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THE DUAL NATURE OF ACID-FASTNESS**

Acid-fastness is usually defined by the Ziehl-Neelsen technique: staining in carbol-fuchsin, differentiation in HCl-alcohol, and counterstaining in methylene blue.[8] As a result, acid-fast substances are red, non-acid-fast materials, blue. Some authors have felt that this property of differential staining resided in one specific type of compound, the mycolic acid type of long-chain fatty acid found only in the acid-fast mycobacteria.[11] Most authorities, however, now consider that the bulk of the evidence supports a structural rather than a chemical explanation of the phenomenon. It is certainly true that grinding[16] or crushing[1] tubercle bacilli abolishes their acid-fastness although no change in chemical composition may be detected. Similar, but not identical, acid-fastness has been produced in a number of usually non-acid-fast bacteria by coating them with lipid not in itself acid-fast.[7] Spores of bacteria are usually acid-fast[9] so long as their protective capsule is present. Finally, mycolic acid, the presumed acid-fast compound in tubercle bacilli, was re-examined[19, 21] and found to be somewhat acid-fast, but not strongly enough to account for the brilliant staining of intact bacilli.

It has been recently noted[2] that under certain conditions spermatozoa are acid-fast. When an explanation of this observation was sought, certain contradictions in the basic theory became manifest. The original report of acid-fastness was by Neisser.[17] He reported that lepra bacilli, once stained, were resistant to acid destaining. This was in contrast to the ease with which other known bacteria were destained. A year later Ehrlich[10] noted a similar property in tubercle bacilli. In these latter organisms, this resistance to destaining was coupled with a resistance to staining and to chemical treatment generally.[10, 15] Out of this generalized resistance of tubercle bacilli has grown the structural theory of acid-fastness.[21] However, neither lepra bacilli[14] nor spermatozoa showed any staining resistance comparable to tubercle bacilli. It therefore appeared questionable whether the structural theory of acid-fastness as presently developed was completely valid. The object of this present paper then is to study both the differences and simi-

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larities of the acid-fast reaction in various cells and compounds, especially as the results may bear on the theory of the staining reaction.

MATERIALS

This study was made possible by the generosity of those who provided the spectrum of acid-fast materials to be tested. With the sources in parentheses, these were:

Formalin-fixed, paraffin-embedded tissue sections containing *M. tuberculosis, var. hominis,* and spermatozoa (surgical and autopsy services, Department of Pathology); *M. tuberculosis (BCG)* in guinea pig tissue and colonies of *M. ranae* (M. I. Bunting and J. Banfield, Department of Microbiology); tissues containing *M. leprae* (sent originally from Dr. G. L. Fite, U.S.P.H.S. to F. A. Putt of this department); rat liver containing *M. leprae murium* (Dr. H. S. N. Greene, Department of Pathology); mycolic and leprosinic acids (Dr. E. Mylon, Department of Pathology). Three colonies of *M. ranae* were used in this work. One was young and brilliantly acid-fast with the standard test. Smears from this colony were stained both in the intact state and after the bacilli had been crushed between glass slides. The two other colonies were old and non-acid-fast by standard tests. One was a rough and the other a smooth variant.

For uniformity, all tissues were fixed in formalin and embedded in paraffin in the usual manner. The detailed changes of acid-fastness produced by various fixatives will be described elsewhere.[6] Here it is sufficient to say that such changes are minor and occur to the same relative extent for all substances examined here.

METHODS

Unless otherwise specified, staining was done in alkaline carbol-fuchsin. Five gms. of new fuchsin (C.I. 678) and 25 cc. of phenol were added to 100 cc. of absolute alcohol. At the time of use the desired amount of this stock solution was diluted with four parts of a 1/20 M. phosphate buffer, pH 7.8. The various differentiating agents are described where used. All slides were differentiated for five minutes since this length of time had been shown to give the most reproducible results in preliminary experiments. The counterstain where used was 0.5 per cent methylene blue in 95 per cent alcohol. Mycolic and leprosinic acids were dissolved in chloroform, then adsorbed by capillary action on to strips of #1 filter paper partially immersed in the solution. This resulted in a band of acid across the strip with a sharp border between this band and adjacent control regions. Because of their thickness, the strips were differentiated for ten rather than for five minutes.

RESULTS

A. DETERMINATION OF STAINING RESISTANCE

As a baseline for subsequent work, the staining resistance of the various test materials was determined under uniform conditions. The slides and strips were stained in saturated aqueous alkaline solutions of new fuchsin (pH 7.8 as elsewhere) for 15 minutes. They were washed in three changes of 95 per cent alcohol and two changes of absolute alcohol, one minute each.

