SINGLE CELL DISSOCIATION OF ACID FAST BACTERIA*

MYCOBACTERIUM OF AVIAN TUBERCULOSIS; MYCOBACTERIUM OF "RAT LEPROSY"

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In a recent study (Kahn, 1929) made of singly isolated viable *Mycobacterium tuberculosis* of the H-37 Saranac Laboratory strain it was found that when these single individuals were grown in microdroplets of Long's medium the resultant multiplication did not take place solely by a process of simple fission but underwent a more complicated type of development process, the more important phases of which were cleavage of the rod into three or more ovoid units, subdivision of these units into minute diplococcoid bodies which later became reduced to the state of fine granules. From these granules, extremely small and delicate rods developed. Later these tiny rods, by a process of elongating and thickening became what may be called the mature *Mycobacterium tuberculosis*.

Differences in the staining reaction of some of the components of the cyclic scheme were also noted, and some of these were found to be non-acid fast. The completed life cycle was observed on individuals of the H-37 strain and also upon single organisms from a so-called wild strain of *Mycobacterium tuberculosis* and there seems to be little question as to the sequence of events, at least under our experimental conditions.

It was next thought advisable to extend those studies to individuals of the avian *Mycobacterium tuberculosis* and about a year was spent in an attempt to study the life cycle of this organism

*This study is part of a group investigation being carried on in conjunction with the Medical Research Board of the National Tuberculosis Association.

with varying results. Petroff and his associates (1927, 1929, 1930) then published their studies having to do with the successful dissociation of the A-1 avian *M. tuberculosis* and also the B-1 strain of the bovine organism. Upon applying Petroff's technic for dissociation to the culture of the avian organism upon which we were working we were able to produce the typical rough and smooth colonies, thus confirming Petroff's findings and also the work of Doan (1931). As will be pointed out in the following sections the number of distinct differences existing between the R and S types is striking to say the least, probably more so than is the case among organisms of more rapidly growing species for which dissociation has been shown. In view of this fact, and also because such variable results had been obtained while attempting to study the life cycle of the organism in undissociated culture, it was considered advisable to determine whether the dissociation phenomenon played a rôle in the cycle of the organism and if such ultimately proved to be the case, to consider if there existed any dissimilarity in this cycle according as to whether the organism was in the R or S phase of this development process.

We considered it probable that if dissociation was to be considered as part of the cyclic phenomenon, a culture from a single bacterial cell from a known R colony form should under certain circumstances give rise to some colony offspring of an S character, while the reverse should be the case with single organisms from initially S cultures. Experiments were designed to determine whether such was the case.

TECHNIC

The cultures of the A-1 avian *Mycobacterium tuberculosis* were dissociated originally on plates of Petroff's egg medium and also on the medium of Proskauer and Beck. The criteria which should determine true dissociation will be discussed in detail in a following section. Suffice it to say here that the S colonies are smooth, round, domed and moistly glistening, while the R colonies are flat, rough in contour and configuration and dull in color. When real dissociation does occur it is a distinct and clear cut phenomenon and no difficulty is encountered in distinguishing the R from the S types.

The typical rough or smooth colony was lifted from the plate with a fine sterile platinum spatula and an emulsion made into suitable culture medium. Single organisms were then isolated with the Chambers micromanipulator which we have found to be admirably adapted to the purpose (Chambers, 1922a, b and c) (Kahn, 1922). Prior to this time investigators have used the single cell method principally for the isolation of rapidly growing organisms, proliferating for the most part diffusely in broth. In our studies it was found necessary to develop the method so that the microdroplets containing the single organisms of this slow growing species could be preserved for a long time in order that periodic observations on change in morphology could be made on identical individuals for several days or weeks.

Observations and descriptions made on preparations stained or otherwise, in which large numbers of organisms are encountered, as has been the case heretofore, must be confusing when life cycle studies are attempted. This is unquestionably the case when the usual hanging drop or agar block technic is employed wherein one encounters a number of living bacteria. With slow growing species it is impossible to keep the eye constantly fixed on the same individual for any length of time and if one glances away from the field only for a brief period one cannot be sure of returning to the organism under original observation. Our technic lends itself readily to making the necessary periodic observations on identical individuals and furthermore it is also of value in obtaining pure line cultures from a single organism such as *M. tuberculosis* which is very difficult to induce to grow in a test tube of liquid medium or on a slant of culture medium such as is usually employed for the more rapidly growing single bacteria of other species.

In view of the fact that with this method a slow-growing single cell may be allowed to develop to colony maturity in the microdroplet before it is transferred to the culture tube, a much larger percentage of successful "takes" are procured than would be the case were the single organism planted in or on a suitable medium. Using the older method of direct transfer an experiment was undertaken involving the separate planting of some 80 single human *M. tuberculosis*, and two successful cultures were obtained.

In a second experiment where the single organisms were allowed to form colonies in the microdroplet before making a transfer, 40 per cent positive cultures developed. This appreciably larger percentage was due solely to the fact that the technic employed made it possible to keep the microdroplets containing the single bacterium intact for many days without drying or spreading and thus the development to colony maturity is permitted under optimal conditions. Time is really saved in the end as the possibility of having planted non-viable forms is precluded.

The method of making the micropipettes as well as the manipulation of the pipette holders have been gone into in detail by Barber (1914) and by Chambers concerning their respective apparatus. Suffice it to say that in these experiments the micropipettes were made from soft glass tubing having an inside diameter of about 1.5 mm. The tubing is cut into lengths of some 14 cm., plugged at both ends with non-absorbent cotton, several of these are placed in wide mouth test tubes and sterilized in the hot air sterilizer. The tubing is then made into a capillary pipette by being drawn out in the Bunsen burner. The fine end of the capillary thus formed is redrawn in the flame of a tiny microburner made from a bit of the above mentioned glass tubing. The opening should be compressed until only the smallest possible flame develops. The flame of this microburner may be further regulated by screw clamps. The fine drawn micropipette is bent at a right angle of from about 3 to 5 mm. This is filled with a dilute suspension of the culture and subsequently mounted into the Chambers micromanipulator. A micromanipulator constructed by Dr. George W. Fitz (1931) is also admirably adapted to this purpose.

The new pattern moisture cell (E. Leitz) having an inside diameter of 5 by 2.3 by 1.5 cm. has been found more serviceable for work of this character than the one originally devised by Barber which is higher and wider.

In view of the fact that this technic involves moving the coverslips on the underside of which the microdroplets containing the single bacteria have been isolated and subsequently mounting these coverslips on hollow ground slides, it is necessary to place

an apron of mica on top of the glass moisture cell and upon this apron the coverslip is placed at right angles, instead of parallel to the top of the moisture cell as has been the case heretofore. (See plate 6, fig. 3.) The mica apron has a slot cut into the surface which lies parallel to the rear of the moisture cell and the droplets are isolated on that portion of the coverslip resting just over the slot. No vaseline or other sealing material is used to hold the coverslip in place but the mica apron is sealed to the rim of the moisture cell with sterile vaseline. The importance of properly clearing the coverslips cannot be overemphasized, and the necessarily elaborate technic directed toward this end has already been described by one of us (Kahn, 1929). Before cleaning the coverslips it has been found of great aid to etch with a diamond point a tiny circle at about the center of the coverslips. The droplets are isolated in and about this circle in such a manner as to form the pattern of a cross. Such an arrangement aids materially in finding the microdroplets both in the fresh condition and when the preparation is stained. No. 1, 24 x 50 mm. is the thickness and size of the coverslip employed. It is necessary to handle the coverslips with forceps throughout the technic. The technic for preparing the coverslips has been worked out after considerable experimentation and has been found most satisfactory for removing every trace of foreign material from the coverslips. Unless this is done foreign particles of one kind or another will cling to either surface of the coverslip and it is easy to see how such a state of affairs would be highly confusing to the observer. A number of coverslips are prepared at a time by the above-mentioned process and are held ready for use. Formerly it was thought that a slight trace of grease would prevent the isolated microdroplets from spreading, but this adds the additional hazard of mistaking the small grease particles for fragments of organisms or granules and accordingly was not employed. With the maintenance of proper moisture and temperature there is very little difficulty in keeping the droplet intact. A viscid medium also aids materially in this regard, but agar or gelatin should not be added as the microscopic particles cannot be removed by clarification.

MEDIA TO BE USED

Naturally, the medium to be used varies with the nutritive requirements of the organism. However, successful adaptation to this technic has for one of its prerequisites a fairly viscid substance. For the work pertaining to the avian strains of *M. tuberculosis* and also to the mycobacterium of "rat leprosy" 365 (11) the following formula was employed for the liquid serum medium in which the single cells or small groups were isolated.

Distilled water, 1000 cc.
Lean chopped beef heart, 750 grams.
Simmer at boiling point until bloody appearance disappears.
Make up loss by evaporation.
Filter through cotton wool.
Peptone, 10 grams.
NaCl, 1. grams.
Adjust pH to 7.0.
Ferrous ammonium citrate, 0.025 gram.
Glycerol C.P., 75 cc.
Place in Arnold sterilizer for forty-five minutes at 100°C.
Adjust pH to 7.4 and filter twice through filter paper.

