# BIOLOGICAL STUDIES OF THE TUBERCLE BACILLUS

## I. Instability of the Organism—Microbic Dissociation

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Plates 18 to 26

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It has become increasingly evident during recent years, that bacteria along with other living forms are subject to modification, embraced under the term of "mutation." The recognition of types of "mutation" is now general among systematic bacteriologists, as is the employment of such terms as "smooth" and "rough" to define differences in colony formation appearing in the "mutating" bacterial species. The literature on this subject has been assembled in papers and monographs and is now readily accessible so that it will not be reviewed in this paper. We refer those seeking the literature to the article by Hadley (1), but it is desirable to direct attention especially to the pioneer work in this field of Lehmann and Neumann (2), Neisser (3), Massini (4), Baerthlein (5), Arkwright (6) and DeKruif (7).

Just as the observations on bacteria in general have shown bacteria to undergo "mutation," so have the studies on tubercle bacilli, in tissue and cultures, shown them at times to be pleomorphic in structure. It is not uncommon to see in preparations "coccoid" granules, rods, beaded and branching rods. Reviews of the literature on this subject may be found in the articles on the tubercle bacillus by Kolle, Schlossberger and Pfannenstiel (8), Kahn (9) and others.

### *Tubercle Bacillus Cultures*

Our purpose in this paper is to deal with the gross cultural characteristics of the tubercle bacillus, with special reference to the topography of colony formation and the virulence according to colony type. No mention will be made of the pleomorphism which may or may not play a part in the dissociation phenomena.

When a pathogenic acid-fast organism is cultivated on favorable media, generally the growth appears white at first. As time goes on, it becomes a cream color at the center of the crowded area and dark orange color as it becomes older. The growth may be wrinkled and

heaped in masses; or show large folds having a smooth surface; or it may be spreading and smooth. Not all cultures develop with the same rapidity; some develop profusely in a few weeks, while for others a much longer time passes before growth becomes visible.

Variations in virulence have been noted by many workers. For the last twenty years we have used the well known Saranac Laboratory human tubercle bacillus culture $H_{37}$ for studies in infection and immunity. Up to a few years ago the virulence of this organism was very constant and apparently a small number of organisms when inoculated into guinea pigs, was sufficient to produce progressive disease. Within the last three or four years a diminution of virulence has been observed such that in some cases we failed completely to infect animals even with a heavy suspension. From time to time we have noticed a variation in antigenic properties of this organism, with special reference to the complement-fixation reaction. Some antigens were anti-complementary and low in fixing properties, while antigens prepared from a different lot of $H_{37}$ cultures were very potent.

When the tubercle bacillus $H_{37}$ is cultivated on liquid media, *i.e.*, veal, synthetic or potato broth, two types of growth may be seen; veil-like filament and small wrinkled heaps. The veil-like growth is confluent, spreads in very thin layers and in time covers the surface of the medium, gradually pushing its way up the side-walls of the flask. The growth coheres and it is very difficult to remove a small portion without disturbing the whole mass. Chromogenicity is generally absent. The second growth appears in small isolated islands connected by a veil-like growth. After some time it becomes very wrinkled and dry. At first it is white, and as it ages, creamy, and finally it assumes an orange color. The production of pigment is much greater in the center of the mass than at the periphery. The growth is fragile and brittle and any portion can be lifted without disturbing the adjoining one. Cultures of the first sort can be triturated to an evenly diffused suspension with no difficulty, while those of the second sort are much more difficult to put in a fine suspension.

If we start with a colony having distinct cultural characteristics, there will probably be a number of variants from the original mother colony upon subsequent cultivation. Two, perhaps three, or more variants may develop from the original colony and these may differ

from each other not only in cultural structure, but also in tinctorial characteristics, virulence and other characteristics.

In the following pages we shall describe in detail the methods used in dissociating various acid-fast organisms. Observations are recorded just as we have seen the various colonies. No attempt will be made to interpret the various stages of their development. For the earlier observations the reader is referred to previous studies by the authors (10).

### *Culture Media for Dissociating the Tubercle Bacillus*

*Gentian-violet-glycerol-egg medium* with a slight modification was used throughout this investigation. The gentian-violet dye exerts some bacteriostatic action on the tubercle bacillus, a loss which is more than compensated for by the usefulness of the dye. The dye has two functions: (a) its dark color makes a satisfactory background, on which white colonies stand out prominently for observation and photography, and (b) it to some extent prevents external contamination. Even with all precautions contamination, especially by the molds, cannot be entirely prevented.

*The Modified Medium.—Meat juice:* 500 gm. of chopped lean veal or beef are infused in 500 cc. of 15 per cent solution of glycerine in water in a refrigerator. After 24 hours the meat is squeezed through cheese cloth and the juice is collected in a sterile beaker. *Eggs:* The shells are washed well and dried, and left in 70 per cent alcohol for ½ hour. They are removed from the alcohol and placed on a sterile towel to dry. They are then broken, emptied into a sterile beaker and mixed well with a sterile glass rod. One part of the meat juice is added to two parts of the eggs by volume. *Gentian-violet:* A 1 per cent alcoholic gentian-violet solution is added to make a final dilution 1 to 30,000 (1 cc. of dye to 300 cc. of the medium). The mixture is well stirred and filtered through sterile gauze to remove coarser particles. 35 cc. of the medium are put in Petri dishes (100 x 15 mm.), and inspissated the first day at 85°C. until completely coagulated, removed from the inspissator and put in the incubator for a gradual cooling (face down). On the second day the dishes are again put in the inspissator at 85°C. for a second inspissation for 1½ hours, after which they are again put in the incubator at 37.5°C. for 3 days to test their sterility. While still in the incubator, rubber bands are slipped around the edge of the plates and they are then removed to room temperature. This step in the preparation of the medium is very important for the following reason: At 37.5°C. the air in the Petri dishes expands and in part escapes with result that when the dish is removed to room temperature room air enters it carrying contaminating bacteria. This difficulty can be eliminated by the use of rubber bands. All plates must be incubated face down to prevent water condensation on the upper lid of the Petri dishes. We cannot emphasize too strongly the importance of this step.

*Preparation of the Rubber Bands* (*See Plate 18A*).—Automobile inner tubes (30 x 3½ inches), preferably new, are cut into bands 1¼ inches wide. These can be shaped into proper form by using wood discs 15 cm. in diameter and 1.5 cm. thick. The bands are stretched over the disc so that one-third will cover the sides of the disc and the remainder will cover the bottom. The discs must have a central hole of ⅜ inch. Twenty-five to thirty such discs over which the rubber bands have been stretched can be threaded on a rod through the hole, a nut put on each end and tightened, autoclaved for 1 hour at 15 pounds pressure, and cooled. The bands when removed from the discs are found to have shaped themselves so that they fit satisfactorily over the Petri dishes.

*Preparation of the Suspension for Plating* (*See Plate 18B*).—A small portion of a recent 12 to 14 day growth, preferably from fluid medium, is removed aseptically with a sterile platinum wire spade and triturated with the same on the inside wall of a sterile Wassermann test tube containing 3 to 4 cc. of sterile saline of pH 7.8. The trituration is done just above the fluid level. From time to time the platinum wire is dipped in the saline to carry more moisture for trituration. After complete trituration the tube is inclined and the triturated material washed into the saline. The suspension is filtered twice through two layers of sterile Whatman No. 5 filter paper. The filtrate is then diluted five to ten times for the human tubercle bacillus, while the bovine and avian require ten to twenty times. The filtrate is inoculated on the surface of five or ten plates made as described above, usually four to six drops from a capillary pipette being used for inoculation. The rubber bands are fitted around the edges of the Petri dishes. Adhesive tape may be used for labels. Rubber bands prevent desiccation of the medium as well as contamination. When a plate is taken out of the incubator, the rubber band should never be removed until it has completely cooled.

*Funnels for filtration* (see Plate 18B) may be made from discarded test tubes, 18 to 20 mm. in diameter. The upper portion of the funnel can be made 1½ to 2 inches long, pulled out in the flame to about 3 to 4 mm. Two layers of Whatman paper No. 5 are inserted in the funnels, suitably folded to make a cup form. For consecutive studies a number of these funnels containing the filter paper can be prepared, placed in a jar, sterilized and used as wanted.