The results are given in Table 1. As was expected,[15] the various types of intact tubercle bacilli were not stained. Also as expected, lepra bacilli,[14] sperm, and crushed tubercle bacilli[1] were stained. The portions of the filter paper strips containing mycolic and leprosinic acids held more dye than the control regions.

TABLE 1. STAINING WITH AQUEOUS DYES

Material	*Stained*
M. tuberculosis var. hom.	No
M. tuberculosis, BCG	No
M. ranae ("acid-fast")	No
M. leprae	Yes
M. leprae murium	Yes
Spermatozoa	Yes
Crushed *M. ranae* ("acid-fast")	Yes
M. ranae ("non-acid-fast")	Yes
Mycolic acid	Yes
Leprosinic acid	Yes

TABLE 2. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS. NO COUNTERSTAIN.

	3% HCl 70% Alcohol	*3% HCl 95% Alcohol*	*5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	4+	4+	4+
M. tuberculosis, BCG	4+	4+	4+
M. ranae, "acid-fast"	4+	4+	4+
M. leprae	0	±	4+
M. leprae murium	0	±	4+
Spermatozoa	0	3+	4+
M. ranae, crushed	0	irregular; many 0	4+
M. ranae, "non-acid-fast"	0	0	2+
Mycolic acid	0	1+	3+
Leprosinic acid	0	1+	4+

B. RESISTANCE TO ACID-ALCOHOLS

The effects of acid-alcohol differentiations were then studied apart from the perhaps confusing effects of counterstains. The test materials were all well stained by 15 minutes in the alkaline carbol-fuchsin. Comparative differentiations were then carried out on each substance. The differentiating solutions were 3 per cent HCl in 70 per cent alcohol, 3 per cent HCl in 95

per cent alcohol, and 5 per cent acetic acid in 95 per cent alcohol.[8, 18] It will be noted in Table 2 that all substances were acid-fast after acetic-acid-alcohol. By contrast, when 3 per cent HCl in 70 per cent alcohol was the differentiating agent, only those substances were acid-fast which had been resistant to staining with aqueous dyes. The results with HCl in 95 per cent alcohol fell between these two extremes.

TABLE 3. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS AND SUBSEQUENT COUNTERSTAINING

	3% HCl 70% Alcohol	*3% HCl 95% Alcohol*	*5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	Yes	Yes	Yes
M. tuberculosis, BCG	Yes	Yes	Yes
M. ranae, "acid-fast"	Yes	Yes	Yes
M. leprae	No	No	Yes
M. leprae murium	No	No	Yes
Spermatozoa	No	No	Yes
M. ranae, crushed	No	No	Yes
M. ranae, "non-acid-fast"	No	No	Irregular
Mycolic acid	No	No	Yes
Leprosinic acid	No	No	Yes

C. THE RÔLE OF THE COUNTERSTAIN

The differentiating agents used above were again tested, but with counterstaining after differentiating and rinsing in 95 per cent alcohol. Excess counterstain was removed by two rapid rinses in absolute alcohol. Table 3 presents the results of this procedure. Acid-fastness after HCl in 70 per cent alcohol was unaltered. Those materials which had retained a certain amount of dye after HCl in 95 per cent alcohol but which had been stainable with aqueous dye took the counterstain and so no longer appeared acid-fast. The only cells which took counterstain after acetic-acid-alcohol were some of the "non-acid-fast" strains of *M. ranae.* Except for this one case, the distribution of acid-fastness now paralleled that of staining resistance as given in Table 1. Those cells resistant to HCl-alcohol had also been resistant to staining. Those cells not resistant to the HCl-alcohol had been stained with aqueous dyes.

D. THE RÔLE OF PHENOL

In the above tests it was noted that crushing, which supposedly destroyed acid-fastness, altered the permeability to dyes and the resistance to HCl-

alcohols but left the organisms still acid-fast to acetic-acid-alcohol. Phenol also appeared to alter the permeability of intact tubercle bacilli to dyes, and it seemed of interest to determine whether, like crushing, the action of phenol was (i) permanent and (ii) non-specific.

Two points were reaffirmed. First, crushing had no effect upon sperm or upon already weakly acid-fast forms of *M. ranae;* it affected staining and destaining only when the cells had been stain-resistant. Second, the addition of phenol to the staining solution had no noticeable effect on the amount of

TABLE 4. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS TO WHICH PHENOL HAD BEEN ADDED. NO COUNTERSTAIN.