Prior to autoclaving, the medium is clarified by allowing it to run twice through a Sharples clarifier. Sterile inactivated rabbit or human serum is then added in amounts of 7 per cent and after suitable sterility tests have been completed the medium is ready for use.

The actual preparation of the culture from which the single bacterium is to be isolated is of utmost importance. It has been our experience that the youngest cultures are the ones best suited to the purpose as they give the largest percentage of successful single cell cultures in view of the fact that the possibility of isolating non-viable organisms is minimized and that the growth potential is higher than in old cultures. Not alone should the culture actually used be a young one, but the stock from which this plant is made should be induced to active growth by several rapid transfers. This procedure is suited to organisms growing on liquid medium or to undissociated cultures on slants of solid medium. In dissociation work, however, where one desires to obtain a single bacterium from the R or S *colonies* as they occur, the

entire colony must be lifted from the plate and an emulsion directly made of this by gently shaking in a sterile test tube containing a few cubic centimeters of the above mentioned medium and a few glass beads. After letting this preparation stand a few minutes to insure settling of the coarser particles the micropipette is inserted and the tip is broken by gently rubbing against the wall of the test tube and sufficient of the suspension is drawn up to fill the micropipette to just beyond the elbow. Thus, with the moisture chamber, mica apron and sterile cover slip properly mounted, the worker is ready to begin operation and with proper dilution of culture, size of drop, etc., some of the microdroplets will be found to contain a single bacterium or a small group of two to six. No accurate observation of the bacterial population is attempted until the coverslip has been removed from the moisture chamber and mounted on a deep hollow ground slide. The method of accomplishing this has already been described (Kahn, 1929).

After the preparations have been mounted on the hollow ground slide the scratched circle on the coverslip is located with the low power objective and a chart is made of the location of each microdroplet. (These droplets should be about 1 low power field apart and of such a size that the entire droplet is visible under a 10a oil immersion lens.) Each droplet is indicated on the chart by a circle which should be of sufficient diameter to permit the accurate and careful drawing of the single cell or small group of bacteria contained therein. As daily observations were made on the preparations in which a study was being made of the life cycle, considerable time was saved by having made a circular rubber stamp, each circle representing a microdroplet.

With a 10a oil immersion lens and a 10× eye piece each microdroplet is carefully scrutinized and an accurate drawing made of the rod or granule contained. Care must be taken to focus in all depths of the droplet and the edges of the droplet must be carefully scrutinized for a possible microörganism. After the initial twenty-four-hour period of incubation it is essential to conduct all microscopical examinations of the preparations in a hot box having a temperature of from 30° to 32°C. Otherwise,

condensation occurs on the underslide of the coverslip and the preparation becomes flooded.

A droplet containing more than 6 microörganisms was discarded as unfit for accurate observation. Generally a few hours were devoted to making these preparations and from a number a few were selected most nearly approaching the optimal and the remainder discarded.

With practice one finds no difficulty in isolating a considerable number of droplets containing one tubercle bacillus or small groups of two or three bacteria.

As the growth rate of this organism is not sufficiently rapid to make constant or hourly observations practical, such observations were made daily. After each observation the oil is carefully removed from the micro-culture with xylene and the preparation resealed with the hot sealing mixture (Kahn, 1929).

Control and other droplets which were proved to be free of any organism by microscopical examination *invariably remained sterile.* A trained observer has no difficulty in recognizing the presence of organisms when such are present or of the various elements which go to make the cyclic scheme when these are known. No débris or other foreign material is to be observed in the micro-droplets if the medium is properly prepared. The addition of agar or gelatin to the medium makes proper clarification impossible.

MICRO-COLONY OBSERVATIONS AND THE MICRO-COLONY METHOD OF OBTAINING CULTURES FROM A SINGLE BACTERIUM

Aside from applying this technic to the observation of growth phenomenon it may also be used in obtaining relatively rapid information as to the character and nature of a colony in regard to R or S features. As has already been reported by us (Kahn and Schwarzkopf, 1931) the R micro-colony grows in a dense network of coarse fibers while the S micro-colony is delicate and lace-like in structure. The S colonies are also slower growing than are the R. Other differences have been pointed out. Intimate immunological phenomena may also be studied by this method.

Aside from this, however, the technic has one of its principal

applications in obtaining a considerably larger percentage of successful positive cultures from single organisms, especially in the case of the slower growing species. This is accomplished by allowing a microcolony to form in the droplet containing the single bacterium while still mounted on the hollow ground slide and before any attempt is made to transfer to culture tubes.

In the case of those bacterial species which form several generations in twenty-four hours, the resultant colony is usually apparent at the end of a day of incubation or less. Not infrequently a period of lag is noted even among individuals of the more rapidly proliferating bacteria, which may extend for as long as twenty-four hours. Naturally a dead organism is sometimes isolated in the microdroplet but such instances are considerably minimized if one takes pains to prepare a young and vigorous stock culture.

Whenever the smallest colony has formed in the microdroplet from a single bacterium, for instance, an aggregate of some ten to fifteen cells, one is almost certain of obtaining growth upon a slant of medium. The replanting is accomplished in the following manner.

With a small, clean, sharp iridectomy scalpel the sealing material holding the coverslip to the hollow ground slide is carefully cut away. After as much of this as possible has been removed, the partially freed coverslip is gently rotated with two fine pointed dissecting needles (one being held in either hand); until the ends of the coverslip are at right angles to the hollow in the slide. Care should be exercised not to expose the microdroplets by allowing the coverslip to be, even in the slightest degree, moved from over the depression; then the coverslip is quickly lifted with sterile forceps to the mica apron which has been previously mounted on the moisture chamber. The droplets are prevented from drying by having placed hot distilled water in the bottom of the moisture cell, which has, of course, been previously mounted on the mechanical stage of the microscope. A micropipette is then prepared with a bore somewhat coarser than is usually employed for single cell isolations. It is filled beyond the elbow with suitable sterile liquid medium. The micro-colony is then

located under the objective and with the micropipette already in place, a little medium is expelled around the tiny colony to facilitate manipulation. Then, by applying very gentle suction the entire colony is drawn into the micropipette and may be expelled upon a slant of suitable medium. Due to the small bore of the micropipette, considerable resistance is encountered in expelling the fluid contained therein. This is much facilitated by attaching a 25-cc. Luer syringe to the end of the rubber tubing. A small colony visible to the naked eye will soon form on the seeded solid medium and this may be spread with a platinum loop to insure obtaining sufficient material from which to make the desired sub-cultures.

The above in a general way is a résumé of the technic employed. There are probably a number of points, however, which may only be gained from *actual* experience with the apparatus.

R and S colony types

At the outset, in order to prevent confusion, it may be well to recall some of the distinctive features of the typical R and S types as outlined by Hadley (1927). A number of these will be found to conform to the two colony types as they occur in dissociated cultures of the mycobacterium of avian tuberculosis, of the mycobacterium of bovine tuberculosis as Petroff has shown, and of the acid-fast organism isolated by Chapin from a leprous rat and recently dissociated in this laboratory. (This last mentioned organism is known to many American bacteriologists as the mycobacterium of rat leprosy, but it is questionable if the organism is the etiological factor in leprosy disease of that rodent.) Some investigators of the dissociation phenomenon have been content to use as a criterion of dissociation the topography and general condition of the growth membrane of a culture as it occurs on a liquid medium. It is claimed that the S type grows as a thin, continuous delicate film, white to faintly cream in color, while the R type of growth membrane consists of islands upon the surface of the liquid which are raised, the surface sometimes corrugated, of darker color, or pigmented, which do not tend to coalesce until the culture is of considerable age. We do not consider these

characteristics of the growth membrane on liquid media an accurate criterion of dissociation of members of the mycobacterium groups for the following reasons. The S types on fluid culture will sometimes, to a more or less degree, assume the characteristics which some observers consider typical for the R type, while subsequent plating on solid medium will yield numerous, and sometimes a preponderance of, S types. Second, young and vigorously growing R types will often give rise to the delicate, white, continuous growth film while colonies obtained from such growth will yield only the type characteristic of the R morphology. The condition of the growth membrane is also dependent to some extent on the surface and volume of fluid used. In other words, the morphology of the individual colony is obviously of foremost importance as a criterion of dissociation; the appearance of the growth membrane as it occurs upon fluid medium or slant cannot be relied upon.

Since Petroff published his method in 1927, workers with the mycobacterium group have been able to cultivate these organisms on plates in single colonies. This method is of great importance, for, as Doan (1931) has pointed out, it has marked a great advance in our conception of the bacteriology of this group, and for the first time biological investigations on dissociation may be undertaken with *Mycobacterium tuberculosis* on the same basis as with other microörganisms.

The method in brief consists in filtering an emulsion made from a culture through a double thickness of sterile No. 5 Whatman filter paper. The resultant filtrate is plated on egg medium plates in various dilutions. Some of those dilutions will be found of sufficient concentration to give rise to widely separated colonies on the petri plates. For the general details of the technic the reader is referred to Petroff's article (1929).

After Petroff published his results on the successful dissociation of the A-1 strain of the avian *Mycobacterium tuberculosis*, a culture of the undissociated strain was kindly sent to this laboratory at the time the writers were studying two other strains of this organism, namely, one procured from Dr. Elise l'Esperance which had been isolated from a tuberculous chicken and a second one from

the late Dr. Krumwiede, Research Laboratories, New York City Department of Health, known as avian tubercle bacillus strain 216.

