BACTERIAL VARIATION IN CULTURES OF FRIEDLÄNDER'S BACILLUS.

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Plates 36 and 37.

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During the course of studies on the biological and immunological properties of Friedländer's bacillus, at least three sharply defined types were found (1) to exist among different strains of the organism. The types were designated A, B, C, and into one Group, X, were placed several heterogeneous strains. That Friedländer's bacillus, moreover, possesses at least two different cellular constituents both of which are important in the antigenicity of the organism was recorded in later communications (2, 3). One of these is now known to be polysaccharide and is chemically different for each of the fixed types (4, 5). The differential specificity of the types appears to depend on the polysaccharides, a class of substances which are analogous to the soluble specific substance of Pneumococcus, originally described by Dochez and Avery (6). The second substance is protein in nature and regardless of type derivation exhibits the undifferentiated characteristics of the species. To this extent, Friedländer's bacillus possesses an antigenic complex which is analogous to that of Pneumococcus (7) as already described in papers from this laboratory.

It has been pointed out by other investigators (8–14) as well as ourselves that there are certain conditions which induce in Friedländer's bacillus the development of variants, a phenomenon which is now considered as bacterial dissociation.[1] The typical colonies of Friedländer's bacillus are now identified as smooth (S) and the variant colonies as rough (R), the organisms in each instance differing not only morphologically, but biologically and antigenically. The most salient

[1] For an analytic and critical review of the literature on microbic dissociation, the reader is referred to Hadley's monograph in the *J. Infect. Dis.*, 1927, xl, 1.

differences are (2) that the S cells form colonies with a smooth surface; they are virulent, encapsulated, and type-specific; and they produce the soluble specific substance. On the other hand, the R cells form colonies with a rough surface; they are avirulent, capsule-free, and not type-specific, but species-specific; and they do not elaborate the soluble specific substance. Furthermore, sera prepared by immunization with S cells are type-specific and passively protect against fatal infection by bacilli of the same type, while anti-R sera are not type-specific and possess no demonstrable protective properties for infected mice.

Further study has since disclosed that there exist among the R variants of Friedländer's bacillus additional dissociates which present definite differences in morphology and antigenicity. Moreover, observations have been made upon some of the conditions conducive to variation, the possible reversibility of the variants to their antecedent forms, and the occurrence of variants in human infection. The present communication comprises a report of these studies.

That several morphological forms or intermediates exist between the extremes of the R and S varieties of the Friedländer's bacillus has been reported by Toenniessen, Baerthlein, and Hadley. The work of the two former writers antedated the use of the present nomenclature.

EXPERIMENTAL.

Without entering into a discussion as to what constitutes a Friedländer's bacillus, the S strains studied in this investigation were all members of the *Encapsulatus* group as determined by cultural and staining reactions. They were all Gram-negative, encapsulated bacilli, which were virulent for mice and showed great variation in sugar fermentation reactions. The strain "SC" (Type A) which was most studied in the present investigation, was isolated from the blood of a fatal case of pneumonia in man. It fermented without gas, dextrose, sucrose, maltose, mannitol, and lactose.

The R strains employed in the previous study were derived by consecutive transfer of S cells in broth to which was added a concentration of 10 per cent homologous immune serum. Plate cultures were made after each transplant until R colonies were grossly visible. In the earlier studies, the R colonies were not examined for structural differences, but at a later period, however, three different forms of R colonies were recognized.

The characteristic colonies of the S and R variety have been observed with each of the serological types of Friedländer's bacillus. A detailed

study, however, has been made of those derived from Type A only, and the data, unless otherwise stated, refer solely to this type.

I. Forms of Variants Encountered.

In addition to the S variety, three different forms of colonies have been recognized. For the sake of convenience and clarity of expression these three R variants may be designated as R1, R2, and R3. Further than gross appearance the R3 colony was not studied, because it was found only rarely and sporadically in mass cultures of R cells and particularly because it was extremely unstable so that the organisms were never obtained in pure culture. The R3 colony was of the "phantom" description, escaping notice when viewed by transmitted light on account of its marked transparency. By reflected light, with most of the light obscured, it was seen as a transparent colony with a smooth surface and an annular margin which appeared slightly raised and fringed.

