Studies on the Effect of Starvation on Mycobacteria

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Ten cultures of *Mycobacterium tuberculosis*, one of *Mycobacterium kansasii* (nonsignificant), and one of *Mycobacterium phlei* were submitted to starvation. As a result they lost first their acid fastness and then all other staining affinities but, in this chromophoric state, they survived for at least 2 years and, after that time, produced cultures of acid-fast bacilli when transferred onto nutrient media. Chromophoric tubercle bacilli similar to those produced experimentally had previously been demonstrated in caseous lesions of lungs surgically removed from patients under chemotherapy. Since it has been shown that experimentally produced chromophoric bacilli can recover their original biological properties, the opinion is warranted that, under suitable conditions, those in the lung could also become reactivated and cause a relapse of the disease.

In previous studies, tubercle bacilli of a new type have been described in the lungs of patients treated by drugs in association with surgery (7, 8). Although morphologically similar to the acid-fast bacilli, these organisms do not stain with either carbol-fuchsin or the counter-stains when applied by the classic Neelsen technique or with any other aniline dyes, and can only be colored by the newly developed oxidative procedures described in these papers. The demonstration of these "chromophoric" bacilli in sections of the lungs of all the patients examined (10) called for their further investigation, aiming at the in vitro development of live bacilli of the same type. Experiments designed to this effect carried out in this laboratory yielded results which are recorded in the present study.

MATERIALS AND METHODS

Bacilli starved in plain agar. Two strains of virulent tubercle bacilli taken from the stock of the clinical laboratory of this hospital were used for the experiment. They were grown in Dubos liquid medium with Tween and albumin, and a series of screw-capped tubes (150 by 25 mm) of each strain was prepared. After 4 weeks the cultures were spun down and washed three times with distilled water to remove all traces of the culture medium, the sediment was suspended in 2 ml of distilled water, and smear preparations were taken for control. Then a series of 50-ml conic glass centrifuge tubes were filled with 15 ml of 3% agar in distilled water, covered with aluminum foil to prevent contamination, sterilized, and put into a 55°C water bath in order to keep the agar fluid. Next the suspension of bacilli of each tube was transferred with a sterile pipette into a tube of agar, the mixture was vigorously stirred with the pipette to secure an even distribution of the bacilli in the jelly, and the tubes were closed with sterilized, new, tightly fitting rubber caps. After solidification of the agar, one tube was set apart for control and the others were incubated at 37°C.

The control tube was processed as follows. The block of agar was taken out of the tube with sterile precautions, transferred into a large petri dish, and cut into slices about 3-mm thick, the largest of which were divided into halves or quarters to obtain the number of cuts needed for the experiment. One slice was fixed directly in 10% formalin after smear preparations had been made with scrapings from its surface. the remaining slices were each dropped into a screw-capped tube (150 by 25 mm) of Dubos broth, and the whole series was incubated for 30 days at 37°C. The tubes were inspected daily and gently shaken individually, and any changes in the agar or the medium were noted. The incubated cuts were processed in a similar way, every other day during the first 2 weeks and thereafter at 4-day intervals until the 30th day. Checks of the medium were taken of tubes incubated 14 days or more. The formalized agar blocks were dehydrated with alcohol, embedded in paraffin, and cut in sections of 5-μm thickness which were stained, together with the smear preparations, overnight at room temperature in carbol-fuchsin, decolorized with 5% HNO₃ in 90% alcohol, counterstained with Loeffler methylene blue, rinsed, dried, and examined. No systematic studies with oxidation were carried out because trial experiments had shown that oxidized sections of agar had a tendency to come off the slides.

