THE FATE OF MYCOBACTERIUM TUBERCULOSIS IN MOUSE TISSUES AS DETERMINED BY THE MICROBIAL ENUMERATION TECHNIQUE

II. The Conversion of Tuberculous Infection to the Latent State by the Administration of Pyrazinamide and a Companion Drug*

By ROBERT M. McCUNE, Jr.,‡ M.D., RALPH TOMPSETT, M.D., and WALSH McDERMOTT, M.D.

(*From the Departments of Medicine and Public Health and Preventive Medicine, The New York Hospital—Cornell Medical Center, New York*)

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In the previous paper of this series (1) it was reported that tubercle bacilli susceptible to various drugs *in vitro* were nonetheless able to survive long continued exposure to these drugs when residing within certain organs of the mouse. This phenomenon was particularly prominent in the spleen. Irrespective of the degree of influence exerted by a particular drug or drug pairing during the first week or two of the chemotherapy, the microbial census in the spleen would then stabilize. Once stabilized, the census would remain constant despite continuation of the chemotherapy for periods as long as 3 to 6 months. This persistence of tubercle bacilli was not a reflection of the emergence of strains drug-resistant in the conventional sense. In the experimental conditions maintained, the trend line of the microbial census during chemotherapy was characteristic for a particular drug or drug pairing and was uniformly predictable for both lung and spleen. Only in the spleen, however, was the phenomenon of microbial persistence easily demonstrable thus indicating a significant difference in the drug-parasite-host relationships which obtained in the 2 organs. It should be noted that this phenomenon of microbial persistence throughout chemotherapy was observed even when the total census of tubercle bacilli in the spleen was quite low, as was the case, for example, during the administration of isoniazid.

The persistence in the spleen during chemotherapy of a constant census of

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‡ James Alexander Miller Fellow of the New York Tuberculosis and Health Association, Inc.

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otherwise drug-susceptible tubercle bacilli, occurred uniformly with 10 antimicrobial drugs[1] known or reported to have antituberculous activity *in vivo* (2). Among the 10 drugs studied were all those commonly used in clinical practice and they were used singly, in various pairs, and in some cases in triple drug regimens. This persistence of tubercle bacilli in the spleen despite appropriate chemotherapy has been significantly altered only by one drug, the nicotinamide derivative, pyrazinamide (1, 3). This influence of pyrazinamide has been uniformly reproducible only when it is administered in association with another antituberculous drug.

The capacity of pyrazinamide and a companion drug to reduce populations of tubercle bacilli in the mouse to below the point of detectability has been designated "vanishing." Observations designed to define this unique phenomenon form the basis of the present report.

Materials and Methods

Microbial Enumeration Technique.—The technique for microbial enumeration employed was that originally described by Fenner, Martin, and Pierce (4), and has been described in detail in the preceding paper of this series (1). In its essentials, the procedure consists of infecting large numbers of mice (male albino mice, Webster Swiss strain) by the intravenous route with strains of tubercle bacilli of human origin. The strains of *M. tuberculosis* used were the H37Rv strain and several others recently isolated from patients with pulmonary tuberculosis. Infection was carried out by intravenous inoculation with 0.2 ml. of a 10^{-1} dilution of culture in 0.1 per cent bovine albumin. Immediately after infection the animals were assigned to various experimental groups. At appropriate intervals after the start of infection, 3 or more mice were removed from each experimental group and sacrificed. The lungs and spleens were homogenized and cultured by quantitative techniques. The actual enumeration of colonies of tubercle bacilli and the calculations involved are described in the previous report (1).

The census of culturable tubercle bacilli within an organ was expressed in terms of viable units per milliliter of homogenate. The logarithms of these numbers were plotted graphically as a function of time after infection. The resulting curves are taken to represent the course of the tuberculous infection within that organ during the experimental period.

Lower Limit of Detectability.—Considering the dilution which is necessary for preparing the tissue emulsion as well as the limitations of the volume of the organ studied, it is obvious that there is a lower limit of the method in terms of ability to detect culturable tubercle bacilli. The lower limitations vary with the organ size, the volume of the homogenizing diluent, and the number of replicates plated. Accordingly these factors will be specified in qualifying the results of certain experiments herein presented.

Drug Administration and Dosage.—Except for streptomycin, the drugs were administered in the diet by the thorough mixing of finely ground pellets of diet with amorphous drug in a McLellan dry batch mixer. Unless otherwise specified, the dosage of pyrazinamide was 2 per cent of the daily diet; the isoniazid dosage was 0.0125 per cent; the para-amino salicylic acid (PAS) dosage was 0.75 per cent. Streptomycin was administered intramuscularly in a single daily injection of 4 mg.

[1] The ten drugs were: isoniazid, streptomycin, para-amino salicylic acid, pyrazinamide, nicotinamide, 4-butoxy-4′(dimethylamino)thiocarbanilide, *p*-isobutoxybenzaldehyde thio-semicarbazone, 2-[o-(2-diethylaminoethoxy)anilino]-*N*-(*p*-tolyl)1,4-naphthoquinoneimine monocitrate, thioisonicotinamide, and hinconstarch.

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RESULTS

Repetition of Observations on Pyrazinamide and Isoniazid Administered Together.—The first experiments consisted of a repetition in greater detail of the observations on the influence of pyrazinamide and isoniazid administered singly and together on the census of tubercle bacilli in the lungs and spleen of mice. Both the infection and the antimicrobial therapy were started on the same day. The results of these experiments may be seen in Text-figs. 1 and 2.

In the lungs of the untreated animals, the population of tubercle bacilli rose steadily during the first 3 weeks of the infection and remained high for the remainder of the 118 day period of observation.

In the treated animals, the microbial populations fell promptly during the first 3 weeks. From that point on, the populations of tubercle bacilli in most of the lungs of the isoniazid-treated animals were below the level of detectability. In some of these animals, however, tubercle bacilli were present even after 118 days of therapy. Tubercle bacilli also persisted in the animals which received pyrazinamide alone even though the micro-organisms could not usually be isolated 8 weeks after the start of therapy. For, in 5 of the 6 pyrazinamide-treated animals, tubercle bacilli were easily detectable in the lungs on the 90th day of treatment and in one animal the census was at the pretreatment level.

In the spleens of the untreated animals (Text-fig. 2), the characteristic early rise in the census with slight fall and then stabilization for the remainder of the 118 day period occurred. Under the influence of isoniazid, the microbial populations in the spleen likewise behaved as in previous experiments (1). A steady fall occurred during the first 3 weeks, and the census stabilized and persisted thereafter for the remainder of the 118 day period.

With pyrazinamide and isoniazid administered together, there were no detectable tubercle bacilli in the lungs of two of three animals examined 23 days after the start of treatment and no tubercle bacilli could be cultured from the lungs of any of the animals during the remainder of the experiment.

In the spleen, during the first 3 weeks of therapy the census curve for the animals which received pyrazinamide alone, dropped more slowly than in the animals with isoniazid alone and remained thereafter at a level higher than in the isoniazid-treated animals.

The behavior of the splenic populations of tubercle bacilli in the animals which received pyrazinamide and isoniazid together was distinctly different from that in the animals which received either drug alone. At the outset (first 16 days of chemotherapy) the pyrazinamide appeared to antagonize the action of the isoniazid. Both drugs were being given together, yet only the same slight fall in census occurred which was observed with the administration of pyrazinamide alone. This was in contrast to the substantial drop in census which occurred with the use of isoniazid alone. By the end of the 3rd week, however, the populations of tubercle bacilli in the spleens of the pyrazinamide-isoniazid animals had begun to fall. Sampling on the 37th day revealed no detectable

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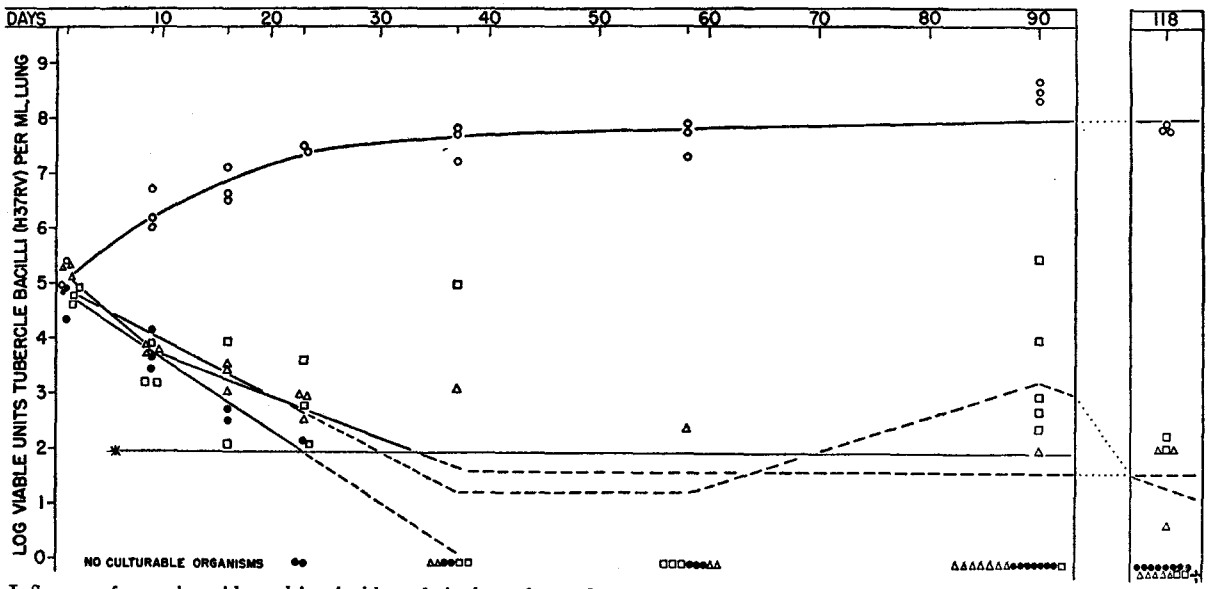

TEXT-FIG. 1. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs during 118 days of therapy. Infecting inoculum; 2.0×10^6 culturable units tubercle bacilli. ○, control; □, pyrazinamide; △, isoniazid; ●, pyrazinamide-isoniazid.

* The techniques used during the first 90 days of the experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.

† The techniques used on day 118 permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

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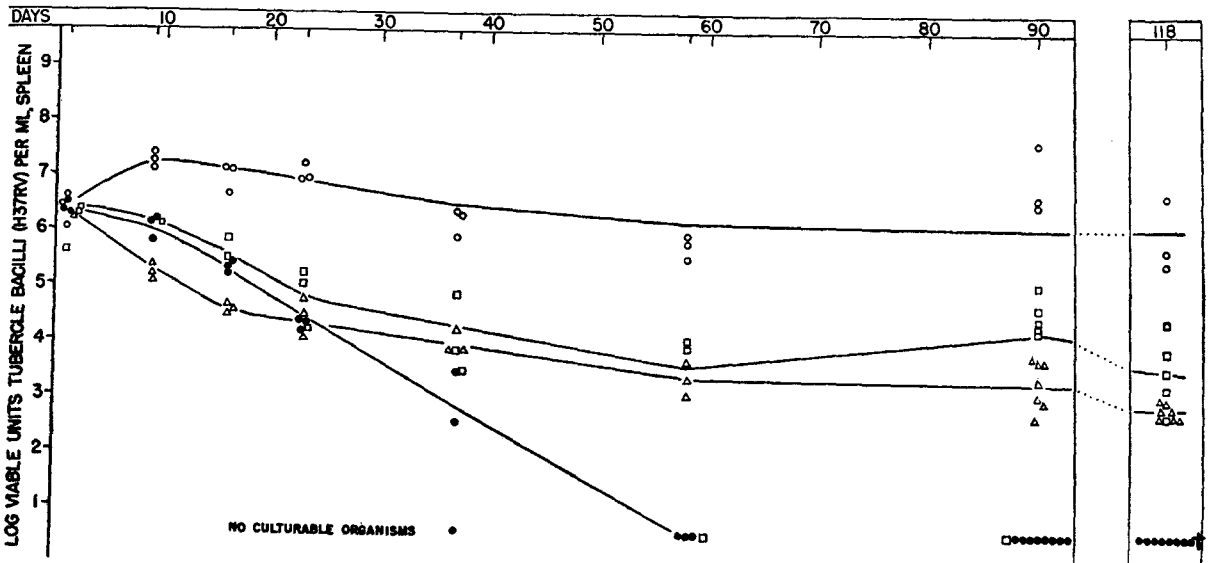

TEXT-FIG. 2. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Text-fig. 1. ○, control; □, pyrazinamide; △, isoniazid; ●, pyrazinamide-isoniazid.

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organisms in the spleen on one of three animals, and thereafter no tubercle bacilli could be cultured from the spleens of any of the animals at any of the observation points.

Pyrazinamide-Isoniazid and Isoniazid-Streptomycin-PAS.—The effectiveness of the pyrazinamide-isoniazid regimen was compared in a single experiment with the effectiveness of a triple drug regimen comprised of all 3 of the antituberculous drugs in general use, *i.e.*, isoniazid, streptomycin, and para-amino salicylic acid (PAS).

The techniques used during the first 90 days of this experiment permitted the detection of 75 to 90 microorganisms per organ. On day 118 of the experiment, however, a technique of considerably increased sensitivity was substituted and is described in detail in a subsequent section. As few as 1 to 3 tubercle bacilli were theoretically detectable in each organ. The results of this experiment may be seen in Text-figs. 3 and 4.