Growth may not appear until the third week. The best time to study the colonies is after 6 to 8 weeks when they have partially matured. For the study of the finer structures of the colonies we have used a Bausch and Lomb low power binocular microscope with drum type objectives. After a plate has been removed from the incubator for study the lid of the plate may become foggy and interfere with vision. This can be remedied by warming the lid of the plate for a few seconds over a Bunsen burner.

## *Dissociation of the Bovine Tubercle Bacillus $B_1$*

The bovine culture which we have successfully dissociated is known as $B_1$. It was isolated by Dr. E. R. Baldwin in 1904. The organism has been cultivated on

glycerine potato, glycerine egg and veal broth. The virulence has been constant for rabbits and guinea pigs until a few years ago since when it has occasionally failed to produce progressive disease in rabbits.

A suspension of 2 weeks' growth was prepared by the method described above and inoculated on the surface of gentian-violet-egg plates. After 4 weeks, isolated colonies appeared on the plates. One of the colonies (a) was perfectly round, raised, opaque, with a smooth surface resembling a moth-ball. It was easily removed from the surface. The second colony (b) was large, spreading, raised at the center, dry and wrinkled. The (a) colony was readily emulsified, while the (b) colony was suspended with difficulty (10).

The separate types of isolated colonies were emulsified, the suspensions filtered and new plates prepared. After due time, colony (a) instead of being like a moth-ball, was flat, spreading and appeared very much like a star. In the middle of each colony there was a small nipple-like growth with a smooth surface, which was surrounded by a darker pigment forming a circle and from this central zone the growth radiated in the form of large folds toward the periphery in a rosette form. The periphery of the colony was very irregular and raised. The colony (b) had a different topography, the central zone being raised with numerous irregular folds and at places appearing like a honeycomb. The periphery was flat and veil-like with perfectly smooth edges spreading over the medium. The colony (a) of the second subculture was difficult to emulsify, while (b) could be prepared in suspension very readily. The different physical properties relating to emulsification probably are due to the lipin content or they may have a different isoelectric point.

The two principal colonies which we have just described were difficult to separate at times and their characteristics were sometimes very confusing. Very often colonies appeared on the plates differing from the two colonies already described. It must be kept in mind that there may be some intermediate colonies which develop from time to time and play some part in the life cycle of the organism.

From the preliminary observations reported here we were convinced that dissociation of the tubercle bacillus does take place and that our methods up to this time were inadequate to make a further study until we could find a medium which would stabilize the development of the organism and retard the dissociation. In other words it was required for further studies that the colonies should be of uniform topography and readily separated.

Recently we have modified the medium with the object of obtaining uniform colony structure. The base of the medium was gentian-violet-egg to which sodium glycocholate and sodium taurocholate were added. Sodium taurocholate in the proportion of 0.25 per cent was found to be most suitable.

When the two colonies dissociated from the bovine tubercle bacillus

were cultivated on this medium, they presented characteristics which could be differentiated without any difficulty. One of the colonies was perfectly round, moist, like a moth-ball, opaque, with smooth surface, easily emulsified and on subsequent subcultures on plates developing a round and flat colony. The other colony was large, flat opaque, changing with age to cream yellow, moist and with smooth surface. It emulsified with difficulty and on subsequent subculturing developed flat colonies (see Plate 19, 1, 2, 34).

The former we shall now call "S" and the latter "R." Organisms of the "S" colony when inoculated into guinea pigs, regardless of the route used, produced generalized tuberculosis in a short time, the disease spreading rapidly throughout the viscera. A few drops of a suspension of 5,000,000 organisms of this colony when introduced into the conjunctiva, without injury, produced conjunctivitis and vitreous opacity with photophobia within 4 weeks. The cervical lymph nodes could be palpated and in many instances the inguinal lymph nodes could be palpated after a short time. The animals reacted to tuberculin within 12 to 15 days and the intensity of the reaction was approximately 15 x 15 mm. with a central necrotic area. Animals inoculated intracutaneously, intraperitoneally, intracardially and intratesticularly developed generalized tuberculosis and death in 3 to 6 weeks. This colony also produced rapid, progressive tuberculosis in rabbits after inoculation with a small number of organisms. The disease was very extensive and tubercle bacilli could be found in the bone-marrow 2 months after the inoculation.

When a suspension of the "R" colony was used in a strength similar to that of the "S" colony, it was found that the disease did not progress but remained localized. When a few drops of a suspension of 5,000,000 bacilli of "R" colony were instilled into the conjunctiva of a normal guinea pig, the result was wholly unlike that observed with the "S" colony. The animal failed to react to tuberculin up to the fourth week and no lesions were observed in the eye. In some animals which were killed for comparison, the only ones found were healed tubercles in the posterior auricular lymph nodes. Intratesticular, intracardiac and intracutaneous inoculations produced lesions which in the majority of instances healed. Lesions were observed in rabbits inoculated by various methods, but they were not like those observed with the "S"

colony. No tubercles were found in the lungs, spleen or liver, up to 8 weeks.

The foregoing observations on guinea pigs and rabbits which were inoculated with the two colonies, were of brief duration (4 months). The results might have been different if we had allowed the animals inoculated with the "R" colony to live longer. We consider this of great importance and believe that some of our animals inoculated with the "R" colony after 12 to 18 months or more might have died of tuberculosis because of a reversion of the bacilli in the animal to the "S" type. This will be referred to in a later paper.

### *Dissociation of the Avian Tubercle Bacillus $A_1$*

The culture of avian tubercle bacillus studied was $A_1$, an organism which has been in our possession for many years. It is slightly pathogenic for chickens and only occasionally produces progressive disease in rabbits.

A suspension from a 2 weeks' growth of a plain egg culture was prepared by the usual method and filtered. The filtrate was picked up with a sterile capillary pipette and six drops seeded on the surface of gentian-violet-egg plates. The ordinary gentian-violet-egg medium may be used satisfactorily in dissociating the avian tubercle bacillus, sodium taurocholate not being necessary for it. After several weeks, a number of single colonies appeared on the plate, the majority of which were flat, slightly opaque, raised in the center, and the periphery appeared translucent. Upon careful examination with a low power lens, a small number of somewhat darker, opaque, round colonies could be seen scattered over the surface.

This second colony is like a moth-ball, moist, glistening and easily emulsified in an even suspension, growing profusely in alkaline synthetic medium but without any diffusion of pigment into the fluid. The first colony is flat, spreading and very similar to the "R" colony of the bovine culture. It grows more profusely in synthetic medium with an acid reaction and produces a slight amount of pigment (see Plate 20, 3, 4, 5 and 6).

The two colonies on gentian-violet-egg medium revealed some very striking changes when kept in the incubator for a period of 8 months. The "S" colony first developed into a moth-ball and after several months spread and assumed a flat appearance, remaining flat for another 3 months. After 7 months from the time of seeding from the mother colonies secondary daughter colonies appeared in the form of papillae. They have, at times, a worm-like appearance sprouting in all directions, the growth being sprinkled with a few moth-ball papillae. The periphery of the mother colony is moist, perfectly smooth and cream-like in color. Subcul-

tures from the periphery developed mostly colonies with typical "R" characteristics, while the worm-like growth or the moth-ball papillae developed some "S" colonies (see Plate 20, 1 and 2).

The "S" organism is highly pathogenic for chickens, $\frac{1}{3}$ mg. inoculated intravenously producing septicemia and death in 30 days. The "R" colony is not very virulent, and will not produce the same type of disease.

### *Dissociation of the Human Tubercle Bacillus*

An attempt was made to dissociate a human type of tubercle bacillus known as $H_{37}$. In the preceding pages we have mentioned that this organism was formerly highly pathogenic for guinea pigs, but that in the last 3 or 4 years in some instances it has failed to produce progressive disease. Such fluctuations of virulence appear to be connected with the media used.