(*a*) *Morphological Differences.*—The S colony, examined by transmitted light after 15–18 hours of growth, appears opaque, white, sharply circumscribed, markedly convex, homogeneous, and circular (see Fig. 1). When seeded heavily the colonies coalesce, and this mucoid coalescent growth is typically characteristic of S organisms. By reflected light the surface of the S colony is glistening and smooth and reflects sharply and accurately the image of objects within focal distance. The colony growth is mucoid and tenacious in consistency, and is elastic to the touch of a needle. The S organism is Gram-negative, and encapsulated (Figs. 2 and 3). The rods are short and thick and most commonly occur in either single or in diplo forms.

The R1 colony by transmitted light appears transparent, pale yellow or tan in color with a slightly indented border; it is flat with a distinct central papilla that becomes more prominent with age; it is not homogeneous, and it is more or less circular in shape (see Fig. 1). By reflected light, the surface appears uneven and glossy and reflects images definitely but in a distorted fashion. Due to the manner in which the light is reflected the center of the colony seems to be raised into a small cone. The colony growth is more discrete, never as tenacious as S, and is readily picked with a needle. Stained preparations exhibits a short, slender, almost coccoid bacillus, which is Gram-

negative and unencapsulated. The forms are often so small as to suggest morphologically *B. influenzæ* (Figs. 4 and 5).

The R2 colony by transmitted light appears transparent, pale yellow or tan in color, with wavy margins; it is flat, not homogeneous, but appears matted simulating a tuft of cotton (see Fig. 1). The shape of the colony varies, but in general it is circular. By transmitted light, the surface markings suggest an oyster shell, *i.e.* irregular concentric rings with rough surface, and the reflection of images is always distorted. The colony growth is not confluent, mucoid, or tenacious and is picked readily. Stained preparations show long, slender, Gram-negative bacilli which, in young cultures, often occur in long wavy chains or threads. The organism is not encapsulated (Figs. 6 and 7).

The size of the S and the R colonies varies considerably, but in a general way, the R2 colonies are the largest, the S next in size, and the R1 are the smallest. Morphologically R2 bacillus is the longest while the R1 is the shortest. The S, on the other hand, shows the greatest dimension in breadth.

Figs. 1 to 7 demonstrate the relative differences of S, R1, and R2, both in colony formation and in microscopical appearance.

(*b*) *Biological Differences.*—Culturally, the S and R forms exhibit as striking differences as they do morphologically. The growth of the S cells in fluid media is viscous and accompanied by the production of soluble specific substance. The S form is always encapsulated and of marked virulence for mice, the intraperitoneal injection of 1/10 millionth cc. of a young culture (6–8 hours) causing death within 24–48 hours. In such instances, the peritoneal exudate is viscous, contains relatively few leucocytes which are frequently surrounded by a clear zone separating them from the bacilli, and phagocytosis of the organism has never been observed in normal animals.

The growth of R1 and R2 in fluid media is diffuse, non-viscous, and not accompanied by the elaboration of specific soluble substance. The organisms are not encapsulated and the virulence of both forms is extremely low, since doses as large as 0.5 cc. of a young culture frequently fail to cause fatal infection in mice. The peritoneal exudate following the injection of R1 or R2 is not viscous and contains numerous leucocytes which are able apparently to phagocyte the bacteria.

The fermentation reactions of the three strains were also studied.

The carbohydrates tested were dextrose, lactose, sucrose, maltose, and mannitol. As will be seen in Table I, both the S and the R strains fermented each of the sugars within the first 24 hours of growth, except that in the case of R1, the fermentation of lactose on the two occasions tested did not occur until the 6th day.