In the lungs with the triple drug regimen (Text-fig. 3) the microbial census fell so that, with a single exception, no tubercle bacilli could be cultured after the 5th week of therapy. Only the less sensitive detection method was used in these particular observations. On the last day of the experiment, however, after 17 weeks of treatment with the triple drug regimens, the more sensitive cultural method revealed the presence of tubercle bacilli in 7 of the 8 animals examined. In contrast, in the animals which received the pyrazinamide-isoniazid regimen no tubercle bacilli were detectable in the lungs even at the termination of the experiment when the more sensitive cultural method was used.

In the spleens (Text-fig. 4), tubercle bacilli persisted throughout the entire 13 week period of therapy with the triple drug regimen of isoniazid, streptomycin, and PAS. With the pyrazinamide-isoniazid regimen, however, no tubercle bacilli could be cultured from the spleens of any of the animals at any time subsequent to the 57th day of the 118 day treatment period.

Effectiveness in Established Infections.—When the start of pyrazinamide or isoniazid, used singly, was withheld for a 21 day period after infecting the mice with tubercle bacilli, there was a reversal in the relative effectiveness of the two drugs on the populations of tubercle bacilli in the spleen (Text-fig. 5). 21 days after infection, the population of tubercle bacilli in the spleens of untreated animals had stopped increasing and the census was beginning to show a fall. By the 5th week after infection, the populations had become essentially stable, but a very slight fall occurred steadily during the remainder of the 100 day experiment.

The initiation of isoniazid therapy 21 days after the start of the infection resulted in a fairly sharp fall in the splenic census of tubercle bacilli with stabilization of the census thereafter. When pyrazinamide therapy was first introduced 21 days after the start of infection, the populations of tubercle bacilli fell promptly and the census reached lower levels than in the isoniazid-treated

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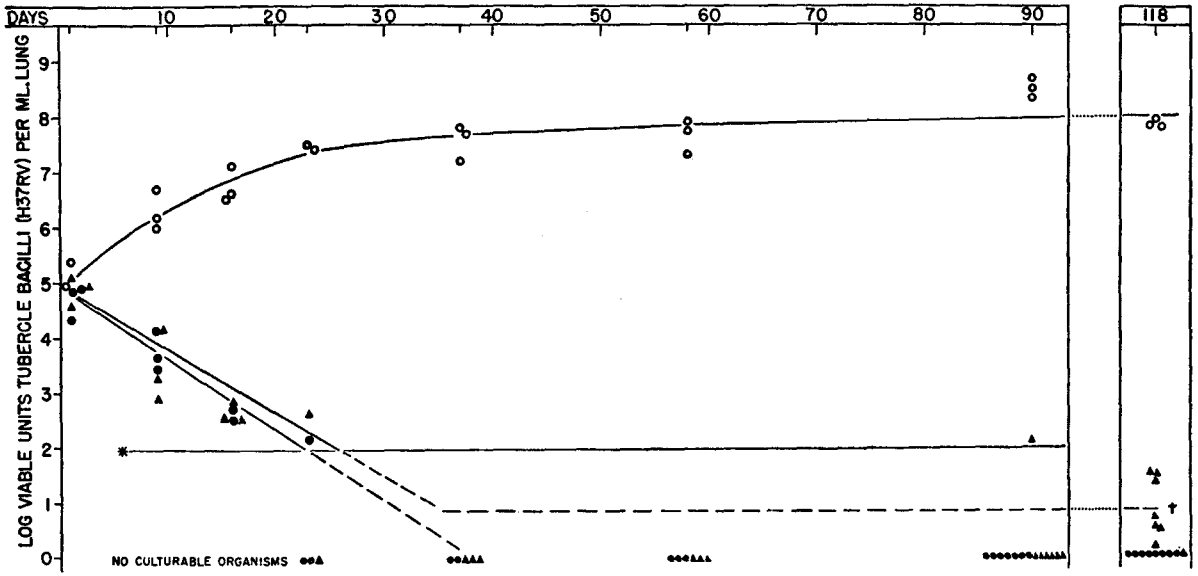

TEXT-FIG. 3. Influence of pyrazinamide-isoniazid and isoniazid-streptomycin-PAS on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 118 day of therapy. Infecting inoculum: 2.0×10^6 culturable units of tubercle bacilli. ○, control; ▲, isoniazid-streptomycin-PAS; ●, pyrazinamide-isoniazid.

* The techniques used during the first 90 days of this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.

† The techniques used on day 118 permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

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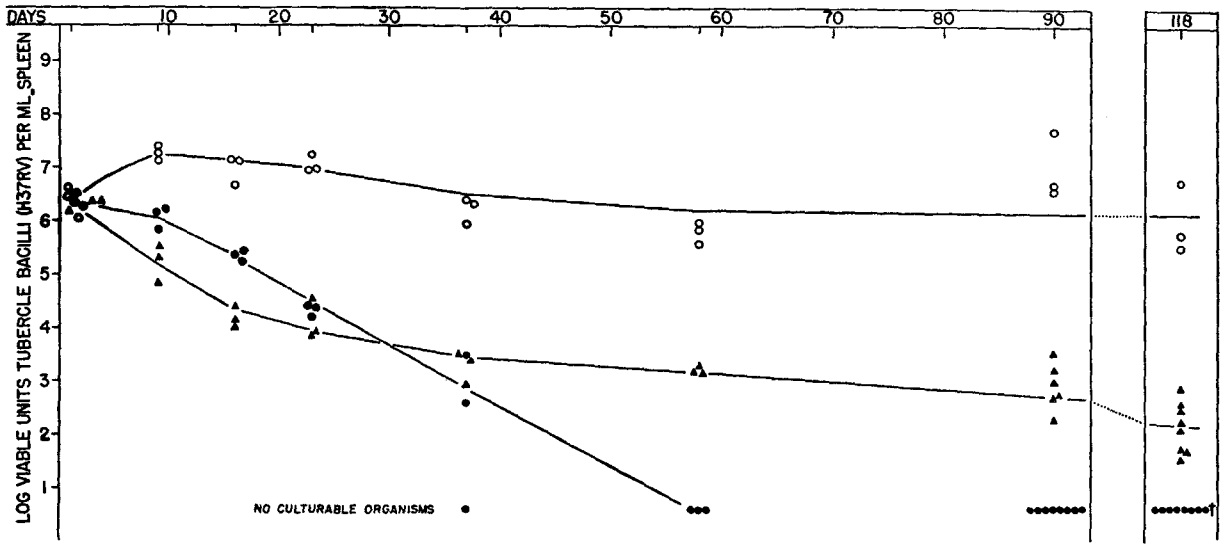

TEXT-FIG. 4. Influence of pyrazinamide-isoniazid and isoniazid-streptomycin-PAS on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung counts are shown in Text-fig. 3. ○, control; ▲, isoniazid-streptomycin-PAS; ●, pyrazinamide-isoniazid.

† The techniques used on day 118 permitted detection of 1 to 3 culturable units of tubercle bacilli per spleen.

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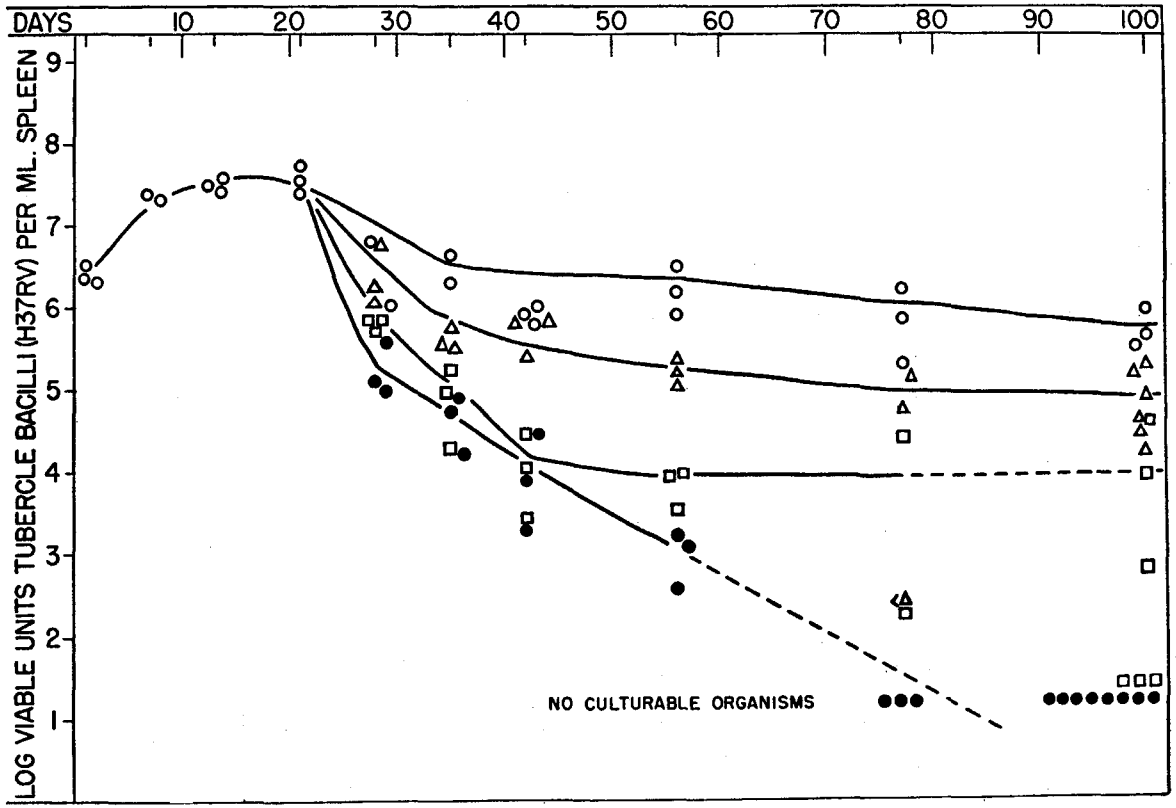

TEXT-FIG. 5. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse spleens in the presence of lesions.

Treatment was started 21 days after initiation of infection. Infecting inoculum 5.7 $\times$ 10^5 culturable units tubercle bacilli. ○, control; △, isoniazid; □, pyrazinamide; ●, pyrazinamide-isoniazid.

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animals. In some of the pyrazinamide-treated animals the census stabilized after the initial fall and remained constant for the period of observation. In other pyrazinamide-treated animals, however, the initial fall continued, and in some of the animals tubercle bacilli could not be recovered at the end of the experiment.

The initiation of pyrazinamide and isoniazid together, 21 days after the start of the infection, was followed by a steady fall in the splenic census of tubercle bacilli. The drop in census could be measured during the first 35 days after the start of pyrazinamide-isoniazid therapy, but fell below the limits of detection thereafter. Throughout the remaining 44 days of the experiment, it was not possible to culture tubercle bacilli from the spleens of any of the pyrazinamide-isoniazid–treated animals, even with the use of the more sensitive cultural technique.

No antagonism of isoniazid action by the pyrazinamide was demonstrated in the 21 day infection. As pyrazinamide exerted the stronger effect in reducing the populations in these circumstances, however, any antagonism of the isoniazid would presumably not have been perceptible.

In the lungs of the untreated animals, 21 days after start of the infection, the populations of tubercle bacilli had stopped increasing and the pulmonic census remained stable throughout the remaining 79 days of the experiment (Text-fig. 6). As in the spleen, the fall in census when pyrazinamide alone or pyrazinamide and isoniazid were first administered at this 21 day point was appreciably greater than occurred with the use of isoniazid alone.

As may be seen in Text-fig. 6, during the first 35 days of treatment of the 21 day old infection, an identical fall in census occurred in the lungs of the animals which received pyrazinamide alone and in those which received pyrazinamide-isoniazid. From this point on, the populations of tubercle bacilli dropped below the limits of detectability in the animals which received the pyrazinamide-isoniazid regimen and the bacilli were likewise undetectable in one-half of the animals which received pyrazinamide alone. In contrast to this disappearance of the tubercle bacilli which occurred with pyrazinamide-isoniazid, and in some instances with pyrazinamide alone, the bacilli could be cultured, at every observation point, from the lungs of the animals which received isoniazid alone.[2] For the final determinations in this experiment (*i.e.* census of day 100), six or seven animals were used in each group except for the untreated group.

Thus, the end result of the influence of pyrazinamide-isoniazid on the census of tubercle bacilli in both the spleen and the lung was identical when started on the 1st day of infection or 21 days thereafter. The relative degree of influence of the individual drugs was different, however, in the 2 infections of different length. Pyrazinamide regularly exerted a greater effect than isoniazid when

[2] The more sensitive culturing technique was used in these studies.