	5% Phenol 3% HCl 70% Alcohol	*5% Phenol 3% HCl 95% Alcohol*	*5% Phenol 5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	0*	0*	3+*
M. tuberculosis, BCG	0*	0*	3+*
M. ranae, "acid-fast"	0*	0*	3+*
M. leprae	0	0	4+
M. leprae murium	0	0	4+
Spermatozoa	0	3+	4+
Crushed *M. ranae*	0	0*	3+
M. ranae, "non-acid-fast"	0	0	2+
Mycolic acid	0	0	4+
Leprosinic acid	0	0	4+

* Differentiation altered by phenol.

dye retained under any circumstances by those cells which were stainable with aqueous dyes. These observations were then extended in the following manner. Sperm, lepra bacilli, and tubercle bacilli were treated for 24 hours with 5 per cent phenol in 20 per cent alcohol at room temperature. The subsequent staining was altered in no way by this treatment and it appeared that phenol had no permanent effect on acid-fastness; it could, however, act elsewhere than in the staining reaction. Five per cent phenol was added to each of the differentiating solutions tested above and differentiation without counterstaining was then repeated. Table 4 shows that phenol had no effect on the differentiation of any substance stainable with aqueous dyes. It did alter the stain retention of intact tubercle bacilli, however, and, just as had crushing, it made these cells slightly less acid-fast than lepra bacilli had been under the same circumstances.

A second experiment was based on the fact[18] that treatment with acids removes the acid-fastness from cells. Table 5 gives the time necessary for complete abolition of acid-fastness from tubercle and lepra bacilli in tissue sections when the sections were treated with 1 N HCl, preheated to 60° C., both with and without added phenol. Again, phenol had no appreciable effect on the reaction of lepra bacilli, but did bring about a change in the reactivity of tubercle bacilli. The generalization can thus be made that phenol, like crushing, acted where—and only where—staining resistance

TABLE 5. TIME NECESSARY FOR REMOVAL OF ACID-FAST MATERIAL FROM TISSUE SECTIONS AT 60° C.

	1 N HCl	*5% Phenol 1 N HCl 10% Alcohol*
M. leprae murium	8 min.	7 min.
M. tuberculosis BCG	12 min.	4 min.

had been present. The difference between the two means of reducing this resistance was that crushing produced a permanent alteration and phenol only a temporary one.

DISCUSSION

From the work reported here, it would appear that the arguments advanced for the structural theory of acid-fastness were valid so long as only strong acid-alcohols were used as differentiating agents. Whatever the nature of this structure, it appears that it may be altered by *prolonged* exposure to fat solvents such as xylene[18] as well as by the methods cited above. Certainly one sample of tubercle bacilli which had been thoroughly defatted during a search for soluble lipid fractions proved to have no structural acid-fastness.

The important fact, however, appears to be the suggestion that there was a second type of acid-fastness present in crushed cells as well as in intact tubercle bacilli, and present also in lepra bacilli and sperm. This second type of acid-fastness, demonstrable only after weak differentiation by such agents as acetic-acid-alcohol, was not altered by any of the mechanisms used to abolish structural acid-fastness. Further, while there was great difference between the reaction of intact tubercle bacilli and mycolic acid from these bacilli, there was little or no difference between the reaction level of crushed

tubercle bacilli and mycolic acid or between lepra bacilli and the corresponding leprosinic acid. Therefore, there appears to be support both for structural (intact vs. crushed tubercle bacilli) and "chemical" (as for mycolic acid) concepts of acid-fastness. While little needs be added to the problem of structure as treated by Yegian and Vanderlinde,[21] further study of the chemical nature of acid-fastness has yielded some interesting results. As was suggested by staining, a mycolic acid-like lipid has been found in spermatozoa.[3] Further, the reaction between mycolic acid and dye during the staining reaction has been studied and found both quantitative and unique.[4] It has then been possible to identify the same reaction in the staining of intact acid-fast cells.[5]

Granting the dichotomy of acid-fastness, it must then be decided which of the two types of reaction would best serve such purposes as the identification of mycobacteria in tissues and tissue fluids. The standard methods[8] (excepting Putt's[18]) use HCl-alcohols as differentiating agents, and so primarily demonstrate structural acid-fastness. Often rapid, incomplete differentiation is advocated so that cells such as lepra bacilli, not possessing structural acid-fastness, may yet retain the dye. An alternative method has been to provide artificial resistance by coating the cells with essential oils.[20] Since structural resistance is labile in any case, there would seem to be distinct advantages in a redefinition of acid-fastness in terms of the more stable and widespread chemical reaction.

A minor problem pointed up by this work concerns the theory of the action of phenol. Classically phenol has been held to act through the formation of a dye-phenol complex.[12] This complex was supposedly more firmly bound in the cell than the dye alone. It is not doubted that such a complex may exist in the staining solution; it is reported here, however, that phenol acts only where structural resistance is present and acts equally well during staining or destaining—it even acts to potentiate extraction of acid-fast material from the cell in a system where no dye is present. It is therefore suggested that phenol acts, not as an alum-like mordant, but to alter the structural permeability of the cell.