Upon applying the technic as outlined by Petroff to these cultures which had been carried for sometime previously on slants of egg medium, it was found that a number of colonies developed which corresponded to the R and S types as noted by Petroff after he had successfully dissociated the A-1 strain of avian *M. tuberculosis*. By applying the single cell technic as previously outlined, successful cultures from one mycobacterium were obtained from the R type of colony of all three strains with little difficulty. The S types, however, presented quite another problem. In a previous communication (Kahn and Schwarzkopf, 1931) we have already shown that in microculture beginning with some fifteen to thirty organisms per droplet, the growth rate of the R is definitely more rapid than the S. This observation has received partial confirmation in the work of Lurie (1928) who demonstrated that virulent *Mycobacterium tuberculosis* proliferates in tissue at a less rapid rate than does the non-virulent organism. Although some 40 per cent of the single R organisms gave rise to successful microculture with subsequent successful gross culture only two successful single cell cultures were obtained with A-1 smooth form after over 100 isolations, while one successful culture from a smooth colony of the L'Esperance strain was obtained after about the same number of isolations and two successful single cell cultures from the smooth colonies of the Krumwiede strain after some forty isolations. In contrast it may be stated that, when some 5 S type bacilli are seeded in the microdroplet, growth takes place in 75 per cent of instances and a microcolony is formed within the minimum time usually employed for this mutant. It is only when a single S cell is isolated that the above-mentioned difficulties are encountered in inducing multiplication. This marked contrast with the R types presents a number of interesting speculations as to why it is that the single S cell has more difficulty in establishing itself especially, as will be shown later, in view of its apparently enhanced pathogenicity to laboratory animals.

In 1920 Churchman separated from a single colony of *B. coli*

two strains, one which grew well in the presence of gentian violet and another which would not grow at all. These two strains were identical by every other bacteriological test known at that time. With the strain of *B. coli* which grew well in the presence of gentian violet when planted by gross methods, single cell studies were made in 1921 by Churchman and Kahn (1921). These showed clearly that *single cell* inoculations of this pure strain would not grow in the presence of the dye (only 1 positive culture out of 148 single cell plants) while about 80 per cent positive results were obtained in the cultures into which single cells of the same strain were inoculated in plain broth, and almost 100 per cent of growth was obtained with groups of about 30 cells inoculated into a series of tubes containing gentian violet broth.

The results which we have obtained in this present study of the growth of avian *M. tuberculosis* S and R strains appear to agree with the suggestion made at that time by Churchman and Kahn that in the growth of some bacteria a phenomenon which was then termed "communal activity" plays a rôle. This would have a direct bearing on the S forms under discussion.

Description of the S form as obtained from a single mycobacterium

The culture grown from a single S organism of the A-1 strain was plated out on 25 plates of Petroff's egg medium according to the technic already outlined, to preclude the possibility of spontaneous dissociation having taken place. The spontaneous S to R dissociation has not been observed in the single cell strains as long as the organisms were kept on Petroff's medium. The colonies as they occur on this medium after six weeks of incubation are from 0.3 to 1.5 mm. in diameter. Little enlargement takes place even after prolonged incubation. In the younger cultures the colonies are dead-white in color assuming a creamy hue after some ten weeks. The colonies are smooth, round, domed, glistenningly moist, compact, regular, seemingly homogenously, dense; no daughter colonies are formed, nor is there an extension of growth at the peripheral base as is the case with the R form. Any further alteration in a true S colony after many weeks of incubation is due to the drying of the medium and is not a matter of growth-continuity as is the case with the R types.

The S colonies go into suspension with great ease in either 0.85 per cent NaCl solution or distilled water, forming a homogeneous type of suspension free of clumps or spontaneously agglutinative particles. As has been shown by us (Kahn and Schwarzkopf, 1931) the mobility velocities of the S forms of this organism are considerably faster than those of the R. For the A-1 strain they average 2.95 micra per second per volt per centimeter as contrasted to 1.53 for the R forms. For the Krumwiede strain the average for the S types was 1.72 micra per second per volt per centimeter while for the R forms it was 3.11. Similar differences have been shown to obtain for R and S types of the B-1 strain of bovine *M. tuberculosis*. These observations have been confirmed by Reed and Rice (1931) using the acid agglutination method of approach.

Microscopic examinations of slides stained by the Ziehl-Neelsen and Krylow acid-fast methods indicate that the S forms retain the carbol-fuchsin somewhat less than do the R. Forms containing intracellular granules are rarer. The extremes of measurement as determined for several hundred S forms from the single cell cultures with the Filer hair micrometer were 3.36μ to 0.55μ in length, the average being 2.04. This is somewhat shorter in general than the R forms, as will be seen. Clumped and filamentous aggregations of organisms are less often seen than is the case with the R culture.

On gross culture (slant of Petroff egg medium) the entire growth gives a moist glistening appearance. As the culture ages it becomes somewhat dryer and wrinkled. This condition may give the false impression of S to R mutation but application of the plating technic to such a culture will soon convince the observer to the contrary.

Description of the R form as obtained from a single mycobacterium

As was the case with the S types, the cultures obtained from the single R organisms were planted out on several plates of Petroff's egg medium and upon none of these 25 plates did an S form of colony develop. Thus, several hundred colonies were observed for varying periods up to eighteen weeks. The R colo-

nies as they occur on these egg medium plates after having been incubated for six weeks are from 1 to 3 mm. in diameter. They are rough, flat, dry, dull, compact and irregular in configuration. At the periphery there is also an irregularity in outline. The color of the six weeks old colonies is about that of old ivory, definitely darker than that of the S types. The picture is that of a nipple surrounded by an areola of somewhat lighter hue. After some nine weeks of incubation the nipple becomes more prominent and assumes an orange coloration while the colony extends at the periphery. The surface inward from the periphery becomes papillated with minute inundations intervening. The nippled area becomes more glossy but material taken from this area and planted upon egg medium plates gives rise only to the R type of colony. Material planted from any other area of the colony also only gives rise to that type of colony. There is the possibility of error in interpreting very young (three to four weeks) R colonies as S colonies. At this time the growth is more glossy than in the older colonies and the central area is also at this time more prominent. Under high power magnification, however, and with careful examination under proper illuminating conditions the dull periphery designating a subsequent R colony may be seen at the base of the colony. Differentiation between R and S types should not be attempted until the plate is at least five weeks old and, preferably, six. After ten to twelve weeks of incubation the orange color in the central nippled area begins to spread while the peripheral growth becomes of a greyer color. The diameter of the colony at this age is 5 to 7 mm. The papillae in the central glossy orange area become much more numerous, but the nippled peak is still distinct although somewhat flattened. At the end of fourteen or fifteen weeks of incubation the colonies may become as large as 10 mm. in diameter; also there is to be seen an extension of the orange colored central area to within about 0.5 to 1.0 mm. of the periphery. The papillae in the heaped up central area spread further toward the periphery, become smaller and more numerous and lose their characteristic waxy luster. This seems to be the end point of growth for the R colony. No further change takes place even after months of incubation save drying and cracking of the colony.

The measurement of several hundred individual rods after staining with the Ziehl Neelsen and Krylow stain revealed an extreme measurement from 5.5μ to 0.5μ. The average was approximately 2.7μ in length. The impression is gained that the rods from the R colonies are on the whole larger or coarser than those from corresponding S colonies of the same age. Beaded forms and those containing the dark-staining (by the Krylow method) intracellular elements seem to be more numerous. As has been already stated the electrophoretic mobility velocity of the R avian forms is considerably slower than that of the S.

The emulsion formed in 0.85 per cent sodium chloride solution or in distilled water is clumpy, coarse, granular or flaky and spontaneously agglutinative. It is not possible to prepare a smooth even type of suspension as with the S colony type, excepting after prolonged shaking with beads in a shaking machine.

