BACTERIOLOGY OF LEPROSY. IV. INFLUENCE OF ENVIRONMENT ON THE PHENOMENON OF ACID-FASTNESS

By A. J. Salle
and J. R. Moser
From the Department of Bacteriology, University of California Berkeley, California

Many attempts have been made to cultivate the true etiological agent of leprosy since Hansen, in 1874, announced the presence of rod-shaped organisms in the lesions of patients suffering from the disease. A large number of organisms have been isolated from lesions in various stages of the disease. However, it is doubtful if the true leprosy bacillus has been cultivated, because it appears that no two workers have isolated the same organism in culture.

A large number of cultivation experiments have been reported. Some of the cultured organisms were acid-fast, while others had both acid-resistant and acid-sensitive stages in their growth. That the tinctorial characteristics of these organisms are decidedly unstable is demonstrated by the facility with which they change from the acid-sensitive to the acid-resistant state. No attempt will be made here to cover the literature on the etiology of leprosy. This has already been reviewed in several excellent papers, among them Wolbach and Honeij (6), Walker (5), Soule and McKinley (4) and McKinley (1).

In a previous communication (3) were reported results of efforts to isolate acid-fast organisms from human and rat leprous lesions by means of embryonic chick tissue cultures. The organisms cultivated were, for the most part, acid-fast in embryonic chick tissues cultures and in minced chick embryo medium, but acid-sensitive on the usual laboratory media. The best growth of acid-fast organisms occurred in from 24 to 48 hours. Usually there were present as many acid-fast as acid-sensitive forms, but after this period of incubation the acid-fast rods gradually disappeared and on or about the tenth day the cultures were almost or entirely nonacid-fast. On transferring the cultures to fresh tissue cultures or minced embryo medium the above picture was repeated. The organisms easily dissociated into the rough and smooth variants on the usual laboratory media. The

cultures obtained from human and rat lesions were morphologically and physiologically identical. It was, therefore, concluded that human and rat leprosy are caused by the same etiological agent.

The purpose of the present paper is to investigate some of the environmental factors playing a part in the phenomenon of acid-fastness and acid-sensitiveness. The organisms used were those referred to above, recently isolated by one of us (Salle) from human and rat leprous lesions.

EXPERIMENTS

1. GROWTH OF ACID-FAST ORGANISMS IN VARIOUS LABORATORY MEDIA

A large number of media were inoculated with the rough and smooth variants of the organisms to determine whether or not living cells were necessary to produce acid-fast organisms. The media used included glycerin veal agar, Petroff's coagulated egg, rabbit blood agar, serum glucose agar, Loeffler's coagulated serum, beef heart broth, brain broth and beef infusion broth.

Growth on these substrates was excellent, becoming evident in from 48 to 72 hours. The morphology of the organisms was similar to that reported by Salle (3) for the nonacid-fast phase of the organisms; they consisted of slender, unbranched, slightly granular diptheroids. In no case was there noted any tendency to resist decolorization with 5 percent or 10 percent sulphuric or by 95 percent ethyl alcohol. When cultivated on synthetic media of varying composition and on media containing phenol or lithium chloride, the organisms showed the same inability to retain the carbol-fuchsin stain. It may, therefore, be concluded that the above organisms do not show an acid-fast phase when grown on lifeless media.

2. GROWTH OF VARIOUS ORGANISMS IN MINCED CHICK EMBRYO MEDIUM

In order to determine whether or not the acid-fast phase is characteristic of bacteria in general when inoculated into minced embryo medium, organisms representing several genera were tested.

The medium consisted of minced chick embryos (9 to 12 days) 1 part, and Tyrode solution 5 parts. The embryos were decapitated, minced in a tissue grinder, and mixed with the Tyrode solution. The heads were removed (2) because the pigment granules present in the eyes may lead to confusion in examining smears. The medium was measured into test tubes (about 3 cc. per tube) after which it was ready for use. For the neisseria and the streptococci, a small amount of normal rabbit serum was added.

