

FATE OF MYCOBACTERIUM TUBERCULOSIS IN MOUSE  
TISSUES AS DETERMINED BY THE MICROBIAL  
ENUMERATION TECHNIQUE\*· †

I. THE PERSISTENCE OF DRUG-SUSCEPTIBLE TUBERCLE BACILLI IN THE  
TISSUES DESPITE PROLONGED ANTIMICROBIAL THERAPY

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An essential element in the pathogenicity of a microorganism is its ability to multiply within the animal host. The degree to which any microbial species can multiply, and the correlation of that multiplication with the production of progressive disease might be expected to depend on a number of factors such as toxin production, effectiveness of host defenses, and similar host-parasite interactions. Nevertheless, it is reasonable to assume that in any particular species, within certain limits, the number of microorganisms within a given tissue would provide an index of the degree of involvement of that tissue.

Specifically with reference to *M. tuberculosis* it is known that various strains vary in their capacity to induce disease in experimental animals. Lurie (2) demonstrated that in rabbits, fully virulent bacilli of bovine origin multiplied progressively to the point of death of the animal. In contrast, human strains (not fully virulent for the rabbit) multiplied initially but soon the increase in numbers was checked. Lurie also showed (3) that the attenuated BCG strain behaved in rabbits in a way similar to that of tubercle bacilli of human origin.

Within the past few years techniques have been developed which permit the precise enumeration of culturable tubercle bacilli in animal tissues on a fairly large scale and with a reasonable degree of safety to personnel. Using these techniques, Pierce, Dubos, and Schaefer (4) have carefully studied in mice the multiplication of various strains of

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avirulent, attenuated, and virulent tubercle bacilli. Among their findings the following are pertinent to the present discussion.

Certain strains were truly *avirulent* and were unable to multiply in mice. Other *attenuated* strains underwent multiplication *in vivo* but could not give rise to progressive disease. With these strains, the maximal numbers of living bacilli recovered from the tissues corresponded directly to the severity and duration of the abortive lesions. In the case of the attenuated and the virulent strains the census of culturable bacilli present in the lungs was at first lower than that in the spleen, but in the former organ it continued to increase throughout the period of observation. In contrast, the microbial population in the spleen rapidly reached a maximum and then decreased progressively. These investigators observed that for a given infective dose and a given interval of time after inoculation, the maximal census of culturable microorganisms attained in the spleen and in the lung proved to be a direct expression of the virulence of the particular strain of tubercle bacilli.

In reviewing the facts set forth above it seemed reasonable to believe that within a well defined experimental situation the bacterial population of tubercle bacilli within tissues of mice might provide a sensitive index of factors influencing the course of tuberculous infection. Specifically with regard to antimicrobial agents used in the treatment of tuberculosis there appeared to be a need for methods of study other than those generally employed. Conventional methods for the assessment of antituberculous drug effectiveness rely on prolongation of survival time of treated animals or on the estimation of size of lesions at autopsy. Both of these methods have many definite advantages but also possess deficiencies, especially when they are utilized for evaluation of the total effect of two or more antimicrobial drugs administered together.

Accordingly, an investigation has been made of the effects of antituberculous drugs, used singly and in multiple drug regimens, on the populations of tubercle bacilli in the lung and spleen of mice with a chronic tuberculous infection. The results of the initial studies on this subject form the basis of the present report.

#### *Materials and Methods*

The test microorganisms used for all experiments were obtained from stock cultures of the virulent strain of *Mycobacterium tuberculosis hominis* (H37Rv) which were maintained by weekly passage in standard tween<sup>1</sup>-albumin medium. The cultures were 7 days old at the time of use and contained from  $2.5 \times 10^7$  to  $1 \times 10^8$  viable units of tubercle bacilli per ml.

Male albino mice (Webster Swiss strain) obtained from Rockland or Carworth Farms were used for all experiments. They weighed 15 to 20 gm. when they were received at the laboratory and infection customarily was carried out 1 week after arrival. The mice were housed in metal cages (not more than ten animals per cage) and were fed whole or pulverized standard mouse pellets with water *ad libitum*.

Infection was carried out by intravenous inoculation of 0.2 ml. of a  $10^{-1}$  dilution of culture in 0.1 per cent bovine albumin.

<sup>1</sup> Tween 80 is the proprietary name for the polyoxyethylene derivative of sorbitan monooleate, obtainable from the Atlas Powder Co., Wilmington.

*Drugs.*—Streptomycin was administered intramuscularly in 4 mg. amounts daily. Isoniazid<sup>2</sup> was administered in the diet by mixing it in a McLellan dry batch mixer with finely ground pellets. In all experiments employing isoniazid a concentration of 0.0125 per cent in the diet was used. This was originally calculated to administer 25 mg. of isoniazid per kg. of body weight daily to a 20 gm. animal that consumes 4 gm. of food per day. Calculations based upon weekly simultaneous measurements of food consumption and body weight demonstrated the actual dosage of isoniazid so administered to be 18 to 20 mg. per kg. of body weight per day throughout the course of the experiments.

Pyrazinamide<sup>3</sup> and para-amino salicylic acid also were administered in the diet by mixing them with pulverized pellets, as described above. Pyrazinamide comprised 2.0 per cent of the diet and PAS 0.75 per cent.

Pyrazinamide is a nicotinamide derivative with the formula:



The antituberculous activity of the drug in man was reported by Yeager and his associates (5).

*Preparation of Tissue Homogenates.*—Mice were sacrificed with chloroform, and aseptic techniques were utilized in removing the organs. Tissue emulsions were prepared by the method described by Pierce *et al.* (4) using a teflon homogenizer, grinding in the presence of 2 per cent bovine albumin. As was the experience of these investigators, the homogenates were found to consist of ruptured tissue cells and intact nuclei, among which tubercle bacilli could be seen to occur for the most part singly or in small aggregates of 2 to 5 bacilli.

Organ measurement was achieved by determining the amount of displacement of a measured volume (5 ml.) of diluent (2 per cent bovine albumin). This value was ultimately used in expressing the population of tubercle bacilli in terms of culturable units per ml. of tissue.

*Enumeration of Tubercle Bacilli.*—The numbers of culturable tubercle bacilli present in the infecting inoculum and the homogenates of tissues of infected animals were determined by inoculating the surface of solid oleic acid albumin agar (6) with appropriate dilutions of the substances, adhering to the principles described by Fenner *et al.* (7).

The oleic acid-albumin agar was incubated for 24 hours at 37.5°C. before use in order to ensure sterility and to effect optimal drying for inoculation of dilutions of the test substances. Cultures as well as tissue emulsions were diluted in 0.1 per cent bovine albumin in distilled water. A 0.2 ml. calibrated serologic micropipette was used to deliver 0.02 ml. of the appropriate dilution onto the surface of the medium. At least three replicates of each dilution were plated. The plates were sealed with adhesive tape (to prevent evaporation) and incubated at 37.5° C.

Colony counts were performed as routine at 2 and 3 weeks after incubation. When absence of growth was a feature, incubation was extended to a minimum of 4 weeks, and generally to 7 weeks. Two observers made independent counts of the plates at the final reading. The calculation of the total number of colonies (expressed as culturable units of tubercle bacilli) per milliliter of tissue was performed by utilizing the dilution factors and the number of colonies (preferably in the range of 5 to 50) counted at the appropriate dilution. The logarithms of these numbers were plotted graphically as a function of time after initiation of infection. The

<sup>2</sup> Kindly supplied as hydrazid by the E. R. Squibb and Sons, Division of the Olin Mathieson Chemical Corporation.

<sup>3</sup> Kindly supplied as aldinamide by the Lederle Division of the American Cyanamid Company.

resulting curves were taken to represent the fate of the tubercle bacillus during the natural course of infection as well as its fate under the influence of treatment with various regimens of antituberculous drugs.

Considering the dilution that is necessary for preparing the tissue emulsion as well as the practical limitation of the volume of the organ enumerated, it is obvious that this method has a lower limit with respect to its ability to detect culturable tubercle bacilli. The lower limits of the sensitivity of the method vary with the organ size and the number of replicates plated, and accordingly will be used to qualify the results of certain experiments herein presented.

All procedures performed with the use of infected material (the homogenization of tissues and the diluting and plating of the infected suspensions) were carried out in specially designed bacteriologic safety cabinets. These cabinets are closed during use except for armholes, and are fitted with exhaust fans which provide a steady slow inflow of air from the room. The air is exhausted through glass wool bacteriologic filters. All pipetting was done with a ring pipettor (8) attached to a vacuum so that no mouth pipetting was performed at any time. After use the interior of the hood was sprayed with 5 per cent phenol and irradiated with ultraviolet light.

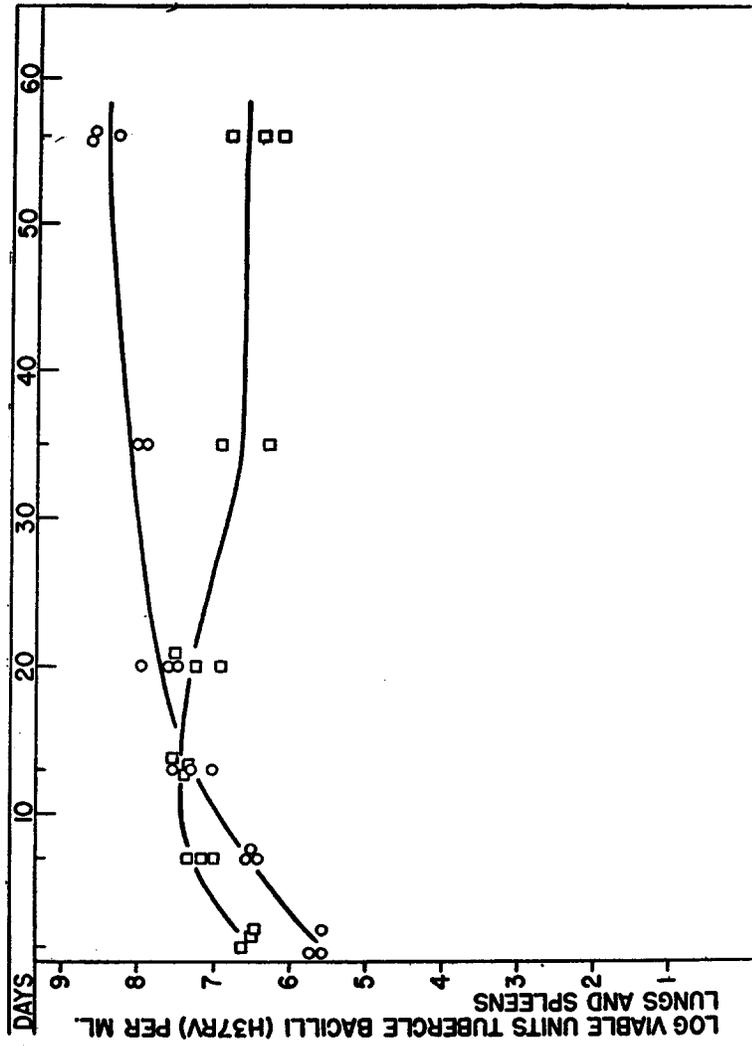
#### RESULTS

*Untreated Animals.*—In Text-fig. 1 may be seen the typical behavior of the populations of tubercle bacilli in the lung and spleen of untreated mice observed over an 8 week period.