It was noted that the diminution of virulence occurred when the organism was cultivated on veal broth with an acid reaction. On the other hand, if cultivated on Proskauer and Beck synthetic medium which is slightly buffered and has an alkaline reaction, no loss of virulence was observed. Ten bacilli from such a culture very often produced tuberculosis in guinea pigs, leading to death in from 100 to 150 days. It was very difficult to dissociate $H_{37}$ by the method used in dissociating the other strains. The difficulty, in all probability, could be explained by the facts (1) that we used a suspension prepared from Proskauer and Beck medium cultures, in which virulent "S" organisms predominate, and (2) that the colonies which appeared on the gentian-violet-egg plates from a filtered suspension, differed decidedly from those seen in bovine, BCG or avian cultures. Each variant developed colonies of different topography, and there was no fixed characteristic which could help us to separate the colonies. They were unstable, and the gentian-violet-taurocholate medium was of no value. Another method had to be devised. Remembering that this organism lost its virulence when cultivated on glycerine-veal broth of acid reaction, we decided to cultivate the organism on Sauton fluid medium buffered at pH 6.6. After a number of cultural passages through this medium, followed by cultivation on gentian-violet-egg plates, we have dissociated two or perhaps three colonies which can be seen in Plate 21, 1, 32, 2, 3, 26 and 4. The supposed "R" colony is very waxy, much raised, with large folds. The periphery is sharply outlined, and emulsifies with difficulty in salt solution. It produces a chromogenic soluble substance in acid synthetic media. When cultivated on solid media, it at first appears creamy-yellow, a characteristic noted of "R" colonies of other bacteria, but as it ages it becomes yellow. The "S" colony is flat, spreading, composed of small wrinkles, appearing at times like ground glass. This colony is

easily emulsified in salt solution of pH 7.2 and grows best on synthetic media of pH 7.6.

The above is the description of the two main colonies. Recently, we have observed other variants which will be described in a subsequent study.

### *Dissociation of Bacillus Calmette-Guerin (BCG)*

The result of dissociation of BCG was published in a recent article (10). At that time the two extreme "R" and "S" colonies were illustrated and fully described.

It has been noted by the workers engaged in the study of this organism that cultures of BCG distributed by the Pasteur Institute several years ago were more virulent for guinea pigs than the recent ones. In a previous study (10) we offered an explanation for the loss of virulence after prolonged cultivation on glycerine-potato-bile medium. We pointed out that only the "R" strain develops readily on that medium and that "S" does not grow well and no visible colonies can be seen in cultures. Therefore, by the process of gradual elimination, the undissociated cultures at present harbor only a very small number of "S" organisms.

Culture BCG 359 (which Petroff obtained in the fall of 1928 from the Pasteur Institute) was more difficult to dissociate than the three cultures which had previously been obtained. Out of some thirty-five plates made from the same suspension, only one plate showed two distinct and one questionable "S" colonies. For this reason we advocate the use of a large number of plates (30 to 50) in attempting to dissociate BCG.

The four following methods have proved successful in our hands in the dissociation of BCG:

1. The original BCG culture was cultivated on Proskauer and Beck's synthetic medium with a reaction of pH 7.4 to 7.6 and subcultures were made every 2 weeks. After six to eight subcultures many "S" colonies could be isolated by the plating method.

2. Rabbits were inoculated intravenously or intraperitoneally four times at intervals of 3 to 4 days with a 5 mg. suspension of undissociated BCG killed in the water bath at 100°C. for ½ hour. 4 weeks from the beginning of the inoculation the rabbits were bled aseptically and the clear serum was added to Proskauer and

Beck's medium in the proportion of 10 per cent. After a preliminary incubation to test sterility, the surface of the flasks was seeded with the original BCG culture and subinoculation made on similar medium every 2 weeks. If a transplant is made from an eight to ten passage on gentian-violet plates, the "S" colony can be separated. At the same time the whole culture acquires virulence for guinea pigs.

3. The virulence of the original culture has been increased, with the "S" colony predominating, by cultivation on synthetic medium containing 10 per cent normal rabbit serum. However, the transformation is much slower in our experience.

4. The "S" colony can be dissociated from abscesses formed in guinea pigs. Guinea pigs were inoculated with 10 mg. of the original BCG culture. 6 to 8 weeks later the same amount was again inoculated subcutaneously. Several weeks after the second inoculation, plate culture prepared from the abscesses revealed a number of "S" colonies.

Dissociation has been accomplished successfully in our hands by any of the four preceding methods. Workers following them will find that single colonies will appear on the plates after from 40 to 60 days. On careful examination, several different colonies with distinct cultural characteristics will be found present in the plates.

We shall describe at first the two widely different colonies which we have studied more extensively than any others.

The "R" colony (see Plate 22, 39) appears waxy, with slightly raised center, sloping gradually towards the periphery. Sometimes the colonies appear in rosette form, but the general characteristic is that of a coil of small intestines. The periphery is clear cut, raised and does not extend into the medium. This colony is at first slightly chromogenic, becomes with age a dark orange, and is very difficult to emulsify in salt solution of pH 7.4. On Sauton's synthetic medium it grows very profusely in small islands. A mass of growth can be lifted very easily without disturbing the surrounding colonies. The organism grows well at pH 6.5 to 6.8. It will grow for the first and second subculturing on alkaline broth for the probable reason that a small amount of the medium is transferred from the original culture; but after the third subculturing the colonies cease to develop.

This colony grows very readily on Calmette glycerol-potato-bile medium. It appears first in small round pebble-like colonies, the surfaces of wh ch are perfectly smooth. They gradually increase in size, occasionally reaching 1 to 2 mm. in diameter. The surface of this colony remains perfectly smooth for the first month. 4 to 6 weeks later a small granular papilla appears at the end, gradually covering the whole surface with secondary growth. At the end of the third month several shoots of coarser papillae develop. Eventually the original colony is covered with this secondary growth, entirely changing its appearance.

The "S" colony is composed of minute irregular wrinkles and the whole structure is much more delicate than the "R" colony. It appears very much like honey-

comb. The periphery is veil-like and extends into the medium, will not grow on glycerol-potato-bile, grows best on Proskauer and Beck medium pH 7.8, is easily emulsified and is more sensitive to gentian-violet-egg medium (see Plate 22, 40).

The pathogenicity of these two colonies has been described in our recent paper (10) and to avoid repetition we have omitted the experimental data on animals.

### *Reversibility of "R" to "S" Virulent Organism*

Recently, we (10) have described in detail the method used in changing the "R" into "S" colonies. Since that time we have repeated the experiments sufficiently often to say here that when the "R" colony is cultivated on media containing anti-"R" serum after the 8th to 10th cultural passages, the offspring of the original colony becomes "S."


## SUMMARY

The recent advances in the study of the other bacteria with application to the dissociation phenomenon, have been applied in the study of acid-fast organisms. For some time, we have realized that the term "dissociation" as employed at present, is not adequate to explain the instability and subsequent variation which occur in cultures. But for uniformity of bacteriological nomenclature, we have adopted the term until a better one is coined. In describing the "R" and "S" colonies, we have had to depart somewhat from the general usage of these terms, that is the "R" meaning rough, and "S" smooth. The colonies of acid-fast organisms are relatively varied and complex. It seems better to employ the letter "R" to indicate greater resistance to environment and relative avirulence; and "S" to indicate colonies which are more sensitive to environment while possessing for certain species relatively great virulence. The terms "rough" and "smooth" apply directly only to avian tubercle bacillus, when cultivated on plain gentian-violet-egg medium. The avirulent colony isolated from this culture is flat and somewhat rough in appearance. The virulent is perfectly smooth, round and resembling a moth-ball. The physical properties are different. They have been fully described elsewhere.

When the bovine "R" and "S" are cultivated on plain gentian-violet-egg medium, differentiation is very difficult. At times they are

almost indistinguishable, but the addition of 0.25 per cent sodium taurocholate to the medium, alters completely the topography of the colony. The "S" appears in perfectly round smooth moth-balls, and the "R" in larger, spreading and somewhat rough colonies.

Lacking suitable media, the human tubercle bacillus $H_{37}$ has been more difficult to dissociate. After 2 years' study, using various media, we have been able to dissociate two types of colonies; but as the animal experiments are not yet completed, very little more than that can be said at present.

We have dissociated two extreme types of colonies from four BCG cultures obtained from various sources. Each of these four cultures has revealed the same types of colonies. For details the reader is referred to a recent paper (10). In this publication we have included photographs taken from time to time in order to keep a record of our observations. When studying the photographs, the reader will notice considerable variation in some of the colonies. Unquestionably, there are more than two types of colonies developing during the life cycle of the organism, but at present we have considered and confined ourselves to only the two extreme types, one which can produce progressive disease, leading to the death of the animal, and the other which is but slightly virulent, and sometimes not at all so for susceptible animals.