Zeihl-Neelsen and Gram stains were used. In the former method the smears were stained in cold carbol-fuchsin for 30 minutes, decolorized with 10 percent sulphuric acid, and counterstained with Loeffler's methylene blue. In every case the smears were prepared from the pellicles to avoid the possibility of artefact from the tissue elements.

The results observed after 72 hours incubation are recorded in Table 1. Stains made at that time and after one week showed no difference. As controls the same organisms were inoculated into beef-heart and infusion broths; in no case were any acid-fast organisms seen in them. With the exception of the yeast *Saccharomyces cerevisiae* and the mold-like organism *Actinomyces violaceus*, none of the organisms retained the fuchsin stain when treated with 10 percent sulphuric acid; the true bacteria were acid-sensitive. The ability to retain the stain in minced chick embryo medium is not a phenomenon characteristic of the true bacteria (Eubacteriales), but is restricted to organisms of other orders (Actinomycetales, etc.).

TABLE 1.—*Growth and staining reactions of various organisms inoculated into minced chick embryo medium.*

Organism	Growth	Gram stain	Acid-fast stain	Morphology
Escherichia coli	good	—	—	No change
Eberthella typhi	good	—	—	No change
Eberthella dysenteriae (Shiga)	good	—	—	No change [a]
Eberthella paradysenteriae (Flexner)	good	—	—	No change [a]
Bacillus subtilis	poor	+	—	No change [b]
Bacillus subtilis, (dissociating strain)	poor	+	—	No change [b]
Bacillus anthracis	poor	+	—	No change [b]
Corynebacterium diphtheriae (No. 8)	good	+	—	Very granular
Corynebacterium xerosis	good	+	—	Very granular
Corynebacterium pseudo-diphtheriticum	good	+	—	Very granular
Staphylococcus aureus	good	+	—	No change
Rhodococcus roseus	good	—	—	No change
Clostridium botulinum (Type A)	good	+	—	No change
Clostridium welchii (Type I)	good	+	—	No change [b]
Clostridium tetani	good	+	—	No change [b]
Streptococcus pyogenes (hemolytic)	good	+	—	
Streptococcus mitior (viridans)	good	+	—	
Neisseria intracellularis	good	—		
Hemophilus influenzae	none		—	
Sporotrichum schenki	good	+		
Monilia krusei	good	+		
Epidermophyton cruris	good	+		
Acladium castellani	good	+		
Blastomyces hominis	good	+		
Actinomyces violaceum	good	+	+[c]	No change
Actinomyces sp. (from air)	good	+	—	No change
Aspergillus niger	good	+	—	No change
Penicillium glaucum	good	+	—	No change
Saccharomyces cerevisiae	good	+	++[d]	No change

[a] Pellicle formed. [b] Few spores. [c] Few acid-fast bacilli. [d] Many acid-fast bacilli.

There is no component of minced chick embryo medium which will universally impart to microorganisms the ability to retain the carbol-fuchsin stain. The property of acid-fastness is an inherent characteristic of the organism, which may be enhanced but not induced by the embryo medium.

TABLE 2.—*Growth and staining reactions of an acid-fast organism inoculated into minced adult animal tissues.*

