ACID-FASTNESS AS A HISTOCHEMICAL TEST*

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INTRODUCTION

Recent work from this laboratory (Berg, 1) has shown that the acid-fastness seen in mycobacteria can reside in two different properties of the cells. First, there was an acid-fastness dependent upon cell structure and shown by intact but not by crushed tubercle bacilli. Secondly, in the same cells there was an acid-fastness not altered by structural changes but rather dependent upon chemical properties. It was lost when and only when mycolic acid was removed from the cells. Further, purified mycolic acid showed the same type of acid-fastness and to the same degree. Lepra bacilli and sperm showed only the second type of acid-fastness and again it was also found in the mycolic-acid-like lipids extracted from these cells (Berg, 1, 2).

Further work (Berg, 3) described the unique reaction that took place between these lipids and one dye which was capable of giving the acid-fast reaction, crystal violet. Among other properties, the dye-mycolic acid complex exhibited intense absorption of light at 3500 Å while such absorption was not shown by the dye alone, the acid alone, or any other type of acid tested in combination with the dye. In this paper, it is shown that the same dye complex was formed in acid-fast cells as in the test-tube when the absorption spectra were determined for materials in tissue stained with crystal violet. In addition, a number of other reactions of the acid-fast material in the cells are described. The separation of the acid-fastness of mycobacteria from that shown by keratohyaline granules, Russell bodies and ceroid gives at least this first type of acid-fastness the character of a histochemical test.

MATERIALS

The types and sources of most of the experimental materials have been detailed in previous papers (1, 2). In addition to various types of mycobacteria and sperm, formalin-fixed paraffin-embedded sections of skin and of lungs containing Russell bodies from cases of pneumonia, carcinoma and sarcoid were examined. Ceroid-containing rat liver was very kindly made available by Dr. R. D. Lillie, National Institutes of Health, Bethesda, Maryland.

METHODS

Modification of the Acid-Fast Technique

The difficulties in determining acid-fastness have already been discussed as far as the strength of the differentiating agents and the use of a counterstain were

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concerned (1). In addition to these problems there was also the question of the dye itself. Admittedly the fuchsins were the most brilliant dyes tested, but their specificity was low. With the usual Ziehl-Neelsen technique the background tissues were stained almost as heavily as the acid-fast material. When the material was pretreated with acid-hydrolysis as in the Feulgen reaction (5) the same regions were subsequently stained with carbol-fuchsin as with Schiff reagent. The same was true after treatment with periodate or other oxidizing agent. Of the 18 basic dyes finally tested, crystal violet appeared the most satisfactory for a number of reasons. It was a relatively brilliant dye. It was removed from normal cells and normal connective tissue by all acid-alcohols. (The materials which remained stained were considered acid-fast and are discussed below.) It did not stain Schiff-positive material. It had worked well in the chemical studies cited above.

The dye proved most useful when it was made up in a saturated alcohol solution and then diluted with four parts of aqueous buffer: the staining proceeded much more rapidly in alkaline than in neutral or acid solutions, and in the pH range 5–8, the rate appeared directly related to the pH. Below a pH of 5 the staining took place very slowly, above a pH of 8 the dye came out of solution. Hence a M/20 phosphate buffer, pH 7.8, was most frequently used as the diluting solution, although a barbital buffer of the same pH worked equally well. The buffered dye solution remained stable for months, although it was best filtered before each use. Phenol in the dye solution was found necessary for the staining of tubercle bacilli and *M. ranae* but not for the demonstration of any of the other materials considered here. It was not used routinely but was added (1 part to 20) when needed because the buffered phenol solution was unstable and could be used for only about 2 hours.

To insure complete staining, the slides were left in the dye for 15 minutes although 5 minutes was adequate in most cases. The slides were then washed in 2 changes of 95% alcohol, ½ minute each, and differentiated for 5 minutes in 5% acetic acid in 95% alcohol. The acid-alcohol was chosen to be as weak as was consistent with removal of the dye from cell nuclei and fibrous tissue. If the differentiation time was shortened, the dye would be present irregularly in the background. After differentiation, the slides were washed in 2 changes of absolute alcohol, 1 minute each, cleared in xylene and mounted in the usual manner.

Since the 3500 Å absorption peak was so close to the visible range, the studies on tissue spectra could be and were done with apparatus designed primarily for work in the visible spectrum. The light source was a tungsten filament. Dispersion was achieved with a grating monochromator. The usual glass lens system was used in this instrument and the microscope. The image was projected onto a ground-glass screen and the light measured with a "Photovolt" photometer. Readings were taken along a linear path that included both the stained material and a region of unstained tissue. The minimum reading in the region of staining was compared with the minimum reading for the unstained portion. Control slides of the same thickness were mounted without staining and studied to insure that there was little difference if any between absorption in the region of mycobacteria and that of tissue cells when the dye was not present. Absorption curves for the

range 3400–7000 Å were determined on several samples of stained rat lepra bacilli, *M. ranae*, sperm (in a spermatocele), keratohyaline granules and Russell bodies. The tissues containing the mycobacteria and sperm were cut at 35 micra so that their absorption at the usual dye peak of 5900 Å was of the same order of magnitude as that of the keratohyaline- and Russell body-containing sections cut at 7 micra. The normal dye adsorption by this method was determined by staining a section of normal liver with the alkaline crystal violet and differentiating not in acid-alcohol but in 95% alcohol so that the non-acid-fast liver cells retained the stain.