Pathogenicity of the R and S single cell strains

As has been reported by Doan (1931) a striking difference exists between the pathological picture produced in chickens and rabbits according to whether the R or S types of the avian *Mycobacterium tuberculosis* are inoculated. The injections were given intravenously. We have confirmed his findings with these single cell strains. In Medlar's (1931) recent studies on the pathology which the avian organism produces when injected intravenously in rabbits, two types of reaction are also described. The first, which he mentions as occurring in his unvaccinated rabbits which as will be seen from the foregoing, is the classical S type of reaction (confirmed by Medlar who has since planted out his avian culture and found it to be an S form) and the second type of reaction, produced with the same avian culture but in rabbits previously vaccinated with subcutaneous doses of human *M. tuberculosis* closely approximates the disease produced when the R form alone is injected. In order to determine the difference in killing time with the R and S types when identical doses of each are employed the following experiment was carried out with thirteen rabbits. While it is realized that this series of animals is a small one, it will be seen that with few exceptions the single cell S strain of avian

M. tuberculosis killed at a more rapid rate than did the R type, although it cannot be denied that some rabbits show great susceptibility to injections of the R mutant as well. Seven rabbits were selected upon which to test the R culture and each animal received 0.5 mgm. of a forty-two day old culture of the single cell strain grown upon Petroff's egg medium plates. The typical R colonies were picked off and suspended in sterile distilled water. The vessel containing this suspension was then vigorously shaken for forty-five minutes in a shaking machine and filtered through

TABLE 1

Rabbits receiving intravenous inoculations with single cell strains of R or S avian Mycobacterium tuberculosis

RABBIT NUMBER	MUTATION TYPE INJECTED	AMOUNT INJECTED	TIME SURVIVED	MUTATION TYPE RECOVERED ON CULTURE
		mgm.	*days*	
47	R	0.5	67	R
67	R	0.5	31	R and S
77	R	0.5	90	R
89	R	0.5	51	R and S
91	R	0.5	92	R
92	R	0.5	28	R and S
93	R	0.5	77	R
63	S	0.5	23	S and R
64	S	0.5	16	S
65	S	0.5	20	S
94	S	0.5	30	S
95	S	0.5	32	S
96	S	0.5	26	S

several thicknesses of sterile cheese cloth to remove the coarse particles. Five-tenths cubic centimeters of the resultant filtrate was then pipetted into small sterile watch glasses, dried over a water bath and weighed in duplicate on a microbalance having a sensitivity of $\frac{1}{400}$ mgm. The injections in all animals were given in the marginal vein of the ear. The animals were fed the usual adequate laboratory ration and each rabbit was kept in a separate cage. At the death of any animal the cage was thoroughly disinfected. Every effort was made to preclude the possibility of cross infection. It will be noted from table 1, that two of these

animals survived for 90 and 92 days respectively, while one survived 77 days, another 67 days and still another 51 days. Two others died within 28 and 31 days. In the case of the animals which survived for 51 or more days, generalized 4+ tuberculosis was noted in each case and the gross pathology impressed us as being so uniform that we will describe these animals as a group. The animals prior to death all showed great loss of weight.

The lungs contained numerous tubercles varying in size from smaller than 1 to 3 mm. in diameter.

Liver: Moderately enlarged with numerous small tubercles and fibrin coated areas.

Kidneys: Both show tuberculous lesions.

Spleen: Enlarged, very dark color: numerous small and large tubercles on surface.

Large numbers of small (1 mm.) tubercles scattered throughout pleura and peritoneum.

Bone marrow of tibia riddled with tubercles.

Axillary and inguinal lymph nodes enlarged and caseous.

Numerous tubercles under skin.

In two rabbits periventricular tubercles were noted.

The organs of each animal were cultured on Petroff's egg medium.

Four gave rise to pure cultures of the R type while rabbits 92, 89 and 67 gave rise to typical S as well as typical R colonies on plates which were seeded with marrow and liver respectively, while the plates from the other organs revealed only the R types. These S mutants from the single cell R strain were found to show the identical pathogenicity of the original S forms. As will be seen from the following data the two animals (nos. 92 and 67) which died within 28 and 31 days (the time usually taken to produce death with the S type of culture) seemingly spontaneously dissociated the R culture and died of an S type of tuberculosis. These animals showed, however, only a small number of S colonies as did the rabbit which died on the fifty-first day after injection. Unfortunately, no accurate counts were kept of this important ratio. These S types, however, were characteristic in every way for the colony when in that stage of its mutation cycle. Their numbers were certainly far less than 1 per cent of the R colonies.

Pathogenicity of single cell S cultures

The method of preparing this culture for injection, including the age of the culture used, was in every way identical with that employed for preparing the R. The six rabbits used to test the pathogenicity of the single cell S type received 0.5 mgm. of culture by the intravenous route. By consulting table 1 it will be seen that all of these animals died within 32 days, the shortest time taken to kill being 16 days. Thus, the average time for death with the S type culture was 24.5 days as contrasted to 62.2 days with the R type.

The gross pathology of the six rabbits which died from the S inoculations and that of the two rabbits receiving the R inoculum, which died after 28 and 31 days, were all practically the same. The most striking feature encountered was the entire absence of *macroscopic* tubercles. The spleen of all of these animals were tremendously enlarged. The lungs revealed congestion and hemorrhagic infiltration with a distinct bloody discoloration. There were no macroscopic tubercles in the bone marrow. Axillary and inguinal lymph nodes were only moderately enlarged. The kidneys revealed no lesions to the unaided eye while the liver was very dark, sometimes almost black, and moderately enlarged. A mottled discoloration of the liver was encountered in two rabbits. No macroscopic tubercles were encountered in this organ. The picture presented was one of a hemorrhagic septicemic process combined with hepatitis.

Cultures made from the organs of these animals showed for the most part a pure culture of S types. There was one exception to this, however. Rabbit 63, which died 23 days after receiving inoculation, yielded one distinct and characteristic R colony on plates made from the liver, while plates made from the other organs revealed only the S types. The R colony thus obtained was, however, characteristic and satisfied the criteria of differentiation.

Thus, it seemed that, in a limited number of cases at least, R to S and S to R mutation of these single cell strains of the avian *Mycobacterium tuberculosis* occurred in the animal body. This confirms the findings of Doan (1931) on non-single cell strains

as regards the former. These tests revealed also that with an identical dose by weight, the S organism kills usually in less time than does the R, but it can by no means be considered that the R types are bereft of pathogenic potentialities. Thomas (1932) has shown that rabbits vary considerably as regards cytological and other constitutional features in their reaction toward the avian and bovine tubercle bacillus. This work would seem to have a bearing on the varying results obtained when inoculations of the R type were made intravenously in rabbits. It seems, however such discrepancies are not so important when the S types are injected, as all of the animals usually succumb after about thirty days.

EXPERIMENTS TO PRODUCE DISSOCIATION OF THE SINGLE CELL R AND S TYPES WITHOUT THE USE OF ANIMALS

No matter how remote the possibility, the objection could be raised that the dissociation of the single cell strains which took place in the animal body (rabbits) might be due to a previously existing infection of the heterologous mutant. To preclude this possibility experiments were undertaken to determine whether or not dissociation could be induced by other means.

For this purpose the single cell strain of the R type avian *M. tuberculosis* was employed.

Effect of reaction of medium

In DeKruif's (1922) pioneer work on bacterial dissociation he found that with *B. lepisepticum* a medium as acid as pH 6.0 retarded the dissociative process, while an alkaline reaction of pH 8.5 often accelerated it. Amoss (1925) reported a pH of 7.8 as the most favorable reaction for the dissociation of pneumococcus while this phenomenon was retarded with an acid medium. Hadley (1927) reports pH 7.8 as the most favorable reaction for dissociating members of the colon-typhoid group. Reed and Rice (1931a) found that a medium of pH 6.5 to 6.8 induced some S to R mutation with a non-virulent bovine *M. tuberculosis*.

Bearing these observations in mind, ten plates each of Petroff's egg medium were prepared at the following hydrogen-ion con-

centrations: pH 5.0, 5.4, 6.0, 6.4, 6.8, 7.0, 7.2. 7.4, 7.8, 8.0 and 8.5. By applying Petroff's filtering technic colonies were induced to grow sufficiently separated so that the morphology of each could be studied when growth occurred. No growth occurred on any of the plates at pH 5.0, and the growth at pH 5.4 was very sparse and insufficient for observation. Fair growth was obtained at pH 6.0 in some of the plates while others showed very scanty colony formation. In our hands the optimal zones extend from pH 6.8 to pH 7.8. In the more alkaline reactions growth was again retarded but a few colonies formed even at pH 8.5. The plates were incubated for a period of twelve weeks, periodic examinations being made as soon as colony formation occurred (three to four weeks with this method) but on none of the plates were S type colonies noted. It may be inferred that alteration of the H-ion concentration within the zones employed did not bring about R to S dissociation with this single cell strain.

Other experiments were then set up whereby suspensions of R organisms were immersed in acid and alkaline fluid media of varying reaction for varying periods and then planted out upon egg plates having a pH of 7.4. No S colonies were induced to form by this process. Plants were also made upon petri dishes of Proskauer and Beck medium containing in addition 1.5 per cent of agar and 10 per cent of sterile inactivated rabbit serum. No R to S conversion took place upon these plates after seven transfers at three-week intervals, the growth on all of the plates being entirely R in character.

Rapid transfer on fluid media rich in normal rabbit serum

Following the work of Soule (1928) and Reed and Rice (1931b) we employed our glycerol broth formula in 300-cc. amounts and also to this added 7 per cent sterile normal inactivated rabbit serum. The single cell R culture was inoculated and transfers made to the same liquid medium at one week intervals. After each transplantation the older culture was plated upon suitable egg medium with the aid of the Petroff filtering technic and examined after growth had taken place for the presence of the S colonies. In these transfers only the youngest, whitest, thinnest

growth membrane was used as inoculum or for planting upon the plates. After the fourth generation, critical examination revealed the presence of a few colonies which were definitely less rough than those on corresponding plates made from cultures taken from slants of Petroff's egg medium. They were small, usually not larger than 1 mm. in diameter, somewhat whiter than the R type, smoother in appearance but still lacking the glistening luster of the typical S form. About the base of these colonies a periphery of irregularly shaped growth occurred which appeared dry and hard. The miscibility of these colonies in normal sodium chloride solution and distilled water was clumpy and R-like in character. When plants from these suspensions were made upon Petroff's egg medium plates, the resultant colony formation was entirely R in character with only a few (less than 1 per cent) simulating the parent form.

