred in large numbers in the center of the foci of necrosis, often forming prominent, dense clumps. There were bacilli of two forms: rather long and beaded, and much shorter and more solidly stained.

Isolation and Bacteriology of the Organism:

The liver and spleen of this frog found dead were treated with 3 per cent solution of sodium hydroxide for 30 minutes at 37°C. and the sediment was neutralized with a 6 per cent solution of hydrochloric acid. After centrifugation it was inoculated on different media, and some tubes were placed in the incubator at 37°C. and the others were left at room temperature (18° to 20° C.). Six days later a number of very small, fine, discrete white colonies were observed in the tubes of Petroff's media which were kept at 37°, while no growth was noticed in the tubes left at room temperature. Smears made from these colonies showed solid staining acid-

and alcohol-fast bacilli * in parallel arrangement. Twelve days after inoculation, growth occurred in all tubes, the most luxuriant being that on Petroff's media; next, the one on Lowenstein's; and, last, that on Dorset's.

When first isolated the colonies appeared small, discrete and white on both Petroff's and Lowenstein's media, turning yellowish after exposure to light. On subculturing, they grew luxuriantly on Lowenstein's or Petroff's media, either with or without glycerin. On glycerinated broth or Sauton's synthetic media the surface film was thicker than on liquid media without glycerin. On plain agar (pH 7) the organism grows more slowly and not so luxuriantly as on glycerinated agar; on the latter, the small, opaque, round colonies appeared seven days after planting. On rabbit's blood agar, colonies appeared in five days as rounded, opaque, and the margins irregular and ill-defined; no hemolysis or change in the blood was noted. When inoculated into plain broth or Sauton's synthetic media there was observed a growth which settled at the bottom of the flask; later, a surface pellicle, which grew thicker as the culture aged, was observed. On subcultures the film was thick and wrinkled.

The organism appears as a slender, pleomorphic bacillus varying from a short coccoid form to longer forms. These organisms are slightly curved and usually packed side by side in parallel arrangement. The organism is non-motile and non-sporogenous; no involution forms have been noted. Numerous granular beaded forms are sometimes seen.

The organism is Gram-positive and is readily stained with carbolfuchsin, not being decolorized by 25 per cent sulphuric acid or 3 per cent hydrochloric acid after one hour's exposure; it is partly decolorized by 30 minutes' exposure to 5 per cent nitric acid, but completely decolorized if exposed one hour or longer.

Growth in Dunham's peptone water was luxuriant but indol was not produced. On litmus milk, growth occurred without changing the media. The organism grew on potato. Gelatin was not liquified. Hydrogen sulphide was formed on lead acetate agar. No acid or gas was produced in

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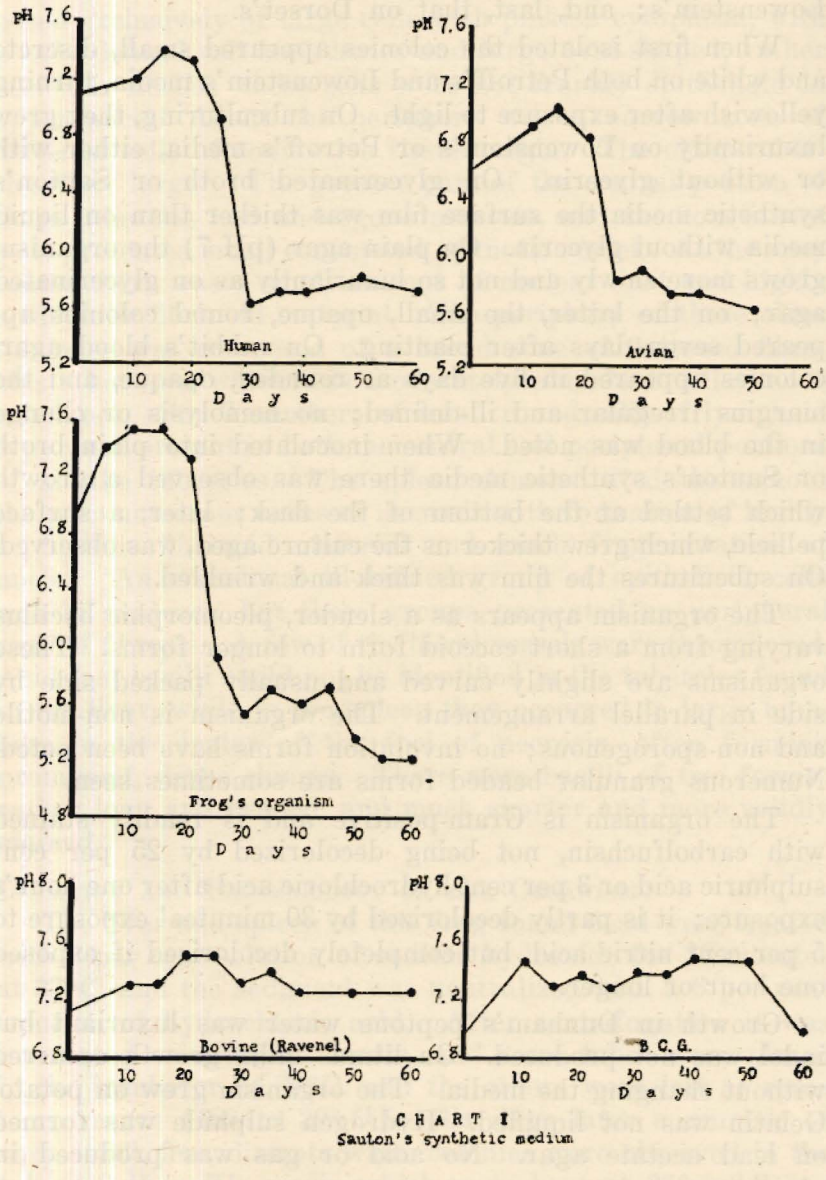