(*c*) *Antigenic Differences.*—It has already been shown in a previous communication (3) that S strains of Friedländer's bacillus induce in rabbits the formation of antibodies which agglutinate type-specifically, precipitate the corresponding specific soluble substance, and protect white mice against infection by strains of the same type. Anti-S sera may in addition contain species-specific antibody depending upon

TABLE I.

Biological Reactions of S, R1, and R2 Strains of Friedländer's Bacillus (Type A).*

Strain	Capsule	Specific soluble substance	Virulence	Phagocytosis	Fermentation reactions				
					Lactose	Dextrose	Sucrose	Mannitol	Maltose
S	+	+	+	−	+	+	+	+	+
R1	−	−	−	+	+x	+	+	+	+
R2	−	−	−	+	+	+	+	+	+

* The S strain was isolated from the blood of a fatal case of pneumonia in man.

+ indicates presence.

− indicates absence.

x, fermentation was delayed to 6th day, whereas all the other fermentations occurred within 24 hours.

the duration and intensity of the immunization. Consequently, anti-S sera may cause agglutination of R cells. Anti-R sera, on the other hand, are lacking in antibodies associated with type specificity and protection, and contain only the common group antibody which reacts with R organisms derived from any of the serological types. The R strains employed in these reactions, however, were mass R cultures and represented one colony arising from the continued growth of an S strain in homologous anti-S serum.

The antigenic character of R1 and R2 was correlated with that of the original S strain from which they had arisen. Antisera were prepared by the intravenous injection of rabbits with heat-killed suspensions (2) of S, R1, and R2, respectively. With the resulting immune

sera, it was established that S was agglutinated in anti-S sera, but not in either R1, or R2, antisera, as is brought out in Table II. R1 and R2 were agglutinated in anti-S serum to a slight extent, the reaction appearing granular in contradistinction to the disc reaction occurring with S in anti-S sera (2).

TABLE II.

Cross-Agglutination Reactions with S, R1, and R2 Strains of Friedländer's Bacillus (Type A).

Strain	Immune serum					
	Anti-S		Anti-R1		Anti-R2	
	1:5	1:10	1:5	1:10	1:5	1:10
S	++++	++++	−	−	−	−
R1	++	+	++++	++++	++++	++++
R2	++	+	++++	++++	++++	++++

In this and following tables ++++ indicates complete agglutination with flocculent precipitate and clear supernatant; +++, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; −, no agglutination.

TABLE III.

Cross-Agglutination Reactions with R1 and R2 Strains of Friedländer's Bacillus (Type A).

Immune serum	Strain	Final dilution of serum									
		5	10	20	40	80	160	320	640	1280	2560
Anti-R1	R1	++++	++++	++++	++++	++++	+++	+++	++	+	−
	R2	++++	++++	++++	++++	+++	+++	++	+	−	−
Anti-R2	R1	++++	++++	++++	++++	+++	+++	++	++	+	+
	R2	++++	++++	++++	++++	++++	++++	+++	+++	++	+

The immune serum of R1 was found to agglutinate both R1 and R2 to about the same extent, and conversely R2 antiserum caused an equally good agglutination of both strains. Agglutination was the typical R variety of granular sedimentation which breaks up readily upon agitation. The antigenic similarity evidenced by the agglutina-

tion reaction was further studied by means of agglutinin adsorption. R1 and R2 immune sera were adsorbed with both strains, and then tested for the presence or absence of agglutinins. The results of the experiments can be summarized briefly: Each strain (R1 and R2) adsorbs from the homologous antiserum agglutinins for both homologous and heterologous organisms; from the heterologous serum, however, antibody is removed only for the strain employed in the adsorption. In other words, R1 and R2 possess mutual agglutinating characters, but not complete, mutual adsorptive properties, as determined by reciprocal adsorption. The data of these experiments are presented in Table IV.

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with R1 and R2 Serum after Adsorption with R1 and R2 Strains.