After nine generations on the glycerol bouillon rabbit serum medium, the usual 10 plates were inoculated by the Petroff method and on two of these definite S colonies grew up after eight weeks of incubation. Unfortunately no count was made to show the proportion of R colonies to the S colonies thus induced to development. It is certain, however, that the S types comprised considerably less than 1 per cent of the total. Several plate cultures were then made from these S colonies and in each instance a pure culture of the S colony type was obtained. These were entirely characteristic for the organism in the S phase of its mutation cycle according to the criteria already outlined. Two rabbits inoculated intravenously with this second generation S culture in 0.5 mgm. doses died after twenty-six and twenty-eight days respectively, the gross pathology simulating that produced with the single cell strains of the known S type already described.

R to S mutation induced by aging the culture at 37.5°C.

As has already been reported by us (Kahn and Schwarzkopf, 1932) a culture of the so-called mycobacterium of rat leprosy 368, Chapin (1912) was received some years ago from the National Tuberculosis Association. After several transfers on petri plates of Petroff's egg medium it was noted that one of these cultures,

initially R in character, had undergone spontaneous dissociation after having been incubated for several weeks at 37.5°C. Typical round, domed, smooth, moist and glistening S colonies had developed among the erstwhile R colony population. Single cell strains were obtained from both of these colony types and, while these bred true for three or more generations, sooner or later spontaneous dissociation occurred in both instances from these known pure line strains. Typical R colonies grew on plates receiving inoculation with an actual S colony from a single cell S strain while S colonies developed on plates which were seeded with an actual R colony from an R single cell strain. The mutation in the case of this species is somewhat more prolific from S to R but the R to S change may not be termed at all infrequent with this culture of "rat leprae."

The dissociation of these single cell strains took place spontaneously, no especial effort being made to induce it.

It could not be said that starvation was the factor concerned in the initial R to S dissociation of this culture. The colonies on the plate were not crowded and it is logical to assume that there was an excess of food material present. It was felt by us that the aging process must have been involved.

It has been the practice in this laboratory to keep the stock cultures of *M. tuberculosis*, single cell strains and others, on slants of Petroff's egg medium. Three previous generations are kept of each strain and these in duplicate. One set is kept in the incubator while another is placed in the ice box. Transplants are made about every six weeks.

In view of the fact that an aged culture of the mycobacterium of "rat lepra" had shown R to S spontaneous dissociation after long time incubation at 37.5°C., an R culture of the single cell strain of avian *M. tuberculosis* which had been incubated for some eighteen weeks was planted on ten Petroff egg medium plates with the purpose of obtaining widely separated colonies. After six weeks of incubation, one typical S colony developed on one of the plates while four such colonies developed on another plate. Of the remaining eight plates, two were so heavily seeded that the colonies coalesced, making cultural observation impossible, while

on the others only the typical R colony type developed. The S colonies thus dissociated from the R single cell strain by aging were entirely characteristic for the usual S colony type. Subcultures made of these S forms revealed pure culture of the S colony. Two rabbits were inoculated with 0.5 mgm. of an emulsion made of these S colony forms. One of the animals died in twenty days while the other succumbed in twenty-nine days. The picture of the gross pathology was that of the S type of tuberculosis and pure cultures of S colonies were recovered from liver and spleen of both of these animals.

S to R dissociation with single cell S strain

The successful cultivation of these single S cells was, as has already been reported, a singularly time-consuming operation and it was only after a number of attempts that microcolonies developed from the single cell. When these had spread for considerable distance in the microdroplet they were transferred with the micropipette to a slant of Petroff's medium. The growth characteristics of this colony type have been described.

Before any deliberate attempt was made to dissociate this single cell S strain, twenty-five plate cultures on Petroff's egg medium were prepared with the aid of the Petroff filtering technic. When preparing plates by this method with the S forms it is to be borne in mind that, with the more homogeneous suspension which these S organisms form in normal NaCl solution or distilled water, a larger number are filtered through the No. 5 Whatman filter paper, and in order to obtain plates containing widely separated colonies, the filtrate should be diluted to a higher dilution than is necessary for the R types. These plates for the most part contained widely separated colonies although in four of them the seeding was a little too heavy and only part of the plate was useful for observation.

All of these cultures were incubated for a total of ten weeks and periodical examinations were made to determine whether any R type colonies had made their appearance. Such was not the case.

S to R dissociation by animal inoculation

One of the rabbits used for testing the pathogenicity of the single cell S strain (no. 63) died in twenty-three days and although the pathological picture presented was a typical S type of tuberculosis, plates made from the liver of this animal gave rise to two separate and distinct R colony types. These were then emulsified in distilled water and plated out upon ten plates of Petroff's egg medium. The resultant growth yielded only the R colony which satisfied the criteria for the organism in this form.

Three rabbits were given intravenous inoculations of this growth in half milligram doses with the following results:

Rabbit 51 survived 30 days:

Lung: Few small tubercles about 0.5 mm. in diameter.

Spleen: A few minute tubercles peppered about the surface.

No other gross abnormalities could be noted.

Rabbit 47 survived for 67 days when it was killed. The animal lost over 300 grams in weight and the gross pathological picture presented was that of tuberculosis caused by the R type avian *M. tuberculosis.*

Rabbit 48 survived for 32 or 33 days. When the carcass was found it was so badly gas blown that little could be made from the autopsy.

It is apparent that this S to R mutant was variable in its killing properties but this same condition is also apparent with other strains of the R organism. These discrepancies may be due to differences in the constitutional make-up of the animal which Thomas (1932) has unquestionably shown or it may be that in these cases a considerable amount of S antigen is carried over when the S to R mutation takes place, although Rice (1931) has shown with complement fixation tests that the S to R change in bovine *M. tuberculosis* is accomplished by a loss in specific antigen activity. All of their preparations from activated cultures in which the R colony has shown to be predominant, have been shown to be much inferior as antigens to similar preparations made from cultures composed of S types. Yet they find also that antisera prepared from virulent S organisms contain S

specific antibodies in addition to species antibodies which also react with R antigens, and group antibodies detected by preparations made from related acid-fast species. Antisera prepared against R organisms have been found by them to contain species and group antibodies, the former being present in higher concentration than in S antisera.

Reed and Rice (1931) have noted that the S type of a bovine strain with which they worked, proved highly unstable on Petroff's medium. This has also been our experience with the S types of the B-1 bovine strain. In this culture the S to R change takes place with frequency and on some occasions after several transfers and subsequent storage at room or ice box temperature for several weeks, an entire slant, previously seeded with an S colony type, will give rise to only R forms on subsequent replating. The same condition was found to pertain when this strain was seeded upon Lowenstein's medium. The stability of these S bovine cultures may be enhanced, however, if the stock cultures are kept on the ordinary egg yolk media.

With our single cell cultures of the S A-1 avian type, spontaneous S to R changes have not been noted either on plates of Petroff's or Lowenstein's media. We were constantly on the alert for this type of reaction as according to the work of a number of investigators on a variety of organisms such a change is one frequently encountered.

We have been successful in demonstrating the S to R mutation by planting the culture on the medium of Proskauer and Beck or that of Long. After four transfers upon plates of 1.5 per cent agar, into which either of these formulae have been incorporated, some typical R colonies were formed from this known pure line S culture. Petroff medium plates were then made from the parent S culture as a control. These gave rise to only the S type of colony while the R colony types from the medium of Proskauer and Beck which had dissociated from the parent culture upon the fourth transfer revealed only R colonies upon subsequently being plated on Petroff's egg medium.

Attempts to induce dissociation were made by applying fixed suspensions of killed organism to the surface of Petroff's egg

medium plates before inoculations were made with the viable form. These plates were allowed to dry for two days in the incubator and were then seeded with the usual filtering technic. Living R forms were seeded upon plates containing dead S or dead R organisms. No dissociation occurred. Living S forms were inoculated upon plates containing dead S or dead R forms with the same negative result.

SUMMARY AND CONCLUSIONS

Our evidence leads us to believe that the culture offspring of a single mycobacterium of the R or S avian *Mycobacterium tuberculosis* or the R or S mycobacterium of "rat leprosy" will under certain conditions give rise to individuals capable of mutation. In other words, that the potential of dissociation is an inherent property of the single cell of each of these two species, regardless as to whether they are in the S or R phase of their mutation cycle.

The R to S mutation of a single cell R culture of avian M. tuberculosis has been induced by the following:

1. Animal passage.
2. Rapid transfers in glycerol broth containing normal inactivated rabbit serum.
3. Aging the culture in the incubator.

The S to R mutation of a single cell S strain of avian M. tuberculosis has been induced by the following:

1. Animal passage.
2. Rapid transfers on the solid medium of Proskauer and Beck.

These findings confirm the work of Petroff, Doan, ourselves and Reed and Rice with non-single-cell strains and remove the objection that dissociation with this species may be merely a juxtaposition of two separate strains as has been suggested. Single cell strains of the R or S types of the mycobacterium of "rat leprosy" 368 mutate spontaneously in each direction on plates of Petroff egg medium.