The remaining tubes of plain agar were processed in the same way as the control tube, at monthly intervals for the first 4 months of the experiment, and then during the following 8 months at bimonthly intervals and every 3 months during the 2nd year. To avoid disruption of the series by contamination of the cultures, very careful technical work was mandatory. Bacilli starved in distilled water. Ten 4-week-old...
cultures of virulent tubercle bacilli (no. 1, 2, 5, 10, 14, 16, 18, 19, 24, 26), one culture of Mycobacterium kansasii (nonsignificant) taken from the stock of the bacteriology laboratory of this hospital, and a culture of Mycobacterium phlei, each grown in two tubes (150 by 25 mm) of Dubos broth, were spun down and washed three times in sterile distilled water. The sediment of each tube was suspended in 15 ml of distilled water and, after a smear preparation was taken from each suspension, the tubes were screw capped and incubated at 37 C. being shaken at intervals to keep the sediment in suspension. To follow the reaction of the organisms to adverse environment, checks were taken from one tube of each suspension every month during the first 6 months, every other month during the following 6 months, and every 3 months during the 2nd year of the experiment. The duplicate tubes were kept in reserve. The preparations were air-dried and stained, and one of the slides taken from each tube was oxidized overnight in 10% periodic acid. The next day the oxidized series was rinsed, first in tap water, and then in distilled water, and then both series were stained with carbol-fuchsin in the usual way, examined under a light microscope and, in the case of the nonoxidized series, also under a phase-contrast microscope. Three additional films of each suspension were examined, one after staining with Gram stain and the others after negative staining with India ink or nigrosin. To determine the proportion of the acid-fast, non-acid-fast, and chromophobic bacilli, 100 cells were counted in three different fields of the films prepared from each suspension, and the average number was calculated. In addition to the microscopy examination, after 6, 12, 18, and 24 months, the suspensions were also planted on 7H10 agar, the development of the cultures was observed, and after 1 month two smear preparations were taken from each culture. These preparations were stained with carbol-fuchsin in the same way as the monthly checks, one directly and the other after oxidation.

RESULTS
Bacilli starved in plain agar: (i) effect of starvation. Sections through the nonincubated control cuts of agar revealed a bacterial population in no way different from that of the original culture, consisting of single and agglomerated acid-fast bacilli interspersed with a few blue cells uniformly scattered throughout the entire sections. In blocks of bacilli starved for 1 month there was a slight increase in the proportion of the non-acid-fast forms as compared with the picture seen in the nonincubated cut. This increase was more accentuated after 2 and 3 months of starvation, at the end of which time the bacilli, whether single or agglomerate, were either red, blue, or unstained, the latter being visible only in light dimmed down by lowering the condenser. Some of the stained rods were colored homogeneously with either fuchsin or methylene blue; in others the staining was segmented or spotty and frequently limited to one or both ends of the cells. Big, dense clumps of bacilli were acid fast, but in loosely structured clusters there was an admixture of blue bacilli. Taking the staining of the bacterial population of the control block as a standard, it can be said that, after 4 months of starvation in plain agar, roughly 60% of the bacilli remained more or less acid fast. 20% became non-acid fast, and about as many remained unstained. With the passing of time the alterations of staining became more extensive, so that after 6 months 50% of the bacilli were acid fast and the remainder were either non-acid fast or unstained. At this stage the originally predominating acid-fast big clumps consisted of red, blue, and clear areas and had a checkered appearance. On close examination these clear areas had a faintly visible fibrillar structure, which also characterized smaller aggregates which were clear in their entirety, unmistakable evidence that they were composed of unstained bacilli. Between 6 and 12 months of starvation, the proportion of stained to unstained bacilli dropped further, being about 10:90% at the end of this time. Only an occasional one of the stained bacilli was well colored with either fuchsin or methylene blue, some of the others appeared as red or blue segments of rods, whereas others still could be identified only by their polar bodies (Fig. 1). Small bunches were predominantly unstained and only in some of the larger unstained clumps could a few acid-fast bacilli be recognized. After 15 months no single stained cells were found, and after 18 months only a few clumps were seen as clear or faintly blue masses dotted with a few red spots. The last 6 months of the experiment brought further loss of the staining capacity of the bacilli, so that after 2 years of starvation only a few of the big clear clumps showed one or several short, slender pink or red rods, which were very likely segments of otherwise unstained bacilli.
(ii) Bacilli in nutrient broth (microscopic). The first signs of growth in the cultures of the control cuts and in those of bacilli that had been starved from 1 to 6 months were observed after 6 days of incubation when the medium started to become opalescent. Henceforth there was a delay in the development of the cultures which increased with the duration of starvation to which the bacilli had been submitted. Bacilli starved for 10 months started growing after 9 days in the incubator, those starved 12 months started growing after 2 weeks, and
those starved 18 months showed signs of growth after 3 weeks. After 24 months of starvation, the medium began to opacify after 4 weeks without reaching the usual turbidity even after 2 months of incubation.