TABLE 1

Separation of acid-fast materials

Substance / Test	Mycobacteria	Spermatozoa	Keratohyaline Russell Bodies	Ceroid
Acid-fast with Ziehl-Neelsen stain	+	+	+	+
Acid-fast with crystal violet acetic acid-alcohol	+	+	+	0
Acid-fastness abolished by hot HCl	+	+	0	
Acid-fastness abolished by hot dichromates	+	+	0	
Absorption at 3500 Å when stained with crystal violet	+	+	0	0

RESULTS

I. The Acid-Fastness of Mycobacteria

The various tests herein described resolved themselves into a pattern of increasing sensitivity and it is thus that they are presented here. The results of all the tests are summarized in Table 1.

The first determination was the modified acid-fast reaction described above. For the material studied here a number of other dyes were also substituted in the carbol-dye or buffered dye solutions and it seemed a general rule that if a substance were acid-fast with crystal-violet, it was acid-fast with the other basic dyes. This of course was not true when carbol fuchsin was the test stain.

Secondly, this acid-fastness was rapidly abolished by treatment with 1N HCl at 60°C. Preheated acid was used and the time for complete removal of acid-fast material ranged between 5 and 15 minutes (1). This was of the same order of time necessary to develop the Feulgen reaction. (Of some interest was the fact that the Feulgen reaction was prevented by previous extraction with 1N NaCl while acid-fastness was unaltered by the same treatment.)

Thirdly, acid-fastness in mycobacteria was completely blocked by treatment for 24 hours at 60°C. with 5% potassium dichromate, ammonium dichromate, or chromium chloride.

Fourthly, in order to dispose of the question of acid-fastness being due to an artificial lipid capsule, as has sometimes been the case (Bienstock, 4), the slides were treated with acetone, alcohol-ether, or pyridine at 60°C. for 48 hours. None of these treatments altered the acid-fastness of the mycobacteria.

Lastly, both lepra and tubercle bacilli, stained with crystal violet and destained in the standard manner, showed the same light absorption at 3500 Å as had been found previously in mycolic and leprosinic acids (Fig. 1). The appearance of this peak was in contrast to the complete lack of absorption in this region in unstained sections of the same material or in non-acid-fast material stained with crystal violet.

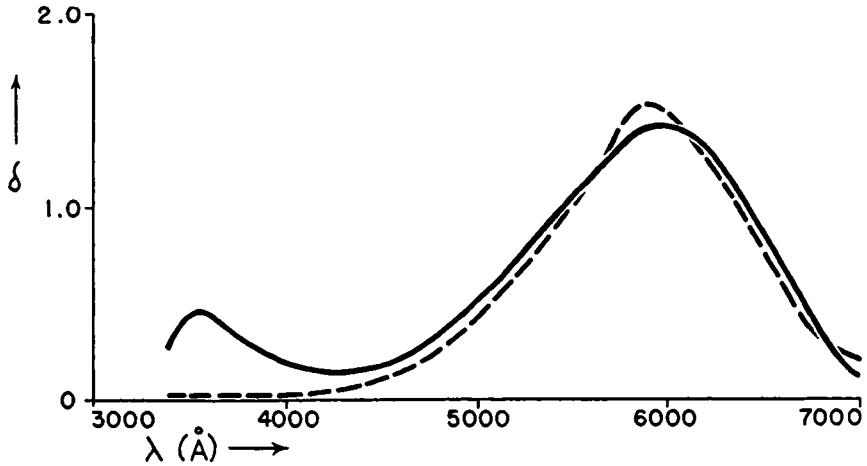

FIG. 1. Light absorption of tissue elements stained with crystal violet. ——— Composite curve for: lepra bacilli, *M. ranae*, spermatozoa. — — — Composite curve for: control liver sections, keratohyaline granules, Russell bodies.

II. The Reactions of Other "Acid-Fast" Substances

Spermatozoa, as might be expected from the presence of the acid-fast lipid (3), gave the same reactions as the mycobacteria. They were acid-fast with crystal violet and acetic-acid-alcohol: this acid-fastness was abolished by treatment with HCl or the chromium-containing compounds, but not by the lipid solvents. Finally they gave the same type of spectral absorption curve as did the mycobacteria and mycolic acids.