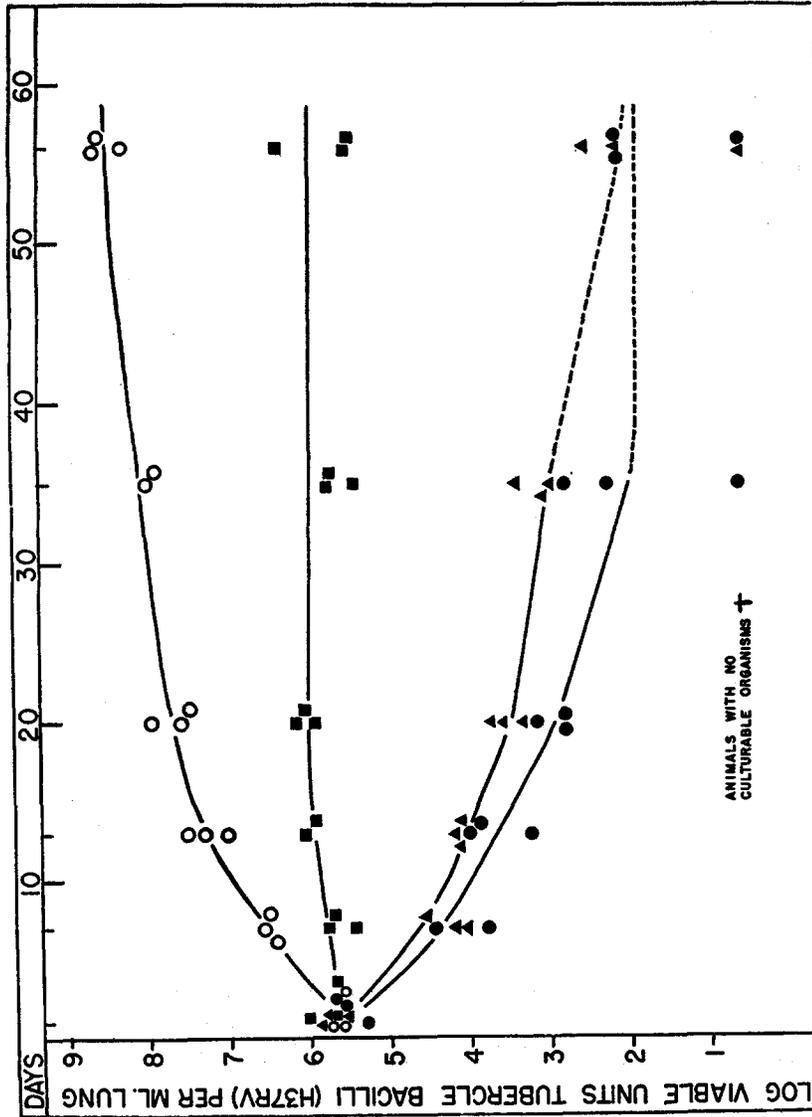
Characteristically, on the day after infection the population in the lung was somewhat lower than in the spleen. Thereafter, the numbers of organisms in the lung progressively increased until they reached a level of  $1 \times 10^8$  per ml. or slightly higher and the census then appeared to stabilize. Tuberculous lesions visible in the gross appeared in the lungs after 6 to 8 weeks of infection. From this point on, although the microbial census appeared to stabilize, the pulmonary disease increased in extent and the animals died between the 4th and 6th month of infection. Autopsy of these animals revealed extensive pneumonic consolidation.

In the spleen, the population increased initially, but quickly reached a peak, distinctly lower than the peak reached in the lung. Following this there was a fall in census and then stabilization of the splenic population at a remarkably constant level for the remainder of the period of observation. These observations appear to confirm in all respects those reported by Pierce *et al.* (4) and will be seen to be reproduced in the control animals of experiments to be presented below.

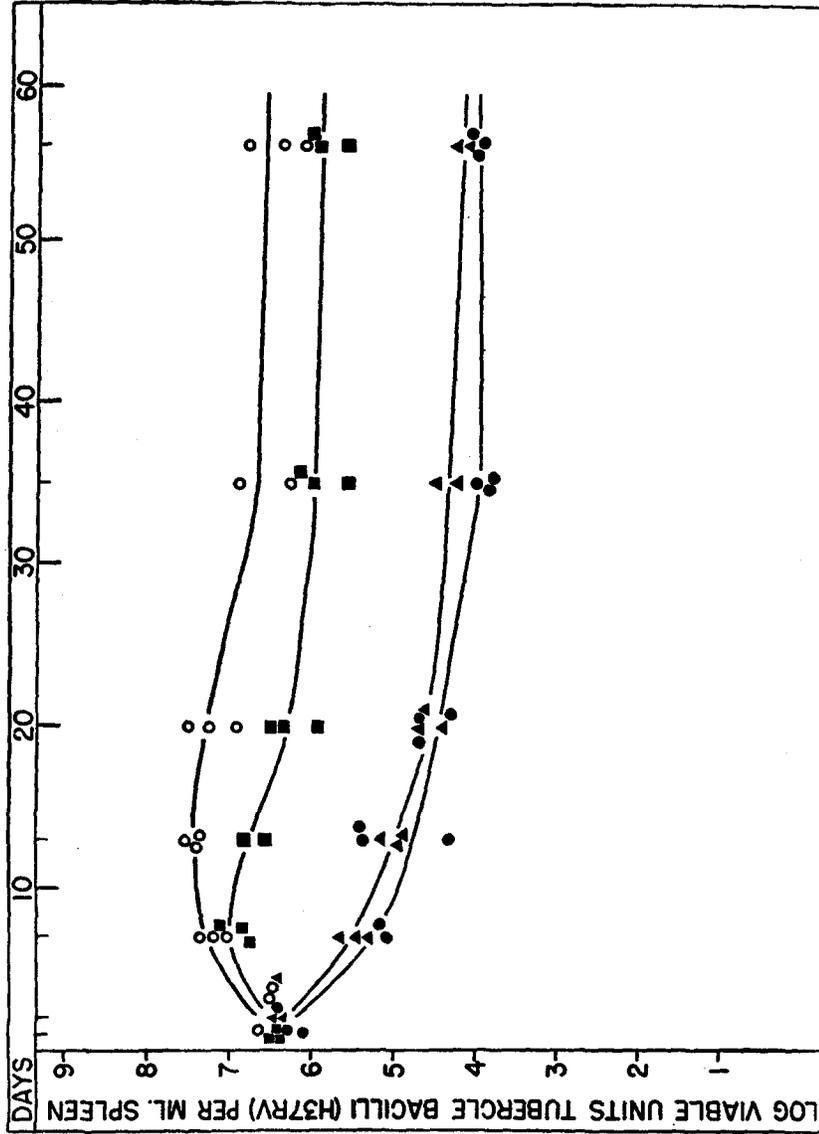
*Streptomycin and Isoniazid.*—In Text-figs. 2 and 3 may be seen the effects of streptomycin and isoniazid administered singly and concurrently. As may be seen in Text-fig. 2 the microbial population in the lungs of the untreated control animals followed the pattern described above. Streptomycin failed to reduce the initial census of tubercle bacilli, but maintained it at this initial level for the duration of the experiment. In contrast, in animals given isoniazid, a fall in the population of tubercle bacilli in the lung began at once and con-



TEXR-Fig. 1. Populations of *M. tuberculosis* (H37Rv) in lungs and spleens of untreated mice. Infecting inoculum:  $2.0 \times 10^6$  culturable units tubercle bacilli. O, lung; □, spleen.



TEXT-FIG. 2. Influence of isoniazid and streptomycin used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs during 56 days of therapy. Infecting inoculum:  $2.0 \times 10^8$  culturable units tubercle bacilli. O, control; ■, streptomycin; ▲, isoniazid; ●, isoniazid-streptomycin. The techniques used in this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.



Text-Fig. 3. Influence of isoniazid and streptomycin used singly and together on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in text-fig. 2. O, control; ■, streptomycin; ▲, isoniazid; ●, isoniazid-streptomycin.

tinued throughout. At 8 weeks, as indicated by the broken line, the population was below the limits of detection in one of three animals. When streptomycin and isoniazid were administered concurrently the reduction in population appeared to be slightly greater than that effected by isoniazid alone.

In the spleens (Text-fig. 3) of the control animals, the populations of tubercle bacilli again showed the initial rise and the subsequent fall and the stabilization previously described. Moreover, in the animals treated with streptomycin an initial rise in the microbial population occurred in the spleen as it had in the untreated animals. The peak census reached, however, was not so high as in the control animals which had received no streptomycin. The microbial census then fell and stabilized after approximately 3 weeks, remaining thereafter at a fairly constant level for the remainder of the experimental period.

In contrast to the effect of streptomycin, in the animals which received isoniazid a fall in the population of tubercle bacilli in the spleen began at once. The rate of fall in the early weeks was rapid but thereafter a notable alteration in the curve ensued, with stabilization of the population at a low level, approximately  $1 \times 10^4$  organisms per ml. of spleen. This phenomenon was quite consistent as will be seen in subsequent experiments.

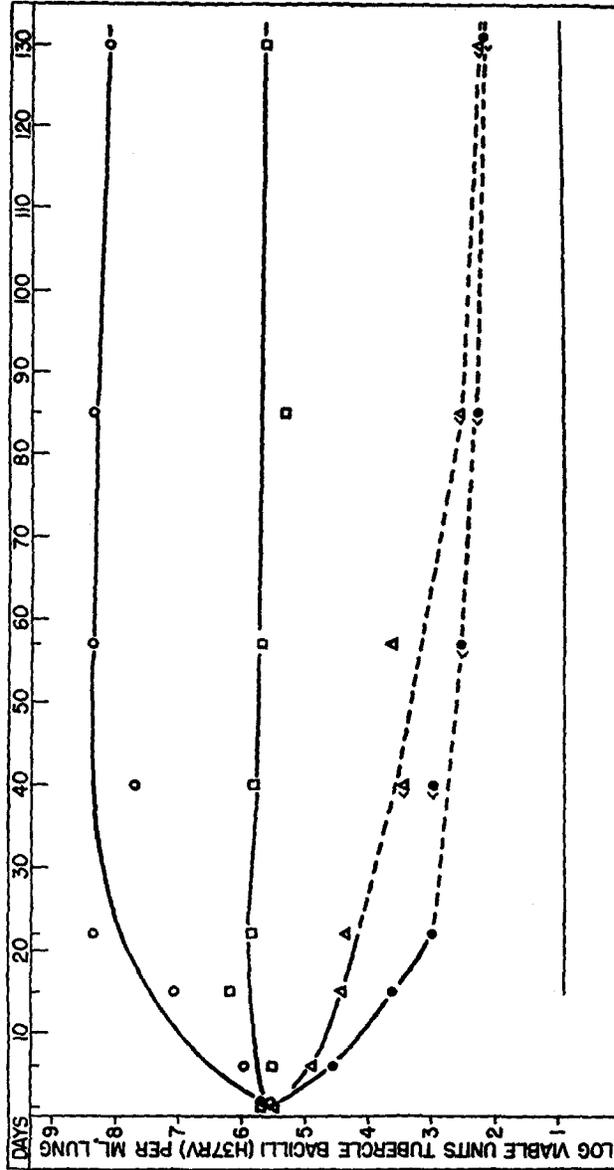
Although the populations in the spleen were always found to be low in the animals treated with isoniazid alone, in no instance was there a failure to recover tubercle bacilli. This is quite a different situation from that encountered in the lung, in which the populations of tubercle bacilli fell below the limits of detection in a number of instances after 8 weeks or more of isoniazid therapy.

In Text-fig. 3 are shown the results obtained in the spleens of animals which received both isoniazid and streptomycin. Again, as in the lung, the values observed seemed consistently lower than in animals which received only isoniazid.

In Text-figs. 4 and 5 are presented data from similar experiment which was extended over a 4 month period and in which more animals were used for each observation period. As may be seen, the results were essentially the same as in the experiments presented in Text-figs. 2 and 3. At every observation point, the populations of tubercle bacilli in the tissues of the animals treated with isoniazid and streptomycin together, were slightly but significantly lower than those from the animals treated with isoniazid alone.

*Drug Susceptibility in vitro of Tubercle Bacilli Which Survived despite Antimicrobial Therapy.*—The strains of tubercle bacilli which had survived in the mouse tissues throughout therapy with isoniazid and streptomycin used singly and together were treated for susceptibility to these drugs *in vitro*.