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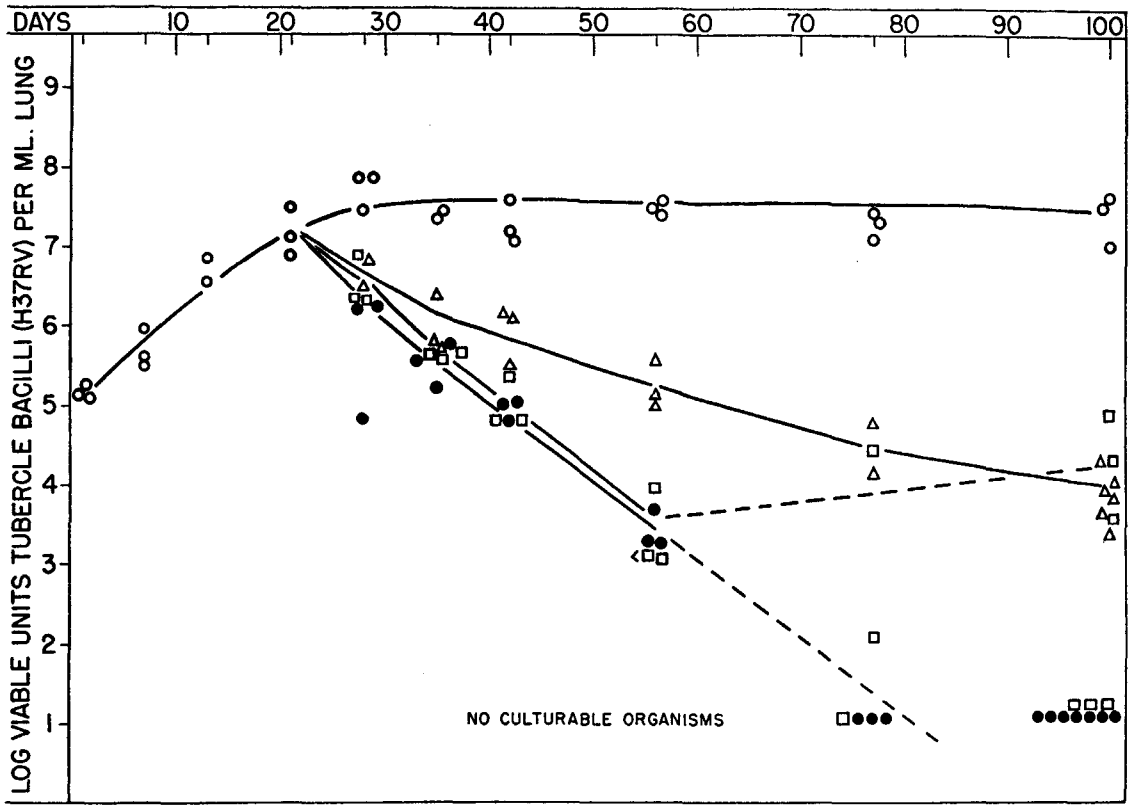

TEXT-FIG. 6. Influence of pyrazinamide and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in lungs of the same animals whose spleen populations are shown in Text-fig. 5.

Treatment was started 21 days after initiation of infection. Lesions were present. ○, control; △, isoniazid; □, pyrazinamide; ●, pyrazinamide-isoniazid.

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the infection had been present for 21 days before chemotherapy was started. The results with pyrazinamide alone were irregular when the infection and the chemotherapy were started on the same day. In some such experiments, the populations of tubercle bacilli in the lungs and spleen remained at the pretreatment census whereas in other experiments the tubercle bacilli "vanished."

Isoniazid-Streptomycin in 21 day Infection.—For a comparison with the pyrazinamide-isoniazid results in the 21 day infection, experiments were conducted in which isoniazid and streptomycin together were first administered 21 days after the start of infection. The results of these experiments may be seen in Text-figs. 7 and 8.

Unlike the situation with pyrazinamide-isoniazid, in no instance did isoniazid, streptomycin, or isoniazid-streptomycin reduce the census of tubercle bacilli to below the limits of detection when chemotherapy was first started 21 days after infection. This was observed regularly in both the spleens and the lungs of the 21 day infected mice treated for 79 days thereafter.

As in the previous experiment, the influence of isoniazid alone in the 21 day infection was quantitatively less than when the administration of the drug was started on the 1st day of infection. This lessened influence of isoniazid in the more advanced infection was apparent both in the lung and in the spleen. The effectiveness of streptomycin on the tubercle bacilli in the lung was not appreciably lessened, however, when the drug was first administered 21 days after the start of infection. Indeed, there was a moderate fall in census during streptomycin administration, in contrast to the stable census observed in previous experiments (1) in which streptomycin was started on the 1st day of infection.

When isoniazid-streptomycin therapy was initiated 21 days after the start of the infection, the behavior of the lung populations of tubercle bacilli was the same as when the chemotherapy and the infection were started on the same day (Text-fig. 7). The census of tubercle bacilli in the spleen did not fall below the limits of detection during the 79 day period of treatment but it showed a slow steady fall. This fall was still apparent at the time the observations were terminated 100 days after the start of the infection (Text-fig. 8). The extent of this fall with isoniazid-streptomycin therapy, however, was not at all comparable with the fall in census observed with pyrazinamide-isoniazid in either the beginning or the older tuberculous infection. It should also be noted in this connection, that in the untreated animals, the populations of tubercle bacilli fell by approximately 1 logarithm unit from the maximum census attained shortly before therapy was started on the 21st day after infection.

Pyrazinamide-Isoniazid Compared with Isoniazid-Streptomycin-PAS in the 21 Day Infection.—An experiment was conducted to compare the effectiveness of pyrazinamide-isoniazid with that of the triple drug regimen, *i.e.*, isoniazid, streptomycin, and PAS, when chemotherapy was first started 21 days after the start of the tuberculous infection. The results of this experiment may be seen in Text-figs. 9 and 10. With the pyrazinamide-isoniazid regimen, no

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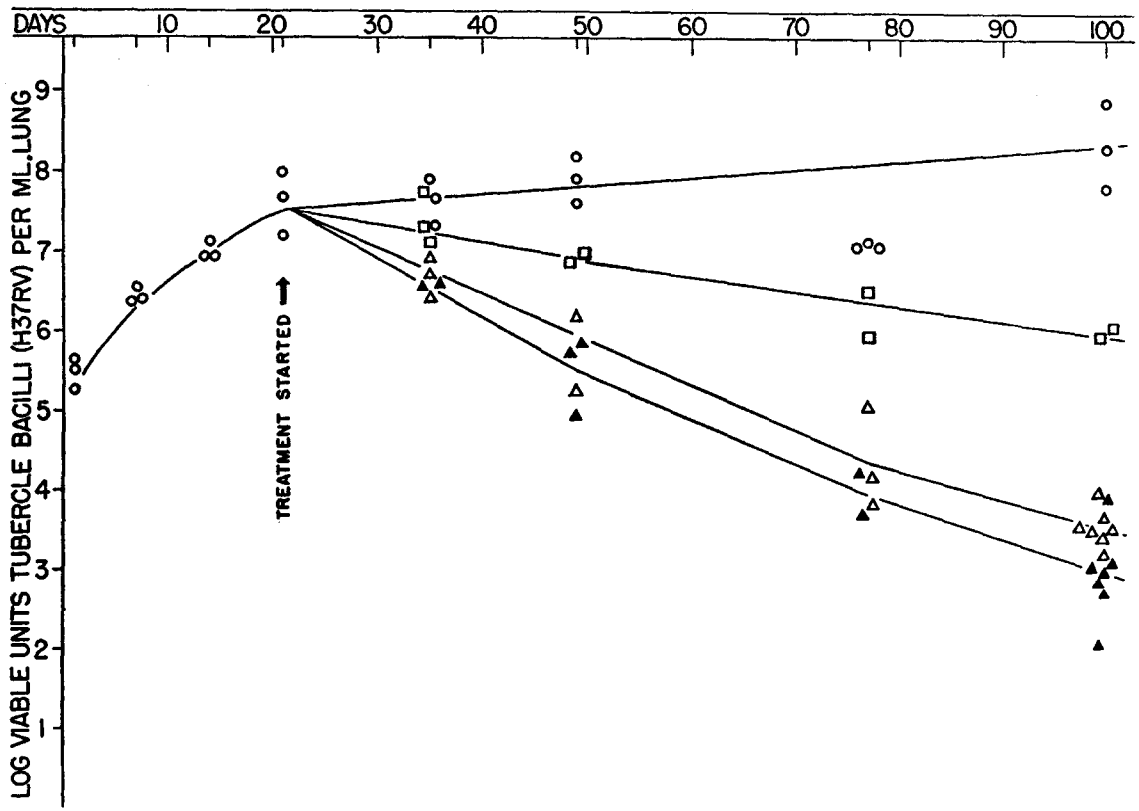

TEXT-FIG. 7. *Influence of isoniazid and streptomycin used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs in the presence of lesions.*

Treatment was started 21 days after initiation of infection. Infecting inoculum 1.8×10^6 culturable units of tubercle bacilli. ○, control; □, streptomycin; △, isoniazid; ▲, isoniazid-streptomycin.

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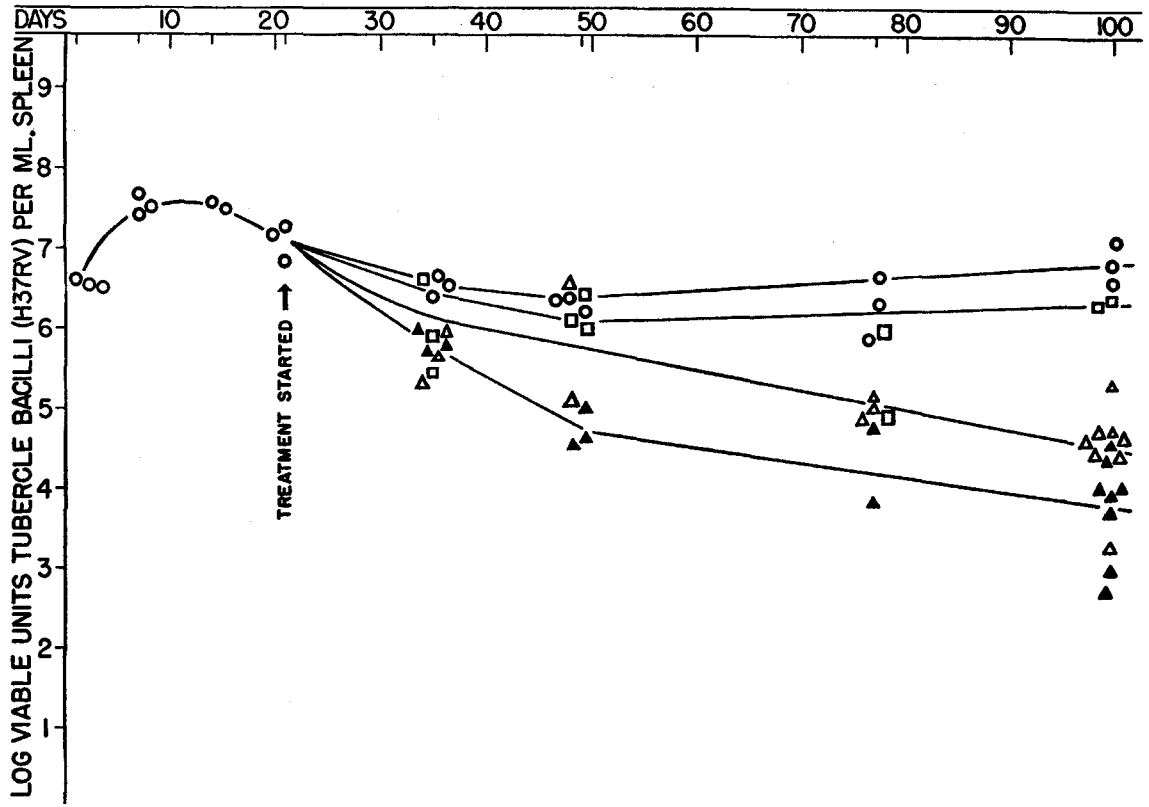

TEXT-FIG. 8. Influence of isoniazid and streptomycin used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung studies are shown in Text-fig. 7.

Treatment was started 21 days after initiation of infection. Lesions were present. ○, control; □, streptomycin; △, isoniazid; ▲, isoniazid-streptomycin.

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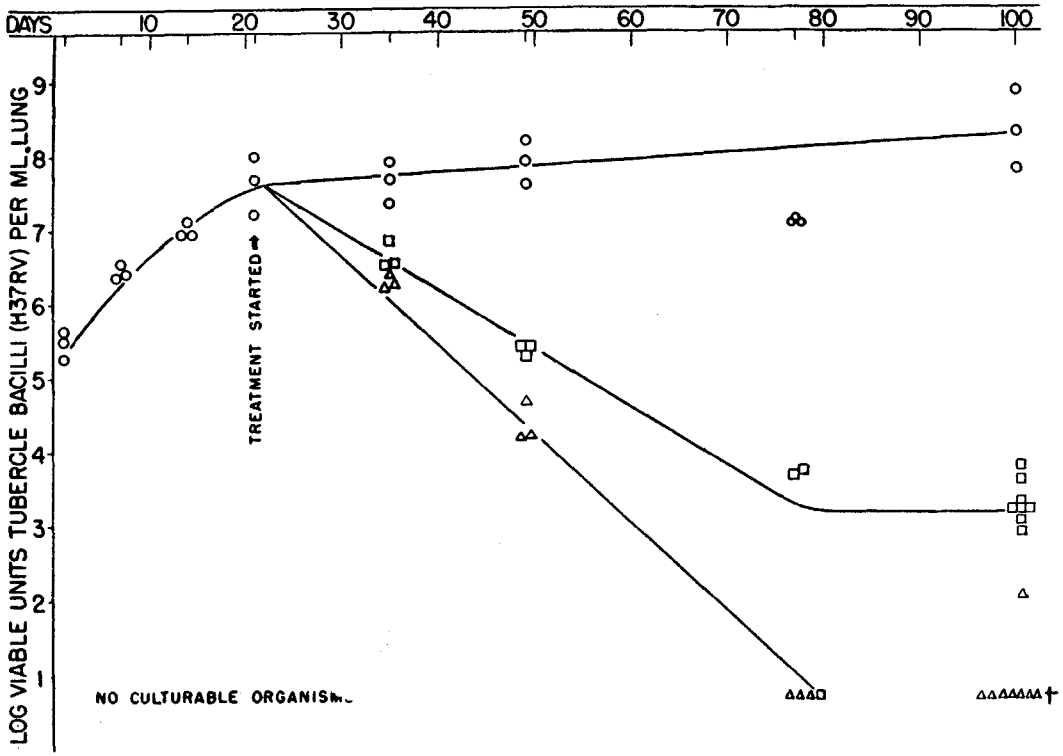

TEXT-FIG. 9. Influence of pyrazinamide-isoniazid and isoniazid-streptomycin-PAS on populations of tubercle bacilli (H37Rv) in mouse lungs in the presence of lesions.