Culture	Lung	Spleen	Liver	Kidney
RABBIT TISSUES:				
H/S Human, smooth variant	Few acid-fast organisms	Few acid-fast forms, single and in clusters	Many acid-fast clusters, some nonacid-fast	Very many acid-fast forms, single and in clusters
H/R Human, rough variant	Fewer acid-fast forms than in H/S	Very few acid-fast forms	Very few acid-fast forms	Many acid-fast forms, mostly in clusters
R/S Rat, smooth variant	Many acid-fast organisms	Very few acid-fast forms	Many acid-fast forms, single and in clusters; larger and more pleomorphic	Very many acid-fast forms, single and in clusters
R/R Rat, rough variant	Very few acid-fast forms	Many large, pleomorphic acid-fast forms	Few acid-fast forms	Many acid-fast forms, mostly in clusters
GUINEA-PIG TISSUES:				
H/S Human, smooth variant	Very many acid-fast forms	Many acid-fast forms, in clusters	Very many acid-fast forms	Very many acid-fast forms
H/R Human, rough variant	Few acid-fast forms	Many acid-fast forms	Many acid-fast forms	Few acid-fast forms
R/S Rat, smooth variant	Same as H/S	Same as H/S	Same as H/S	Same as H/S
R/R Rat, rough variant	Same as H/R	Same as H/R	Same as H/R	Same as H/R

3. GROWTH OF AN ACID-FAST ORGANISM IN MINCED EMBRYONIC TISSUES

In this experiment rabbit, guinea-pig and rat embryos were removed aseptically and minced in tissue grinders. The ages of the embryos were 21, 15 and 10 days, respectively. The media were prepared in the same manner as given above.

The results were the same as those obtained when the chick embryo medium was used. Acid-fast organisms predominated in young cultures. After the second day the acid-fast forms gradually gave way to the acid-sensitive ones. It may be concluded that mammalian embryonic tissues are just as effective as avian cells for the development of acid-resistant forms of the organisms studied.

4. GROWTH OF AN ACID-FAST ORGANISM IN MINCED ADULT TISSUES

The question that naturally arose at this point was whether or not the organisms in question became acid-fast when inoculated into tissues obtained from laboratory animals.

Organs from full grown rabbits, guinea-pigs and rats were removed aseptically, minced, and suspended in Tyrode solution in the same proportion as given above. The media were inoculated and examined at the end of 48 hours incubation.

The results are given in Table 2. Growth occurred within 48 hours in all of the tubes, with the exception of those containing minced rat tissues, which gave much poorer growths than those from the other animals. Only a few acid-resistant forms were observed, when any, in these poor growths. In the growths in minced guinea-pig and rabbit tissues the numbers of acid-fast organisms present were even greater than when minced chick medium was employed, except with lung tissue, in which growth was not good.

There was no apparent difference in the morphology of the organisms growing in the different media, with the exception of the smooth variant isolated from a rat and inoculated into minced rabbit liver. In that instance a large number of the organisms were greatly increased in size and assumed a variety of shapes. Some had large club-shaped terminations; others were pronouncedly curved, and many "Y" forms were seen. In general, more acid-fast forms appeared in media prepared from adult animal organs than in the chick embryo preparations. As the cultures aged the same progression to "coccoid" forms and the concurrent diminution of acid-resistant organisms was noted. After about five days of incubation the organisms reverted to the acid-sensitive types.

The results show that living embryonic tissue was not necessary for the development of the acid-fast forms; minced dead sterile tissues obtained from adult rabbits and guinea-pigs were equally good.

5. EFFECT OF HEATED TISSUE MEDIA ON ACID-FAST ORGANISMS

Minced chick embryos and kidney, liver and spleen tissue from an adult rabbit were each suspended in Tyrode solution in the same proportion as in previous experiments. One lot of each of the preparations was heated to 60° C. for 30 minutes; the other was autoclaved at 20 pounds pressure (126° C.) for 30 minutes.

The organisms cultivated in the heated preparations exhibited the same morphological and tinctorial pictures as those grown in the unheated media. This experiment demonstrated that the phenomenon of acid-fastness was not due to the living condition of the tissues, dead cellular material being equally as effective. Therefore, the ability of the minced tissues to support the growth of acid-fast organisms must be due to the presence of a constituent not affected by a temperature of at least 126° C. for 30 minutes.