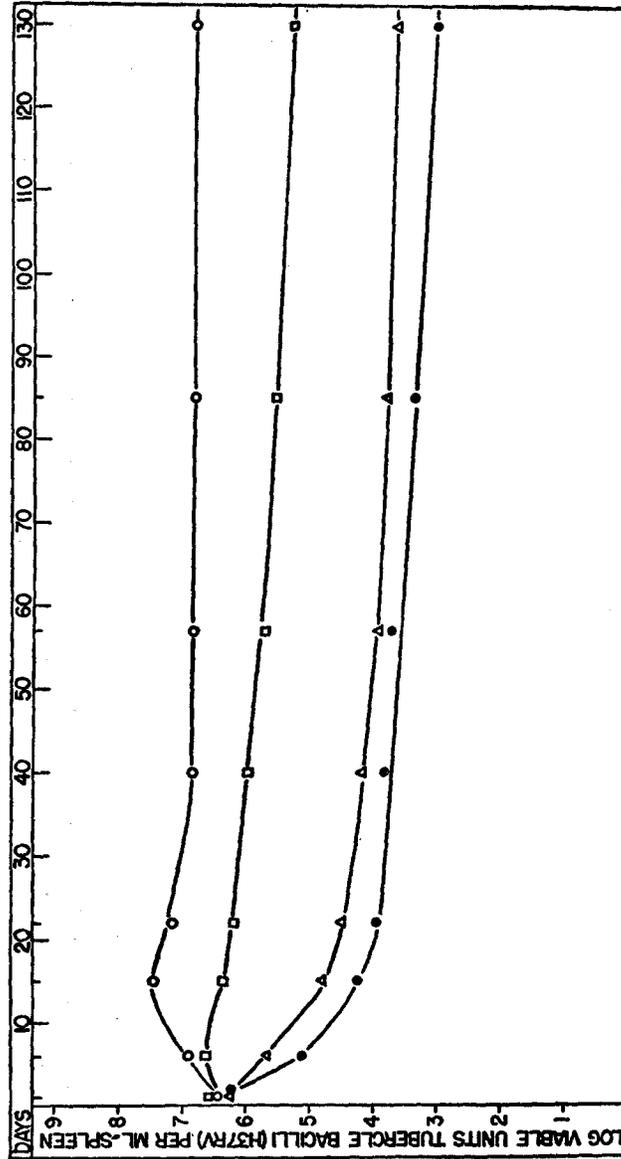
A quantitative method was employed which consisted of inoculating measured aliquots of the tissue homogenates onto culture medium containing isoniazid concentrations of 0.1, 1.0, and 10.0  $\mu\text{g}$ . per ml. or streptomycin concentrations of 1.0 and 10.0  $\mu\text{g}$ . per ml. This procedure was performed as routine in the latter phases of the experiments.



Animals with no culturable organisms†

△ 2/5  
 ● 6/7  
 △ 4/6  
 ● 3/6  
 ● 2/3  
 △ 1/3  
 ● 1/3

TEXT-Fig. 4. Influence of isoniazid and streptomycin used singly and together on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 130 days of therapy. Infecting inoculum:  $2.1 \times 10^6$  viable units tubercle bacilli. Each symbol represents the average bacterial population of 3 to 7 animals. O, control; □, streptomycin; △, isoniazid; ●, isoniazid-streptomycin. † The techniques used in this experiment permitted detection of 70 to 90 culturable units tubercle bacilli per lung.



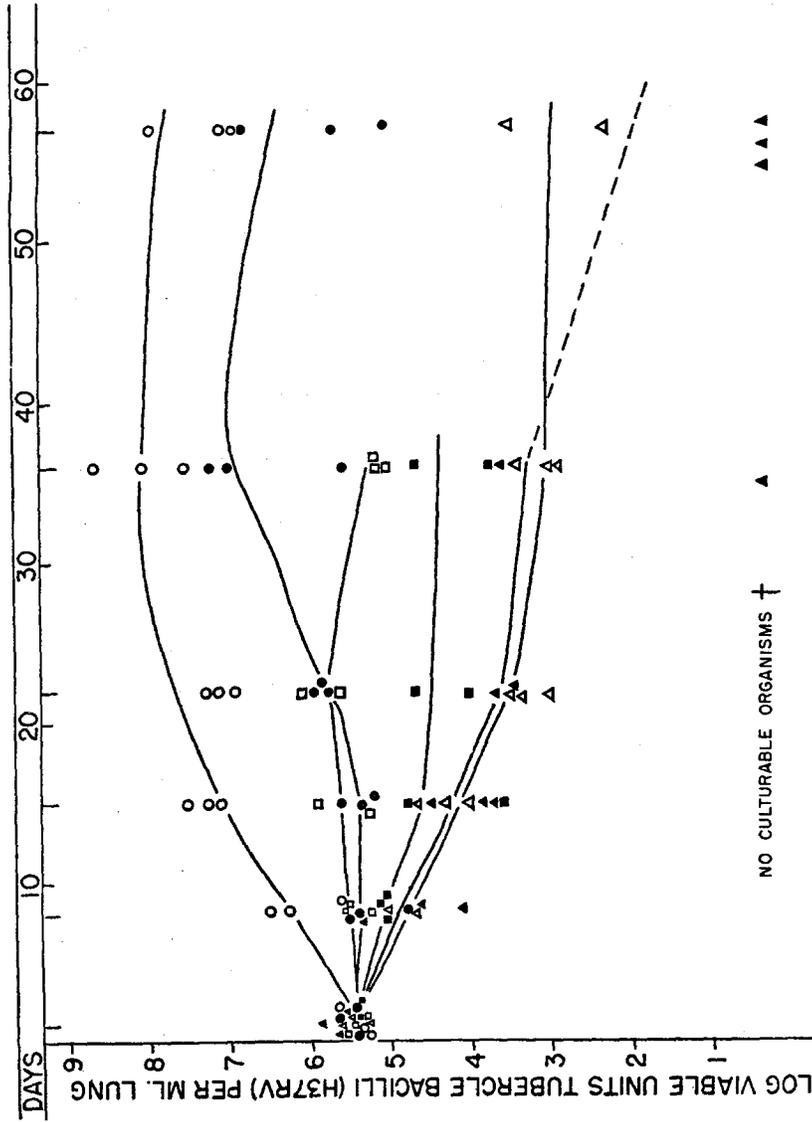
TEXT-FIG. 5. Influence of isoniazid and streptomycin used singly and together on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung studies are shown in Text-fig. 4. O, control; □, streptomycin; Δ, isoniazid; ●, isoniazid-streptomycin.

Tubercle bacilli resistant to streptomycin (*i.e.* capable of growth in drug concentrations of 1.0  $\mu\text{g}$ . per ml.) represented less than 2 per cent of any individual population of tubercle bacilli in the animals which received streptomycin therapy for the 12 week period. With respect to isoniazid, the individual populations of tubercle bacilli were likewise almost wholly composed of drug-susceptible cells with the exception of the strains isolated from four animals. 2 of these 4 strains were approximately evenly divided among tubercle bacilli which were susceptible to isoniazid concentrations of 1.0  $\mu\text{g}$ . per ml. and bacilli which were resistant to this concentration. The other two strains consisted entirely of tubercle bacilli resistant to the 10.0  $\mu\text{g}$ . per ml. concentration of isoniazid. 1 of these 2 completely resistant strains was isolated from an animal in which an apparent resurgence to a high population had occurred despite continued therapy with isoniazid alone. The other strain was found in an animal in which a drug-induced fall in census was apparently being maintained. In the observations as a whole, therefore, there was no correlation between the persistence of tubercle bacilli in the tissues despite continued antimicrobial therapy and the resistance of these tubercle bacilli *in vitro* to the drug used in the antimicrobial therapy.

*Para-Amino Salicylic Acid with Streptomycin and Isoniazid.*—In Text-figs. 6 and 7 the data are presented on the populations of tubercle bacilli in the lung and spleen of animals which received para-amino salicylic acid (PAS) administered either alone or with streptomycin or isoniazid.

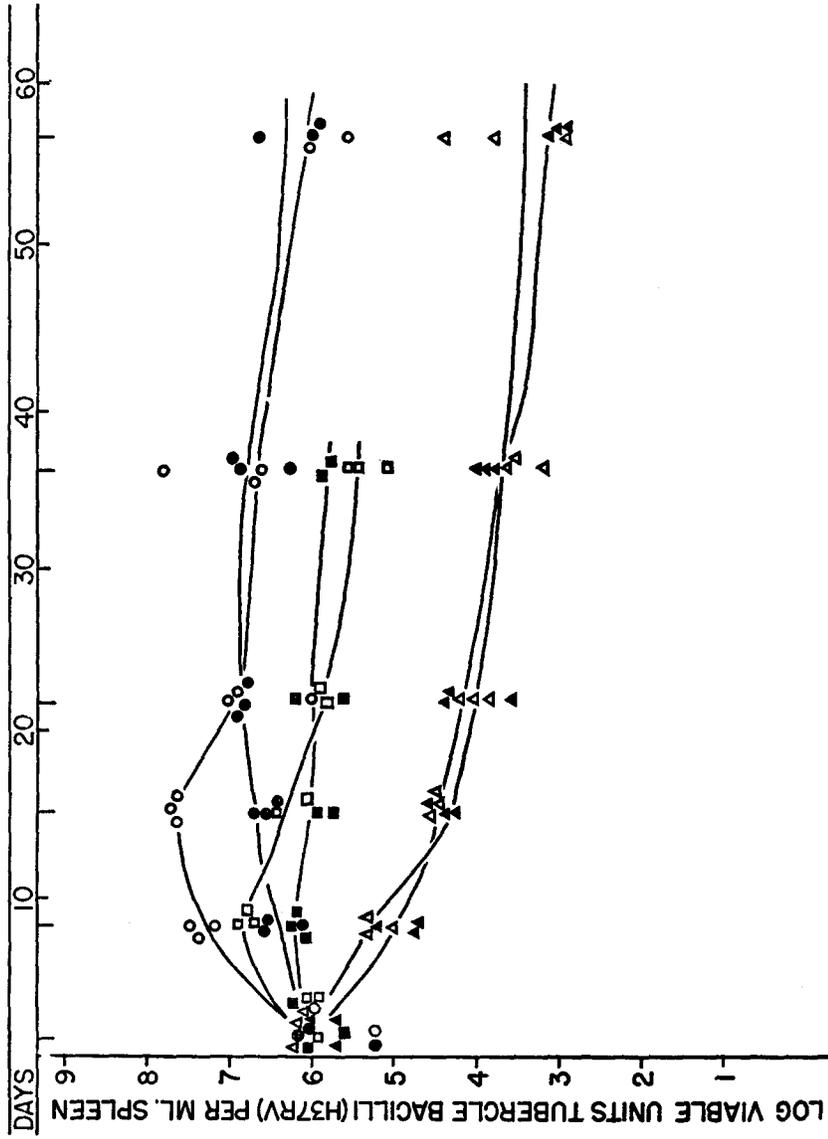
The results observed in the lungs of the control animals (Text-fig. 6) and in those of the animals given isoniazid or streptomycin may be seen to be closely similar to those previously presented. The administration of PAS alone prevented an increase in the total population of tubercle bacilli during the first 3 weeks. Thereafter despite continuation of the PAS therapy there was an increase in the population of tubercle bacilli above the original level. The administration of PAS and streptomycin together notably enhanced the total antimicrobial action and this two-drug regimen effected a distinct reduction in the microbial population in the lung. The administration of PAS and isoniazid together, however, did not significantly increase the total antimicrobial effect over that produced by the latter drug when used alone.

In the spleen (Text-fig. 7), the microbial populations in the untreated animals and in those which received streptomycin or isoniazid singly were similar to the results in previous experiments. In the animals which received PAS alone there was a slow, steady increase in the number of tubercle bacilli so that by the end of the experiment the populations in the PAS-treated animals approximated those present in the control animals. The enhancing effect when PAS and streptomycin were given together was notable in that the initial multiplication which occurred with streptomycin alone failed to occur when PAS was administered along with the streptomycin. As in the lung, the ad-

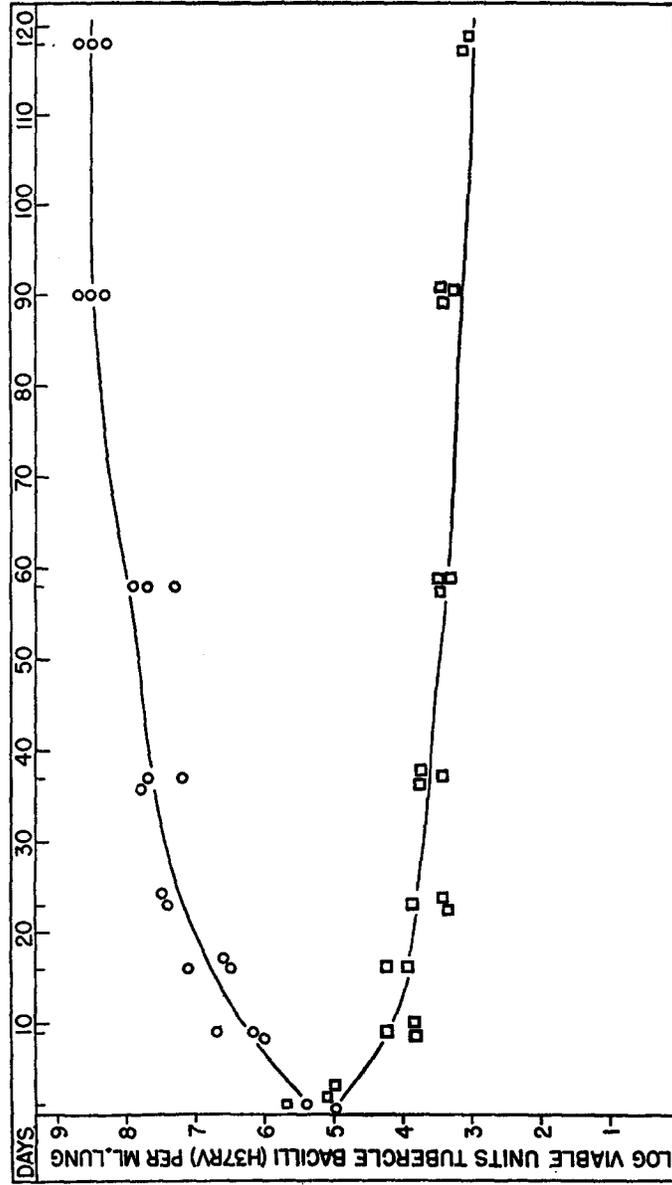


TEXT-FIG. 6. Influence of drugs on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 57 days of therapy. Comparative effects of isoniazid, streptomycin, and PAS used singly and isoniazid-PAS and streptomycin-PAS used together. Infecting inoculum:  $5.7 \times 10^5$  culturable units tubercle bacilli. O, control; ●, streptomycin; ▲, streptomycin-PAS; Δ, isoniazid; ■, isoniazid-PAS.