Immune serum	Adsorbed with	Antigen	Final dilution of serum							
			20	40	80	160	320	640	1280	2560
Anti-R1	R1	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−
	R2	R1	++++	++++	++++	+++	++	++	+	−
		R2	−	−	−	−	−	−	−	−
Anti-R2	R1	R1	−	−	−	−	−	−	−	−
		R2	++++	++++	++++	++++	+++	+++	++	+
	R2	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−

As was to be expected from previous results (2) neither R1 nor R2 immune sera caused agglutination of the S strain from which they originated, nor did they cause precipitation of specific soluble substance, nor passively confer immunity upon mice infected with the antecedent S strain.

II. The Reversibility of R to S.

The R strains studied in this investigation have been remarkably stable during the 2 years they have been under observation. Since

the permanency of R becomes of paramount importance when viewed in terms of the problems of infection and epidemiology, experiments were planned to determine the reversibility of R to S. The older literature particularly with other species offers some evidence in favor of reversibility, but the objection has been raised that mass cultures were studied instead of pure line strains. More recently, however, it has been shown unimpeachably that single cell cultures of R may be caused to revert to S under proper cultural conditions. Thus Jordan (15) and Soule (16) showed interconvertibility of *B. paratyphosus* B, Levinthal (17), and Dawson and Avery (18), of Pneumococcus, Soule (19), of *B. subtilis*.

In the present study of reversibility, single cell strains were obtained by the technique of Avery and Leland (20). Since all experiments with pure line strains uniformly failed to bring about reversion, mass R cultures were studied instead, because such cultures might contain individual organisms with greater potentialities for reversion than the single R cells chosen at random. The observations were made with cultures derived from each of the three serological types. The methods adopted for reversion were (1) rapid transfer through meat infusion broth, (2) rapid transfer through dextrose broth, (3) growth in the supernatant culture fluid of the parent S strains, (4) growth in anti-R sera, (5) passage through normal white mice both before and after preliminary transfer through anti-R sera. The greater part of the experiments were carried out before our recognition of the two distinct forms of R variants and the mass cultures studied may have been mixtures of both forms. The results obtained with each method are briefly summarized below.

(*1*) *Rapid Transfer through Meat Infusion Broth.*—One strain each of both mass and single cell R cultures, derived from the three serologically different types, was carried through 90 transfers in meat infusion broth. Transplants were made two or three times daily and from time to time plates were streaked to examine colony formation and the cultures were tested for agglutination by the homologous anti-S serum. The reversion of R to S was not observed by this method.

(*2*) *Rapid Transfer through Dextrose Broth.*—It had been noted earlier in the study that a number of R strains which fermented

dextrose, grew in this medium in conglomerate clumps or masses strongly suggestive of a thread reaction. It is interesting to note in this connection that this phenomenon was never noticed in acid fermentation by S strains. Two mass cultures of R forms derived from a single colony were transplanted once daily in 1 per cent dextrose broth for 35 transfers. By the sixth subculture, clumped growth no longer occurred, although dextrose was still fermented. No evidence, however, was obtained of reversion.

(*3*) *Growth in the Supernatant of the Parent S Strain.*—18 hour broth cultures of S strains were centrifuged and the supernatant was withdrawn and rendered sterile by heating at 56°C. for 30 minutes. This was added in 10 per cent concentration to infusion broth alone and to infusion broth containing 10 per cent anti-R serum. In such media mass R cultures were transplanted twice daily for 90 transfers. At no time during the period of observation was reversion encountered.

(*4*) *Growth in Anti-R Sera.*—Both mass and single cell R strains derived from each of the serological types were carried twice daily through 10 per cent anti-R serum broth for 90 generations. The anti-R serum used in the different experiments was both homologous and heterologous and later, mass cultures of R organisms derived from Type A were carried through 40 transplants in 1 per cent and 5 per cent anti-R serum broth. In the earlier transplants growth always appeared in thread formation, that is, clumped in the bottom of the tube with a clear supernatant fluid. After 20 to 40 or more transplants this reaction disappeared and growth was uniformly diffused. Although in some instances the colony growth seemed to be somewhat less rough, nevertheless, reversion did not occur.