6. EFFECT OF REMOVAL OF CELLULAR ELEMENTS ON ACID ORGANISMS

Minced chick embryo and kidney, liver and spleen tissues from an adult rabbit were each suspended in Tyrode solution as before. The media were divided into four lots. (*a*) The first lot was centrifugalized at 3,500 R.P.M. for 45 minutes. The supernatant liquid was carefully removed and distributed into test tubes. This procedure removed practically all of the cells from the supernatant fluid. (*b*) The second lot was filtered through a Berkefeld N cylinder. (*c*) The third lot was passed through a Chamberland L3 filter. (*d*) The fourth lot served as a control. The tubes were inoculated, incubated for 48 hours, and examined.

The centrifugalized supernatant fluid and the filtrates from the Berkefeld and the Chamberland filters showed the presence of numerous acid-fast forms. This experiment indicated that the acid-fast property was due to some substance or substances present in tissue extracts, not destroyed by heat or removed by filtration.

7. EFFECT OF BLOOD CONSTITUENTS ON ACID-FAST ORGANISMS

The organisms were acid-sensitive on coagulated serum and on blood agar. Because of the presence of appreciable amounts of blood in the minced tissue media it was decided to perform additional experiments with blood.

Rabbit blood was defibrinated and centrifugalized, and the serum was removed. The red cells were washed five times with Tyrode solution to remove as much of the serum as possible. The red-cell suspension was prepared by mixing one part of cells with five parts of Tyrode solution. The serum solution was prepared by dissolving one part of serum in five parts of the same solution.

Growth of the organisms in both media was very good. Numerous acid-fast forms were present in the serum medium after 48 to 72 hours of incubation. On the other hand only acid-sensitive forms were observed in the red cell medium.

The serum suspension, when heated to 100° C. or autoclaved and then filtered, gave the same results. It is believed that the

presence of blood serum in the various minced tissues was responsible for the phenomenon of acid-fastness in those cultures. Guinea-pig rat and human blood sera produced similar results. Blood agar failed to show the presence of acid-fast forms, probably because the organisms were not bathed by the serum.

When rabbit serum was added in 10 percent concentration to beef heart broth, brain medium and infusion broth and then inoculated, many acid-resistant forms were observed in 48 hours. This also demonstrated that some factor present in serum was responsible for the appearance of the acid-resistant phase of the organisms.

8. EFFECT OF SERUM CONSTITUENTS ON ACID-FASTNESS

The characteristic of acid-fastness is due to the high content of lipoidal material surrounding the organisms. For this reason the two principal lipoidal constituents of serum, namely, cholesterol and lecithin, were added to various culture media in a concentration of 0.5 percent. The preparations were inoculated and examined after 48 hours. The results are given in Table 3.

The results showed that beef heart, brain and infusion broth media containing 0.5 percent cholesterol yielded as many acid-fast organisms as the minced chick embryo mixture. On the other hand, the same media with the addition of lecithin instead of cholesterol showed very few, if any, acid-resistant forms. The synthetic medium containing both cholesterol and lecithin also failed to produce a growth of the acid-fast rods.

It may be concluded that one of the factors involved in the phenomenon of acid-fastness is the lipoidal alcohol cholesterol. It is not the only factor, however, because no acid-fast rods were observed in the synthetic medium with cholesterol. Also no such forms were found in brain medium alone. Brain tissue is relatively rich in fatty substances, including cholesterol.

9. GROWTH OF VARIOUS ORGANISMS IN CHOLESTEROLIZED BEEF HEART MEDIUM

Beef heart medium containing 0.5 percent cholesterol was inoculated with the same organisms used in the above experiments. Control tests were made in beef heart and beef infusion broth without cholesterol. Smears were prepared after an incubation period of 72 hours. The results are given in Table 4.