(iii) Bacilli in nutrient broth (microscopic). Microscope examination of the broth in 21-day-old cultures induced by nonincubated cuts of agar revealed the usual bacterial population consisting of acid-fast bacilli interspersed with a few blue rods. Checks taken from cultures resulting from bacilli starved from 1 to 12 months showed no change in either their morphology or staining. However, from then onward there was a slight but progressive decrease in the density of the opacification of the broth accompanied by an increase in the proportion of blue cells, noticeable in cultures resulting from bacilli starved for 18 months and striking in those exposed for 2 years to starvation.

(iv) Bacilli inside agar cuts (macroscopic). The macroscope follow-up of the agar blocks themselves was as follows. Cuts incubated in nutrient broth immediately after solidification of the agar and those seeded with bacilli that had been starved from 1 to 6 months remained unchanged during the first days, but after a week they became opaque at the periphery, remaining clear in the center. During the follow-

Fig. 1. Chromophoric bacilli. On the left side, below crystalloid, group of refringent rods of normal shape and size. Note that, although bathed by methylene blue, the cells remained unaffected by that dye just as they had been by carbol fuchsin. On the right side, cluster of faintly visible bacilli with prominent polar bodies. Smear preparation of agar taken after 10 months of starvation of the bacilli, stained with carbol fuchsin, and counterstained with Loeffler methylene blue. ×1,100.

ing days the opaque marginal zone resolved progressively into a multitude of tiny, grey, ill-defined flakes, distinctly visible with the naked eye after 10 days of incubation. After 2 weeks the flakes became larger and better defined, and after 4 weeks they appeared as sharply outlined bodies, either rounded, elongated, or angular. Densely structured at the periphery of the blocks, which had been bathed by the broth, they became less numerous and less compact as their distance from the margin increased. Similar observations were made with bacilli that had been starved for 12 and 18 months, but in those instances the first flakes were observed after 2 and 3 weeks, respectively, and at the end of the incubation those seen in the latter were smaller than those in the former. Finally, in cuts with bacilli starved between 18 to 24 months, flakes appeared only after 1 month and were smaller and far less numerous than those developing from bacilli starved for shorter periods.

(v) Bacilli inside agar cuts (macroscopic). Sections through control blocks of agar incubated in nutrient broth taken during the first few days revealed the usual bacterial population consisting of single and aggregate predominantly acid-fast bacilli. After a week there was a slight increase in the number of blue bacilli close to the margin of the section, which after 2 weeks was strewn with a multitude of small colonies of blue bacilli interspersed with a few acid-fast rods. After 3 weeks the colonies had increased in size and after a month they had reached their full development. At that time the large colonies strewn throughout the periphery of the sections and the increasingly smaller ones located in their depth, as well as the flakes located in the center, also consisted of blue bacilli with a small admixture of red cells. Sections through blocks seeded with bacilli starved for 1 month and then incubated in nutrient broth showed, during the first week, no changes in the bacterial population other than those due to starvation, i.e., an increased number of blue bacilli. With the passing of time the bacilli continued to grow, and, after 2 weeks, colonies of predominantly blue bacilli developed which continued to increase in size and which, after 4 weeks in the incubator, resembled in all respects those described in the previous series. There was no significant difference in the development of bacilli starved for 1 month and those kept under starvation for 12 months, but from then onward there was a progressive decrease in the number and size of the colonies. Discernible after 18 months, these alterations
became further accentuated in sections through cuts of agar seeded with bacilli starved for 18 to 24 months. However, even the few small colonies found in sections through cuts of bacilli starved for 2 years and incubated in nutrient broth for 1 month or longer also consisted of blue cells with an occasional red rod enmeshed in them.