Full details of the technique employed by us have been described in the test. Anyone attempting to duplicate the work must strictly adhere to the technique described. *Departures from it may lead to failure.*

The underlying factors favoring dissociation are not yet clearly understood. We believe that every single bacillus contains the two components, "R" and "S." If the environment is favorable for the development of the "R" component, the offspring will be "R's," although the original organism may be "S." Conversely, if the environment is favorable for the "S" and not for the "R" component the "S" will develop. For example, if an avirulent "R" colony obtained from the avian bacilli is cultivated on egg medium, which is favorable for the organism, the offspring after a suitable length of time will develop "S" colonies.

## REFERENCES


1. Hadley, P., *J. Infect. Dis.*, 1927, **40**, 1.
2. Lehmann, K. B., and Neumann, R., Atlas und Grundriss der Bakteriologie, 1896.
3. Neisser, M., *Centralbl. f. Bakteriol., 1. Abt., Ref.*, 1906, **38**, suppl., 98.
4. Massini, R., *Arch. f. Hyg.*, 1907, **61**, 250.
5. Baerthlein, K., *Arb. a. d. Kaiserl. Gsndhtsamte*, 1912, **40**, 433.
6. Arkwright, J. A., *J. Path. and Bact.*, 1921, **24**, 36.
7. DeKruif, P., *Proc. Soc. Exp. Biol. and Med.*, 1921, **19**, 34; *J. Exp. Med.*, 1921, **33**, 773.
8. Kolle, W., Schlossberger, H., and Pfannenstiel, W., *Deutsche. Med. Wchnschr.*, 1921, **47**, 437.
9. Kahn, M., *Amer. Rev. of Tuberc.*, 1929, **20**, 150–198.
10. Petroff, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1927, **24**, 633, 956, 958; Petroff, S. A., Branch. A., and Steenken, Wm., Jr., *Ibid.*, 1927, **25**, 14; *Am. Rev. Tuberc.*, 1929, **19**, 9.


## EXPLANATION OF PLATES

### PLATE 18

A, process used in preparation of the rubber bands. a, properly cut rubber band 1½ inches wide. c, front view of rubber band stretched over the wooden disc. d, same, back view. e, number of such discs with stretched bands are strung on iron rod, tightened and autoclaved for 1 hour at 121.3°C. b, properly shaped rubber band.

B, process used in the preparation of suspension of organism for seeding. a, funnels. b, the organism is triturated just above the liquid level. c, Petri dish with the rubber band properly applied.

For details, the reader is referred to the text.

### PLATE 19

Colonies of bovine tubercle bacilli $B_1$. 1, 2, 34 and 38 are on 0.25 per cent taurocholate gentian-violet-egg medium plates, 37 and 3, without taurocholate, ⅛ normal size.

1, the moth balls are "S," the flat are "R" colonies, age 150 days. 38, "S" colony, age 298 days, secondary variants have developed. 34, typical "R" colony, age 68 days. 2, typical "S" colony, age 68 days. 37 and 3, are "R" and "S" colonies cultivated on medium from which the taurocholate has been omitted; differentiation is very difficult.

### PLATE 20

Colonies of avian tubercle bacilli $A_1$, on gentian-violet-egg medium plates; 1, 3, 4, ⅛ normal size; 2, 5, 6, magnification 7 ×.

1, "S" colony, secondary growth and papillae, age 286 days. 2, same as (1), magnified 7 ×. 3, typical "S" colony, a subculture from the papillae, 1-a, age 40

days. 4, typical "R" colony, a subculture from the periphery of 1-b, age 40 days. 5, higher magnification of (3). 6, higher magnification of (4).

PLATE 21

Colonies of human tubercle bacilli $H_{37}$ on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

1, undissociated mostly "S," few typical "R" colony structure has developed after 3 months, age 168 days. 32, ? "R" colony with autolyzed center, age 86 days. 2 and 26, "R" colonies, age, former 168, latter 100 days. 3, "S" colony which developed "R" in the center periphery is "S," age 86 days. 4, typical "S" colony, age 86 days.

PLATE 22

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

39, Strain 3 "R" colony, age 67 days. 40, Strain 3 "S" colony, age 86 days. 1, Strain "Park," secondary veil-like growth (a) can be seen shooting under the mother colony. Subcultures from such growth developed "S" like structure, age 242 days. 4, Strain 3 undissociated. The structure is similar to (1), the secondary growth can be seen in (b), age 210 days. 3, Strain "Park," probably intermediate colony, age 242 days. 14, Strain 3 undissociated. This is very similar to (3), age 210 days.

The original cultures, dissociated and illustrated in Plates 22, 23, 24, and 25 were obtained from the following sources. No. 3, Pasteur Institute strain obtained in the spring of 1927. No. 359, Pasteur Institute strain, given in person to one of the authors in the fall of 1928. No. "Park" strain, a culture sent to Dr. Baldwin by Dr. W. H. Park, New York City.

PLATE 23

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

20, subculture from undissociated Strain 3 organisms which has been cultivated through 7 passages on synthetic medium containing 10 per cent normal sera. Majority of the colonies are "S." Few of them have developed in the center an "R" structure, age 283 days. 21, Strain 359, at the beginning these were typical "S," as they aged "R" structure developed in the center, periphery continues to be "S," age 215 days. 22, this is unexplainable at present, some parts are "S" and the others look like "R," age 110 days. 9, Strain 359, undissociated, secondary growth can be seen developing from the original colonies, age 240 days. 16, Strain 3 undissociated, consisting of "R," "S" and intermediate, age 240 days. 5, Strain 359 undissociated, the growth is moist with autolyzed zone, age 110 days.

PLATE 24

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

12, Strain "Park" S?, colony, age 130 days. 2, Strain 359, undissociated secondary veil-like growth, a and b. 18, Strain 3 undissociated; this plate was

seeded from a synthetic anti-sera medium culture. Majority of colonies on this plate are "S," age 210 days. 6, Strain 359. This colony erroneously has been described by Kraus, Tzekhnovitzer and Zeyland as "S" colony. Although it is smooth in structure, it is not the true "S" colony which we have described in the text, age 110 days. 17, Strain 3 "S" colony which has been cultivated for a long period on acid medium. The organisms after such treatment have mutated and changed into "R," age 110 days. 13, Strain "Park" "S" colony, age 110 days.