The results are identical with those obtained in Study II. With the exception of *Saccharomyces cerevisiae* and *Actinomyces violaceum*, none of the organisms was acid-resistant. It can again be stated that the ability of an organism to retain the acid-fast stain is not

TABLE 3.—*The effect of some serum constituents on the acid-fast property of the microorganisms isolated from human and rat leprosy.*

Medium	Cultures used			
	Human, rough	Human, smooth	Rat, rough	Rat, smooth
Beef heart with cholesterol	Many acid-fast organisms	Very many acid-fast organisms	Same as human, rough	Same as human, smooth
Brain broth with cholesterol	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth
Infusion broth with cholesterol	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth
Synthetic medium with cholesterol	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth
Beef heart with lecithin	Very few acid-fast organisms	Several acid-fast organisms	Same as human, rough	Same as human, smooth
Brain broth with lecithin	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth
Infusion broth with lecithin	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth
Synthetic medium with lecithin	No acid-fast organisms	No acid-fast organisms	Same as human, rough	Same as human, smooth

a phenomenon characteristic of the true bacteria (Eubacteriales) but restricted so far to organisms grouped into other orders (Actinomycetales, etc.). There is no component or components in either

TABLE 4.—*Growth and staining reactions of various organisms inoculated into cholesterinized beef heart medium.*

Organism	Growth	Gram stain	Acid-fast stain	Morphology
Escherichia coli	good	—	—	No change
Eberthella typhi	good	—	—	No change
Eberthella dysenteriae (Shiga)	good	—	—	No change
Eberthella paradysenteriae (Flexner)	good	—	—	No change
Bacillus subtilis	good	+	—	No change
Bacillus subtilis, (dissociating strain)	good	+	—	No change
Bacillus anthracis	good	+	—	No change
Corynebacterium diphtheriae (No. 8)	good	+	—	No change
Corynebacterium xerosis	good	+	—	No change
Corynebacterium pseudo-diphtheriticum	good	+	—	No change
Staphylococcus aureus	good	+	—	No change
Rhodococcus roseus	good	+	—	No change
Clostridium botulinum (Type A)	good	+	—	No change
Clostridium welchii (Type I)	good	—	—	No change
Clostridium tetani	good	+	—	No change
Streptococcus pyogenes (hemolytic)	good	+	—	No change
Streptococcus mitior (viridans)	good	+	—	No change
Neisseria intracellularis	good	—	—	No change
Hemophilus influenzae	—	—	—	—
Sporotrichum schenki	good	+	—	No change
Monilia krusei	good	+	—	No change
Epidermophyton cruris	good	+	—	No change
Acladium castellani	good	+	—	No change
Blastomyces hominis	good	+	—	No change
Actinomyces violaceum	good	+	+	No change
Actinomyces sp. (from air)	good	+	—	No change
Aspergillus niger	good	+	—	No change
Penicillium glaucum	good	+	—	No change

minced embryo or cholesterolized beef heart medium that will universally impart acid-fastness to microorganisms.

DISCUSSION

In a previous communication (3) one of us (Salle) reported the isolation of organisms from human and rat leprous lesions by the tissue culture method. The organisms were acid-fast in such cultures and in minced embryo medium but entirely acid-sensitive on the usual laboratory media. The organisms from both sources revealed the same morphological and physiological characteristics. Therefore, it was concluded that human and rat leprosy are caused by the same etiological agent. The best growth of acid-fast organisms occurred in from 24 to 48 hours. There were usually present as many acid-fast as acid-sensitive organisms. After this period of incubation the acid-fast rods gradually disappeared and on or about

the tenth day the organisms were all, or almost all, acid-sensitive. On transferring the growth to fresh tissue cultures or minced embryo medium the process was repeated.

In order to determine whether or not the acid-fast phase is characteristic of organisms in general when inoculated into minced embryo medium twenty-eight organisms were tested. The results showed that, with the exception of the yeast *Saccharomyces cerevisiae* and the mold-like organism *Actinomycetes violaceum*, none of the organisms employed retained the fuchsin stain when stained by the Ziehl-Neelsen method. The ability of an organism to retain the stain after growth in minced chick embryo medium is not a phenomenon characteristic of the true bacteria (Eubacteriales), but is restricted to organisms of other orders (Actinomycetales, etc.) It is evident that the property of assuming an acid-resistant phase under the influence of various environmental factors is an inherent characteristic which is enhanced but not acquired by contact with minced chick embryonic tissue.