It is worth mentioning that the irregular shape and ill-defined jagged outline of the small colonies developing deep in the agar cuts were due to the pattern of growth of the peripheral bacilli, many of which were projecting into the surrounding agar. Some of these cells were arranged into segmented rows, each segment being the length of one average sized tubercle bacillus, which penetrated deep into the surrounding jelly (Fig. 2, inset). It is of interest that the constituent bacilli in these rows were either red or blue and that there was no definite order in their location within the rows, a red rod being in continuity with one or two blue ones and vice-versa, with either a red or a blue cell at the beginning or end of the row.

During processing, some of the colonies inserted into the agar were partly or entirely disintegrated and their bacterial content was spilled out from their original site and scattered throughout the surrounding jelly. These spills consisted of morphologically normal and perfectly stained blue bacilli and a few equally well-stained, acid-fast rods. Cocco-bacillary and coccoid forms were few, filamentous forms have never been observed, and there was no trace of clumping of the bacilli (Fig. 3).

**Bacilli starved in distilled water:** (i) *M. tuberculosis*. The most striking alteration of the tubercle bacilli starved in distilled water was the rapid decline of their acid fastness. Noticeable after 1 month, it was very extensive after 3 months of exposure, when only about \( \frac{1}{3} \) of the bacilli were more or less well stained with carbolfuchsin, an about equal number were colored with methylene blue, and the remaining ones were unstained. Most of the single acid-fast bacilli were homogeneously stained a deep or pale red, the others were segmented or granular, and the general picture of the bacterial population was reminiscent of that seen in aged cultures on solid medium, the more so as the same irregularity of staining was observed among the non-acid-fast bacilli. The chromophobic forms, visible only in dimmed light or under the phase microscope, appeared as homogeneous, translucent streaks, identifiable by their sharp outline, normal length, and characteristic curvature (Fig. 1). Small bunches of bacilli were predominantly non-acid fast, bigger clusters consisted of both acid-fast and non-acid-fast bacilli, and big dense clumps were either predominantly or entirely acid fast. Not infrequently clusters and clumps of bacilli con-

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**Fig. 2.** Section through an agar cut processed after 10 months of starvation of the bacilli and after 1 month of incubation in nutrient broth. Colonies of blue bacilli scattered throughout the marginal zone of the section. Note the great difference in number, shape, size, and structure between the marginal colonies and those developing in depth of the cut. The free space surrounding the big dark clump is due to retraction of the agar. Stained as before. \( \times 450 \). Inset: One of the small blue colonies developing in the center of the cut, with rows of bacilli protruding into the agar. Same section, same staining. \( \times 1,100 \).
sisted of red, blue, and unstained areas, having the characteristic checkered appearance described in the agar series. It is worth emphasizing that, at this early stage of starvation, the acid fastness of strain number 26 was slightly better preserved than that of the other strains. After 6 months, roughly 20% of the bacilli were more or less acid fast, whereas after 9 months only 10% retained carbolfuchsins, about as many were blue, and the remainder were unstained. After 18 months only about 20 red cells could be counted in a high-power magnification field, whereas in suspension number 26 roughly 20% of the bacilli were normally acid fast. In smears of nine suspensions of bacilli starved for 2 years, about 10 cells per high-power magnification field were counted. Strain numbers 19 and 24 showed an occasional additional bunch of short acid-fast rods and strain number 2 showed an occasional additional bunch of normal-sized bacilli, either fully or weakly acid fast. However, in films of suspension number 26 about 10% of the bacterial population was still acid fast.