CHART I
Sauton's synthetic medium

mannite, lactose, glucose, saccharose or maltose; while acid, but no gas, was formed in arabinose and xylose.

When glycerin broth with an initial pH 7.2 was used, its reaction changed to pH 7.5 after five days, where it remained until the thirtieth day when it dropped to pH 7, and later to pH 6.7, gradually returning to pH 7. This glycerol curve is similar to that previously observed for *M. thamnopheos* [3]. The reaction on Sauton's synthetic media with an initial pH of 6 9 changed to pH 7.5 in 10 days, after the twentieth day suddenly dropped to pH 5.9, reaching pH 5.2 on the sixtieth day when the experiment was discontinued. The glycerol curve resembles more the human than the bovine type of tubercle bacillus (chart I).

The organism isolated from this frog grew more luxuriantly at a temperature of 37°C. when first isolated, but subcultures developed best at about 25° to 30° C. Under anaerobic conditions at room temperature (250° to 28° C.) no growth was observed.

CHROMOGENICITY:

Initial cultures grown in the dark developed no pigment, or a pale creamy color. However, when the cultures were exposed to light, they were found to turn a pale yellow or orange color in twenty-four hours. The chromogenesis was not influenced by having the cultures on atmospheres of different gases such as oxygen, hydrogen, or carbon dioxide, or even a partial vacuum.

This chromogenic substance did not diffuse throughout the medium; it is soluble in absolute ethyl alcohol and slightly soluble in ether, acetone, 95 per cent ethyl alcohol and synthol, the solubility being increased by heating the solvent to boiling point. It is insoluble in cold or boiling xylol, and in chloroform. The alcoholic solution, after evaporation, leaves an amorphous orange colored residue with the odor characteristic of tuberculin (O. T.) and old cultures of tubercle bacilli.

PATHOGENICITY:

Five frogs (*Bufo marinus*) were inoculated with 0.5 ml. of a suspension of the organism under study; three intraperitoneally and two subcutaneously in the hind legs. One frog died twenty-one days after being injected, the others were killed at different periods after inoculation. They had

acid-fast bacilli in all organs and in the bone marrow; tubercles were demonstrated in the lungs, spleen, liver and kidneys. Sections made from various organs showed the bacilli in the typical clump arrangement.

Several iguanas (*Ameiba exul*) were inoculated intraperitoneally with 0.2 ml. of a suspension of the bacillus from cultures obtained from the original frog: they were found dead from eight to sixteen days after injection. Tubercles were seen in all organs and acid-fast bacilli were demonstrated in smears and sections.

Lizards (*Anolis cristatellus*) died usually six or seven days after receiving 0.2 ml. intraperitoneally of the suspension of the organism under study. Acid-fast bacilli in great numbers were seen in all organs. In the liver there were large aggregations of acid-fast bacilli with early focal epithelioid cell grouping.