Treatment was started 21 days after initiation of infection. Infecting inoculum: 1.8×10^6 culturable units of tubercle bacilli. ○, control; □, isoniazid-streptomycin-PAS; △, pyrazinamide-isoniazid.

† The techniques used on the last day of the experiment permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

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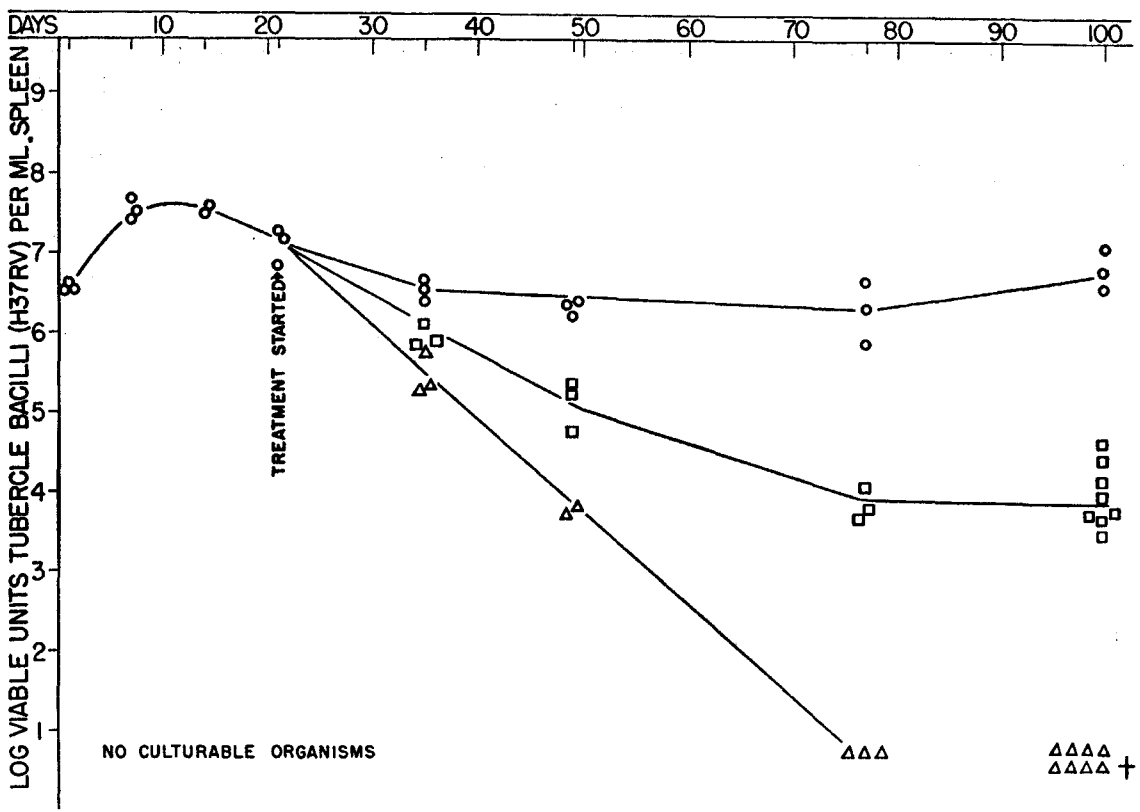

TEXT-FIG. 10. Influence of pyrazinamide-isoniazid and isoniazid-streptomycin-PAS on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in Text-figure 9.

Treatment was started 21 days after initiation of infection. Lesions were present.

† The techniques used on the last day of the experiment permitted detection of 1 to 3 culturable units of tubercle bacilli per spleen.

○, control; □, isoniazid-streptomycin-PAS; △, pyrazinamide-isoniazid.

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organisms could be cultured from either the lungs or the spleens of any animals subsequent to the 56th day of treatment, with the exception of the lung of one of eight animals cultured on the 79th day of treatment. In contrast, the populations of tubercle bacilli in the animals which received the triple drug regimen remained above the limits of detectability in both lung and spleen with but a single exception throughout the entire 100 day experimental period.

Attempts to Establish the Presence of Tubercle Bacilli in the Mouse Tissues Yielding No Tubercle Bacilli on Culture.—Four procedures were employed in an effort to increase the recovery of tubercle bacilli from the spleens of the animals which received pyrazinamide and a companion drug. The procedures were: an elaboration of the basic technique by the inclusion of more animals, a reduction of the quantity of diluents used for homogenizing, and the additional practice of culturing virtually all of an organ rather than aliquots; the inoculation of guinea pigs with homogenates of the spleens of the pyrazinamide-treated mice; the continued incubation *in vitro* (for 9 months) of cultures of the homogenates following a technique introduced by Hobby *et al.* (6); and the maintenance of the animals for a 90 day treatment-free period before sacrifice and examination of the tissues.

Elaborations of Technique.—

The methods customarily employed in the present study for enumeration of tubercle bacilli in the animal tissues permit detection of 70 to 90 organisms per lung or spleen depending upon the size of the organ. Accordingly, the amount of diluent used for homogenizing the organs was reduced, and as much of each total homogenate as was practically possible was spread on the surface of the culture medium. As a consequence, the sensitivity of the technique was increased to the point at which the recovery of as few as 1 to 3 culturable tubercle bacilli per lung or spleen was possible. The possibility existed that the tissue extracts in the more concentrated homogenates might contain substances inhibitory for tubercle bacilli. A tenfold dilution of these homogenates, however, yielded a proportionate tenfold difference in counts. Moreover, when known numbers of tubercle bacilli were added to splenic and pulmonic homogenates, from 32 uninfected animals, it was possible to obtain positive cultures with regularity when so few as 1 or 2 viable units per individual spleen had been added. Eight of these uninfected animals had received pyrazinamide and isoniazid either singly or together until the time of sacrifice.

In the latter phases of the experiments when it was no longer possible to culture tubercle bacilli from the pyrazinamide-isoniazid-treated animal tissues, the numbers of animals sampled from these groups were increased from the usual 3 to 8, thus increasing the significance of the disappearance of the microorganisms from the tissues.

An example of the results of these elaborations of the technique may be seen in the experiment presented in Text-fig. 3. With the use of the original less sensitive technique, no tubercle bacilli could be cultured from the lungs of any of the animals after the 3rd week of treatment with the triple drug regimen of isoniazid, streptomycin, and PAS. When the more sensitive technique was employed after 17 weeks of treatment, however, it was possible to culture tubercle bacilli from the lungs of all of the animals examined. Nevertheless, when

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pyrazinamide and isoniazid were used together, no tubercle bacilli were demonstrable in the lungs or spleens of any of the animals after the 8th week of treatment even when the more sensitive technique was employed.

Guinea Pig Inoculation.—A 6-month experiment, to be described in detail below, was set up to determine the length of the period of pyrazinamide administration in the pyrazinamide-isoniazid regimen, which was necessary to result in the disappearance of the tubercle bacilli.

Aliquots of splenic homogenates from representative animals of each treatment group used in this experiment were inoculated subcutaneously into guinea pigs. The cutaneous reaction of the guinea pigs to tuberculin was determined 30 days after infection, and the animals were examined post mortem 2 months after infection. Gross and histopathologic studies were performed.

In the guinea pigs inoculated with tissues from mice which had received isoniazid or pyrazinamide singly or pyrazinamide-isoniazid for only 2 weeks with isoniazid alone thereafter, the cutaneous reaction to tuberculin became positive and the characteristic lesions of tuberculosis were demonstrable on postmortem examination. In contrast, both the tuberculin reaction and postmortem examination failed to reveal any evidence of tuberculous infection in the guinea pigs inoculated with spleens from mice which had received the pyrazinamide component of the pyrazinamide-isoniazid regimen for as long as 8 or 12 weeks.

Long Continued Incubation in Vitro of Splenic Homogenates from Pyrazinamide-Treated Animals.—A series of splenic homogenates from which no tubercle bacilli could be cultured by customary techniques was subjected to long continued incubation in tween-albumin medium by a technique reported by Hobby and her associates (6). Subcultures of these emulsions after 9 months of incubation failed to reveal any tubercle bacilli.

Prolonged Posttreatment Observation.—The results of the experiments in which prolonged posttreatment observation was employed to determine the presence of tubercle bacilli may be seen in Table I.

Groups of mice received 90 or 120 day periods of pyrazinamide-isoniazid therapy starting, on the day of infection with tubercle bacilli. At the completion of the antimicrobial therapy microscopy and culture of the lungs and spleens of sample subgroups failed to reveal any tubercle bacilli even with the use of the more sensitive technique. The remaining animals in the groups were then observed for a 90 day treatment-free interval before sacrifice and culture of the tissues.

In the animals treated for 90 days, tubercle bacilli were cultured from spleens of twelve of the thirty animals examined after the 90 day treatment-free interval. In one of these thirty animals, culture of the lungs likewise revealed tubercle bacilli. Of the eleven animals treated on the 120 day regimen, tubercle bacilli were cultured from both the lung and spleen of one animal, and the remaining animals yielded no tubercle bacilli on culture of either lung or spleen.

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In the thirteen of the total of forty-one animals from which tubercle bacilli could be recovered, the total populations were small, a mean of 2.5×10^2 culturable units per lung and 6.4×10^4 culturable units per spleen. All 9 of the strains which were tested under appropriate conditions *in vitro* (5, 8) were susceptible to pyrazinamide and to isoniazid to the same degree as the parent strain used to produce the infection.

TABLE I

Microbial Populations (H37Rv) in Spleens and Lungs of 41 Mice 13 Weeks after Discontinuing Pyrazinamide-Isoniazid Treatment

Animal No.	Spleen	Lung
90 day treatment		
1	3.3×10^5	0
2	3.1×10^5	0
3	1.1×10^5	3.5×10^3
4	5.6×10^3	0
5	1.9×10^3	0
6	1.9×10^3	0
7	1.4×10^3	0
8	1.4×10^3	0
9	1.2×10^3	0
10	1.0×10^3	0
11	9.6×10^2	0
12	1.3×10^3	0
13–30	0	0
120 day treatment		
1	6.8×10^4	1.5×10^3
2–11	0	0

Populations of *M. tuberculosis* (H37Rv) in mouse tissues 13 weeks after cessation of a 13 and 17 week course of pyrazinamide-isoniazid treatment. Infecting inoculum: 2.8×10^6 culturable units of tubercle bacilli.

This method of prolonged posttreatment observation was the only method discovered by which the continued presence of tubercle bacilli in certain of the animals which received pyrazinamide-isoniazid could be demonstrated.

Examination of Tissues Other Than Lung or Spleen.—The possibility existed that the failure to demonstrate any tubercle bacilli in the spleens and lungs of mice which received pyrazinamide-isoniazid therapy did not reflect the disappearance of the microorganisms from other tissues of the same animals. In this case, posttreatment relapse need not necessarily occur *in situ* but might merely represent a reseeding of the spleen with tubercle bacilli from some focus persistent elsewhere.

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Accordingly a group of ten mice infected intravenously with tubercle bacilli were treated for 90 days with pyrazinamide and isoniazid and were sacrificed. At autopsy, in addition to the lungs and spleen, the liver, the kidneys, and the inguinal, axillary, cervical, and tracheo-bronchial lymph nodes were removed and homogenized. With the use of the more sensitive culturing technique, there were no tubercle bacilli demonstrable in any of these tissues by culture or by microscopy.

Infection with a Wild Type Strain of M. tuberculosis.—As all of the experiments presented thus far and all those in the preceding report (1) were conducted with the stock H37Rv strain of tubercle bacilli, the influence of pyrazinamide and isoniazid on a wild type strain of tubercle bacilli was studied.

The used strain was isolated from the sputum of a previously untreated patient with far advanced pulmonary tuberculosis. Subcultures of the strain were regularly inhibited by isoniazid concentrations of 0.063 μg. per ml. The wild type strain was as virulent for mice as the H37Rv strain. In fact, it was perhaps slightly more virulent as judged by the failure of any untreated animals to survive 12 weeks after infection.