(ii) **M. kansasii.** This microorganism, which appeared better stained than *M. tuberculosis* at the end of 3 months of starvation, showed general weakening of its staining after 6 months, but about 50% of the cells still appeared faintly acid fast. After 18 months, 30% of the organisms were weakly acid fast, and after 2 years 20% of the cells were red, 30% were blue, and 50% remained unstained.

(iii) **M. phlei.** The alterations of the acid fastness of *M. phlei* went parallel to those of *M. tuberculosis* except for strain number 26.

During the first 18 months of the experiment there was no parallel between the loss of acid fastness and the viability of the tubercle bacilli in all suspensions, which yielded cultures of acid-fast bacilli in the usual time when planted on 7H10 agar, and only during the final 6 months were signs of a decline in their viability observed. After 2 years of starvation, subcultures of strain numbers 1, 10, 14, 19, 24, and 26 showed a delay of 1 week in the appearance of growth on nutrient agar as compared with their original development on this same medium, whereas strain numbers 2, 5, 16, and 18 showed a delay of 2 weeks and produced less abundant cultures than the first group. In contrast to *M. tuberculosis*, *M. kansasii* and *M. phlei* showed their normal growth throughout the entire course of the experiment. In subcultures of all the suspensions under study the bacilli were similar both in morphology and staining to their original cultures, and all responded well to oxidation.

Three months of starvation did not alter the reaction to oxidation of any of the suspensions under study, but from then onwards only the weakly acid-fast and non-acid-fast cells responded to the treatment, whereas the great majority of the chromophobic ones remained unaffected.

Attempts to stain the entire bacterial population present in the smears in order to gain a general idea of the number of bacilli actually present in the films were not very successful because of the extraordinary resistance of the chromophobic forms to aniline dyes. To date, the most satisfactory results have been obtained with Gram stain after which the bacilli, all gram-negative, were faintly stained with safranin. More helpful than the Gram mixture or phase contrast was negative staining with India ink or nigrosin. Although not differential, this procedure gave valuable information about the number and morphology of the bacilli which, even after 2 years of starvation, were no different from those grown on the usual culture media.

**DISCUSSION**

The most conspicuous alteration of the starving bacilli was the progressive loss of acid fastness. This peculiarity of staining was first observed in lepra bacilli (6) and a year later also
in the newly discovered tubercle bacilli (4). Since that time great efforts have been made to determine its nature and significance, evidenced by the long list of publications on the subject, which are analyzed in classic reference books of bacteriology. At the present time this special staining affinity is generally ascribed to mycolic acid (1). However, this opinion has been questioned on the grounds that mycolic acid is too weakly acid fast to account for the brilliant red staining of the bacilli (11), and that it is an oversimplification to ascribe their acid fastness to any single substance developed by the mycobacterial cells (5).

The loss of acid fastness became noticeable very soon after the beginning of starvation, when an increasing number of bacilli turned non-acid fast and stained blue, and then lost all their staining affinities and became chromophobic, a state reached by the majority of them after about 6 months of exposure. From then onwards the transformation progressed at an increasingly slower pace, so that even after 2 years of exposure a few bacilli still exhibited some degree of acid fastness. This sequence of events, in addition to showing the slow, progressive changes in the staining affinities of M. tuberculosis, also brings the demonstration that the non-acid-fast or blue bacilli are not artifacts or degenerating cells as has been suggested by some authors (12), but are intermediate stages between the acid-fast and the chromophobic bacilli.

A key to the understanding of the chemistry of these alterations is provided by the demonstration that weakly acid-fast and even chromophobic mycobacteria can be made fully acid fast by oxidation (8), and that acid-fast bacilli can be made chromophobic by reduction and have their acid-fastness restored by oxidation (9). These experiments provide evidence that oxygen plays a decisive role in the resistance of mycobacteria to acid destaining, and that acid-fastness hinges on the oxygen potential of the cells. It can be postulated, therefore, that the waning of the acid fastness of starving bacilli is caused by a progressive inhibition of their metabolic activity which depresses and eventually arrests the oxidative processes accompanying it, resulting in depletion of their oxygen potential.