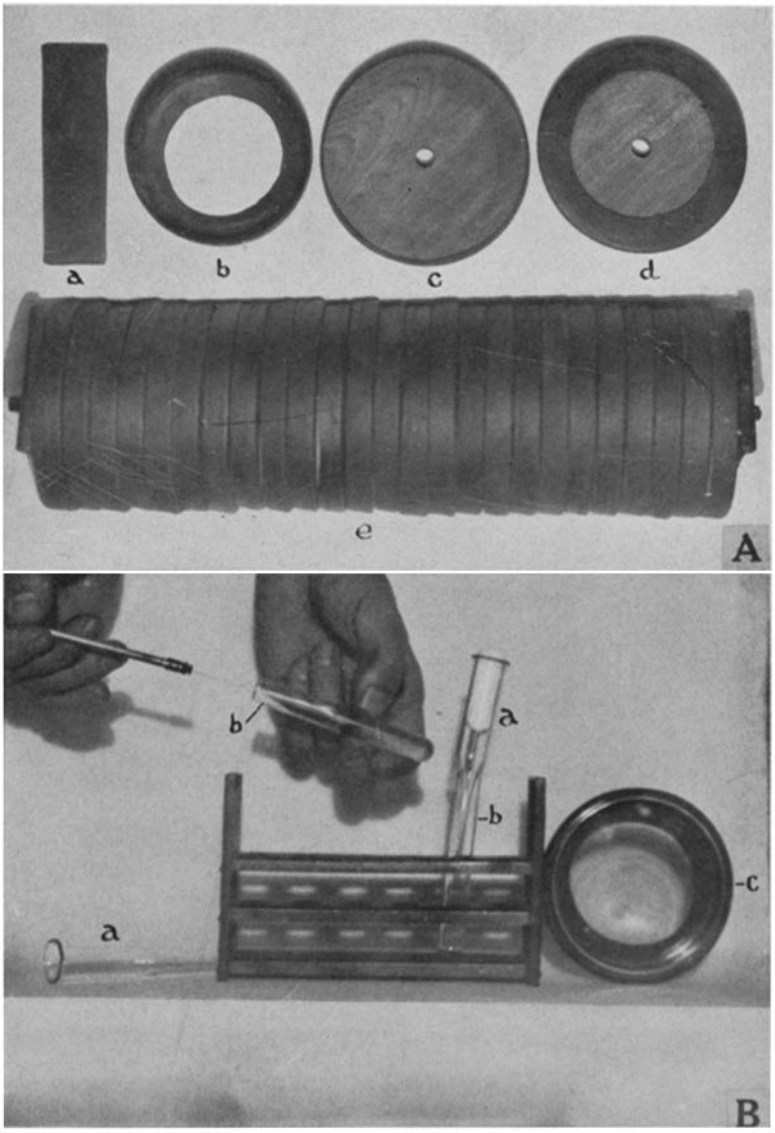
PLATE 25

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

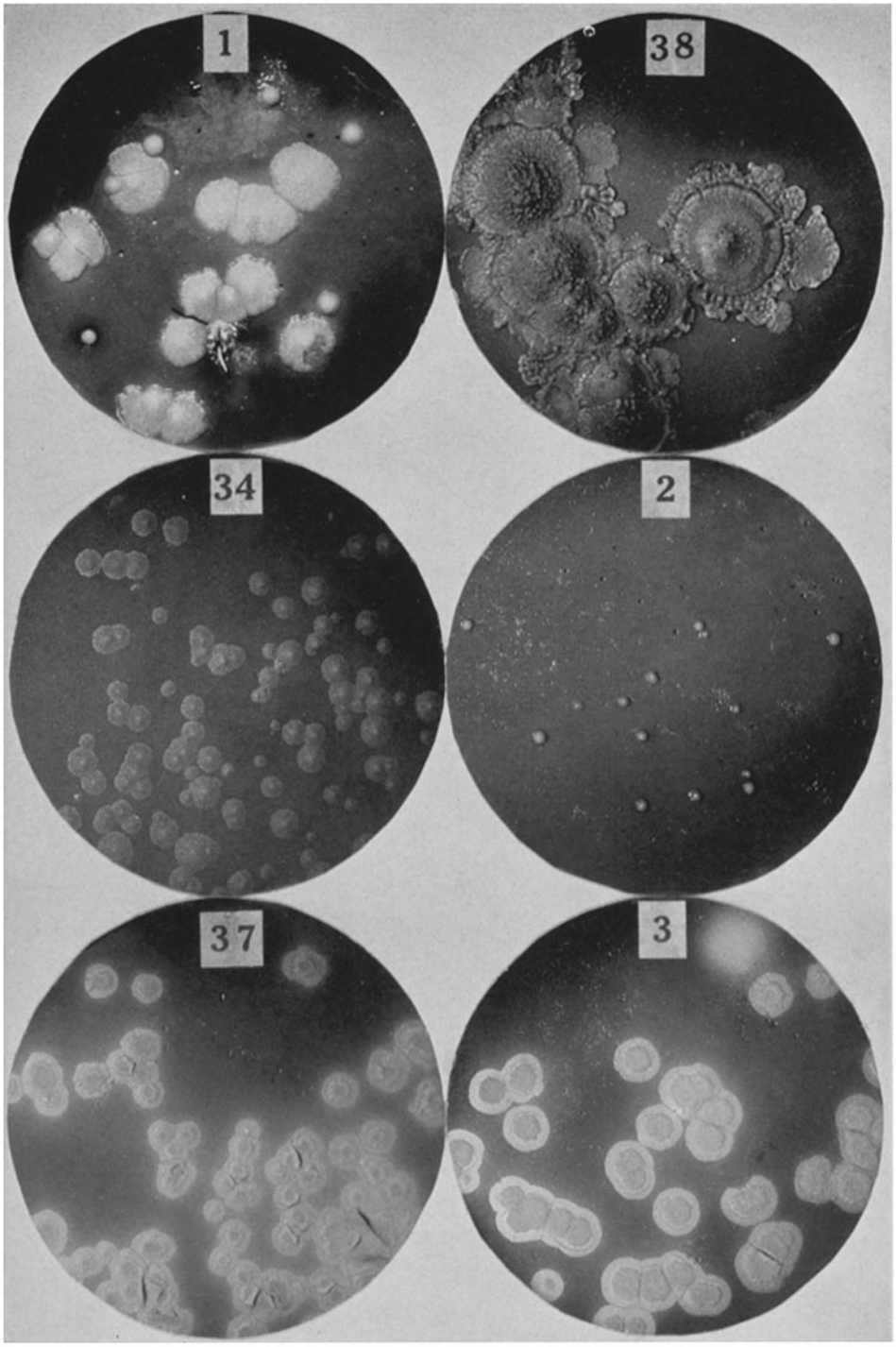
10, Strain 3 "S" colony which first developed "S" structure and as aged developed "R" structure in the center, age 281 days. 19, Strain 3 undissociated. It is a subculture from anti-sera growth; periphery "S" and the center is "R" structure, age 281 days. 11, Strain 3 "S" colony. Three zones can be seen, the center is "R," periphery is "S," the intermediate zone is undetermined at present, age 154 days. 7, Strain 3 undissociated organisms, giving same characteristics as (11), age 156 days. 8, Strain 359, colonies on sodium taurocholate medium, age 150 days. 4, Strain "Park." Note the secondary veil-like growth (a) shooting under the mother colony. Such growth upon subculturing develops many "S" colonies, age 210 days.

PLATE 26

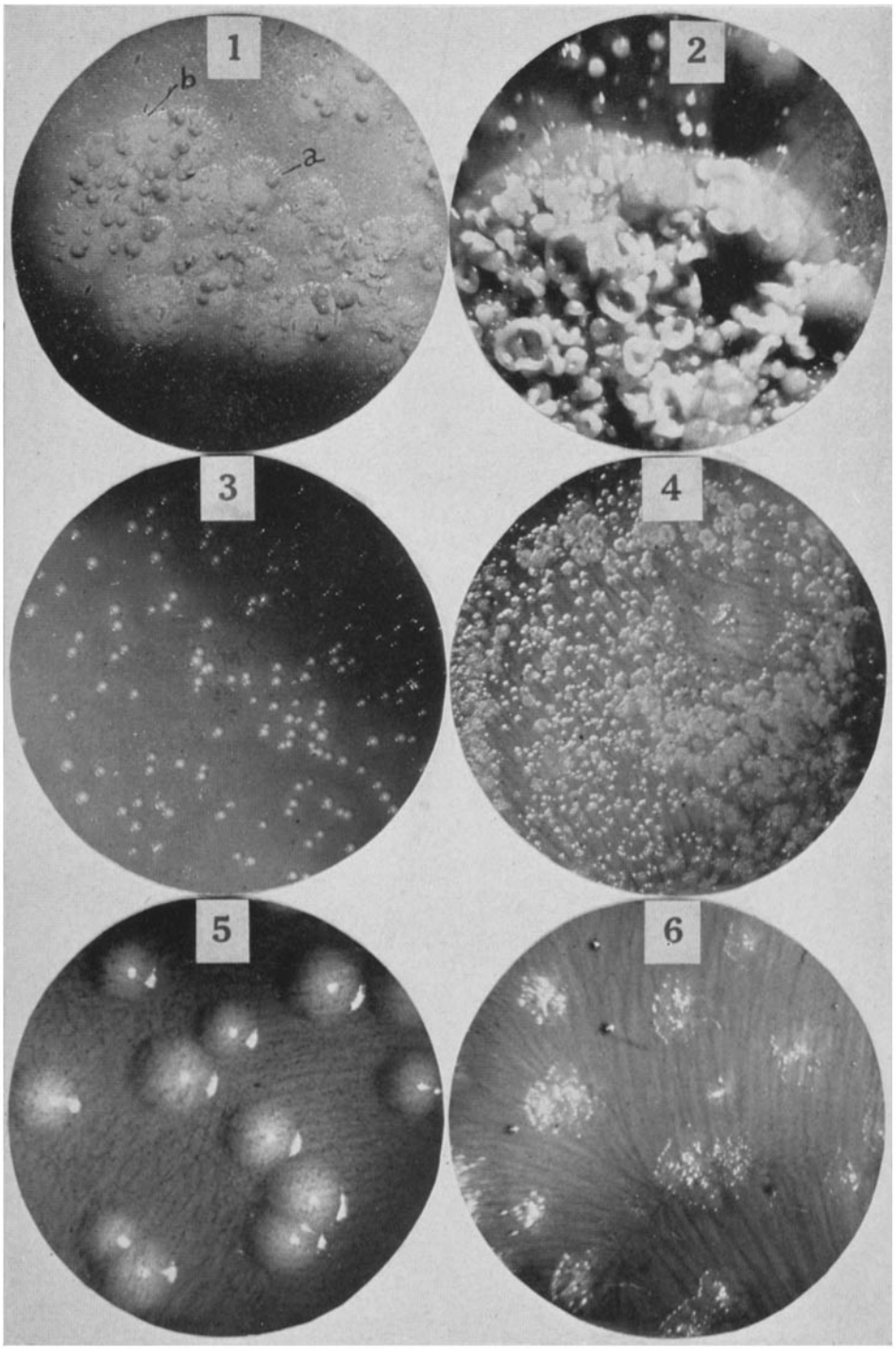
Cultures of tubercle bacilli isolated from six patients, directly on gentian-violet-egg medium plates, using sodium hydrate digestion. Every one of these cultures revealed two types of colonies. Magnification 7 ×.