These observations substantiate the work of Jordan (1928) on *Bacillus paratyphosus* B, and also the observations of Dawson (1928) on the pneumococcus. Reiman (1925) was able to induce S to R mutation with single cell strains of the pneumococcus but

could not induce the R to S. Amoss (1925) found that a single cell strain of the pneumococcus gave rise to avirulent forms when grown in immune serum broth, bile broth and slightly acid broth. More recently Pinner and Voldrich (1932) have demonstrated the important fact that non-pathogenic *Staphylococcus albus*, *Staphylococcus citreus* and *Staphylococcus roseus* may split off spontaneously from a single cell strain of a pathogenic *Staphylococcus aureus* culture. From the *Staphylococcus albus* strain, which had mutated from the single cell *Staphylococcus aureus* culture, they were able to again induce *Staphylococcus aureus* formation, with the reacquired virulence peculiar to the latter.

In this experiment of Pinner and Voldrich, as well as in the experiments on the pneumococcus alluded to above, the lack or presence of the lethal qualities of the culture seems to have been definitely correlated with type mutations. In our hands the single cell strains of the S or R mycobacterium of rat leprosy 368 were not pathogenic for white rats or rabbits. In these experiments 3 cc. of a heavy emulsion of either R or S types were injected intraperitoneally into the rats and as much as 5 cc. injected intravenously into rabbits without in either instance producing untoward symptoms. These animals were sacrificed after twelve weeks and no lesions were noted which suggested tuberculosis or leprosy of the rodent type.

As far as the pathogenicity of the single cell strains of the R and S types of the mycobacterium of avian tuberculosis is concerned, it may be stated even from our small series of animals, that the R mutant or dissociant is definitely not bereft of tuberculogenic properties. The tuberculosis thus induced has invariably terminated fatally. The average time taken to produce fatal results in rabbits when 0.5 mgm. doses of the R organism were given intravenously was 62.2 days as contrasted to 24.5 days with the S mutant employing identical doses. It cannot be denied, however, that some of the rabbits receiving the R inoculation die well within the time usually taken to produce death with the S mutant. On the other hand, in our series of rabbits receiving 0.5 mgm. doses of the S single cell cultures none of the rabbits have survived as long as some of the animals receiving the R

inoculum. Practically all of the former died within 30 days while some of the animals receiving the R survived 92, 90, 77 and 67 days.

As has been pointed out the type of morbid anatomy produced by the R or S type varies considerably. Animals receiving the R culture, if surviving for sufficient time, show an abundance of macroscopic tubercles in a number of the organs while in the animals dying from tuberculosis induced by injections of the S organism, macroscopic tubercles are seldom noted.

These conditions are found to pertain also to animals receiving S cultures mutated from a single cell R avian M. tuberculosis and R cultures mutated from the S colony type. That is to say, the mutant is in all respects typical for its non dissociated homologue and not for the culture from which it was dissociated.

In interpreting these results we must take into consideration the possibility that the difference in pathogenicity with this species may be more apparent than real. Thomas has unquestionably shown the vast differences in susceptibility of individual rabbits. This may be anticipated, as he has shown, by a detailed study of the cytology of the blood. We have attempted to obviate this difference to some extent by using litter mates in all of our experiments as indicated by the numbers. Doan[1] has shown that a given weight of S culture may show upon count, as many as four times the number of organisms contained in a similar weight of R culture and of course such a condition might account for the difference in pathogenicity, as one set of rabbits would be receiving a much more massive dose than the other. Such, however, has not been the case, as the following work of Doan has clearly shown.

Working with single cell strains of R and S avian M. tuberculosis supplied by us, Doan injected into various laboratory animals 15,000,000 carefully counted organisms from cultures of the R or S type. These cultures were of the same age and also from parent cultures of the same age. The first animal of his series to die was a rooster which had received inoculation of the S culture intravenously. Death occurred 25 days after inoculation. The

[1] Personal communication.

fowl showed marked emaciation, cyanosis and massive microscopic tubercle formation without gross lesions. On sectioning liver, spleen and bone marrow Doan found small discrete tubercles made up of epithelioid cells containing vast numbers of acid fast organisms. The second animal to die in Doan's series was a chicken which had also received intravenous inoculation with the avian S culture. Death occurred on the thirty-fifth day after inoculation. The pathological picture was identical with that of the first chicken. In addition, in both of these chickens, Doan found that the lymphocyte count had fallen to about one per cent while the monocytes were respectively 37 per cent and 66 per cent. There was progressive anemia in both, approximately 2 million red cells at death. Two chickens which received the R organisms at the same time and in the same dosage as in the fowl above mentioned were still alive and without external signs of tuberculosis 71 days after inoculation.

The rabbits in Doan's series survived longer than did ours but this may be accounted for by the difference in the size of the dosage employed, ours being by far the larger. The first of Doan's rabbits died 67 days after inoculation with the counted suspension of S avian *M. tuberculosis*. The cause of death was fulminant, generalized miliary tuberculosis. The spleen was greatly enlarged and weighed 25 grams. One of the R rabbits was killed on the same day for comparison and showed no gross evidence of tuberculosis.

These differences cannot entirely be due to accidental dissimilarity in constitution of the animals. The indication strongly suggests itself that there is also an inherent difference in the pathogenicity of the R and S types of this single cell strain of the avian *M. tuberculosis*. These mutation types also differ in other important characteristics such as in the configuration of the colony, in the electrophoretic mobility velocities, in the miscibility in normal sodium chloride solution or distilled water, in the rate of growth, and, as Richardson, Shorr and Loebel (1931) have shown, in the rate of respiration. Experiments are now under way to determine whether there is also a difference in the life cycle of these mutants of the A-1 strain of avian *M. tuberculosis*.

It is of interest to note that with this species it was not found necessary to add anti-R serum to the liquid medium in order to produce the R to S mutation. Normal serum sufficed. It may well have been, however, that the addition of anti-R serum would have enhanced the process as has been found to be the case with a variety of organisms.

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PLATE 1

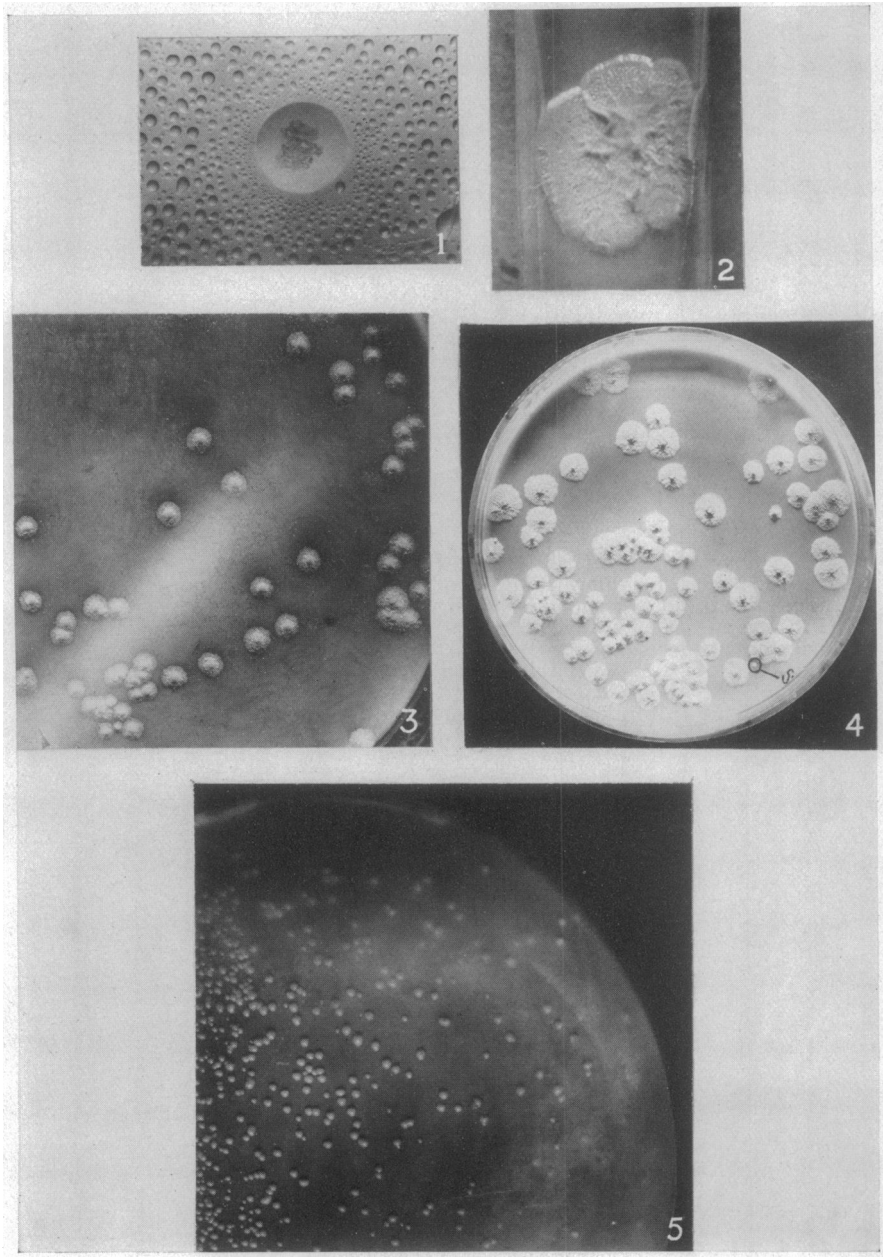
FIG. 1. Microcolony formed from single R Mycobacterium of avian tuberculosis growing in microdroplet of medium.