Fresh-water fishes (*Fundulus fonticola*) inoculated intraperitoneally survived from eight to thirty days; all showed acid-fast bacilli in their internal organs with the same typical grouping observed in other injected animals. Sections made from the organs demonstrated sharply defined tubercles. At one point in the section there was a sharply defined tubercle occupying a portal space and consisting mainly of cells with clear nuclei and sparse cytoplasm, few lymphocytes centrally placed and large cells with vesicular nuclei and abundant cytoplasm.

A turtle injected intraperitoneally with 0.05 ml. of a thirty-day broth culture of the frog's organism was found dead twenty days after; tubercles were observed in the liver, lungs and peritoneum. In the affected organs the tubercles were very numerous and consisted of small, not very sharply outlined, accumulations of small medium-sized cells with rounded, somewhat vesicular nuclei and moderately abundant pinkish cytoplasm without any definite cell boundaries. Occasional lymphoid cells occurred among the epithelioid ones, but there was no peripheral fibrosis and giant cells were never in evidence. In some organs like the spleen, most of the tubercles were undergoing necrosis with the formation of fibrinoid material and nuclear dust.

Nine rabbits were inoculated: three subcutaneously, three intraperitoneally and three intravenously, each with one ml.

of a heavy suspension prepared from the initial culture. The rabbits which were inoculated subcutaneously developed an abscess at the site of the injection, which was found healed thirty days later. No lesions in the internal organs were noticed. The animals which received intraperitoneal inoculation showed some enlarged and caseous mesenteric nodes, and smears made from these glands demonstrated acid-fast bacilli. The omentum was studded with large groups of epithelioid cells amongst which were numerous, large, giant cells of the Langhan's variety. Between the nodules in this organ, loose, edematous, fibrous tissue, moderately infiltrated with plasma cells and lymphocytes, were noticed. In the capsule of the liver there were small isolated and large confluent nodules and plaques composed of prominent, polygonal, epithelioid cells, and at times a central area of necrosis filled with pinkish staining débris and nuclear dust. In some of these nodules there were numerous, very large, giant cells of both the Langhan's and foreign body type. Macroscopically, no pathologic lesions were visible in the other organs. A microscopic study of sections made from the lungs, liver, spleen and kidneys demonstrated that they were essentially normal. The tubules of the testes and epididymis were normal, but in the fibrous tissue surrounding the epididymis and in the tunica vaginalis there were large conglomerations of epithelioid cells with occasional, fairly dense collections of lymphocytes and foci of necrosis. The three rabbits injected intravenously, when killed thirty days later, showed no lesions in the internal organs, but the testes exhibited numerous tubercles and abscesses. The scrotum was covered with nodules; acid-fast bacilli were present in smears made from the organs, and cultures made on Petroff's media developed colonies of an organism having the same characteristics as the original frog bacillus. Sections made through the testes exhibited numerous, isolated and conglomerated groups of epithelioid cells accompanied by numerous lymphocytes that formed dense focal collections. Some of these foci presented a central area of caseation necrosis. The tubules in relation to these areas have undergone degeneration and necrosis. In the epididymis there were two or three similar areas, but they were more numerous in the fatty tissues surrounding the organ.

Guinea pigs were inoculated with one ml. of a suspension of the frog's bacillus, corresponding in density to number two in McFarland's nephelometer; some were injected intraperitoneally and others, subcutaneously. All animals when injected into the dermis with 0.1 ml. of the filtrate of an eight-week-old glycerol broth culture of the organism under study, or with 0.1 ml. of a 10 per cent solution of O. T. (human) showed a positive skin reaction forty-eight hours after the injection. All guinea pigs were killed thirty days after inoculation. The thickened omentum was covered with pus and the lymph nodes contained acid-fast bacilli. No other evidence of infection was seen in the other internal organs. The histopathology of the lymph nodes revealed that the lymphoid tissue had been replaced by a diffuse growth of epithelioid cells which did not form distinct tubercles. There were numerous, multinucleated cells but no typical giant cells and no frank caseation.