Keratohyaline granules and Russell bodies were studied since they had long been known to be brilliantly acid-fast after carbol-fuchsin (see Rich, 6). They are considered together here because they reacted to the series of tests in the same manner. They were both definitely acid-fast with crystal violet and the other basic dyes as with fuchsin. This reaction however was not altered by 15–20 minutes in hot HCl or by 24 hours in the chromium solutions. Finally, neither showed any light absorption at 3500 Å when the absorption at 5900 Å was of the same magnitude as that of the mycobacteria (Fig. 1). Ceroid and the pigment found in epididymides in old age represented yet another group of substances brilliantly acid-fast after carbol-fuchsin. However the reaction of these substances was found to be *dye-specific*. They were not acid-fast with any of the other basic dyes. The same was true of nuclear material and red cells which were irregularly acid-fast with the Ziehl-Neelsen technique. The reactions of these substances therefore could not be studied by the other tests.

III. Differences Between the Reactions of Mycolic Acid and the Acid-Fast Materials in Cells

Mycolic acid is known to possess different solubilities in the intracellular and purified states. When pure, it is soluble in chloroform and acetone (Stodola et al., 8), but it cannot be removed from cells by these solvents, only by acid-hydrolysis. As described above, acid-fast material from sperm was also removed by acid-hydrolysis but not by lipid solvents (2). In addition there was another difference between the reactions of the material in the cell and the purified lipids which should be ascribable to changes in the properties of the complexes in which the acid-fast lipids are found. The acids were sudanophilic while defatted bacilli (still acid-fast and still containing mycolic acid) were not. This was even more obvious in spermatozoa where the acid-fast material was in the posterior portion of the heads yet this region was completely unstained by Sudan dyes.

The picture was clarified somewhat by examination of these phenomena in material extracted from a large volume of sperm. Instead of allowing acid-extraction to go to completion as had been done previously (2), the reaction was stopped after 7 minutes by simultaneous neutralization and cooling of the HCl solution. The extracted material was concentrated by adsorption on filter paper. It was found that this material could be divided into two fractions. One portion was soluble in acid but not in chloroform or sodium hydroxide solution, and it was not sudanophilic. The other fraction was sudanophilic as well as acid-fast, and was soluble in chloroform or base but not in water or HCl. Further treatment of the fractions resulted in the disappearance of all acid-soluble, non-sudanophilic material and an increase in the chloroform-soluble lipid fraction. Thus it appeared that acid hydrolysis had been changing material which did not act like a fatty acid to material that did.

DISCUSSION

When this work is considered together with previous observations, it appears that there are not one but many types of reactions bearing the common name of "acid-fastness." In addition to the dye-specific acid-fastness of ceroid, the reaction of keratohyaline granules, and the reactions of mycobacteria and sperm, there is the acid-fastness produced by a lipid coating (Bienstock, 4) or a chitin capsule (Ruppel, 7). The aim of the present series of experiment has been to characterize one type of acid-fastness and to prove it a chemical reaction. Acid-fast lipids have been found in every cell type showing an acid-fastness like that in lepra bacilli. These lipids react with basic dye in stoichiometric proportions and with the production of unique complexes. The most characteristic property of this complex, its U-V absorption, has served to identify a similar complex in stained acid-fast bacilli or sperm. The differences between the reactions in the mycobacteria or sperm and in the fatty acids were not differences in acid-fastness but in ancillary reactions which in large part may be specifically attributed to changes in the reactivity of mycolic acid and its analogues. For instance it was apparent that the fatty acid responsible for acid-fastness in sperm must be

present in the sperm head in such a way that a lipid phase, capable of dissolving Sudan dyes, is not formed.

One further point can be made concerning the form of the dye bound in acid-fast cells. The spectra of the pure complexes showed that the U-V peak was approximately 4 times the height of the visible absorption maximum. Unless the sensitivity and the transparency of the optical system used here were at fault, the U-V peak was lower than the visible peak, indicating that there was dye in the cells not bound to the acid-fast lipid. Still, the presence of any absorption at 3500 Å was specific and differentiated the reaction in mycobacteria and sperm from that in keratohyaline granules. Whether the acid-fastness of this latter material and of Russell bodies was due to another specific chemical compound had not yet been determined. If the work described herein is an indication, however, this too, as a specific staining reaction may be found to have a true histochemical basis.

SUMMARY AND CONCLUSIONS

A study was made of the acid-fast reaction in cells and tissues, with the reaction in mycobacteria as the standard of comparison. For these bacilli, the reaction was obtained with all basic dyes. It was eliminated by pretreatment with 1N HCl or with chromium-containing compounds but not by lipid extractions. The dye remaining in the cells after differentiation showed the same U-V absorption band which had been found to be unique and characteristic for the dye-mycolic acid-complex. Spermatozoa gave the same reactions as did tubercle and lepra bacilli. Keratohyaline granules and Russell bodies were acid-fast with all dyes but the acid-fastness was not altered by treatment with chromium compounds or acid nor was the 3500 Å absorption peak present. Ceroid and epididymal pigment were acid-fast only with the fuchsins. The differences in the reactions of the acid-fast lipids in the cell and in the free state are described.

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