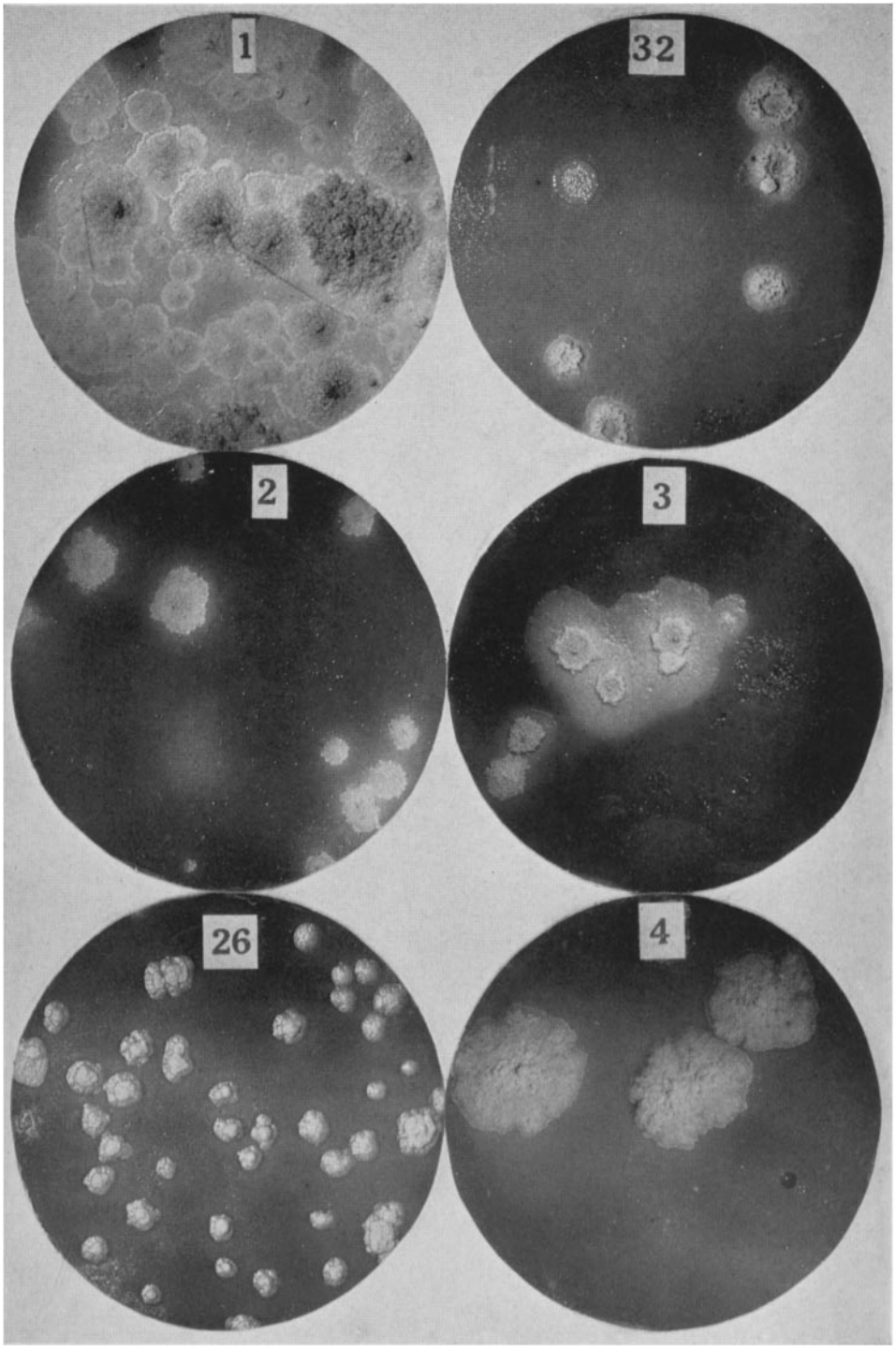

(Petroff and Steenken: Tubercle bacillus. I)



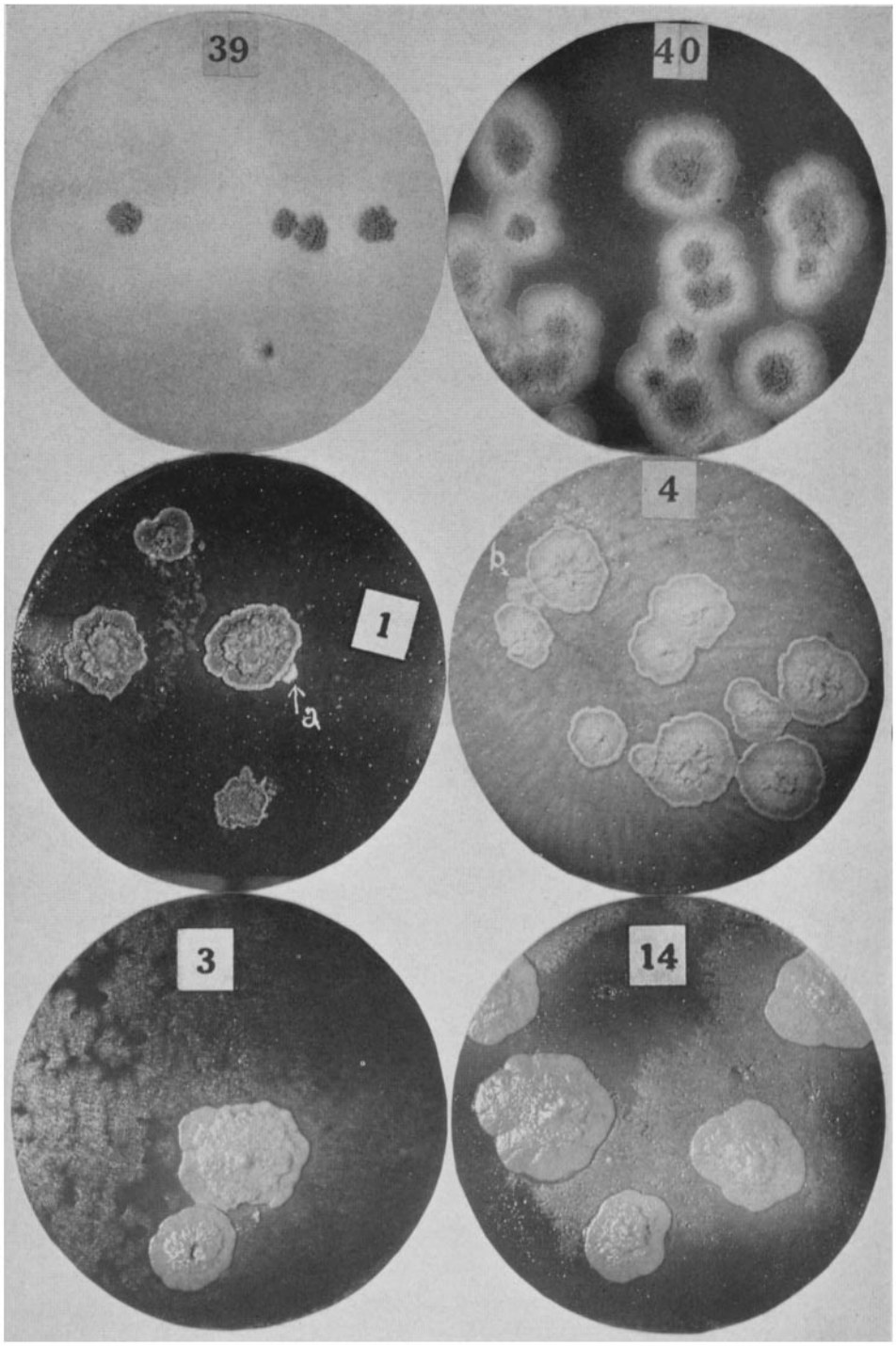

(Petroff and Steenken: Tubercle bacillus. I)