(*5*) *Passage through Normal White Mice.*—Each mass culture from the preceding experiment (*i.e.* after 90 transplants in anti-R serum broth) was passed through normal mice by intraperitoneal injection. As controls, two other R strains—one derived from Type A and the other from Type B—were passed through mice without preliminary growth in anti-R sera. Mice were injected with large amounts of young R cultures and the peritoneal washings reinjected into other normal mice. This was carried out with each strain through a series of 22 mice but in no instance did reversion occur.

(6) *Experiment with R1 and R2.*—The foregoing experiments on the reversion of R to S were carried out as stated with either mass cultures or pure line strains without regard to the particular form of the R variant studied. It seemed possible, however, that the question might now be answered more accurately and completely by a study of the two well defined variant forms R1 and R2. Cultures of each variety, therefore, were transferred twice daily in broth to which had been added in one series 10 per cent homologous immune serum, and in another series 10 per cent heterologous immune serum. The strains were grown in this way for 60 transplants and after 30 to 38 transfers the thread reaction had disappeared. Under these conditions it was possible to induce R1 to change to R2 but reversion of either variant to the S type was not observed.

In summarizing, then, the results of the study of reversion, it may be stated that none of the methods employed, succeeded in bringing R forms back to the S type.

This does not mean, however, that R forms are irreversible, but that under the conditions stated, the methods employed were not adequate to effect the change.

III. Some Incitants to Variation.

(a) *Experimental Derivation of R Forms.*—Mass R cultures may be experimentally derived by the continued subculture of S cells in broth to which has been added homologous immune serum, R organisms gradually appear as the S forms disappear. It is an old observation among earlier workers, however, that Friedländer's bacillus upon aging gives rise to variant colonies which differ strikingly in certain characters, the authors reporting on some or all of the properties of virulence, agglutination, and colony appearance. Our experience corroborates these results and included the isolation of R forms from aged colonies on plates and occasionally from broth cultures stored for several weeks, in which the change has spontaneously occurred.

(b) *Occurrence of R Forms in Disease.*—It is definitely known that R variants may be experimentally derived *in vitro* from S cells. The phenomenon of bacterial dissociation, however, would acquire greater significance if it could be demonstrated that the process actually takes place in the animal body during the course of infection. In order to

study this possibility, a survey was made of strains freshly isolated from a number of different pathological conditions and a careful search was made for the presence of R variants. In all, cultures from seventeen different sources were examined and these included seven cases of human pneumonia, one case of pneumonia in a guinea pig, two of liver abscesses in man, two of acute and fatal abscesses in guinea pigs, two of cystitis in man, one of infected antrum in man, and two cases of infected adenoid tissue. In five instances R forms were iso-

TABLE V.

The Occurrence of R Variants in Infections Associated with Friedländer's Bacillus.

Case No.	Source	Type	Presence of R
1	Sputum, Autopsy } pneumonia	A	Not found
2	Sputum—pneumonia	A	Not found
3	Sputum—pneumonia	A	Not found
4	Sputum—pneumonia	A	Not found
5	Sputum—pneumonia	A	Not found
6	Abscess—guinea pig (fatal)	A	Not found
7	Abscess—guinea pig (fatal)	A	Not found
8	Liver abscess	A	Not found
9	Adenoid tissue	A	Present
10	Adenoid tissue	A	Present
11	Autopsy—pneumonia (guinea pig)	B	Not found
12	Sputum—pneumonia	B	Not found
13	Infected antrum	C	Present
14	Sputum—pneumonia	Group X	Not found
15	Urine—cystitis	Group X	Not found
16	Urine—cystitis	Group X	Present
17	Liver abscess	Group X	Present

Except where stated, the strains were derived from human infections.

lated and in each they were present in mixtures of R and S. Since the occurrence of the two distinct variants, R1 and R2, was recognized only after this survey was completed, it is impossible to state the relative frequency of these two forms. However, of the R strains isolated, two were present with S organisms of Type A, one with those of Type C, and two others in association with S cells of Group X. Interestingly enough, the R strains were found not in acute infections but in chronic conditions. Thus R forms were present twice in cultures from adenoid

tissue, twice in cases of chronic cystitis, and once from a subacute antrum. Suggestive as the data are, no generalization, however, can be made from so few observations. The details of this study are recorded in Table V.