FIG. 2. Macroscopic colony on slant of Petroff egg medium formed from microcolony in figure 1.

FIG. 3. Pure culture of R colonies on Petroff egg medium plate formed from macroscopic colony in figure 2. Six weeks old.

FIG. 4. Single S colony obtained after injecting single cell R culture intravenously in rabbit. Material for plating obtained from marrow of tibia. Nine weeks old.

FIG. 5. Pure culture of S colonies developing on Petroff egg medium plate. Culture obtained from single S colony in figure 4. Five weeks old.



(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 2

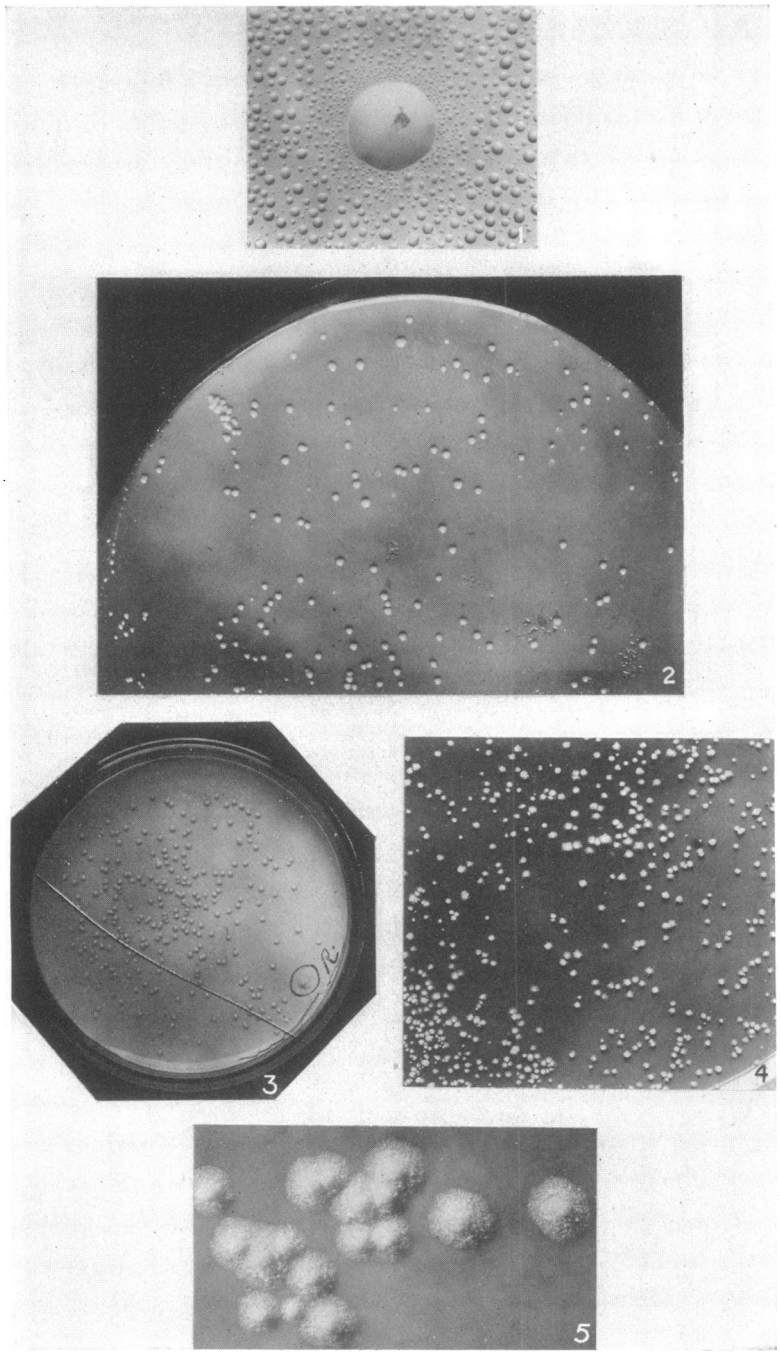
FIG. 1. Microcolony developing from single S Mycobacterium avian tuberculosis growing in microdroplet of medium.

FIG. 2. Pure culture of S colonies developing on Petroff egg medium plate. Culture obtained from microcolony in figure 1.

FIG. 3. Single R colony developing on Petroff egg medium plate after injection into rabbit of S colony suspension from single cell S strain.

FIG. 4. Pure culture of R colonies obtained from single R colony in figure 3.

FIG. 5. R colonies six weeks old (higher power) from single R colony in figure 3.



(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 3

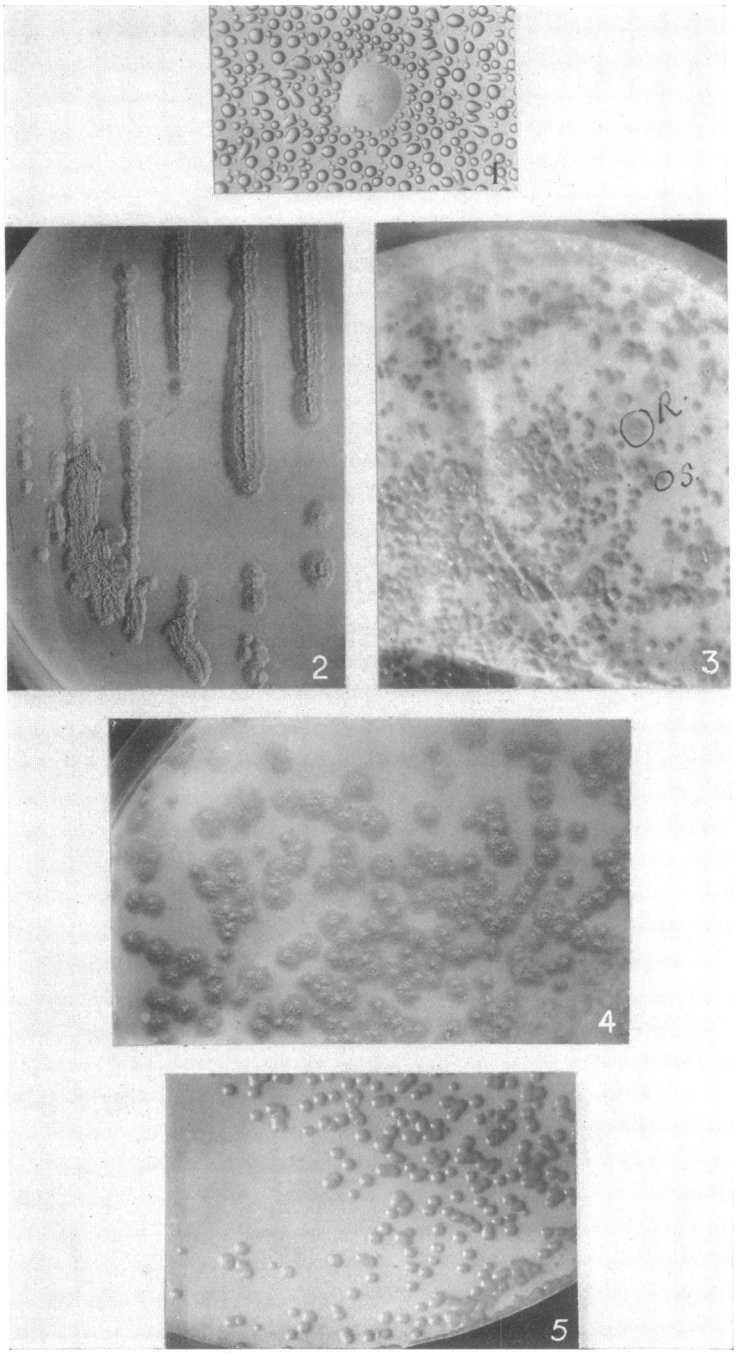
FIG. 1. Microcolony from single R Mycobacterium of rat leprosy developing in microdroplet of medium, slightly out of focus.

FIG. 2. R type of growth obtained from culture of microcolony in figure 1. Three weeks old.

FIG. 3. Spontaneous dissociation Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Three weeks old.

FIG. 4. Pure culture of R type Mycobacterium of rat leprosy obtained from R colony in figure 3.

FIG. 5. Pure culture of S type Mycobacterium of rat leprosy obtained from S colony in figure 3.