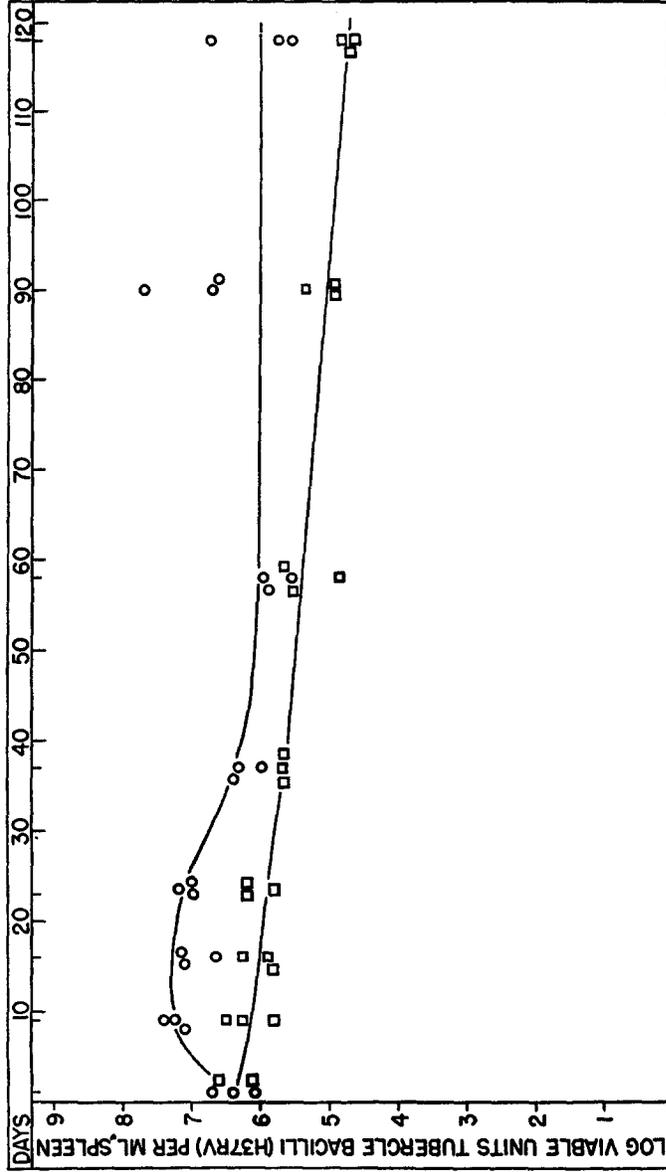
† The techniques used in this experiment permitted detection of 70 to 90 culturable units tubercle bacilli (H37Rv) per lung. See text for explanation of abbreviated streptomycin and streptomycin-PAS curves.



TEXT-FIG. 7. Influence of drugs on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in Text-fig. 6. O, control; ●, PAS; □, streptomycin; ■, streptomycin-PAS; △, isoniazid; ▲, isoniazid-PAS.



TEXT-FIG. 8. Influence of streptomycin-PAS on populations of tubercle bacilli (H37Rv) in mouse lungs during 118 days of therapy. Infecting inoculum:  $2.0 \times 10^6$  culturable units of tubercle bacilli. O, control; □, streptomycin-PAS.



TEXT-FIG. 9. Influence of streptomycin-PAS on populations of tubercle bacilli (H37R<sup>A</sup>) in spleens of the same animals whose lung studies are shown in Text-fig. 8. O, control; □, streptomycin-PAS.

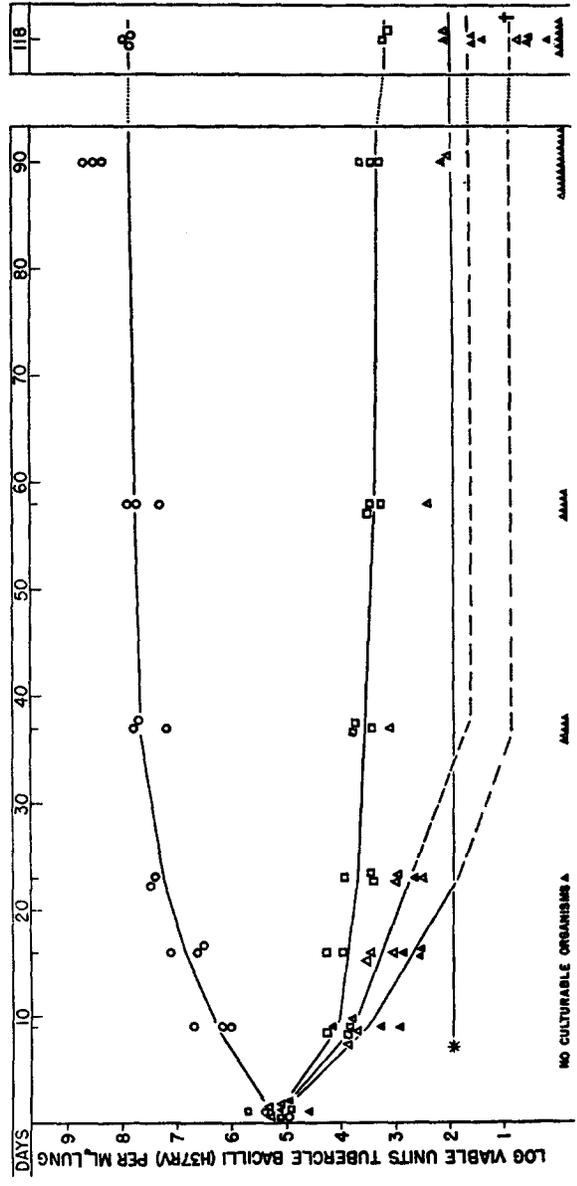
ministration of PAS along with isoniazid had no greater effect on the populations of tubercle bacilli in the spleen, than was the case when isoniazid was given alone.

It will be noted in Text-figs. 6 and 7 that the curves of the population data for the animals which received streptomycin are abbreviated. Due to an accident which occurred in the animal quarters a number of the mice in this experimental subgroup were inadvertently killed and thus insufficient numbers remained for sampling. An additional experiment was done therefore (Text-figs. 8 and 9) in which animals received streptomycin and PAS together for an 118 day period. As may be seen in Text-figs. 8 and 9 the populations of tubercle bacilli in both lung and spleen followed the same course under chemotherapy as was observed in the present experiment portrayed in Text-figs. 6 and 7. After 3 weeks of treatment with both streptomycin and PAS, the census of tubercle bacilli in the spleen had become stable and persisted unchanged thereafter throughout the remainder of the 118 day period of therapy.

*Isoniazid-Streptomycin-PAS Administered together.*—The principal object of the next experiment (Text-figs. 10 and 11) was to determine the effects on the populations of tubercle bacilli in the mice when three drugs, isoniazid, streptomycin, and PAS were administered together. As mentioned above, an additional subgroup of animals in this experiment received streptomycin along with PAS and another subgroup received isoniazid alone.

As may be seen in Text-fig. 10, administration of all three drugs together resulted in a reduction in the census of tubercle bacilli in the lung comparable with that observed with isoniazid alone. On the last day of this experiment (*i.e.* the 118th day of chemotherapy) a technique of considerably increased sensitivity was used for detection of the surviving tubercle bacilli. Whereas the technique used previously had permitted the detection of 70 to 90 culturable units per lung, the new technique permitted the detection of 1 to 3 culturable units per lung. By use of the more sensitive technique it was possible to demonstrate the persistence of tubercle bacilli in the lungs of all of the animals which had received the 3 drugs together. Only the less sensitive technique was used in the determinations made in the animals which received isoniazid alone and bacilli could be detected in only three animals.

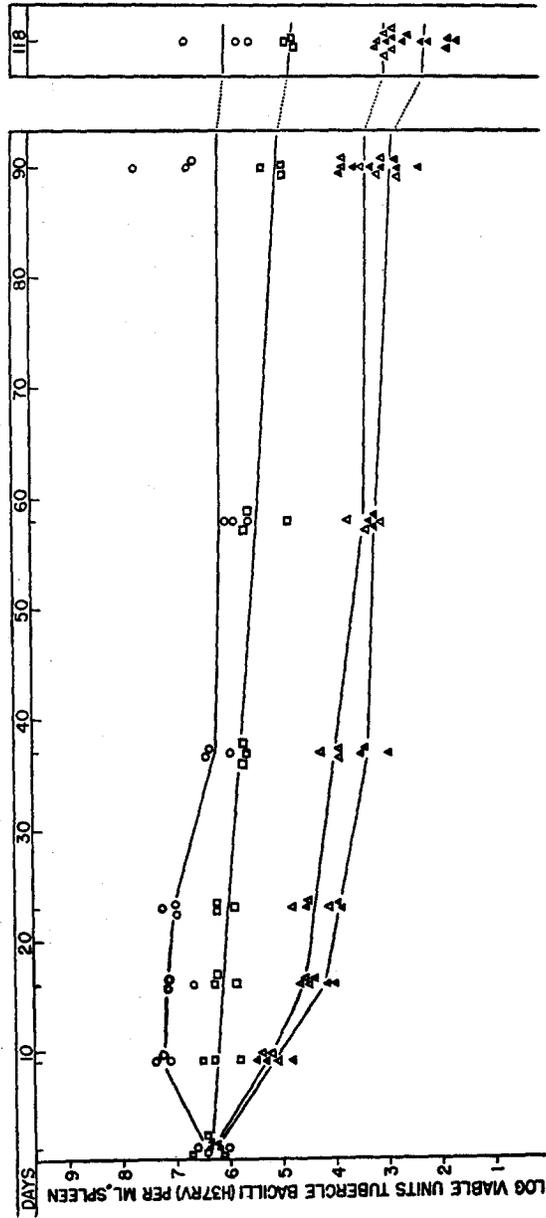
In the spleen (Fig. 11) the administration of isoniazid, streptomycin, and PAS together, was followed by a reduction in the census of tubercle bacilli to a point slightly below that produced by isoniazid alone but comparable with the levels previously observed with isoniazid-PAS or isoniazid-streptomycin. Of particular interest was the fact that stabilization of the population of tubercle bacilli in the spleen occurred during the triple drug therapy. The microorganisms persisted throughout the entire period of antimicrobial therapy in a fashion identical with that observed with the previously studied single and multiple drug regimens.



TEXT-Fig. 10. Influence of drugs on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 17 weeks of therapy. Comparative effect of isoniazid alone, streptomycin-PAS and isoniazid-streptomycin-PAS during 17 weeks of therapy. Infecting inoculum:  $2.1 \times 10^6$  viable units of tubercle bacilli. O, control; □, streptomycin-PAS; ▲, isoniazid; ◆, isoniazid-streptomycin-PAS.

\* The techniques used in this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli (H37Rv) per lung.

† The techniques used to enumerate the microbial census of lungs from animals treated with isoniazid-streptomycin-PAS on the last day of the experiment permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.



TEXT-FIG. 11. Influence of drugs on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in Text-fig. 10. O, control; □, streptomycin-PAS; Δ, isoniazid; ▲, isoniazid-streptomycin-PAS.