The influence of pyrazinamide and isoniazid alone and together on the pulmonic and splenic populations of tubercle bacilli in mice infected with this wild type strain may be seen in Text-figs. 11 and 12. The more sensitive culturing technique was used on day 56 and day 84 for the organs from the groups which had received pyrazinamide alone and pyrazinamide-isoniazid. There were no tubercle bacilli demonstrable in the lungs of any of the treated animals after 8 weeks. The behavior of the populations of tubercle bacilli in the spleens was similar to that observed in animals infected with H37Rv. 8 weeks after the start of pyrazinamide and isoniazid, no tubercle bacilli were demonstrable in either the lungs or the spleens of any of the animals examined. In contrast, persistent tubercle bacilli were demonstrable at the 12 week observation point in the spleens of some of the animals which had received either drug alone.

Pyrazinamide Alone and Isoniazid Alone.—The respective influence of pyrazinamide alone and isoniazid alone on the populations of tubercle bacilli in the spleens of mice in the beginning infection and in the 21 day infection have been described previously. The administration of isoniazid alone never resulted in the disappearance of tubercle bacilli from the mouse spleens. In contrast, such a disappearance from the spleens (as well as the lungs) was observed not infrequently following the administration of pyrazinamide alone. In the 21 day infection, approximately one-half of the animals which received pyrazinamide alone would fail to reveal culturable tubercle bacilli on sacrifice. When antimicrobial therapy and infection were both started on the same day, the administration of pyrazinamide alone for a 12 week period resulted in the disappearance of the tubercle bacilli from the spleens of 19 of 32 animals examined.

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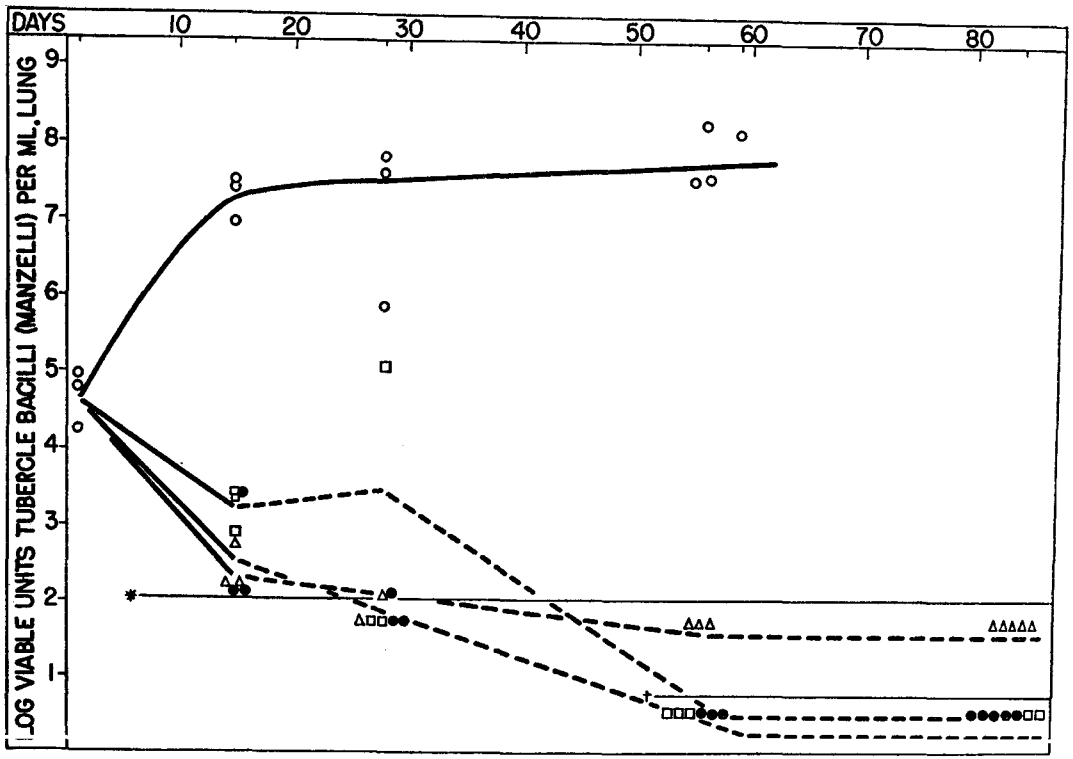

TEXT-FIG. 11. Influence of pyrazinamide and isoniazid used singly and together on isoniazid-susceptible (>0.032 <0.063) populations of tubercle bacilli in mouse lungs during 12 weeks of therapy. Infecting inoculum 2.6 × 10^5 culturable units of tubercle bacilli. ○, control; △, isoniazid; □, pyrazinamide; ●, pyrazinamide-isoniazid.

* Symbols below this line signify no culturable tubercle bacilli.

† On day 56 and 84 the techniques used for culturing the lungs from pyrazinamide alone and pyrazinamide-isoniazid-treated animals permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

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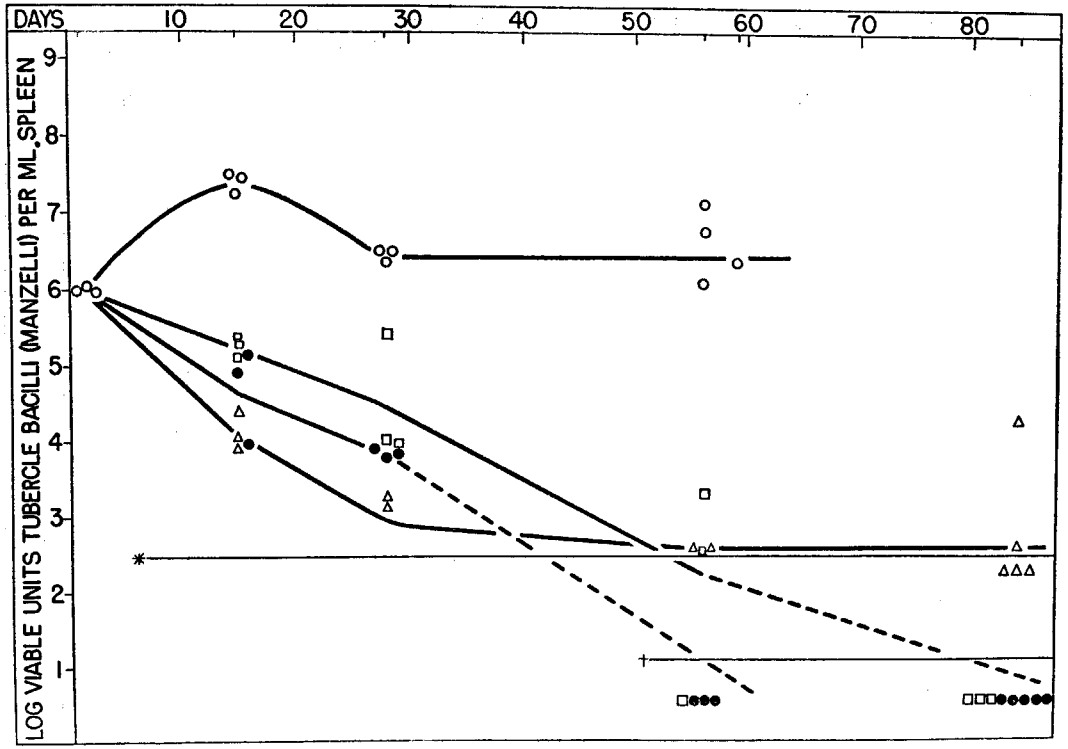

TEXT-FIG. 12. Influence of pyrazinamide and isoniazid used singly and together on isoniazid-susceptible (>0.032 <0.063) populations of *M. tuberculosis* in spleens of the same animals whose lung studies are shown in Text-fig. 11. ○, control; △, isoniazid; □, pyrazinamide; ●, pyrazinamide-isoniazid.

* Symbols below this line signify no culturable tubercle bacilli.

† On day 56 and 84 the techniques used for culturing the lungs from pyrazinamide alone and pyrazinamide-isoniazid-treated animals permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

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Antagonism of Isoniazid by Pyrazinamide or Nicotinamide.—In Text-fig. 2 it may be noted that during the first 2 weeks of pyrazinamide-isoniazid administration (when infection and chemotherapy were started the same day) the fall in microbial population for the pyrazinamide-isoniazid animals closely resembled that observed with pyrazinamide alone, rather than that observed with isoniazid alone in the same experiment. Accordingly, experiments were conducted to discover whether nicotinamide, the parent compound from which pyrazinamide is derived, could likewise antagonize the influence of isoniazid. This experiment was conducted in the same manner as the previously described experiments and both the infection and the chemotherapy were started on the same day. The results may be seen in Text-figs. 13 and 14.

The administration of nicotinamide alone showed no effect on the splenic population until the 2nd week; there then occurred a very slight fall which continued as a gradual descent, throughout the 12 week treatment period. The populations of tubercle bacilli in the lungs of the animals treated with nicotinamide alone were comparable with those present in previous experiments with streptomycin. When nicotinamide was administered along with isoniazid, the effectiveness of the latter drug was significantly less during the 1st month of treatment than when the isoniazid was administered alone (Text-figs. 13 and 14).

Thus nicotinamide antagonized the action of isoniazid in the early days of the infection in the same way as did pyrazinamide. Nevertheless, the administration of nicotinamide-isoniazid was not followed by the vanishing of the tubercle bacilli from the tissues as had been observed with pyrazinamide-isoniazid. During the last 2 months of treatment with nicotinamide-isoniazid, the microbial censuses in both the lungs and spleens of the treated animals were similar to those determined in the animals which had received isoniazid alone.

Companion Drugs Other Than Isoniazid.—Experiments were conducted in which other antimicrobial drugs were substituted for isoniazid as the companion drug for pyrazinamide. The drugs used were streptomycin, para-aminosalicylic acid (PAS), and oxytetracycline. The conditions of these experiments were otherwise identical with those described above in which infection and chemotherapy were started on the same day.

The populations of tubercle bacilli in the spleens of the animals which received pyrazinamide-streptomycin, pyrazinamide-PAS, or pyrazinamide-oxytetracycline all showed the same steady fall to below the limits of detection as was the case when isoniazid had been used as the companion drug for pyrazinamide. Thus, in the experimental system employed (infection and chemotherapy started together but without a posttreatment drug-free interval) any one of the 4 drugs isoniazid, streptomycin, PAS, or oxytetracycline appeared to be indistinguishable from the others in terms of effectiveness as a

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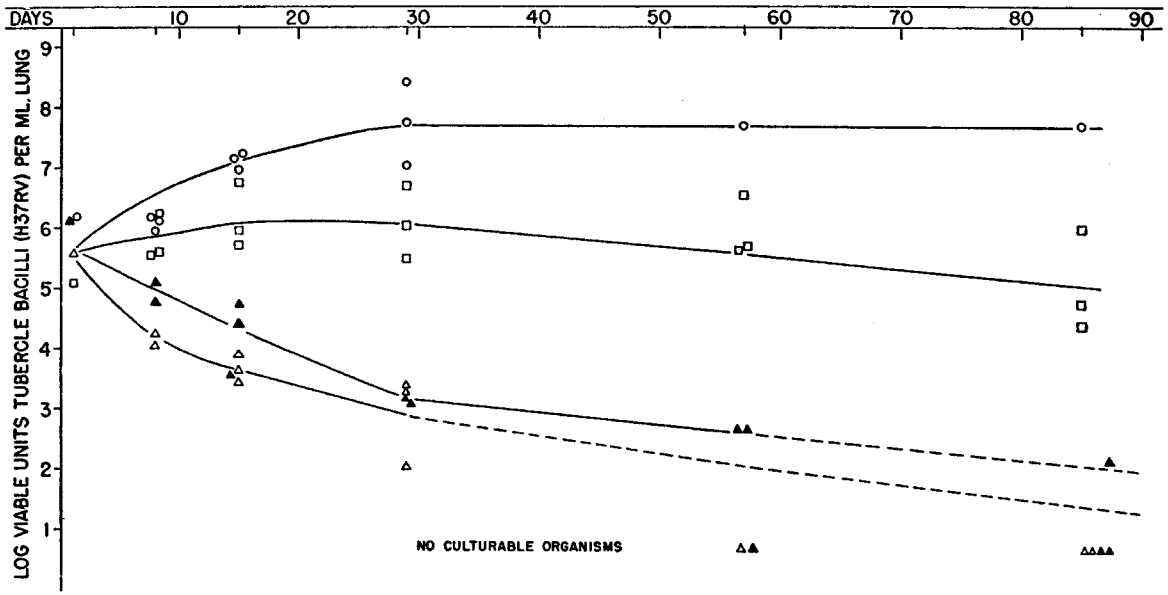

TEXT-FIG. 13. Influence of isoniazid and nicotinamide used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs during 12 weeks of therapy.

Infecting inoculum; 8.8×10^5 culturable units of tubercle bacilli. ○, control; □, nicotinamide; △, isoniazid; ▲, isoniazid-nicotinamide.