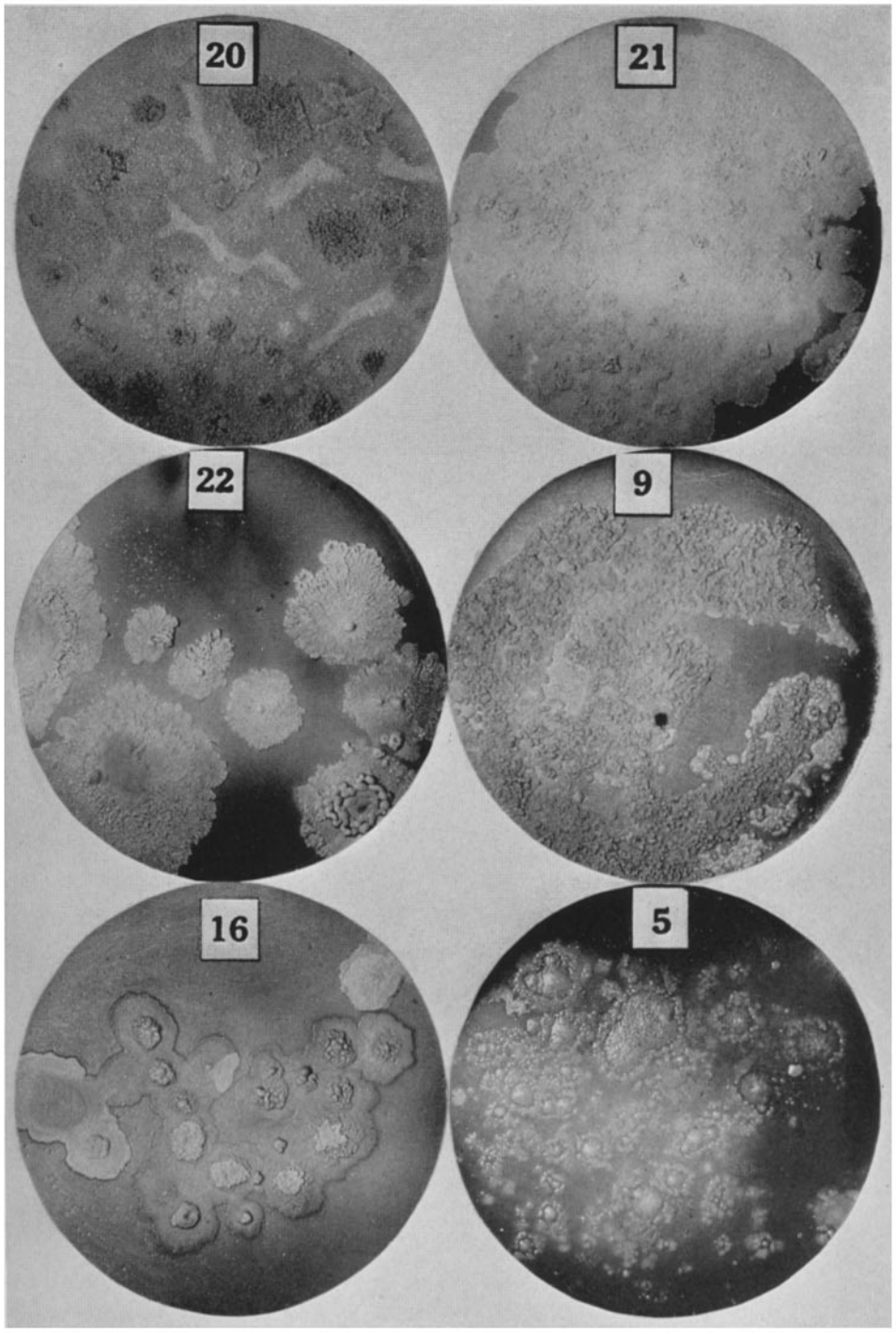

(Petroff and Steenken: Tubercle bacillus. I)



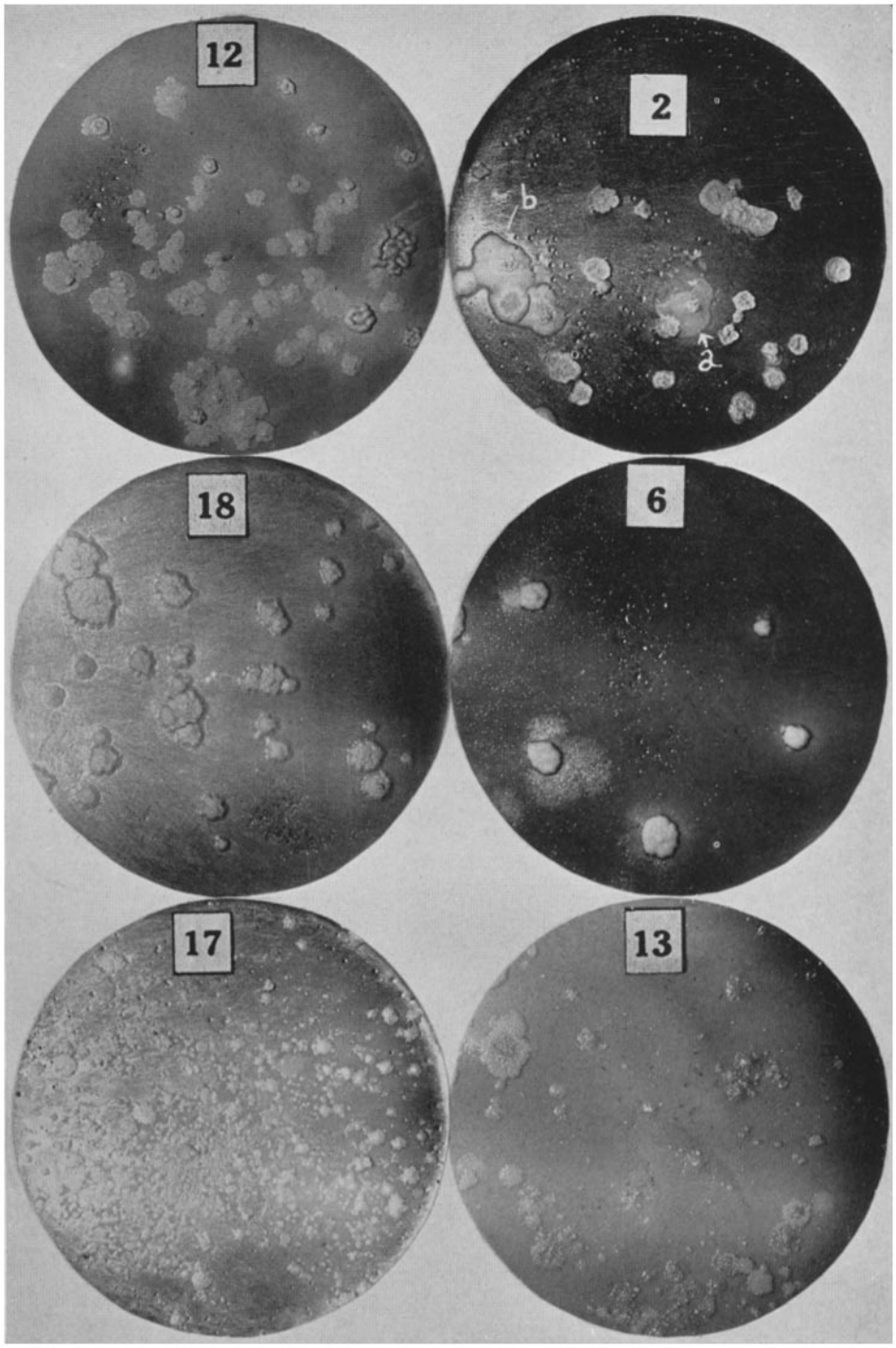

(Petroff and Steenken: Tubercle bacillus. I)