*Pyrazinamide and Isoniazid.*—In Text-fig. 12 may be seen the effects of pyrazinamide and isoniazid on the populations of tubercle bacilli in the lung. The behavior of the microbial populations in the untreated control animals and in those which received isoniazid alone was the same as seen previously. Pyrazinamide alone proved surprisingly effective during the first 2 months of administration at the end of which time no tubercle bacilli could be cultured from the lungs of any of the three animals tested. At the end of the 3rd month, however, there had been a marked increase in the populations of tubercle bacilli in two of the three animals. (The less sensitive technique for detection of the tubercle bacilli was used in these experiments.) The apparent resurgence of the microbial populations in the lungs during therapy with pyrazinamide alone was of particular interest because the time relationships roughly coincide with those observed in the clinical relapse of tuberculosis in patients treated with this drug (5).

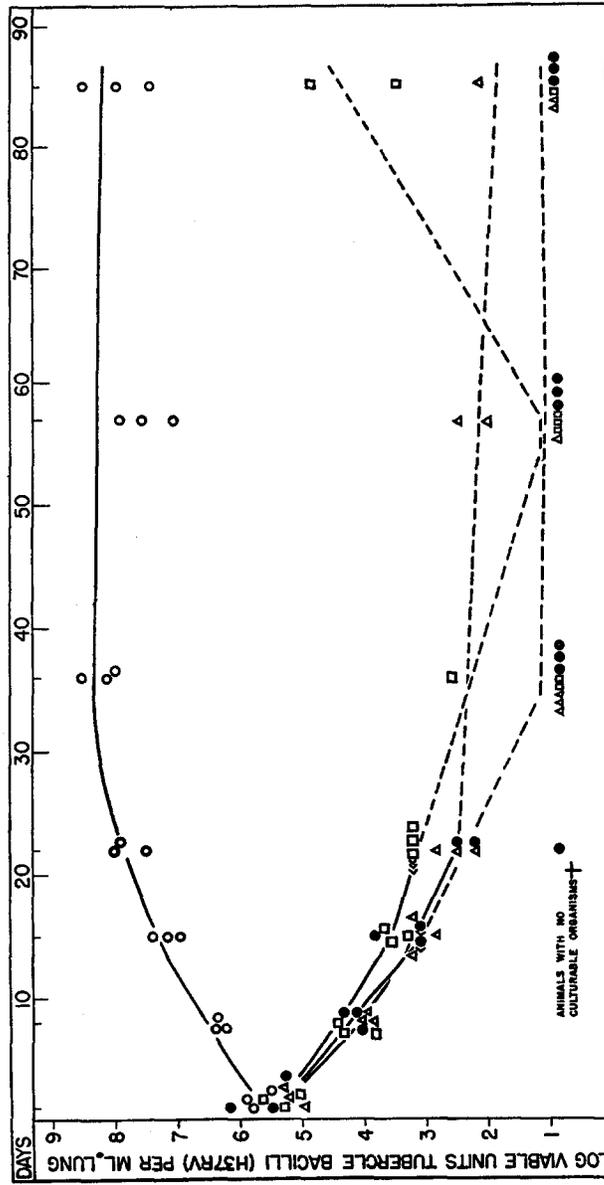
The administration of pyrazinamide and isoniazid together was more effective than with either drug alone. No tubercle bacilli could be cultured from the lungs of one of the three animals examined at 3 weeks, and throughout the remainder of the experiment no tubercle bacilli could be cultured from the lungs of any of the animals at any of the observation points.

The fate of the bacilli in the spleen in this pyrazinamide-isoniazid experiment was of particular interest as may be seen in Text-fig. 13. When pyrazinamide and isoniazid were administered together, there was first an apparent antagonism of the isoniazid, for the census of tubercle bacilli fell less rapidly than in the animals which received isoniazid alone. After the first 2 weeks of therapy, however, the situation was reversed and the microbial census in the animals which received pyrazinamide and isoniazid together fell steadily until it eventually fell below the limits of detection. 5 weeks after the start of therapy, no tubercle bacilli could be cultured from the spleen of one of two animals, after 8 weeks there were no culturable bacilli in the spleens of two of three animals and after 12 weeks there were none in any of the three animals examined.

The course of the microbial populations in the untreated control animals and in those which received isoniazid alone, was the same as in the previous experiments. Pyrazinamide when administered alone, prevented any increase in the census of tubercle bacilli in the spleens and after 2 weeks of therapy, the populations showed a slight fall.

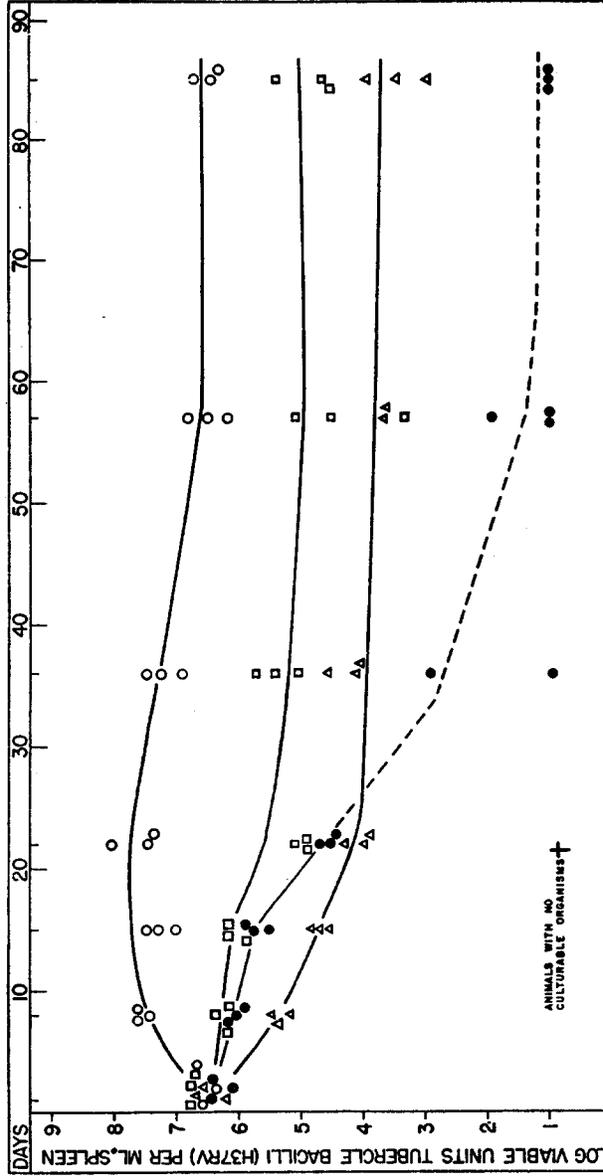
This effect of a marked reduction in the populations of tubercle bacilli in the spleen after the administration of pyrazinamide and isoniazid together, was in sharp contrast to the effects noted with the other drugs used either singly or in the various multiple drug regimens. With all the other chemotherapies studied the populations of tubercle bacilli invariably persisted in the spleen with a stable census after an initial fall of greater or lesser magnitude.

In the experiment shown in Text-figs. 12 and 13, the number of animals sacri-



TEXT-FIG. 12. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs during 85 days of therapy. Infecting inoculum:  $2.4 \times 10^6$  culturable units tubercle bacilli (H37Rv). ○, control; □, pyrazinamide; △, isoniazid; ●, pyrazinamide-isoniazid.

† The techniques used in this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.



TEXT-FIG. 13. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Text-fig. 12.

† The techniques used in this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per spleen. O, control; □, pyrazinamide; △, isoniazid; ●, pyrazinamide isoniazid.

ficed at each observation point was relatively small. Nevertheless, the phenomenon was so unique that it received further intensive study the results of which are presented in an accompanying report (9). Suffice it to say at this point that the basic observation has been repeatedly confirmed, namely, that tubercle bacilli uniformly disappear from the spleens only in those animals which have received pyrazinamide along with another drug.

*Histopathologic Studies.*—Any of the drugs or multiple drug regimens studied in the preceding experiments, provided sufficient antimicrobial action to prevent the development of macroscopic lesions when chemotherapy and infection were started on the same day. Nevertheless, the number of tubercle bacilli present within the infected organs showed considerable variation depending upon the chemotherapy employed. The particular pattern of the microbial population change produced by any one of the drugs or multiple drug regimens was characteristic for that drug regimen within any one of the sets of circumstances studied. It seemed important, therefore, to examine the tissues microscopically to determine whether the tissue changes of the tuberculous lesions could be related to the pattern of the microbial population changes characteristic for the individual chemotherapies. Accordingly, sections of lung and spleen were made from the animals of various groups at the termination of the experiments and the results of the examination of this material may be briefly summarized.

The control untreated animals showed the characteristic tissue changes associated with experimental tuberculous infections of mice. The lungs (Fig. 1) showed dense confluent areas of cellular infiltration, with marked proliferative inflammation and small areas of necrosis. The spleens were markedly enlarged, and on microscopic section showed intensive hyperplasia, with many focal areas of proliferation. Acid-fast bacilli were numerous in both organs.

In animals treated with PAS (Fig. 2) the lungs showed considerably less cellular infiltration, especially involving the alveoli. Nevertheless, large areas of proliferative infiltration persisted, particularly involving the alveolar septa. The spleens were somewhat less hyperplastic than in the untreated animals, but the difference was not marked. Tubercle bacilli were numerous in both organs.

Figs. 3 to 9 are photomicrographs of the lungs of animals given isoniazid, streptomycin, and pyrazinamide singly, as well as the following multiple drug regimens: streptomycin and PAS, isoniazid and PAS, isoniazid, streptomycin and PAS, and isoniazid and pyrazinamide. Minor differences in the amount of residual inflammation can be detected from one animal to another, but the striking feature is how little infiltration could be detected in any of the lungs from the animals treated on these various regimens. Some thickening of the alveolar septa was present in all of the groups except for those animals treated with pyrazinamide and isoniazid. In this particular group, after 12 weeks of treatment, the lungs appeared entirely normal. In the spleens, from all groups of animals, some slight hyperplasia was present, the least amount again being noted in the animals which received pyrazinamide and isoniazid together.

A prolonged search was made for acid-fast bacilli in the sections of both lung and spleen from those groups of animals represented in Figs. 3 to 9. In general, this search was most unrewarding. A rare tubercle bacillus was found in an organ with a concentration of bacilli

as low as  $1 \times 10^4$  per ml. The difficulty in finding acid-fast bacilli increased markedly, however, as the concentration fell below  $1 \times 10^6$  per ml., and the bacilli could not be found with any degree of regularity when the total population was below  $1 \times 10^6$  per ml.

#### DISCUSSION

From the above observations it may be seen that the influence of antimicrobial drugs on populations of tubercle bacilli (H37Rv) persisting in mice is considerably different depending on whether the tubercle bacilli are situated in the lung or in the spleen. This difference between the 2 organs in terms of drug-parasite relationships is analogous to the difference which exists between them with respect to host-parasite relationships. The latter phenomenon was reported previously by Pierce, Dubos, and Schaefer (4) and has been observed repeatedly in the untreated control groups of animals in the present investigation.

In the spleen, the infection appeared to be somewhat less active than in the lung as judged by the height of the maximal splenic populations attained and the slow steady fall in census which occurred thereafter. By the same token, in the spleen the influence of most antimicrobial drugs was considerably less marked than when the same drugs were acting on tubercle bacilli situated in the lung.

Thus the infection appeared to be subjected to stronger natural limiting influences in the spleen but this was not accompanied by any increase in the effectiveness of the antimicrobial drugs. It is clear, therefore, that the drug-parasite and the host-parasite reactions are distinctive for each of the two organs. It is not clear, however, how the particular environment which each organ provides for the tubercle bacilli, exercises its influence on what may be attained therein by antimicrobial therapy.

Comparative examinations of the influence of the various antimicrobial drugs on the number or character of the tuberculous lesions appeared to be a far less sensitive index of antimicrobial drug effectiveness than determinations of the census of culturable tubercle bacilli within the organs. For example, the lungs from many of the various drug therapy groups were indistinguishable one from another in terms of the extent of lesions or their appearance. In terms of the size of the populations of tubercle bacilli within these same lungs, however, there were marked differences which were characteristic and uniformly predictable for the particular drug or drug regimen employed. This similarity in histopathologic appearance despite wide variation in population size may simply reflect differences in the time required to effect the separate processes of reducing the microbial population and healing the lesions. Finally, in this connection it is of interest to note that the population of tubercle bacilli does not have to be increasing in order for the lesions to continue to progress. Once a population of tubercle bacilli attained a sufficiently high level, as in the

untreated animals, the resulting pulmonary lesions showed a steady progression thereafter even though the size of the population remained stable.