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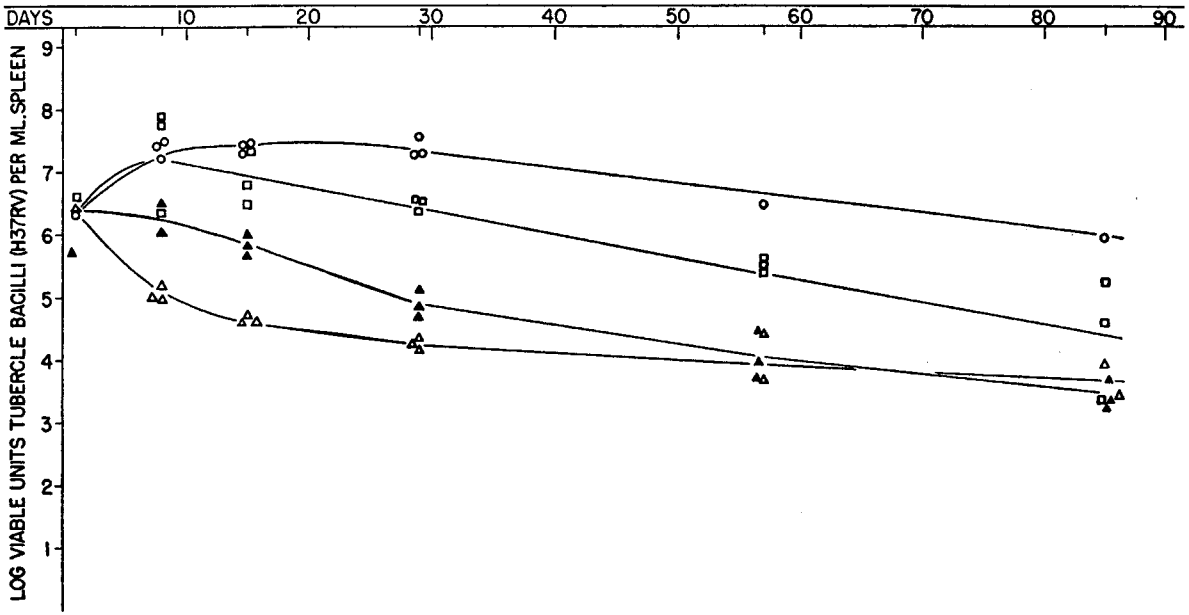

TEXT-FIG. 14. Influence of isoniazid and nicotinamide used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung studies are shown in Text-fig. 13. ○, control; □, nicotinamide; △, isoniazid; ▲, isoniazid-nicotinamide.

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companion drug for pyrazinamide. It is of interest that oxytetracycline when administered alone showed little if any influence on either the lung or spleen populations of tubercle bacilli in the experimental system. Moreover, the uniform disappearance of the tubercle bacilli from the spleens of the animals

TABLE II A

Comparative Effects of Varied Intervals of Pyrazinamide in Combination with Isoniazid on Microbial Populations (H37Rv) in Mouse Lungs during 13 Weeks of Treatment

	Control	Isoniazid 13 wks.	Pyrazinamide 13 wks.	PZA—2 wks. INH—13 wks.	PZA—4 wks. INH—13 wks.	PZA—8 wks. INH—13 wks.	PZA—13 wks. IHN—13 wks.
	5.5×10^8	1.1×10^4	1.4×10^8	0	0	0	0
	9.4×10^7	1.5×10^1	0	0	0	0	0
	6.2×10^7	0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	—*	0
		0	0	0	0		—*
		0	—*				
		0	—*				
		0	—*				
Percentage of animals with no culturable organisms.....	0	85	92	100	100	100	100

The comparative effects of varied periods of pyrazinamide administration (isoniazid administration kept constant) on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 13 weeks of therapy. Infecting inoculum: 2.3×10^5 culturable units of tubercle bacilli.

* Contaminated culture plate.

which received pyrazinamide-PAS occurred at a time after the start of infection (5th to 8th week) at which when PAS was used alone it has apparently lost its effectiveness (1). The respective values of these 4 drugs as companion drugs for pyrazinamide has not yet been studied in the 21 day infection or in the experimental system with the 90 day posttreatment observation period.

Strains of Tubercle Bacilli Resistant to Isoniazid in Vitro.—The influence of isoniazid as a companion drug for pyrazinamide was studied in animals infected with isoniazid-resistant strains of tubercle bacilli.

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The two strains used had both been isolated from patients with pulmonary tuberculosis who had continued to discharge tubercle bacilli after prolonged therapy with isoniazid alone (7). In conventional drug susceptibility tests *in vitro*, strain *Rh* was resistant to isoniazid concentrations of 0.25 μg. per ml. and strain *Th* was resistant to concentrations of 6.3 μg. per ml. Quantitative studies revealed that 68 per cent of strain *Rh* consisted of tubercle bacilli

TABLE II B

Comparative Effects of Varied Intervals of Pyrazinamide in Combination with Isoniazid on Microbial Populations (H37Rv) in Mouse Spleens during 13 Weeks of Treatment

	Control	Isoniazid 13 wks.	Pyrazinamide 13 wks.	PZA—2 wks. INH—13 wks.	PZA—4 wks. INH—13 wks.	PZA—8 wks. INH—13 wks.	PZA—13 wks. INH—13 wks.
	1.4×10^6	5.2×10^3	2.1×10^5	5.5×10^3	6.8×10^2	0	0
	1.2×10^6	4.6×10^3	1.1×10^5	2.1×10^3	1.0×10^2	0	0
	1.2×10^6	2.8×10^3	4.5×10^4	2.0×10^3	6.9×10^1	0	0
		2.7×10^3	4.3×10^4	1.8×10^3	5.2×10^1	0	0
		2.3×10^3	—*	1.7×10^3	5.2×10^1	0	0
		2.2×10^3	0	1.3×10^3	0	0	0
		1.6×10^3	0	5.2×10^2	0	0	0
		1.3×10^3	0	3.9×10^2	0	0	0
		1.2×10^3	0	3.2×10^2	0	0	0
		5.2×10^2	0	3.1×10^2	0	0	0
		5.2×10^2	0	2.8×10^2	0	0	0
		3.9×10^2	0	2.4×10^2	0	0	0
		3.6×10^2	0	7.5×10^1	0	0	0
		2.0×10^2	0	—*	0	0	0
		—*	0	—*	0	0	0
Percentage of animals with no culturable organisms.....	0	0	69	0	67	100	100

The comparative effects of varied periods of pyrazinamide administration (isoniazid administration kept constant) of populations of *M. tuberculosis* (H37Rv) in mouse spleens during 13 weeks of therapy. Infecting inoculum: 2.3×10^5 culturable units of tubercle bacilli.

* Contaminated culture plate.

susceptible to isoniazid concentrations of 1 μg. per ml. whereas all the cells of strain *Th* were resistant to this concentration of drug.

The results observed from the use of pyrazinamide alone in these isoniazid-resistant infections were in no way different from those in the isoniazid-susceptible infections previously described. The use of isoniazid as a companion drug to the pyrazinamide, however, failed to result in the disappearance of the tubercle bacilli from the spleens of the animals infected with either of these drug-resistant strains.

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Time-Dose Relationships of Pyrazinamide Necessary to Produce Pyrazinamide-Companion Drug Phenomenon.—In all of the experiments in the present and preceding investigations (1) in which the administration of pyrazinamide and a companion drug resulted in the disappearance of the tubercle bacilli from the mouse spleens, the daily dosage of pyrazinamide had been 2 per cent

TABLE II C

Comparative Effects of Varied Intervals of Pyrazinamide in Combination with Isoniazid on Microbial Populations (H37Rv) in Mouse Lungs during 27 Weeks of Treatment

	Control	Isoniazid 27 wks.	Pyrazinamide 27 wks.	PZA—2 wks. INH—27 wks.	PZA—4 wks. INH—27 wks.	PZA—8 wks. INH—27 wks.	PZA—27 wks. INH—27 wks.
	3.0×10^8	3×10^0	6.9×10^5	1.9×10^1	0	0	0
	1.5×10^8	0	3.1×10^3	0	0	0	0
	4.3×10^7	0	5.2×10^1	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
					0		—*
Percentage of animals with no culturable organisms..........	0	93	75	92	100	100	100

The comparative effects of varied periods of pyrazinamide administration (isoniazid administration kept constant) on populations of *M. tuberculosis* (H37Rv) in mouse lungs during 27 weeks of therapy.

* Contaminated culture plate.

of the diet and the duration of the pyrazinamide therapy had been at least 12 weeks. Accordingly, a series of experiments was conducted in an attempt to obtain information on the minimal pyrazinamide daily dosage and the minimal duration of pyrazinamide therapy which were necessary to produce the uniform disappearance of tubercle bacilli from the spleens of animals receiving pyrazinamide and a companion drug.

The chemotherapy and the infection were both started on the same day in these experiments. Isoniazid was the companion drug used and it was administered daily for a 13 or 27

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week period. The pyrazinamide administration, however, was terminated in various groups of animals at 2, 4, 8, and 13 weeks respectively. The results of the 27 week experiment may be seen in Tables II A to II D.

As may be seen in Tables II A to II D, 8 weeks of pyrazinamide with isoniazid concurrently and thereafter resulted in the uniform disappearance of the

TABLE II D

Comparative Effects of Varied Intervals of Pyrazinamide in Combination with Isoniazid on Microbial Populations (H37Rv) in Mouse Spleens during 27 Weeks of Treatment

	Control	Isoniazid	Pyrazinamide 27 wks.	PZA—2 wks. INH—27 wks.	PZA—4 wks. INH—27 wks.	PZA—8 wks. INH—27 wks.	PZA—27 wks. INH—27 wks.
	3.0×10^7	8.4×10^2	9.7×10^4	3.1×10^2	8.1×10^0	0	0
	1.1×10^7	1.6×10^2	4.7×10^4	2.5×10^2	8.1×10^0	0	0
	4.2×10^6	1.5×10^2	8.9×10^2	1.2×10^2	8.1×10^0	0	0
		1.4×10^2	7.5×10^3	1.2×10^2	8.1×10^0	0	0
		1.4×10^2	6.2×10^3	1.0×10^2	0	0	0
		1.2×10^2	4.8×10^3	9.5×10^1	0	0	0
		1.0×10^2	0	7.1×10^1	0	0	0
		5.4×10^1	0	6.8×10^1	0	0	0
		5.4×10^1	0	4.2×10^1	0	0	0
		4.6×10^1	0	2.7×10^1	0	0	0
		2.7×10^1	0	1.9×10^1	0	0	0
		2.7×10^1	0	0	0	0	0
		2.3×10^1			0		0
		8×10^0			0		0
Percentage of animals with no culturable organisms.....	0	0	50	8	73	100	100

The comparative effects of varied periods of pyrazinamide administration (isoniazid administration kept constant) on populations of *M. tuberculosis* (H37Rv) in mouse spleens during 27 weeks of therapy.

tubercle bacilli from the spleens at the completion of the total 13 week period of chemotherapy. When the total 13 week period of chemotherapy included only a 4 week period of pyrazinamide, the results were not uniform; when the pyrazinamide was given for only 2 weeks the results were indistinguishable from those observed with the use of isoniazid alone. The necessity for administering the pyrazinamide for at least 8 weeks was the same whether the total period of isoniazid therapy was 13 weeks or 27 weeks.

It should be noted that in all experimental situations in the total study

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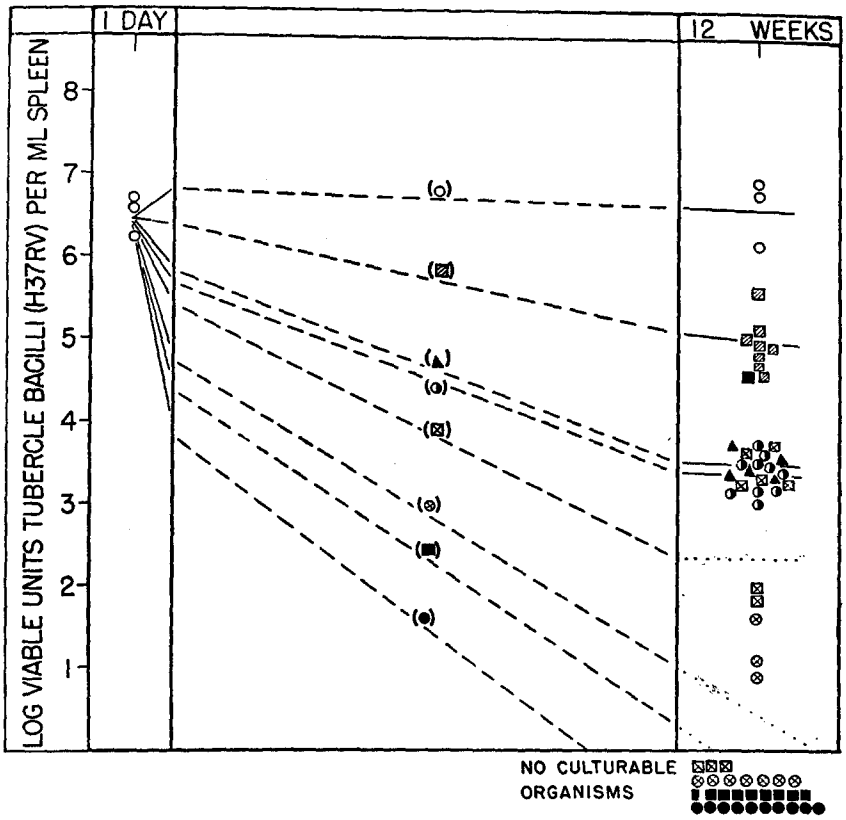
(including those just presented), in which the populations of tubercle bacilli uniformly disappeared from the lung and spleen, the total period of antimicrobial therapy has been at least 12 weeks. No experiments have been performed



TEXT-FIG. 15. Influence of various dosages of pyrazinamide used alone and with isoniazid on populations of tubercle bacilli (H37Rv) in mouse spleens after 84 days of therapy.