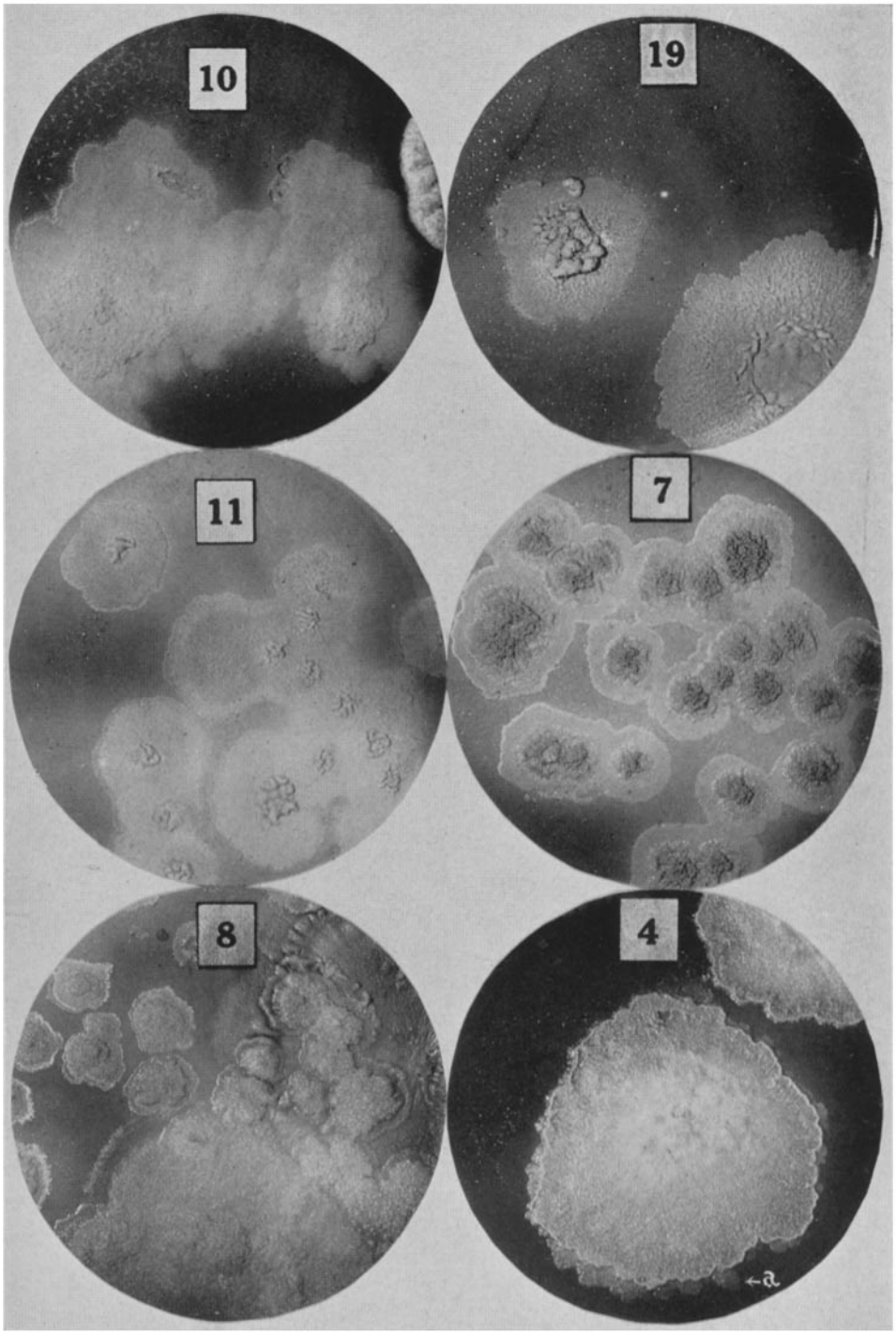

(Petroff and Steenken: Tubercle bacillus. I)



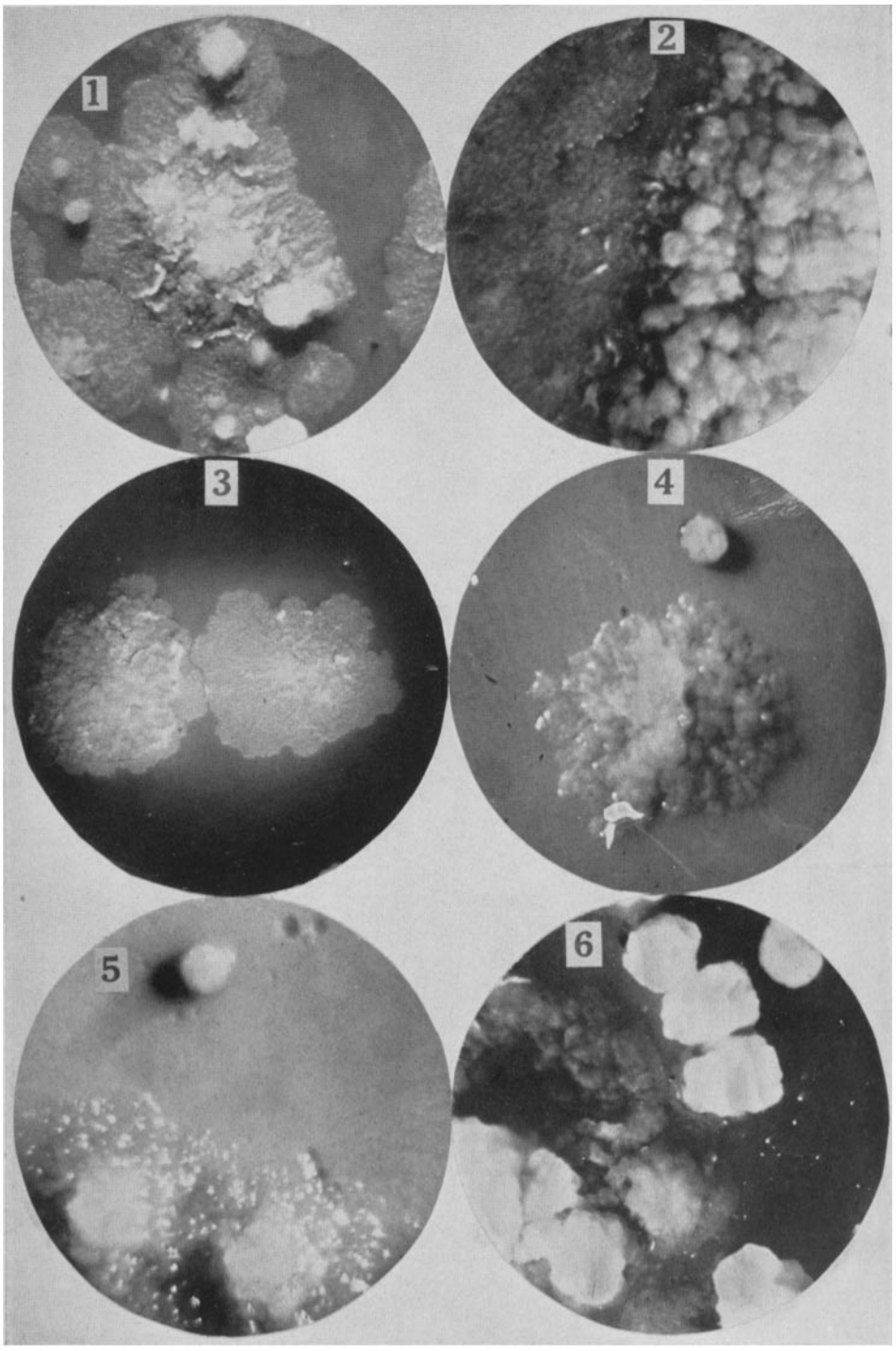

(Petroff and Steenken: Tubercle bacillus. I)




(Petroff and Steenken: Tubercle bacillus. I)




(Petroff and Steenken: Tubercle bacillus. I)




(Petroff and Steenken: Tubercle bacillus. I)