DISCUSSION.

The study of variation in cultures of Friedländer's bacillus reported in the present communication discloses three different forms of R variants. Two of the variants (R1 and R2) have been studied in detail, and they may be recognized grossly by colony formation or microscopcally by the size and arrangement of the individual cells. Moreover, it has been possible to differentiate the dissociates further by serological reactions. Both variants (R1 and R2) are agglutinated in antisera prepared by injection of rabbits with either strain, but they lack the capacity of complete reciprocal agglutinin adsorption. The two R strains are markedly different from their antecedent S strain in colony appearance, morphology, virulence, and antigenicity.

A number of methods have been adopted to induce reversion of R to S. Whether the technique or its application was inadequate, the results were uniformly negative. This does not imply, however, that all R forms of Friedländer's bacillus are irreversible, but that in the case of the strains studied, the proper stimulus was not supplied by the methods used. In this connection the work of Dawson and Avery (21) offers an interesting comparison. They found one R strain of Type I pneumococcus irreversible by the identical methods which caused other R strains of the same and different types to change to the S form. In the present study, R2 has been converted to R1, while, on the other hand, R1 itself has remained unchanged following numerous transplants in homologous immune serum. Conversion of R2 to R1 and the less rough appearance of R1 colonies make it not unlikely that R1 is an intermediary form between S and R2.

The spontaneous development of R variants in S cultures of Friedländer's bacillus has been found to accompany the process of aging. Growth in immune sera *in vitro* also converts the S cells into R forms. That variation, however, is more than an *in vitro* or cultural degradation gains support from the fact that R forms have been found in cultures taken directly from foci of infection in the animal body caused

by Friedländer's bacillus. It is an interesting observation that in the cases studied R variants were found only in chronic infections and always in conjunction with S forms.

CONCLUSIONS.

1. Under proper conditions mass R cultures of Friedländer's bacillus may give rise to a number of variants which are dissimilar in colony appearance and morphology. Three such forms have been described. In two varieties, differences have been observed not only in colony formation and morphology, but also in cultural and antigenic characters.

2. None of the methods employed were adequate to cause reversion of any of the R variants to the S type. Growth of the R2 variant in its own antiserum, however, induced a change to the R1 form.

3. R forms of Friedländer's bacillus may be derived from S strains by aging or by growth in anti-S serum of the homologous type.

4. R strains may be isolated in culture directly from infection. In the cases where R forms were found, S cells were also present, and the pathological condition was of a chronic nature.

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EXPLANATION OF PLATES.

PLATE 36.

FIG. 1. Plate culture of Type A, Friedländer's bacillus, taken by transmitted light. The S colony and the two variant forms, R1 and R2, are labelled. Note opacity of S and transparency of both R varieties.

FIG. 2. Smear of peritoneal exudate of mouse infected with S. Stained with Gram, × 1000. Note absence of leucocytes and presence of large capsules.

FIG. 3. Smear of S grown on agar. Gram stain, × 1000. Capsule is greatly diminished.