The oxidation-reduction procedure (9) also provides information about the significance of acid fastness. Since the oxygen involved in acid fastness can be removed from the bacilli by reduction and restored to them by oxidation without any noticeable damage to the cells, it is not likely to be chemically bound with their substance but rather loosely connected with it. It seems, therefore, not to be directly concerned with the growth of the bacilli but rather with their respiration. This hypothesis finds support in the observations of earlier writers on the respiration of tubercle bacilli, which led them to believe that the consumption of oxygen and the synthetic processes involved in the growth of the microbes depend on different metabolic systems (5).

There were significant differences in the resistance of the acid fastness of the various strains of mycobacteria to starvation, and there was no parallel between the loss of acid fastness of starved bacilli and their viability. Tubercle bacilli starved in agar were but slightly diminished in their viability at a time when only 10% of them were acid fast, whereas those kept in distilled water yielded normal cultures after 18 months, when only 20 red cells were counted per microscope field in the inoculum, and viable bacilli have been demonstrated after 2 years of starvation in all the suspensions of bacilli under study. In the distilled water series, nine strains of tubercle bacilli and M. phlei lost their acid fastness more readily than strain number 26 of M. tuberculosis, which showed 10% of acid-fast bacilli after 2 years but initiated growth of subcultures at about the same time as the other strains of M. tuberculosis. M. kansasii, on the other hand, with 20% of acid-fast bacilli at the end of the experiment, and M. phlei, with only a few red cells left, both yielded subcultures as originally. This shows that there were great biochemical and metabolic differences not only between the various species of mycobacteria under study, but also between individual cells of the same suspension, some becoming chromophobic during the first months of starvation and others showing some degree of acid fastness even after 2 years of exposure. Similar differences are regularly observed not only in aged cultures of tubercle bacilli, as has previously been mentioned, but also in routinely stained films of regular cultures, which always show variable proportions of acid-fast, non-acid-fast, and chromophobic bacilli, thus reflecting the metabolic state of the cells at the time of processing.

In summary, the acid fastness of mycobacteria seems to be due to the oxygen potential of the bacterial cells, which is the result of the active oxidative processes accompanying their metabolism and varies with their metabolic activity. It is primarily related to the respiration of the cells, which is closely connected with
their general metabolism. Actively metabolizing mycobacteria with uninhibited respiration are fully acid fast, less active cells are weakly acid fast or non-acid fast, and those with arrested metabolism are chromophobic. The opinion is warranted, therefore, that acid-fast staining, currently used for coloring mycobacteria, is also a test indicative of the metabolic state of the cells.

The most important result of the starvation experiments is the demonstration that metabolically inactivated tubercle bacilli lose their staining capacity but remain alive for a long period of time and recover their original biological properties when transferred into a favorable environment. Their remarkable resistance to adverse conditions and their reversibility are of fundamental importance for the bacteriology, pathology, and treatment of tuberculosis. In bacteriology, they show that M. tuberculosis is a two-phase microorganism which, depending on its environment, can appear either in its metabolically active acid-fast form or in its inactive chromophobic form. This biological characteristic is reflected in the pathogenesis of tuberculosis. During the acute, exudative phase of the infection, which provides a favorable environment for their development, the bacilli are acid fast, but in the caseous lesions, a notoriously poor culture medium characterizing the chronic phase of the disease, they become predominantly chromobiotic in both treated and untreated lungs (2). The indifference to drugs of the chromobiotic bacillus has been proven by their demonstration in all the lungs of treated patients examined, no matter what drugs or drug regimens were used, whatever the duration of the treatment or whether the bacilli were originally drug sensitive or drug resistant (10). In the chemotherapy of tuberculosis the biphasic nature of M. tuberculosis is reflected in the well-known favorable influence of the drugs in fresh exudative lesions, where they interfere with the metabolism of the acid-fast bacilli, and their limited success in chronic productive tuberculosis in which the metabolically inactive chromobiotic forms predominate (3). Finally, the reversibility of the bacilli comes into play when, in suitable conditions, the chromobiotic forms can recover their original properties and cause relapse of the disease.