The present method of rapid staining at room temperature was derived from a study of Putt's[18] acid-fast technique. He attributed the accelerating action of lithium carbonate (pH 10) to a "mordanting" effect. However, it was found that any other means of providing an alkaline staining medium was equally effective, and a buffer solution was used only for convenience. Alkali, of course, had been used originally in the stain for tubercle bacilli by Koch,[15] but its value was lost sight of after phenol had been introduced by Ziehl.[22]

SUMMARY AND CONCLUSIONS

It has been shown that acid-fast materials may be considered in two groups. One group includes intact tubercle bacilli of various types; these do not stain with aqueous dye solutions but only in the presence of alcohol and phenol. Once stained, they are resistant to differentiation in hydrochloric-acid-alcohol.

The second group includes lepra bacilli, sperm, crushed tubercle bacilli, and the mycolic acids. These substances may be stained with aqueous dye solutions. They are acid-fast after differentiation in acetic-acid-alcohol but not after HCl-alcohol.

The first type of acid fastness is labile and is not only abolished by crushing, but it disappears in the presence of phenol and in old colonial forms; it fulfills the description of structural acid-fastness. The second type of acid-fastness is not labile and is altered only when the *chemical* composition of the cell is altered. It appears more suitable for diagnostic purposes than the first, structural, labile type which is shown by present methods.

Ancillary findings included: (i) counterstain may obscure acid-fastness, and (ii) acid-fast material may be rapidly stained at room temperature if the fuchsin or carbol-fuchsin is made alkaline.

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REFERENCES

1 Benians, T. H. C.: Observations on the gram-positive and acid-fast properties of bacteria. J. Path. Bact., Lond., 1912, *17,* 199.

2 Berg, J. W.: Differential staining of spermatozoa in the testis. Am. J. Clin. Path., 1953, *23,* 513.

3 Berg, J. W.: Acid-fast lipid in human spermatozoa. Arch. Path., Chic., in press.

4 Berg, J. W.: The chemistry of acid-fastness. Proc. Soc. Exp. Biol., N. Y., 1953, *84,* 196.

5 Berg, J. W.: Acid-fastness as a histochemical reaction. J. Histochemistry & Cytochemistry, 1953, in press.

6 Berg, J. W.: A new method for the staining of mycobacteria in tissue sections. Arch. Path., Chic., in press.

7 Bienstock, B.: Auf Frage der sog. Syphilisbacillen-und der Tuberclebacillenfärbung. Fortsch. Med., 1886, *4,* 193.

8 Conn, H. J. and Darrow, M. A.: *Staining procedures used by the Biological Stain Commission.* Geneva, N. Y., Biotech. Publications, 1951, pp. $IIIA_2$-8 & $IIIB_311$.

9 Dubos, R. J.: *The bacterial cell.* Cambridge, Mass., Harvard University Press, 1945, p. 90.

10 Ehrlich, P.: Neue Methode zum Entdeckung des Tuberkelbacillus. Deut. med. Wschr., 1882, *8,* 269.

11 Fethke, N. and Anderson, R. J.: Chemistry of the lipids of tubercle bacilli. LXXIV. A contribution to the study of acid-fastness of acid-fast bacilli. Am. Rev. Tuberc., 1948, *57,* 294.

12 French, R. W.: Chemical structure as affecting the staining and therapeutic properties of dyes. Stain Techn., 1926, *1,* 74.

13 Grimme, A.: Die sichtigsten Methoden der Bacterienfärbung in ihrer Wirkung auf die Membran, den Protoplasten und die Einschlüsse der Bakterienzelle. Zbl. Bakt., 1902, *32,* 161.

14 Hansen, G. A.: Bacillus leprae. Virchows Arch., 1880, *79,* 32.

15 Koch, R.: Die Aetiologie der Tuberculose. Berl. klin. Wschr., 1882, *19,* 221.

16 Koch, R.: Ueber neue Tuberkulin Präparate. Deut. med. Wschr., 1897, *23,* 209.

17 Neisser, A.: Weitere Beiträge zur Aetiologie der Lepra. Virchows Arch., 1881, *83,* 514.

18 Putt, F. A.: A modified Ziehl-Neelsen method for demonstration of leprosy bacilli and other acid-fast organisms. Am. J. Clin. Path., 1951, *21,* 92.

19 Rich, A. R.: *Pathogenesis of tuberculosis.* Springfield, Ill., Charles C Thomas, 1951, p. 15.

20 Wade, H. W.: Demonstration of acid-fast bacilli in tissue sections. Am. J. Path., 1952, *28,* 157.

21 Yegian, D. and Vanderlinde, R. J.: The nature of acid-fastness. J. Bact., Balt., 1947, *54,* 777.

22 Ziehl, F.: Zur Färbung des Tuberkelbacillus. Deut. med. Wschr., 1882, *8,* 451.