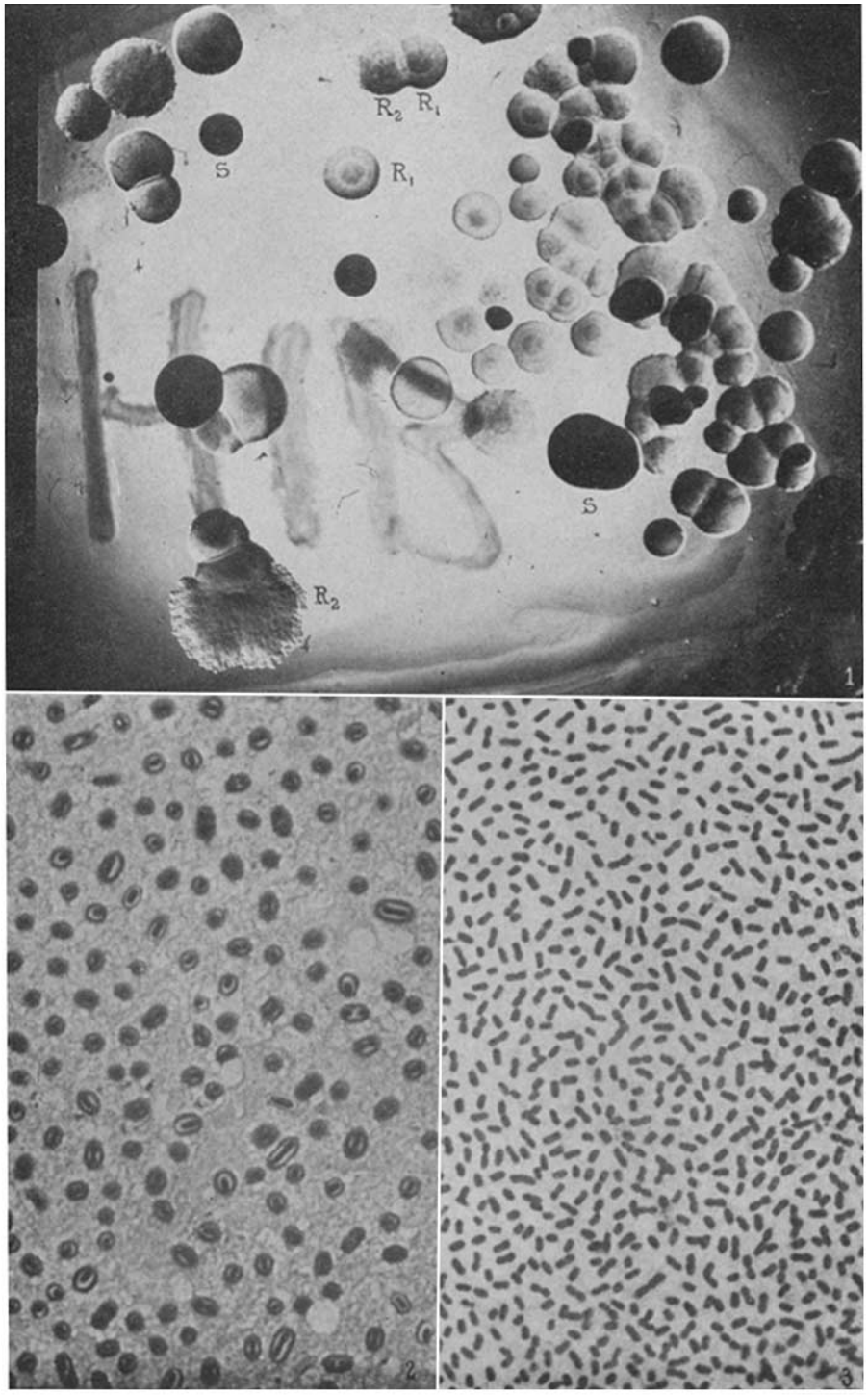
PLATE 37.

FIG. 4. Smear of peritoneal exudate of mouse injected with R1. Stained with Gram, × 1000. Note phagocytosis and lack of capsules.

FIG. 5. Smear of R1 grown on agar. Gram stain, × 1000. Note size and arrangement as contrasted with S and lack of capsules.