In the present studies, isoniazid was clearly the most effective single drug in reducing the number of tubercle bacilli in both lung and spleen. Pyrazinamide was surprisingly effective and for short periods its potency approached that of isoniazid. Subsequently, however, resurgence of growth occurred in some of the pyrazinamide-treated animals. The effectiveness of streptomycin in reducing the total microbial population was appreciably less than that of isoniazid or pyrazinamide. Indeed, during the first 10 days of streptomycin therapy the population of tubercle bacilli in the spleen regularly showed an increase. As might have been anticipated, para-amino salicylic acid (PAS) showed a relatively low order of effectiveness. It is of interest, moreover, that the period during which PAS appeared to maintain its full antimicrobial effectiveness was limited to the first 3 or 4 weeks of therapy.

The technique of microbial enumeration has also seemed to offer a useful method for the evaluation of the effects of simultaneous administration of 2 or more drugs. For example, the population studies in both lung and spleen provided evidence that the effect of streptomycin was enhanced by PAS. In contrast, PAS did not enhance the action of isoniazid. Moreover, in all the regimens tested including the administration of all three drugs together, the characteristic pattern of stabilization of the population in the spleen was observed.

Current concepts of the biology of tuberculous infection stress the importance of the ability of small numbers of tubercle bacilli to remain viable in relatively quiescent areas of disease for long periods of time. This ability to survive is generally considered to be a major factor in the marked tendency to recrudescence exhibited by tuberculous lesions. Clinical experience with the generally accepted chemotherapeutic regimens provide ample evidence that none of these therapies is eradicated, and that recrudescence of apparently quiescent lesions remains an important problem. In the studies herein reported the phenomenon of persistence of culturable tubercle bacilli in the mouse spleen was regularly observed during therapy with isoniazid, streptomycin, and PAS given singly, in pairs and all 3 simultaneously. Moreover, when subsequently tested *in vitro*, the persisting strains of tubercle bacilli, with few exceptions, were susceptible to isoniazid or to streptomycin.

In striking contrast to these effects were those observed with the combination of pyrazinamide and isoniazid. With this therapy, reduction in the population of tubercle bacilli below detectable levels occurred earlier in the lung than with isoniazid alone and moreover, it occurred with uniformity. A more unique effect, however, was the uniform disappearance of the tubercle bacilli from the spleen. It is obvious that it is extremely difficult to establish that the tubercle bacilli had actually been eliminated from the tissues. Nevertheless, the tech-

niques employed in the present study are capable of detecting tubercle bacilli when present in very small numbers. The mechanics of this phenomenon of the complete disappearance of tubercle bacilli from the lung and spleen of mice which received pyrazinamide and isoniazid have been subjected to detailed study and the results are presented in the succeeding report of this series (9). In other studies in this laboratory (10) it has been established that tubercle bacilli are not uniformly eliminated from the tissues of man by the concurrent administration of pyrazinamide and isoniazid although the treatment is highly effective.

#### SUMMARY

Observations are presented on the behavior of populations of tubercle bacilli in the tissues of mice during the administration of antimicrobial drugs. The behavior of the populations during therapy with any particular drug was different depending upon whether the tubercle bacilli were subsisting in the lung or in the spleen. Moreover, the pattern of microbial behavior was distinctive and predictable for each drug studied. Changes in the size of the populations of tubercle bacilli in the tissues appeared to be a more sensitive reflection of drug influence than microscopic study of the number and character of the tuberculous lesions. Nevertheless, in untreated animals, pulmonary lesions evolved and progressed steadily to a fatal outcome despite the fact that the populations of tubercle bacilli had stabilized at a relatively high census early in the course of therapy.

The uniform persistence of tubercle bacilli in the spleen throughout prolonged drug administration was demonstrated with every drug or multiple drug regimens except for pyrazinamide when accompanied by isoniazid. Cultures of the bacilli which survived in the tissues despite antimicrobial therapy were highly susceptible to the drugs employed when tested *in vitro*. Thus the survival of the tubercle bacilli in the tissues represented microbial persistence rather than drug resistance.

When pyrazinamide and isoniazid were administered together, it was not possible to detect the microorganisms in the spleen or lungs of treated animals. A detailed investigation of this apparent abolition of microbial persistence forms the subject of an accompanying report.

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#### BIBLIOGRAPHY

1. Tompsett, R., McCune, R. M., Jr., Ormond, L., Deuschle, K., Muschenheim, C., and McDermott, W., *Tr. Assn. Am. Physn.*, 1954, **67**, 224.
2. Lurie, M. B., *J. Exp. Med.*, 1928, **48**, 115.
3. Lurie, M. B., *J. Exp. Med.*, 1932, **55**, 31.

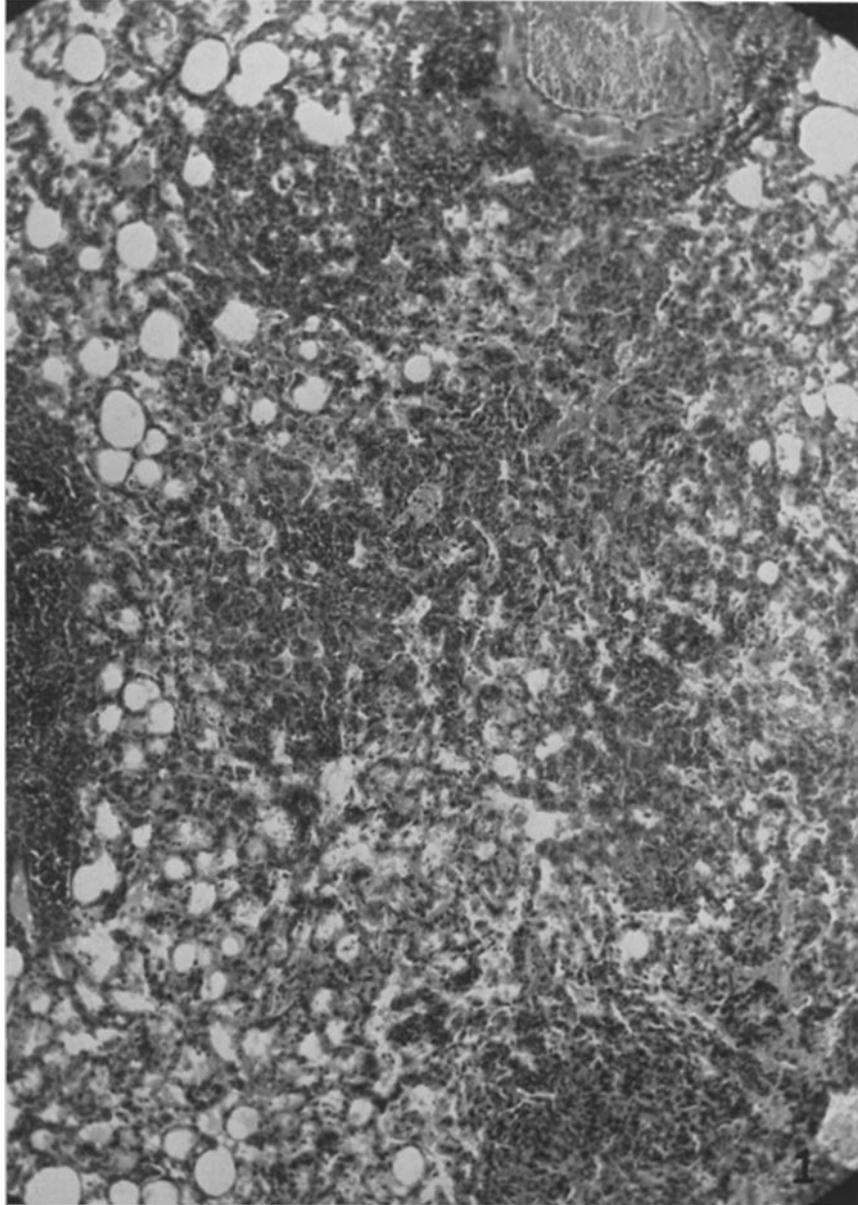
4. Pierce, C. H., Dubos, R. J., and Schaefer, W. B., *J. Exp. Med.*, 1953, **97**, 189.
5. Yeager, R. L., Munroe, W. G. C., and Desau, F. F., *Am. Rev. Tuberc.*, 1952, **65**, 523.
6. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56**, 334.
7. Fenner, F., Martin, S. P., and Pierce, C. H., *Ann New York Acad. Sc.* 1949, **52**, 761.
8. Wedum, A. G., *J. Lab. and Clin. Med.*, 1950, **35**, 648.
9. McCune, R. M. Jr., Tompsett, R., and McDermott, W., *J. Exp. Med.*, 1956, **104**, 763.
10. McDermott, W., Ormond, L., Muschenheim, C., Deuschle, K., McCune, R. M., Jr., and Tompsett, R., *Am. Rev. Tuberc.*, 1954, **69**, 319.

#### EXPLANATION OF PLATES

FIGS. 1 to 9. The series of plates are photomicrographs of lungs of mice from various experiments described in the text. In each instance, the mice were infected by intravenous inoculation of approximately  $2 \times 10^6$  culturable units of tubercle bacilli (H37Rv). Treatment was started on the day of infection and at the interval stated in the individual legend mice were sacrificed, the tissues were obtained for section, and enumerations of the populations of tubercle bacilli were performed on the homogenized tissues of groups of animals as described in the text. In each legend is indicated the drugs administered and the average population of tubercle bacilli in the lung at time of sacrifice. The magnification in each plate is approximately  $100\times$ . Hematoxylin and eosin stain.

#### PLATE 56

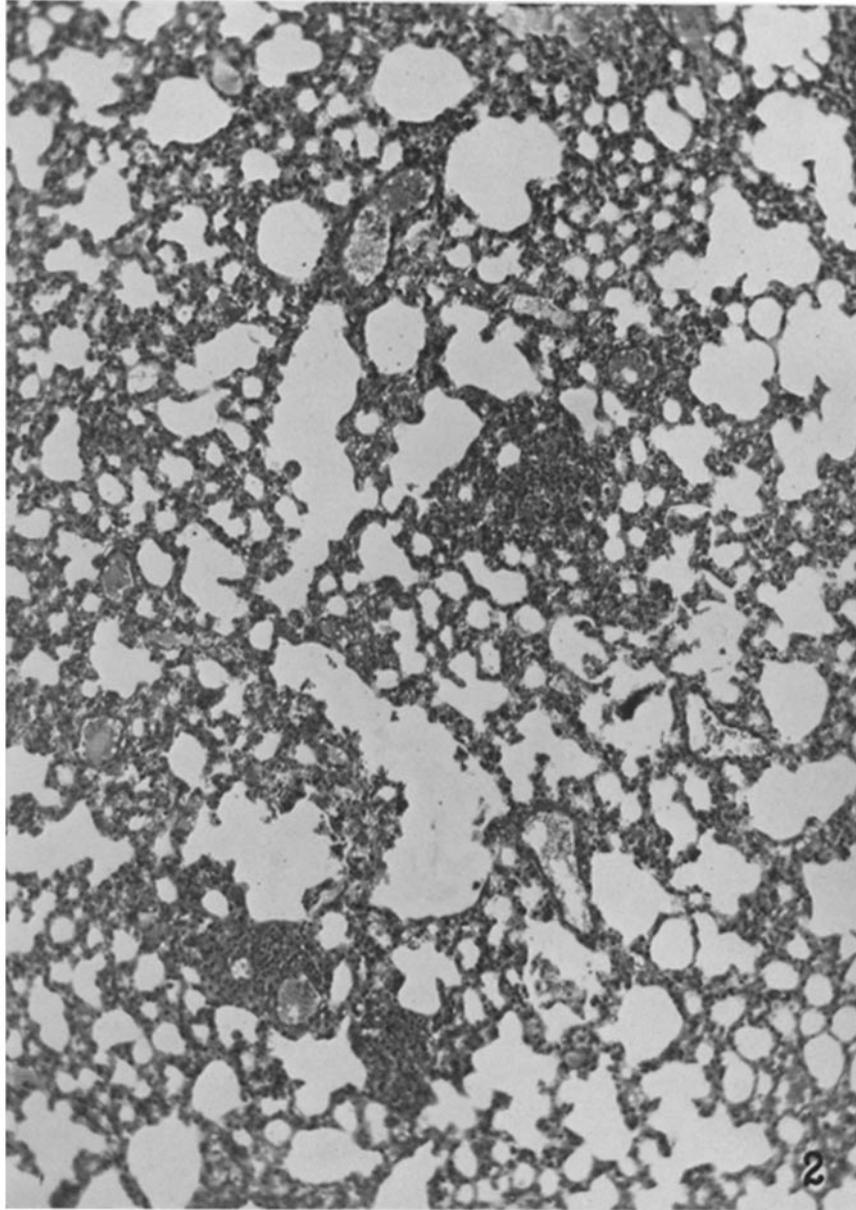
FIG. 1. Untreated control, 12 weeks after infection. Microbial population:  $6.5 \times 10^7$  culturable units of tubercle bacilli per ml. of homogenized tissue.