Mice of different ages—14, 33, 45, 60 days and more than a year old—were inoculated intraperitoneally with one ml. of a suspension of the frog's organism corresponding in density to number three in McFarland's nephelometer. The same number of control animals of the same litter and under analogous conditions of feeding and housing were kept at the same time. Two or three weeks after being injected all the inoculated animals of less than 60 days of age began to show on their tails the formation of nodules filled with pus; acid-fast bacilli were demonstrated in smears made from this pus. Later these nodules appeared on the legs, and in a few animals on the ears. These nodules usually ruptured and developed into necrotic patches which gradually healed leaving a scar. None of the control animals showed signs of a similar infection. One of the sixty-day-old animals died 26 days after inoculation and the others were killed from 30 to 60 days after receiving the injection of the bacillary suspension. In all inoculated animals the internal glands were involved more or less extensively. In most cases the iliac and inguinal glands were 2 or 3 mm. in diameter and filled with pus, showing acid-fast pleomorphic bacilli ranging from short bacilli to longer forms, suggesting chains or filaments. The mesenteric lymph nodes and the glands of the omentum were in numerous cases enlarged and caseous. There were abscesses in the testes of many of the male animals. Of the

other internal organs the liver was the most frequently involved; nodules were observed occasionally in it. The microscopic study of the sections made from the organs of some of the mice showed the following: In the liver there were small compact groups of mononuclear and polymorphonuclear cells in the sinusoids and portal spaces. There were also occasional groups of epithelioid cells and tubercles exhibiting a definite zone of fibrosis about the periphery and some fibrinoid material at the center. Minute nodules were sometimes seen in the pleura, composed of epithelioid cells. The male mice that had the testes infected showed large abscesses in the epididymis, which were limited by a narrow wall of fibrous tissue, inside of which, at times, groups of large mononuclear or epithelioid cells were observed. In the fallopian tube of a female mouse studied, there was a nodule showing advanced coagulation necrosis throughout a large part of the center, and an ill-defined zone of vacuolated large mononuclear cells about the periphery; outermost was fibrous tissue. The microscopic study of the tail of the experimental mice showed in the subcutaneous tissue and between muscle bundles large foci of dense infiltration with cells that had undergone necrosis and fragmentation. Conglomerate groups of epithelioid cells were sometimes observed. Faint staining, rather long, beaded, acid-fast filaments were sometimes seen in the necrotic foci of the tail. Distinct acid-fast bacilli, slender, long and beaded or else stouter and more solid were found more often in the epithelioid cells in the tail.

From the organs of the infected mice acid-fast bacilli were isolated, exhibiting the same characteristics as the original organism isolated from the frog. Cultures thus isolated were found to be capable of infecting frogs and mice.

Three albino rats inoculated intraperitoneally with one ml. of the suspension made from the frog's bacillus showed no gross lesions in the internal organs when killed sixty days later.

Two pigeons were injected, one intraperitoneally, and the other intravenously, each with one ml. of the bacterial suspension made from the organism isolated from the original frog, and were found two months later to show no evidence of tuberculosis. Similarly, two chickens were injected with the same negative results. Five weeks after inoculation, the chickens were tested intradermally in the wattle with 0.1 ml.

of a 10 per cent solution of O. T. and 0.1 ml. of the filtrate of an eight-week-old broth culture of the organism under study, and were found negative to O. T. dilutions, but positive to the filtrate of the frog's bacillus.

TUBERCULIN AND TOXIN PRODUCTION:

The cultures of the bacilli isolated from the frog were grown for eight weeks on glycerol broth and on Sauton's synthetic medium. The broth and the synthetic medium with the cultures were heated for two hours in an Arnold sterilizer and then filtered through a Berkefeld candle. These two filtrates caused definite redness and edema when 0.1 ml. was injected into the dermis of tuberculous guinea pigs. A more severe reaction and in some cases even necrosis, was observed when 0.1 ml. of these filtrates was injected intradermally into guinea pigs which four weeks previously had been inoculated with 1 ml. of a heavy suspension prepared with cultures of the organism isolated from the original frog (suspension corresponding to No. 5 in McFarland's nephelometer). The injection of 0.1 ml. of a 10-per-cent solution of O. T. into the dermis of these guinea pigs caused redness and edema.

The filtrates prepared from eight-week-old glycerol broth cultures of the frog's organism was used to determine if a soluble toxin was produced. Different amounts of this filtrate varying from 1 to 10 mls. were injected intraperitoneally into tuberculous guinea pigs. Death occurred in animals that received more than 5 mls. of the filtrate. Normal guinea pigs which received as much as 10 mls. remained well.