(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

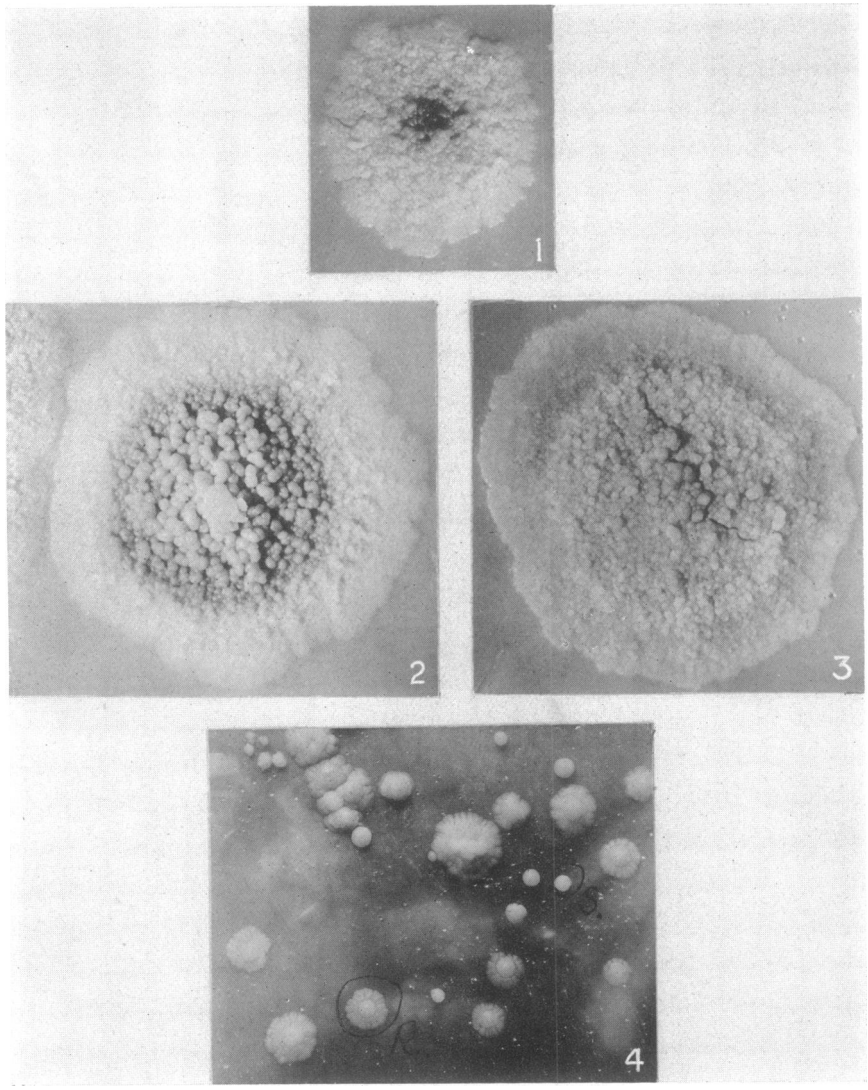
PLATE 4

FIG. 1. Higher magnification R colony Mycobacterium of avian tuberculosis Nine weeks old. Note central orange colored area beginning to develop.

FIG. 2. Higher magnification R colony Mycobacterium of avian tuberculosis Ten weeks old. Note extension of orange colored area toward periphery.

FIG. 3. R colony sixteen weeks old.

FIG. 4. Spontaneous S to R dissociation B-1 strain bovine Mycobacterium tuberculosis occurring on plate of Petroff egg medium. Non-single cell.

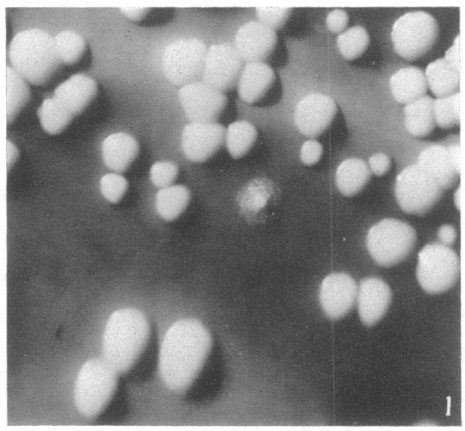


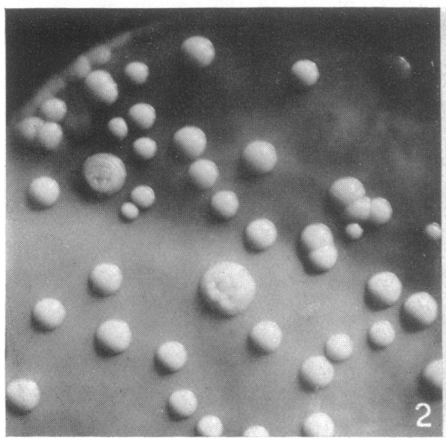
(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

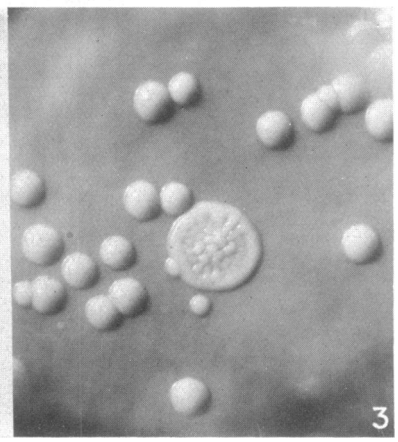
PLATE 5

FIG. 1. R colony developing from single cell S strain Mycobacterium avian tuberculosis after injection into rabbit 63. Material for plating obtained from liver of this animal.

FIGS. 2 AND 3. Intermediate "flat S" or O colony type developing from single cell S strain.







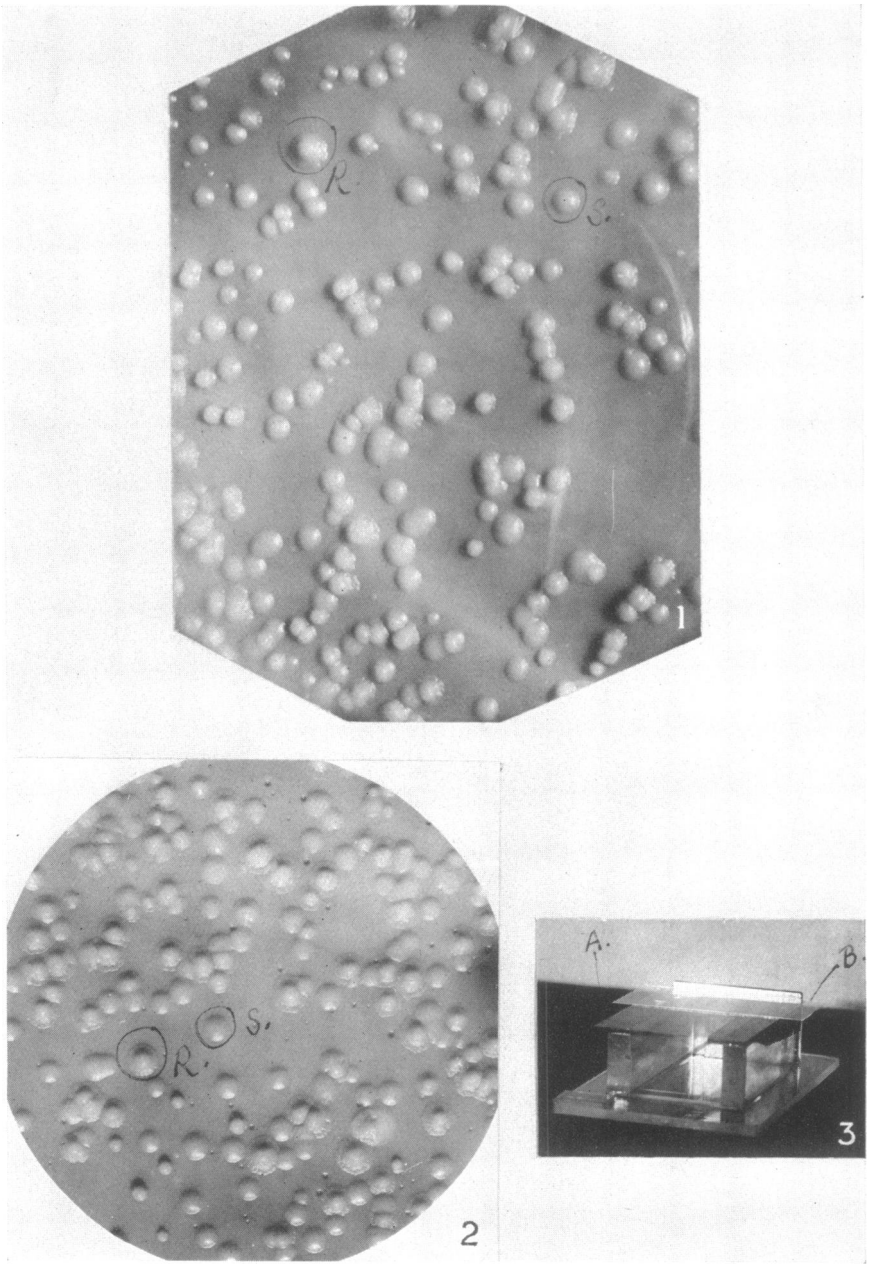
(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 6

FIG. 1. Spontaneous S to R dissociation of Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Single cell S strain employed in seeding this plate. Plate nine days old.

FIG. 2. Spontaneous R to S dissociation of Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Single cell R strain employed in seeding this plate. Plate twelve days old.

FIG. 3. Moisture chamber used for isolating single cell in microcolony culture technic. Showing relative position of mica apron (*a*) and cover slip (*b*).



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