Infecting inoculum 5.3 × 10^6 culturable units of tubercle bacilli. ○, control; ▲, isoniazid; ■, pyrazinamide 0.125 per cent plus isoniazid; ◑, pyrazinamide 0.125 per cent plus isoniazid; ⊠, pyrazinamide 0.5 per cent; ⊗, pyrazinamide 0.5 per cent plus isoniazid; ■, pyrazinamide 2.0 per cent; ●, pyrazinamide 2.0 per cent plus isoniazid.

in which pyrazinamide and isoniazid were administered together for an 8 week period with no chemotherapy thereafter.

Daily Dose of Pyrazinamide.—In another experiment, the daily dose of pyrazinamide was reduced to one-fourth and one-sixteenth the dose used in the previous experiments. Infection and chemotherapy were started on the same day and the pyrazinamide-isoniazid was continued for the total period of 12 weeks. The results of this experiment may be seen in Text-fig. 15.

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TABLE III

Influence of Sequential Administration Pyrazinamide and Isoniazid on Microbial Populations (H37Rv) in Mouse Tissues during 12 Weeks of Treatment. Effect of Discontinuing Treatment for an Additional 12 Weeks

Group	8 wks.		12 wks.		24 wks.	
	Spleen	Lung	Spleen	Lung	Spleen	Lung
A						
Pyrazinamide 8	6.5×10^3	0	7.0×10^2	0.6×10^1	6.1×10^4	0
wks.	0	0	2.4×10^2	0	3.9×10^3	4.3×10^6
Isoniazid 4 wks.	0	0	0	0	3.4×10^3	1.2×10^3
					2.7×10^3	0
					2.2×10^3	2.6×10^6
					1.9×10^3	—*
					0	0
B						
Pyrazinamide 4	1.3×10^3	0	7.3×10^1	0	7.1×10^5	2.9×10^5
wks.	0	0	2.7×10^1	1.6×10^2	1.6×10^5	2.8×10^1
Isoniazid 8 wks.	—*	—*	1.1×10^1	0.3×10^1	6.1×10^4	1.4×10^4
					2.7×10^4	7.5×10^3
					2.5×10^4	1.2×10^5
					1.4×10^4	1.8×10^4
					5.5×10^3	5.5×10^4
C						
Isoniazid 8 wks.	3.3×10^3	2.0×10^3	1.1×10^1	0	1.9×10^5	5.2×10^3
Pyrazinamide 4	2.9×10^3	3.4×10^3	0.8×10^1	0	3.2×10^4	0
wks.	2.6×10^3	1.7×10^3	0	0	1.6×10^4	0
					3.7×10^3	0
					1.9×10^3	7.1×10^5
					1.3×10^3	0
					0	0
D						
Isoniazid 4 wks.	8.9×10^2	0	0	0	6.5×10^4	0
Pyrazinamide 8	0	0	0	0	4.2×10^4	5.7×10^4
wks.	0	0	0	0	1.6×10^3	0
					0	0
					0	0
					0	0
					0	0
					0	0

The influence of sequential administration of pyrazinamide and isoniazid on populations of *M. tuberculosis* (H37Rv) in mouse tissues during 12 weeks of treatment and the effect of discontinuing treatment for an additional 12 weeks.

Infecting inoculum: 1.8×10^5 culturable units of tubercle bacilli.

* Contaminated culture plate.

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As may be seen in the figure, when the daily dose of pyrazinamide was reduced by 75 per cent and administered with isoniazid, there were no detectable organisms in seven of ten animals. When the daily dose of pyrazinamide was lowered by 94 per cent, it was minimally effective when given alone, and when administered with isoniazid the results were identical with those seen with isoniazid alone.

It appeared, therefore, from these 2 sets of experiments that within a total period of 13 weeks of chemotherapy the minimal duration of the pyrazinamide administration must be approximately 8 weeks and the minimal daily dosage must be 2 per cent in the diet in order to produce the pyrazinamide companion drug phenomenon of the vanishing of the tubercle bacilli with uniformity. A similar period of pyrazinamide administration also appeared to be necessary in the 21 day infection. For, tubercle bacilli could frequently be cultured from the mouse spleens 4 weeks after the start of pyrazinamide-isoniazid therapy even though the bacilli uniformly vanished thereafter.

Pyrazinamide and Isoniazid Administered in Sequence.—The respective roles of pyrazinamide and the companion drug were studied in a series of experiments in which pyrazinamide and isoniazid were each administered singly, but in sequence for periods of varied length.

The antimicrobial therapy was started on the 1st day of infection and was continued for a total period of 12 weeks irrespective of the particular drug regimen employed. There were 4 experimental groups of animals: group A received pyrazinamide for 8 weeks and isoniazid for 4 weeks thereafter; group B received pyrazinamide for 4 weeks and isoniazid for 8 weeks thereafter; group C received isoniazid for 8 weeks and pyrazinamide for 4 weeks thereafter; and group D received isoniazid for 4 weeks and pyrazinamide for 8 weeks thereafter. The results are presented in Table III.

As may be seen in Table III, the tubercle bacilli had disappeared from both the spleens and the lungs of only one group of animals (group D) after completion of the 12 weeks of chemotherapy. From the beginning of the infection, this group had received 4 weeks of isoniazid followed by 8 weeks of pyrazinamide. In the group of animals which differed only in the fact that this sequence of administration of the 2 drugs was reversed (group A), tubercle bacilli could be cultured from the spleens of two of the three animals and the lungs of one of the three, examined at the 12 week observation point. Tubercle bacilli were also demonstrable in the spleens of all but one of the animals in the 2 groups (B and C) which had received the pyrazinamide for only 4 weeks. After 12 weeks' posttherapy observation, tubercle bacilli could be cultured from the spleens (three of eight animals) and from the lungs (one of eight animals) of the animals from the group in which no tubercle bacilli were demonstrable at the completion of the chemotherapy (group D).

DISCUSSION

From the above observations it may be seen that populations of tubercle bacilli of human origin, subjected *in vivo* to pyrazinamide and a companion

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drug, vanished from the tissues in so far as could be determined by microscopy, culture, or guinea pig subinoculation. In actuality, the vanishing of the bacilli did not represent a complete elimination of all tubercle bacilli from the tissues of all of the animals. In fact, 90 days after cessation of drug treatment, tubercle bacilli could be isolated from approximately one-third of the animals studied.

The tubercle bacilli which survived in the animals, despite exposure to pyrazinamide and a companion drug, were not resistant either to pyrazinamide or to isoniazid in the conventional sense except in one instance. In experiments to be published elsewhere (8), it was observed that 9 of the 10 strains subjected to testing which had been derived from pyrazinamide-isoniazid animals after a 90 day treatment-free interval were inhibited by pyrazinamide when tested in an acidic environment *in vitro*. Nine of these same strains were also inhibited by isoniazid concentrations of 0.063 μg. per ml. when tested *in vitro*.

It appears, therefore, that, despite the marked quantitative difference between the pyrazinamide–companion drug regimen and the other chemotherapies with respect to the abolition of tubercle bacilli in mouse tissues, the nature of the survival of the bacilli in the two sets of circumstances is the same. In both situations, some bacilli, whose descendants were drug-susceptible when tested *in vitro*, were nonetheless able to persist in the mouse tissues despite continued chemotherapy.

In the case of the non-pyrazinamide regimens, however, the "persisting" tubercle bacilli could always be detected. The consistent failure of these bacilli to increase in numbers indicated that the infection would meet the criteria for the designation "dormant" in the sense of being demonstrably present although inactive. With the pyrazinamide-containing regimens, the presence of any "persisting" tubercle bacilli could not be detected after an appropriate period of therapy. Instead, the entire population vanished and the presence of "persisters" in a portion of the animals could only be shown retrospectively after a 90 day treatment-free interval. Thus at the time of the completion of treatment, the tuberculous infection would be said to have been in a truly *latent state*, meaning that it was undoubtedly present in some animals but was hidden beyond the reach of the available methods of detection. By its very nature the only proof of the presence of true latency is the subsequent reappearance of the infection in some animals.

In essence, the difference between the *latent* and *dormant* state typifies the difference between the behavior pattern of the populations of tubercle bacilli during pyrazinamide–companion drug therapy and all the other chemotherapies which are studied.

This situation has many parallels in the antimicrobial therapy of other infections. For example, in the pharyngitis of man produced by Group A hemolytic streptococci, the administration of sulfonamide renders the infection dormant whereas penicillin renders it truly latent. With sulfonamide, the presence of the streptococci can generally be demonstrated by culture throughout therapy, but the infection is inactive as

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judged by its absence of progression and possibly lessened communicability. With penicillin, the streptococci "vanish" soon after the onset of therapy and cannot be cultured from the nasopharynx at the time chemotherapy is stopped. After a treatment-free interval, however, the same serologic type of streptococci reappear in the pharynx of a minority of persons treated. The size of this minority can be steadily diminished by prolongation of the period of chemotherapy. Presumably with a substantial prolongation (*e.g.* 6 or 8 weeks), the "persisters" might be eliminated altogether. The last named phenomenon, however, might represent natural death of the parasites after prolonged "physiologic imprisonment" as much as a complete and direct eradicating action of the drug.

The difference between rendering an infection dormant and rendering it latent is thus clear cut. What is not clear, however, is whether this difference is merely quantitative or whether it reflects the operation of a qualitatively different type of chemotherapeutic action or even some major change in the state of the parasite.

In the present investigation, the true latency of the experimental tuberculous infection was established only for the tissues examined, which in most of the experiments consisted of only the lungs and spleens. It is possible, therefore, that tubercle bacilli were present in a readily detectable state in some other tissues. In a single experiment in which tubercle bacilli had vanished from the lung and spleen, examination of hepatic, renal, and lymphatic tissue likewise failed to reveal any bacilli. Moreover, the spleen represents an organ in which the survival to drug exposure by tubercle bacilli was easily demonstrable for all of the non-pyrazinamide chemotherapies which were studied.

It should also be recognized that virtually all the results of the present investigation are subject to the defects implicit in sampling techniques. Nevertheless, it is believed that the uniform predictability of the results with respect to the disappearance of the tubercle bacilli from the tissues provides considerable assurance that the observations made on samples are valid for the groups in their entirety.

The further possibility cannot be denied that the vanishing of the populations of tubercle bacilli represents a widespread alteration of the ability of individual cells to grow on artificial media or in guinea pigs, rather than a reduction to an extremely low census. A bacterial population subsisting in tissue must be fairly large to be detectable by microscopy of tissue samples. Consequently, a population just below the microscopic limits of detection, if rendered non-culturable with sufficient uniformity, would vanish even though the census were still fairly high. With the technics used in the present studies, however, tubercle bacilli could be cultured regularly from the animals receiving any form of chemotherapy which did not include pyrazinamide. Moreover, when known numbers of tubercle bacilli were added to tissue emulsions from drug-treated but uninfected animals, it was possible to obtain positive

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cultures with regularity when so few as one or two culturable units per individual spleen had been added. In the latter instance, however, it must be recognized that the added bacilli had not undergone any drug exposure when introduced into the drug-containing tissue emulsions.

This question of an actual census reduction *versus* a widespread alteration of the ability of individual cells to grow *in vitro* cannot be definitely settled at present. Whichever mechanism is responsible for the vanishing, it is sufficient for the purposes of the present discussion that this phenomenon of drug-induced latency is unique to the pyrazinamide-companion drug regimen.

Two questions arise with respect to the mechanics of the disappearance of tubercle bacilli from tissues during chemotherapy: First, is the role of isoniazid as a companion drug a specific one. Second, are those actions of pyrazinamide and the companion drug, which are essential for the phenomenon, exerted independently on separate microbial cells or are they mutually dependent and hence necessarily exerted on the same microbial cell.

From the present data, a possibly specific role of isoniazid in the process cannot be completely excluded for the majority of the experiments were made with isoniazid as the companion drug. In the course of these experiments, a definite interrelationship among the antimicrobial activities of isoniazid, pyrazinamide, and nicotinamide was observed. Both nicotinamide and pyrazinamide antagonized the population-reducing effects of isoniazid during the first 2 weeks of infection. Unlike pyrazinamide, however, the administration of nicotinamide thereafter along with isoniazid was not associated with the vanishing phenomenon. These 3 compounds, particularly nicotinamide and pyrazinamide, have a relatively close structural similarity to one another.

The apparent degree of specificity in the interrelationships among the 3 drugs might indicate that isoniazid likewise played a specific role in the vanishing phenomenon when administered as a companion drug to pyrazinamide. Nevertheless, 3 other drugs, streptomycin, para-amino salicylic acid, and oxytetracycline, likewise were effective as companion drugs. These 3 drugs and isoniazid are of diverse chemical nature and exert widely different degrees of inhibitory activity against tubercle bacilli, both in the particular experimental system employed and in all other comparative tests. Indeed, in the present experimental system, the anti-tuberculous activity of oxytetracycline when used alone was barely detectable. Despite their heterogeneity, these drugs all serve to render a pyrazinamide effect, which occurs irregularly with pyrazinamide alone, into a uniformly predictable phenomenon. It seems unlikely, therefore, that the role of isoniazid as a companion drug in the vanishing phenomenon is a specific one. By the same token it should be noted that the four companion drugs used with pyrazinamide were demonstrated to be comparable only in the terms of rendering the infection latent when administered along with pyrazinamide. Whether the rate of reappearance after a particular posttreatment

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interval would be the same irrespective of companion drug has not yet been studied.