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 57

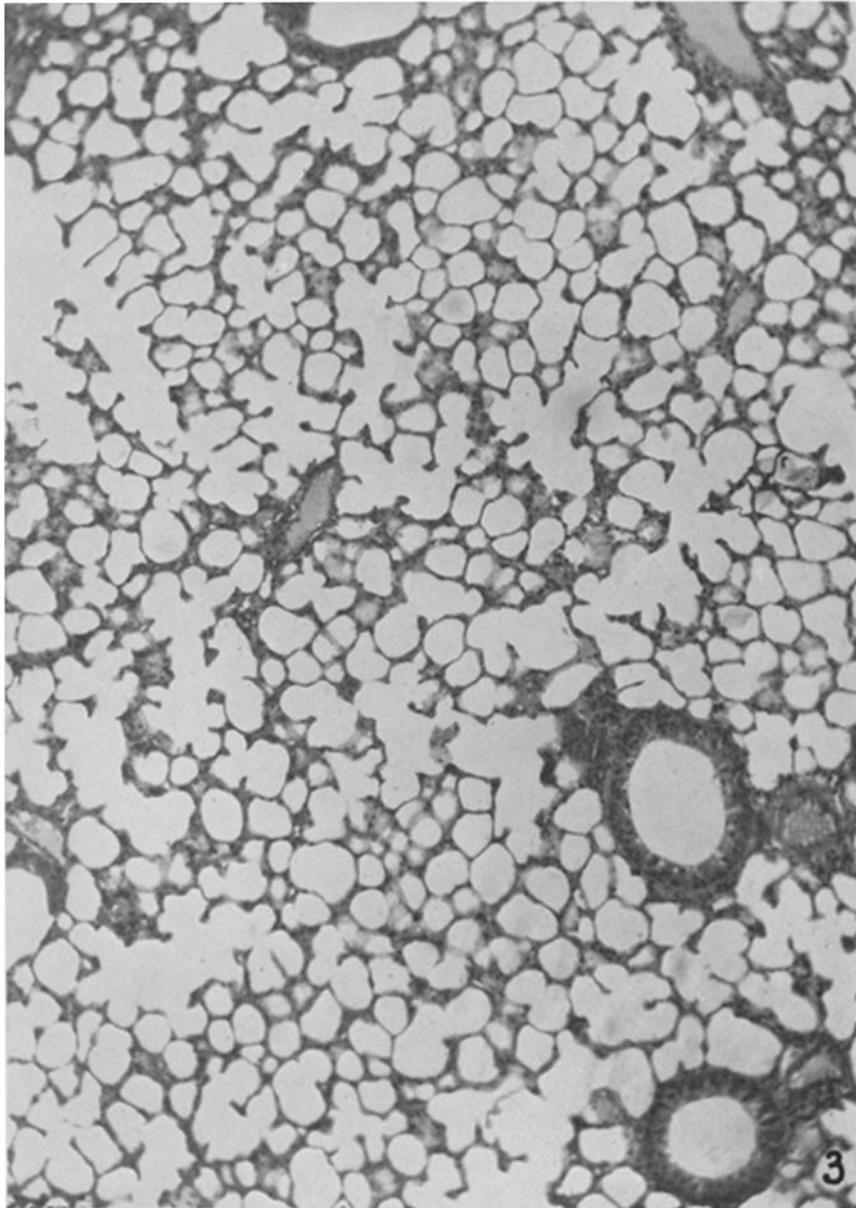
FIG. 2. Treatment: PAS, 8 weeks. Microbial population:  $2.9 \times 10^6$  per ml.



(McCune and Tomsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 58

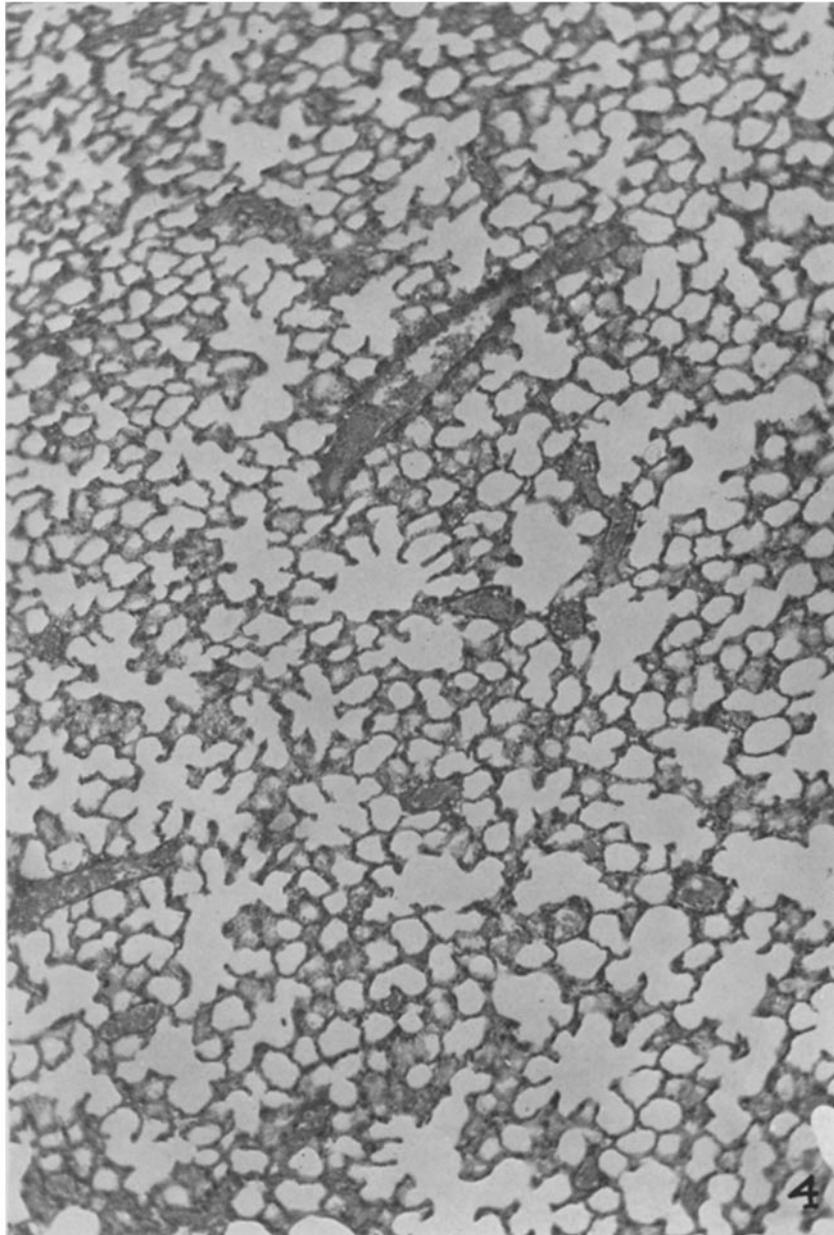
FIG. 3. Treatment: Streptomycin 12 weeks. Microbial population:  $2 \times 10^5$  per ml.



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 59

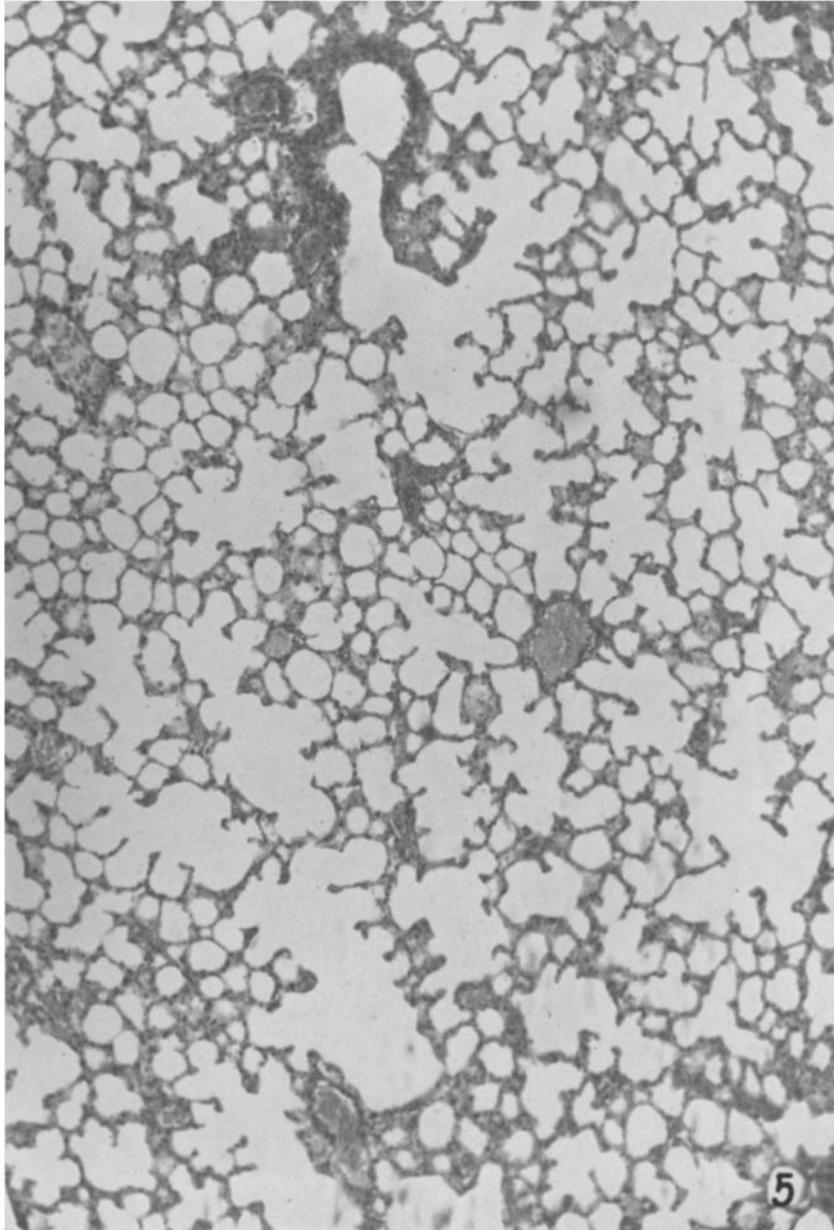
FIG. 4. Treatment: Streptomycin and PAS 16 weeks. Microbial population:  $1.3 \times 10^8$  per ml.



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 60

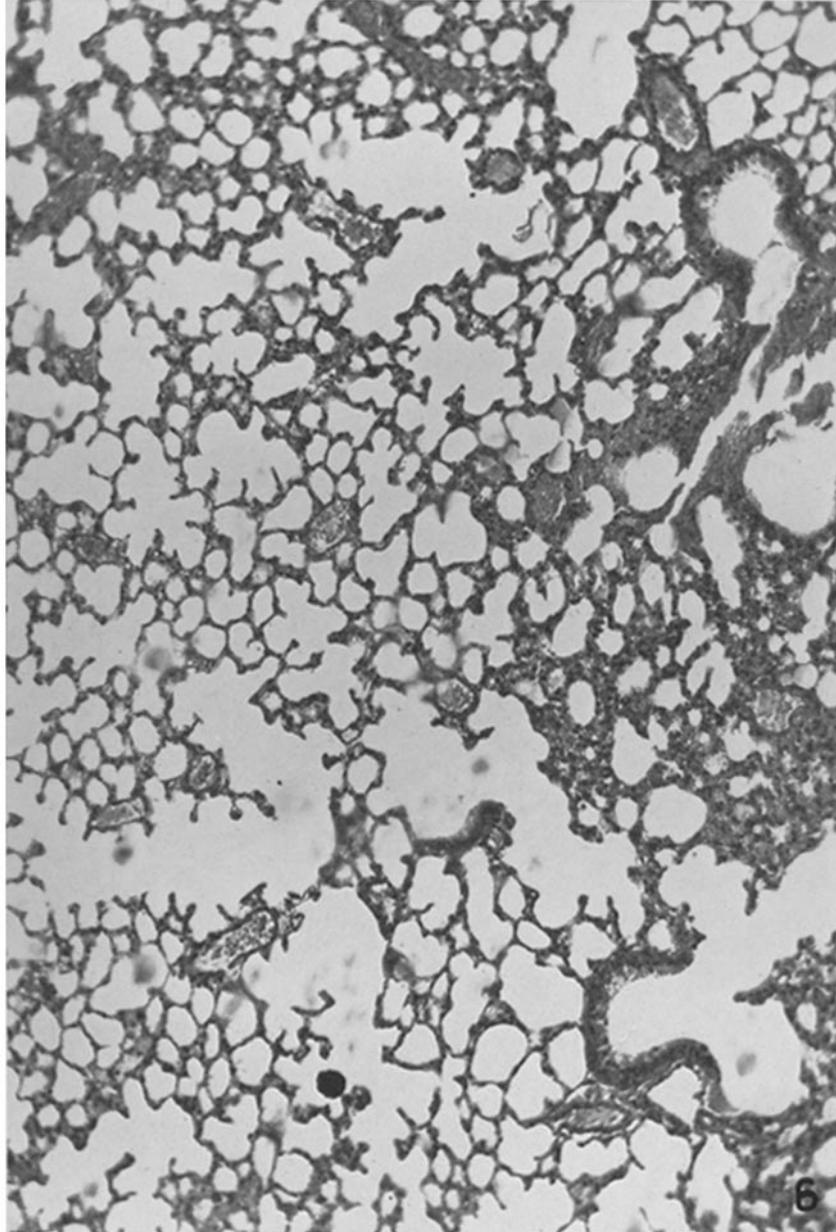
FIG. 5. Treatment: Isoniazid 12 weeks. Microbial population:  $4.3 \times 10^3$ .