SEROLOGIC REACTIONS:

Antiserum was obtained from rabbits that had been injected repeatedly intravenously, intraperitoneally and subcutaneously with the acid-fast bacilli isolated from the frog. Using this serum prepared from rabbits inoculated by different routes, agglutination tests were performed; it was found that the culture originally obtained from the frog as well as after it had been passed through mice was agglutinated. A different strain of *M. ranae* was also agglutinated with the serum, but not a strain of *M. marinum*. Agglutination tests were conducted using as antigen suspensions of the original frog's bacillus and sera of tuberculous guinea

CHART II

Organisms of Cold-Blooded Tuberculosis	Morphology	Acid-Fastness	Gelatin	Litmus Milk	Blood Agar (Rabbit)	Action on Carbohydrates	Oxygen Growth	Optimum Temperature	Chromogenicity	Pathogenicity
Organism isolated from frog	Slender rods, pleomorphic, short coccoid to long bacilli. Clumps beaded	Acid-fast.....	No liquefaction	Unchanged....	No hemolysis	Acid but no gas in arabinose and xylose	Aerobic...	25°C. to 30°C.	Pale creamy in the dark. Yellow to orange when exposed to light	Frogs, lizards, iguanas, fresh water fishes, turtles and mice
Myco. ranae (Kuster)	Slender rods.....	Acid-fast when stained in cold solution. In young cultures not acid-fast	No liquefaction	Becoming thin clear, peptonized, yellow, alkaline	.....	.....	Aerobic...	25°C....	Grayish white...	All cold-blooded animals
Myco. piscium (Kral and Dubard)	Slender rods singly or in thread branching	Acid fast.....	No liquefaction	Thickened. No coagulation	.....	.....	Aerobic...	25°C....	White, creamy-like	Carp, frogs, lizards. Not for guinea pigs or pigeons
Myco. marinum (Aronson)	Short, thick,.....	Not decolorized by 3% acid alcohol or Gabbet's	No liquefaction	Acid coagulation	No hemolysis	No action.....	Aerobic facultative	18°C to 20°C	Grayish white, later yellow to deep orange	Pigeons, mice, gold fish and frogs
Myco. thamnospheos (Aronson)	Slender rods, beaded, barred	.....	Slight surface growth	Unchanged....	.....	No action.....	Aerobic facultative	20°C. to 25°C.	Pinkish becoming salmon-colored	Garter snakes, frogs, gold fish, chameleons and lizards
Myco. chelonei (Bergey et al.)	Slender rods.....	Not strongly acid-fast	.....	.....	.....	.....	Aerobic...	25°C. to 30°C.	Yellowish white	Cold-blooded and guinea pigs

pigs, persons negative to O. T. and leprous patients, but all gave negative results.

DISCUSSION:

In the following chart the characteristics of the various cold-blooded acid-fast bacilli and those of this organism isolated from a frog are tabulated. As can be observed, the acid-fast bacillus under investigation is essentially different from *Myco. ranae* (Kuster).

The cultures of this organism are moist and glistening, and have a creamy consistency on solid media; in liquid media they grow as a flocculent precipitate on the bottom of the container, but approximately thirty days later begin to form a surface pellicle which gradually becomes thicker and wrinkled. It produces a chromogenic substance soluble in absolute alcohol, but insoluble in xylol and chloroform. The organism forms hydrogen sulphide, but no indol. Acid, but no gas is produced in arabinose and xylose.

The cultures on broth produced tuberculin, which when injected into the dermis of tuberculous guinea pigs or guinea pigs previously injected with the same organism, caused a positive skin reaction. Guinea pigs inoculated with this acid-fast bacillus gave a positive skin reaction when injected with 0.1 ml. of a 10-per-cent solution of O. T. The filtrate of cultures of this organism was less toxic to tuberculous guinea pigs than dilutions of O. T.; nevertheless, more than 5 mls. of an eight-week-old glycerol broth culture caused death to tuberculous guinea pigs when injected intraperitoneally. Despite the fact that tuberculous guinea pigs were killed with 5 cc. of the filtrate, it should not be indicative of specific relationship and toxicity since it is well recognized that tuberculous guinea pigs are more sensitive to many non-specific substances and that death may be induced in tuberculous animals with doses which are innocuous to normal animals.