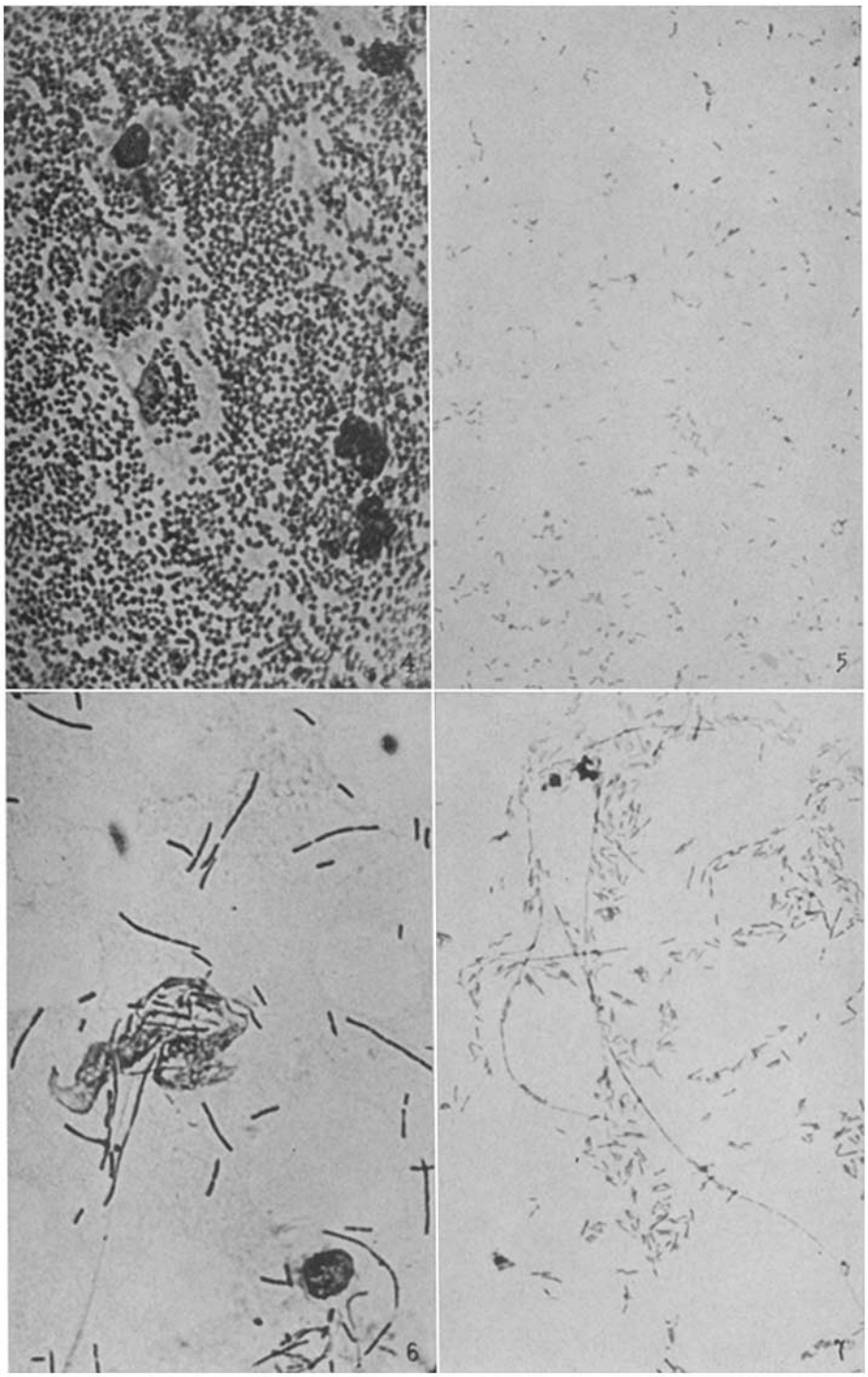
FIG. 6. Smear of peritoneal exudate of mouse injected with R2. Stained with Gram, × 1000. Note phagocytosis, lack of capsules, and length of rods.

FIG. 7 Smear of R2 grown on agar. Gram stain, × 1000. Note size and arrangement and lack of capsule.



Photographed by Louis Schmidt.

(Julianelle: Cultures of Friedländer's bacillus.)



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