Minced rabbit, guinea-pig and rat embryo media also showed the presence of many acid-fast organisms; the growth of such forms was not limited to chick embryonic tissue alone. Minced organs from adult rabbits, guinea-pigs and rats also showed the presence of numerous acid-resistant organisms. In general, more forms of that kind appeared in such media than in the minced embryo preparations. Living embryonic tissue was not necessary for the development of the acid-fast forms; dead organs from adult animals functioned equally as well.

Heat and filtration had no effect on the property of minced embryonic and adult tissues of producing acid-fastness. This indicated that the substances responsible for this phenomenon are not injured by heat or removed by filtration. Also, the results were not due to the presence of either living or dead tissue cells.

Acid-fast forms were produced in rabbit and guinea-pig serum diluted with Tyrode solution. When serum was added to several laboratory media, acid-fast organisms were produced in abundance. Some factor or factors present in serum was responsible for the tinctorial characteristic in question. One of these factors is the lipoidal alcohol cholesterol. It is not the only factor, however, because no acid-fast rods were observed in a synthetic medium with cholesterol; also, no such rods were formed in brain medium without added cholesterol, in spite of the fact that brain tissue is relatively rich in fatty substances, including cholesterol.

Beef heart medium containing cholesterol was not successful in producing acid-fast forms of organisms that ordinarily are acid-sensitive. With the exception of *Saccharomyces cerevisiae* and *Actinomyces violaceum*, none of the organisms were acid-resistant when grown in this medium. The true bacteria, or Eubacteriales, were acid-sensitive. There are no constituents in either minced embryo or cholesterolized beef heart medium which will impart to such microorganisms the ability to retain the carbol-fuchsin stain. That property may be enhanced but not induced by the medium.

SUMMARY

1. Four nonacid-fast diphtheroids isolated from human nodules and rat granuloma are acid-fast in tissue culture and in minced embryo medium but not on the usual laboratory media.

2. Minced liver, kidney and spleen of adult rabbits and guinea-pigs served as satisfactory substitutes for minced chick embryo medium for the production of acid-fast forms.

3. The property of adult and embryonic tissues to induce the acid-fast stage of the diphtheroids is not destroyed by heating or removed by filtration. Therefore, neither living nor dead tissue is necessary for the production of acid-fast forms.

4. Acid-fast forms can be produced in serum.

5. The organisms are capable of assuming the acid-fast stage in a medium composed of beef heart and cholesterol.

6. It is concluded that for the production of acid-fast forms from these organisms two factors are essential: (*a*) The medium must be of such composition as to foster the growth of the organisms to the stage at which they are capable of becoming acid-fast. (*b*) Cholesterol or some other substance must be supplied in the medium when this stage is reached.

7. A change in the lipoid metabolism of these organisms is believed to take place concurrently with the change in morphology as followed by microscopic observation.

8. A group of microorganisms of the order Eubacteriales inoculated into minced tissue medium and also into 0.5 percent cholesterol beef heart medium did not show any acid-fast forms.

9. The phenomenon of acid-fastness appears to be restricted to members of the order Actinomycetales.

REFERENCES

(1) McKinley, E. B. Medicine **13** (1934) 377.
(2) Rivers, T. M. Jour. Exp. Med.

(3) SALLE, A. J. Jour. Inf. Dis. **54** (1934) 347.
(4) SOULE, M. H. and MCKINLEY, E. B. American Jour. Trop. Med. **12** (1932) 1.
(5) WALKER, E. L. Jour. Prev. Med. **3** (1929) 167.
(6) WOLBACH, S. B. and HONEIJ, S. B. Jour. Med. Res. **29** (1913) 367.