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 61

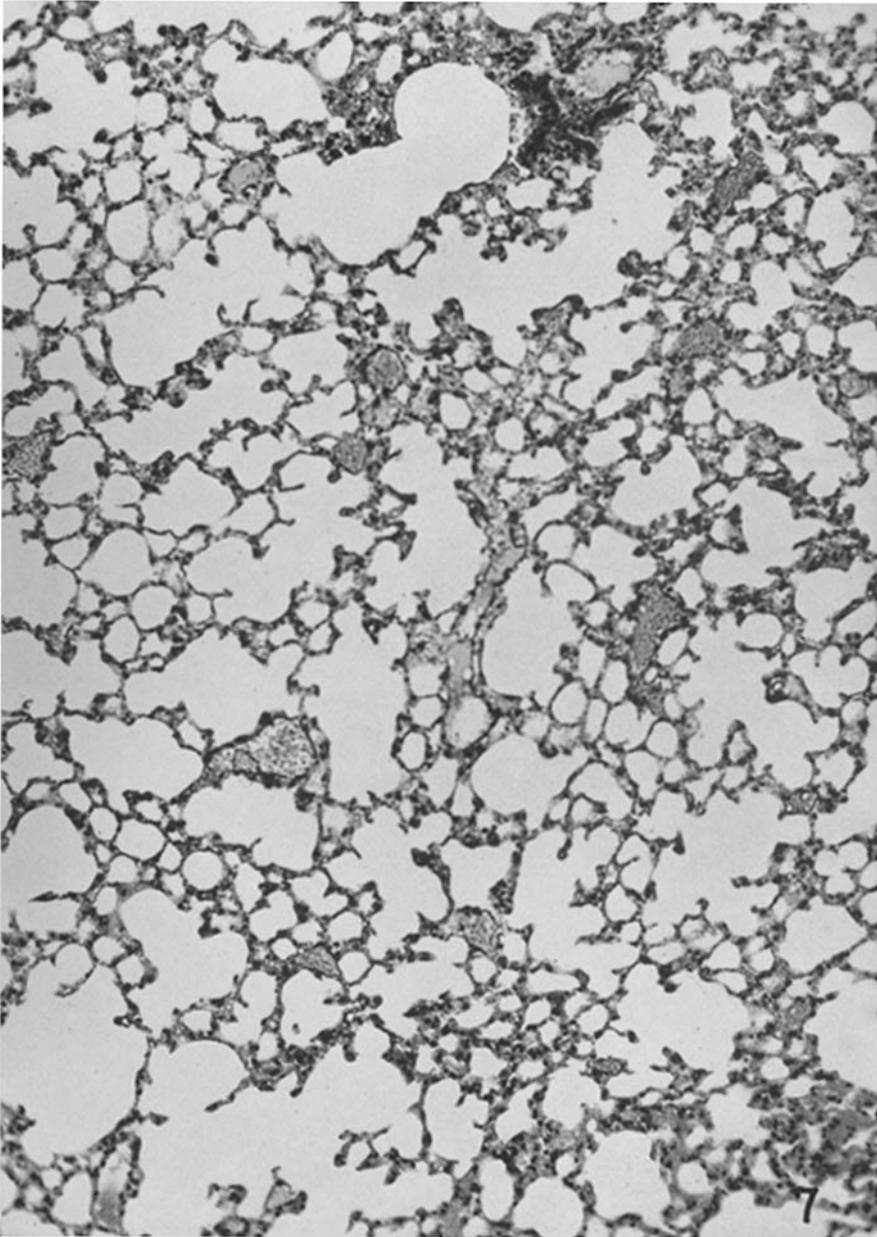
FIG. 6. Treatment: Isoniazid and PAS 8 weeks. Microbial population: no recoverable organisms (*i.e.*, less than  $1.5 \times 10^2$  per ml.)



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 62

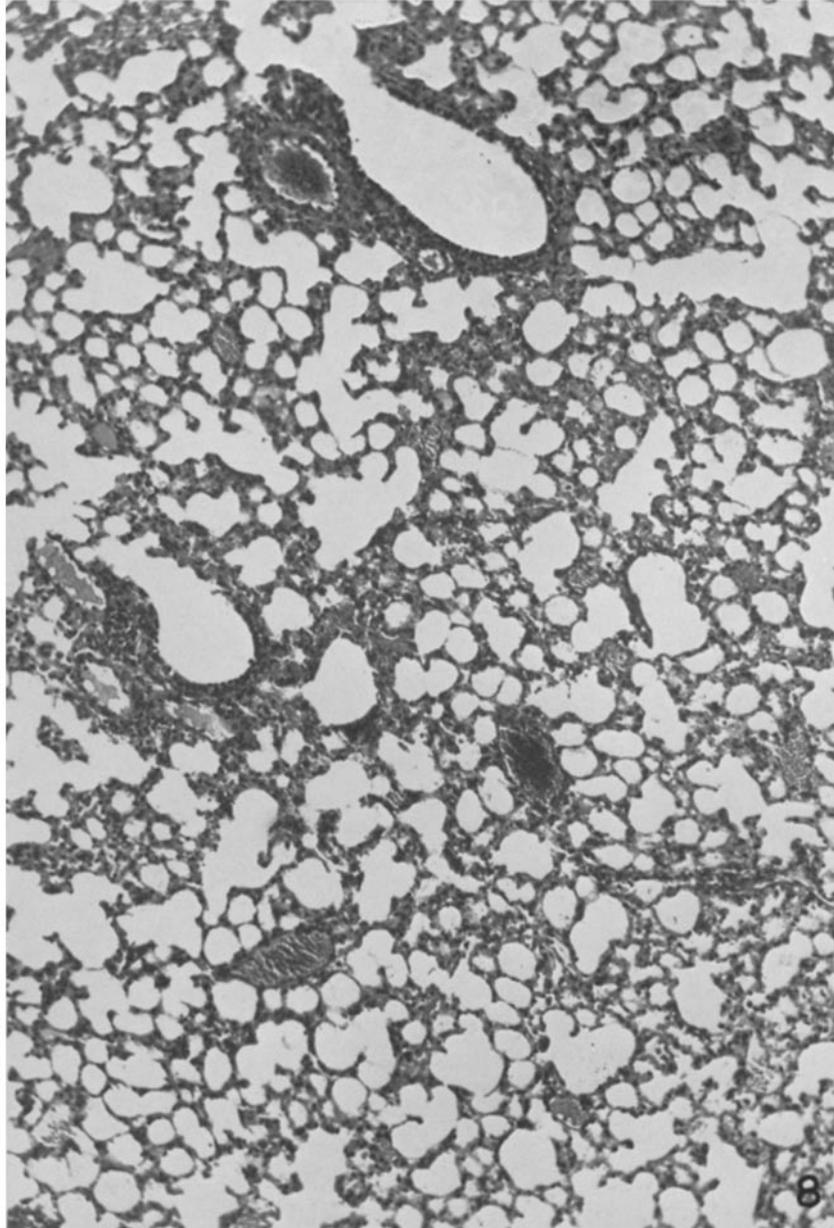
FIG. 7. Treatment: Isoniazid, Streptomycin and PAS 16 weeks. Microbial population  $1.5 \times 10^0$  per ml.



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 63

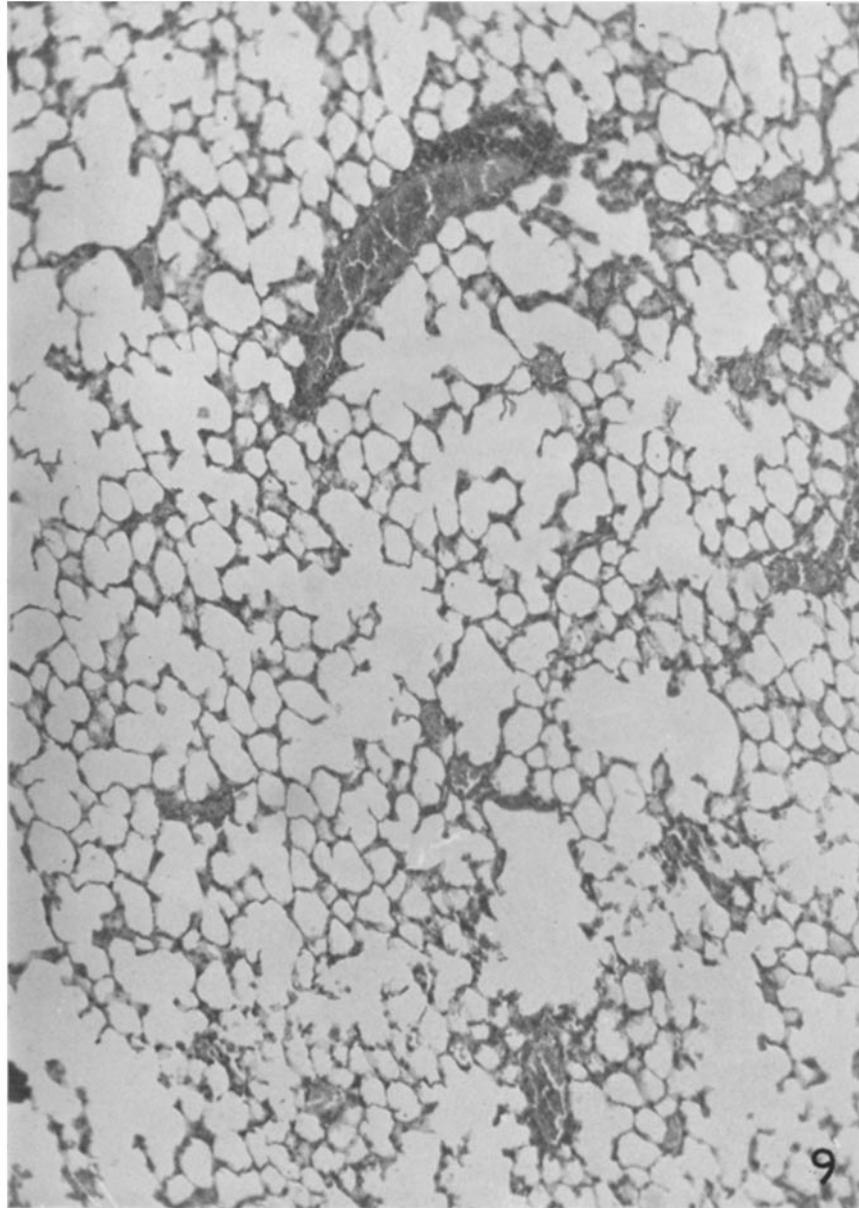
FIG. 8. Treatment: Pyrazinamide 8 weeks. Microbial population:  $2.3 \times 10^4$  per ml.



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)

PLATE 64

FIG. 9. Treatment: Isoniazid and pyrazinamide 12 weeks. Microbial population: no recoverable organisms (*i.e.* less than  $1 \times 10^2$  per ml.)



(McCune and Tompsett: Fate of *Mycobacterium tuberculosis* in tissues. I)