Interpretation of the experimental observations is difficult on the question of whether the disappearance of tubercle bacilli during chemotherapy principally represents action of pyrazinamide and the companion drug on the same microbial cell or whether the two drugs act separately on different cells.

It seems clear that at least a portion of the action is on different cells. A small minority of isoniazid-resistant cells are present in the H37Rv strain of tubercle bacilli. Small numbers of pyrazinamide-resistant cells are also presumably present in the H37Rv strain or at least they emerge from this strain *in vivo*. Hence it is reasonable to assume that such drug-resistant cells were originally present in some of the pyrazinamide-isoniazid experiments and were rendered dead or latent either by one of the drugs acting alone or by the host defenses.

It is tempting to invoke this same explanation for the "vanishing" phenomenon as a whole. Pyrazinamide is the major drug in the phenomenon as evidenced by the facts that the effect could not be produced without pyrazinamide and occasionally occurred with pyrazinamide alone. It could be postulated that the principal role of the companion drug might consist of acting on the tubercle bacilli which escaped pyrazinamide action because they were pyrazinamide-resistant. With a sufficiently powerful impact of the pyrazinamide on the initial population, even a relatively weak companion drug might be able to control the residual population as long as the chemotherapy were continued.

The results of a single experiment in which the pyrazinamide and the isoniazid were administered in sequence have some bearing on this point. In this experiment, the survivors of 4 weeks of isoniazid therapy "vanished" during the succeeding 8 weeks of pyrazinamide, whereas the survivors of 4 weeks of pyrazinamide failed to vanish during a comparable period of isoniazid administration. This occurred despite the fact that at the 4 week point when the drugs were exchanged, the populations of pyrazinamide survivors to be subjected to isoniazid were appreciably smaller than the populations of isoniazid survivors to be subjected to pyrazinamide.

The major portion of the tubercle bacilli in these residual populations represented "persisters" with respect to the drug involved and not tubercle bacilli resistant to that drug in the orthodox sense. More than 99 per cent of the isoniazid survivors at the 4 week period would be expected to be isoniazid-susceptible on the basis of repeated observations with the H37Rv strain of tubercle bacilli in this experimental system (1). A precise estimate of the number of pyrazinamide-resistant cells to be expected among the pyrazinamide survivors cannot be stated. Nevertheless, the pyrazinamide-resistant cells should have been only a small minority as judged by observations at the 4-week point and thereafter with pyrazinamide alone, and the lack of prominence of

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pyrazinamide-resistant cells among the tubercle bacilli cultured 90 days after the cessation of pyrazinamide-isoniazid therapy. To a great extent, therefore, the difference in effectiveness between the two drugs after they were exchanged in the sequential experiments, consisted of their capacity to affect tubercle bacilli which had been subjected to the influence of the other drug but were not resistant to it. In short, pyrazinamide was highly effective on isoniazid persisters. To this extent, therefore, the observations support the premise that both the pyrazinamide and the isoniazid act on the same microbial cell in causing the disappearance of tubercle bacilli.

The observations also suggest that the timing of the exposure of the tubercle bacilli to the two drugs is important and that exposure to the isoniazid must precede or accompany, rather than follow, exposure to the pyrazinamide. This is of interest in view of the recent observations of Koch-Weser on the action of isotopically labelled isoniazid in the presence of other antituberculous drugs (9). He has shown that the effect produced by isoniazid on tubercle bacilli (H37Rv) *in vitro* in the presence of streptomycin is distinctly different when exposure to the streptomycin precedes isoniazid exposure than when the two drugs and the bacilli are brought together at the same time.

In experiments in which pyrazinamide and isoniazid were given sequentially the fact that the tubercle bacilli had been subsisting in the animals for 4 weeks when pyrazinamide was started did not appear to be a determining factor in the production of the vanishing phenomenon. For the phenomenon could be produced when pyrazinamide was started on the 1st day of infection provided that a companion drug was also given. By the same token, a conspicuous feature of pyrazinamide action was the fact that it was equally effective against populations of tubercle bacilli which had been subsisting in the mice for 21 days as it was against freshly introduced bacilli. Indeed there was an appreciable difference between pyrazinamide (with or without a companion drug) and isoniazid in this respect.

There are certain prominent characteristics of the 21 day infection which might conceivably influence the over-all effectiveness of an antimicrobial drug. The census of tubercle bacilli is higher at that time, the inflammatory response and the microbial population are both more advanced and the host-parasite interaction has attained a crude equilibrium as evidenced by the stabilization and ensuing slight fall in the microbial census. The influence of any of these factors might be exerted directly on the parasite by rendering it more or less susceptible to a particular drug; in addition, the inflammatory environment might exert a direct influence on drug activity.

The size of the microbial population may have influenced the action of isoniazid in the 21 day infection, but population size *per se* had no discernible influence on the action of pyrazinamide. In the 21 day experiments, the populations of tubercle bacilli had just reached the peak size in both lung and

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spleen at the time pyrazinamide was started. In experiments giving the single drugs in sequence, however, at the time pyrazinamide was started, the populations were relatively low because of a preceding 4 week period of isoniazid. Nevertheless, in both experimental situations, the pyrazinamide appeared to exert maximal activity as judged by production of the vanishing phenomenon.

The role of the biochemical environment of the infected lesion with respect to host resistance (10, 11, 13) and to antimicrobial drug action (5, 12) has been the subject of several recent discussions. In the particular case of pyrazinamide it has been observed that the action of the drug on tubercle bacilli of human origin became demonstrable *in vitro* only in environments comparable in their acidity with those presumably present in necrotic lesions or the interior of monocytes (5). Such acidic environments did not cause the Ravenel strain of bovine origin to become susceptible to the drug.

Mackaness has studied this question in a previously reported experimental system (14, 15) for the observation of tubercle bacilli situated within monocytes *in vitro*. He has found (16)[3] that tubercle bacilli of human origin, subsisting intracellularly *in vitro*, were completely inhibited by pyrazinamide concentrations of 12.5 μg. per ml. in the extracellular environment. The Branch strain of bovine tubercle bacilli was not inhibited by pyrazinamide in these circumstances even when considerably higher pyrazinamide concentrations were tested. The extracellular fluid environment in the Mackaness system is maintained at a pH of 7.0 throughout the experiment. In this environment, the monocytes remain viable and capable of phagocytizing tubercle bacilli during the 28 day experimental period. In this situation, therefore, pyrazinamide is introduced into an environment (extracellularly) at a pH range in which it should not have demonstrable activity for human tubercle bacilli. The drug then diffuses from that extracellular environment into the cell and in these circumstances is inhibitory for tubercle bacilli of human, but not of bovine, origin which are is residence there.

Thus there are two environments in which the inhibitory action of pyrazinamide on tubercle bacilli of human origin can occur; an extracellular environment which is sufficiently acidic; and the interior of monocytes in which there presumably is a low pH range. Whether the two environments operate by the same mechanism, *i.e.* the influence of acidity on drug or the tubercle bacilli, cannot be stated at present.

Pyrazinamide is thus capable of exerting an effect on tubercle bacilli of human origin: in acidic environments; within monocytes; after 21 days of growth in mice; and after previous or concurrent exposure to isoniazid in mice. Moreover, with a companion drug, pyrazinamide is as effective against tubercle bacilli of human origin in the mouse spleen as in the mouse lung. The drug is not effective against tubercle bacilli of bovine origin in acidic environments, within monocytes, or in experimental infections in animals. Moreover, it is not effective against human tubercle bacilli *in vitro* when tested within the physiologic range of pH. Pyrazinamide is also not effective, even in the acidic environments *in vitro* against tubercle bacilli of human origin which had resumed multiplication in the mouse during prolonged therapy with pyrazinamide alone.

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[3] The writers are indebted to Dr. G. B. Mackaness of the Australian National University at Canberra for making these observations and permitting their mention in the present report.

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These observations suggest that the pyrazinamide susceptibility of an individual tubercle bacillus is closely related to its capacity to undergo some type of specific alteration in response to environmental influences including the other antituberculous drugs present in the environment. The two strains of bovine origin studied, and a tiny minority of the H37Rv strain of human origin, lack or lose this capacity to undergo the specific alteration. The majority of the cells in human strains maintain this capacity to assume the altered state even after a 21 day period of maturation in the mouse. The alternative possibility that it is the drug itself which is "activated" in some way by the environmental influences cannot be excluded. This does not seem likely however, both because of the apparent diversity of the environments of demonstrable effectiveness and the lack of demonstrable activity on tubercle bacilli of bovine origin.

With respect to the vanishing of tubercle bacilli the only influence which has been demonstrated to determine pyrazinamide effectiveness has been the previous or concurrent exposure of the tubercle bacilli to another antituberculous drug, which in most cases, was isoniazid. It has been reported (9) that isoniazid-resistant tubercle bacilli are unusually susceptible to pyrazinamide *in vitro*. Only two isoniazid-resistant strains were used in the present study *in vivo* and in neither case did the tubercle bacilli disappear from the mouse tissues during the administration of pyrazinamide alone.

It has not yet been determined whether the observed relapse rate of approximately one-third of the infections 90 days after completion of therapy could be significantly raised by prolongation of the posttreatment period of observation or lowered by prolongation of the treatment. Thus, it has not yet been established whether the tuberculous infection had actually been eliminated, by pyrazinamide and a companion drug, in two-thirds of the animals and persisted in latent form only in the remainder, or whether the infection remained latent in all. Studies directed to these questions are now in progress with particular emphasis on the use of methods known to be capable of producing the emergence of infections from the latent state (17, 18).

SUMMARY

Populations of tubercle bacilli of human origin exposed *in vivo* to pyrazinamide and a companion drug, vanished from the tissues of the mouse in so far as could be determined by microscopy, culture, or guinea pig subinoculation. The vanishing did not represent a complete elimination of the tubercle bacilli from all the animals. 90 days after the completion of treatment, tubercle bacilli could be cultured from approximately one-third of the animals examined at that time. This complete disappearance of the tubercle bacilli thus meets the definition of a truly *latent* infection in that the infection is present but is hidden beyond the limits of diagnostic reach. All but one of the strains of tubercle

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bacilli which survived in the animals and were detectable in the posttreatment period, were susceptible to pyrazinamide when tested under appropriate conditions *in vitro*.

Only two factors could be identified which were essential for the uniform occurrence of the disappearance of tubercle bacilli: the administration of the pyrazinamide in a high daily dosage for at least eight of a total of 12 weeks of antimicrobial therapy; and the concurrent or prior exposure of the microbial populations to isoniazid or in some cases to other antituberculous drugs.

The observations suggest that the ability of pyrazinamide-containing chemotherapies to bring about the disappearance of a tubercle bacillus is closely related to the occurrence of some alteration in the bacillus, essential for maximal pyrazinamide action, in response to environmental influences, including other antituberculous drugs present in the environment.

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BIBLIOGRAPHY

1. McCune, R. M., Jr., and Tompsett, R., *J. Exp. Med.*, 1956, **104,** 737.
2. Unpublished observations in this laboratory.
3. McDermott, W., Ormond, L., Muschenheim, C., Deuschle, K., McCune, R.M., Jr., and Tompsett, R., *Am. Rev. Tuberc.*, 1954, **69,** 319.
4. Fenner, F., Martin, S.P., and Pierce, C. H., *Ann. New York Acad. Sc.*, 1949, **52,** 751.
5. McDermott, W., and Tompsett, R., *Am. Rev. Tuberc.*, 1954, **70,** 748.
6. Hobby, G. L., Auerbach, O., Lenert, T. F., Small, N. J., and Comer, J. V. *Am. Rev. Tuberc.*, 1954, **70,** 191.
7. Deuschle, K., Ormond, L., Elmendorf, D., Jr., Muschenheim, C., and McDermott W., *Am. Rev. Tuberc.*, 1954, **70,** 228.
8. Lee, Seung Hoon, McCune, R. M., Jr., Tompsett, R., and McDermott, W., unpublished experiments in this laboratory.
9. Koch-Weser, D., *J. Clin. Inv.* 1956, **35,** 718.
10. Dubos, R. J., Biochemical Determinants of Microbial Diseases, Cambridge, Harvard University Press, 1954.
11. Dubos, R. J., *Lancet*, 1955, **269,** 1.
12. McDermott, W., in Bacterial and Mycotic Infections of Man (R. J. Dubos, editor), Philadelphia, J. B. Lippincott Co., 2nd edition, 1952.
13. Wood, W. B., Jr., Year Book of Pathology and Clinical Pathology, Chicago, The Year Book Publishers, 1950, 17.
14. Mackaness, G. B., *J. Path. and Bact.*, 1952, **64,** 429.
15. Mackaness, G. B., *Am. Rev. Tuberc.*, 1954, **69,** 690.
16. Mackaness, G. B., personal communication to the authors.
17. LeMaistre, C., and Tompsett, R., *J. Exp. Med.*, 1952, **95,** 393.
18. Smith, J. M., and Dubos, R. J., *J. Exp. Med.*, 1956, **103,** 119.

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