It is generally believed that only the acid-fast bacilli, the vegetative form of M. tuberculosis, can reproduce on artificial media. This opinion is refuted by the development of bacilli starved in plain agar after transfer of the cuts into nutrient broth. Soon after immersion the chromophobic bacilli enclosed in the agar began to grow, but instead of yielding acid-fast bacilli they developed predominantly blue forms. The main stages of this development are illustrated by Fig. 1 to 3 taken from color films which, unfortunately, had to be reproduced in black and white. The agar technique makes it possible to follow the development of the colonies step by step, showing such features as the multiplication of the bacilli by transverse fission and the development and composition of the blue colonies. The frequently observed disruption of blue colonies by processing and the dispersion of their bacterial content indicate that blue bacilli growing in the conditions described do not develop any cord substance, which is one of the features differentiating them from colonies of acid-fast bacilli.

The in vitro development of blue bacilli is a demonstration of the decisive role of nutrition not only in the growth of tubercle bacilli but also in the development of their acid fastness. When a block of agar seeded with bacilli made chromophobic by starvation is transferred into nutrient broth, two different cultures develop concurrently in the same tube. One is that induced by bacilli spilled off by the broth from the surface of the cut and the other by those developing inside the jelly. Both originate from chromobiotic bacilli but, whereas the former are in direct contact with the medium and are provided with all the nutrients they need for both growing and developing their special staining affinities, and become acid fast, the latter are separated from it by the agar enveloping them and are either reached by the broth in an insufficient amount or receive it altered in its composition through loss of some ingredients and, therefore, grow predominantly non-acid fast. That this difference is due to nutrition and not to the direct action of the agar or to lack of contact with the air is demonstrated by as yet unpublished studies on the growth of bacilli incorporated into solid nutrient media, which develop small colonies of acid-fast bacilli inside the jelly. These observations show the extreme selectivity of the growth requirements of the bacilli. They also indicate that, on the standard media now in use, mycobacteria do not grow acid fast because they are inherently so, but because they find there the nutrients they need for both their growth and the development of acid fastness. It can be assumed, therefore, that in adequately adjusted media it might be possible to grow M. tuberculosis in vitro not only in its acid-fast form but also in its non-acid-fast and even chromophobic forms.
Various morphologically abnormal forms of microbes such as the L-forms and others have been described during the past decades but, since their meaning has not been determined, it is difficult to tell whether the chromophobic tubercle bacilli belong to that group. It is certain, however, that striking similarities exist between these and the abnormal forms which are found in the late or subclinical stage of infectious diseases such as syphilis, brucellosis, and typhus, which the present writer has also had the opportunity of studying. For instance, in a plaque of a syphilitic aorta, forms of spirochaetes were found which were so different from the classic shape of Treponema pallidum that their true nature could only be determined thanks to the presence of a few recognizable parasites scattered among them. Their ability to maintain a subclinical infection for many years and their indifference to drugs suffice to show the importance of these abnormal forms which, to underline their development by adaptation to an increasingly adverse environment, could be called "adaptation forms," or "a-forms" for short. Since the transformation of the original microorganisms into their a-forms is due to inhibition of their metabolism, as a result of which they lose some of their non-essential features and become indifferent to drugs, microbes should be investigated and tested not only in their actively metabolizing state but, to determine all their potentialities, also when their metabolic activity is at its lowest. Such studies are particularly indicated at the present time when the increasing resistance of the microbes to drugs threatens to develop into a serious challenge to the chemotherapy of infectious diseases.

LITERATURE CITED