Antiserum prepared from rabbits that had been injected with cultures of the bacillus isolated from the frog agglutinated the same organism and another strain of *Myco. ranae*, but not *Myco. marinum*. The original frog organism was not agglutinated by serum of tuberculous guinea pigs or serum of leprous patients.*

* In a future paper the immunological reactions of this bacillus under investigation will be fully discussed.

The bacillus when first isolated grew faster at 37°C., but after subculturing it multiplied better at 25°C. to 30°C.

This strain of acid-fast bacillus is pathogenic for frogs, lizards, turtles, Puerto Rican iguanas, fresh-water fishes and mice; but it is non-pathogenic for pigeons, chickens or rats. Injected in large doses, it produces enlargement of the omentum in guinea pigs, and the lymph nodes of the organ may be purulent. In the rabbit, when injected intravenously, it causes tubercle and abscess formation in the testes; on intraperitoneal inoculation both omentum and testes might be involved.

SUMMARY:

Tubercles were found in various organs of a frog found dead in an aquarium tank; acid-fast bacilli were seen within the tissues showing a marked tendency to pack together inside the cells.

An acid-fast, pleomorphic bacillus was isolated, which grows best from 25°C. to 30°C. and which exhibited the property of acquiring a yellow to orange pigment when exposed to light. It is pathogenic for cold-blooded animals and mice, but not so for birds or rats. Under certain conditions it may produce abscess in the testes and omentum of rabbits and in the omentum of guinea pigs.

Cultures of a different strain of *Myco. ranae* were agglutinated with antiserum prepared with the organism under study, but the cultural and biological characteristics were different.

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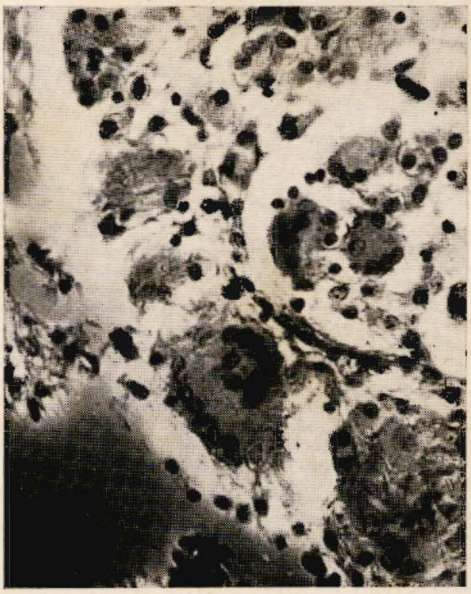

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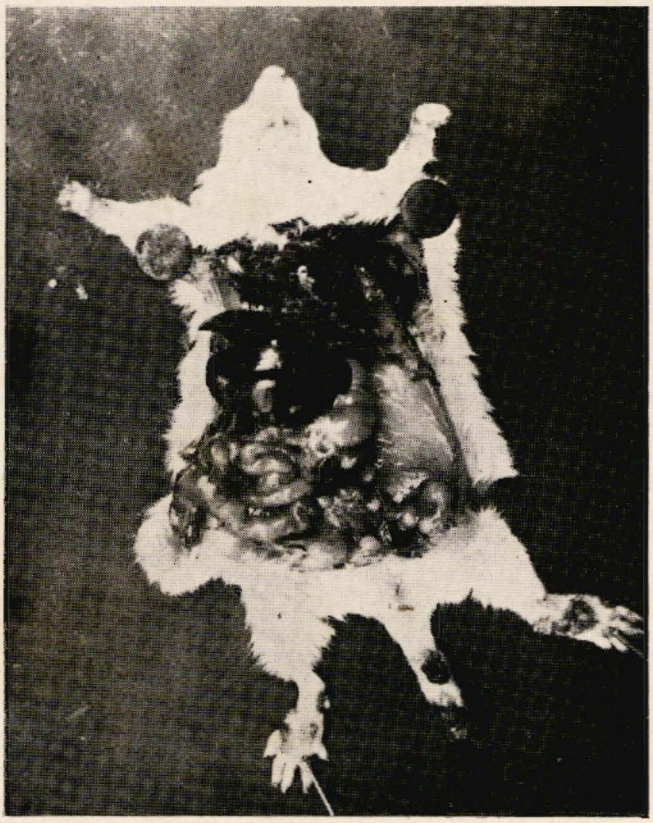

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