STUDIES OF THE COMMON AEROBIC SPORE-FORMING BACILLI

I. STAINING FOR FAT WITH SUDAN BLACK B-SAFRANIN

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In a classification of the aerobic spore-forming bacilli proposed by Smith and Clark (1937) one of the important differential points is the presence of reserve fat in the cells of organisms of the "B. cereus-B. megatherium group" when grown on glucose agar. In contrast, bacilli of the "B. subtilis-B. mesentericus group," and some other sub-groups, are said not to store fat. The authors do not describe their method for fat staining, but presumably they used Sudan III, or one of the related red dyes, in wet preparations.

The obvious practical value of so simple a differential test led us to investigate the matter of fat storage more fully, and since our fat-staining preparations made with Sudans II, III, or IV were not satisfactory, we sought a better technique. We soon confirmed the findings of Hartman (1940), who first pointed out that Sudan black B, previously recommended for histological work (Lison, 1934; Leach, 1938), is much superior to the red Sudans for demonstrating fat in bacterial cells.

Hartman used wet preparations only, suspending the bacteria in a solution of Sudan black B made in 70 per cent alcohol or in ethylene glycol. The latter was regarded as the more suitable solvent, since it does not evaporate rapidly. The fat droplets were recognized as blue-black bodies in a colorless cytoplasm.

It occurred to us that the stained fat granules would probably

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be shown even more clearly in dried smears counterstained with a contrasting color. After some experimentation we found that when a drop from a suspension of the bacteria in alcoholic Sudan black B solution is carefully dried upon a slide a field reasonably free of precipitate may be obtained, and that when this dried smear is counterstained with aqueous safranin, a permanent preparation is secured in which even the most minute fat droplets are brilliantly revealed.

TECHNIQUE

Stock Sudan black B solution. A saturated solution is made by adding an excess (i.e., about 0.3 gram) of the dry stain (obtained from the National Aniline and Chemical Company, N. Y.) to 100 ml. of 70 per cent alcohol in a screw-top bottle. The solution is ready for use after standing at room temperature, with occasional shaking, for 24 hours. It keeps without loss of staining power for at least one month.

 Cultures. To test the aerobic spore-bearers for capacity to store fat the organisms are grown upon slants of glycerol infusion agar or glucose infusion agar. If fat is accumulated at all by the cells it appears in approximately equal amounts on either medium within 24 hours and usually is at a maximum after about 48-hours' incubation. The fat granules in smears from sugar agar cultures usually stain somewhat less intensely than those in preparations from glycerol agar slants.

Procedure. Just before use, the stock Sudan black B solution is filtered into small test-tubes in about 0.5 ml. amounts. The bacteria from the slant cultures under examination are emulsified directly in the staining solution, and the emulsions are allowed to stand at room temperature for 15-20 minutes. During this time the precipitate present largely settles out. A loopful of the emulsion is then removed from the top of the fluid and smeared with a circular motion upon a clean slide so that it dries quickly, leaving precipitated particles at the periphery of the drop. The slide is not heated. A 1 per cent aqueous solution of safranin is now applied briefly, and the smear is then washed with water and dried in the usual manner.
The cytoplasm of the bacterial cells is thus stained pink, while the fatty material contained in it takes on a bluish-gray or bluish-black color. The stained smears, without coverglasses, when stored in an ordinary slide box remain unchanged for at least six months.

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In spore-forming bacilli stained with Sudan black B-safranin in this way the fat appears in the form of dark, bluish-black granules, ranging in size from tiny dots to large round bodies wider than the cells, or as transverse bands and rounded areas which color a fainter bluish-gray. The smallest of the granules can be recognized only by close examination under the oil immersion lens.

In order to trace the development of the fat particles and to compare the appearance of methylene-blue-stained organisms with those stained by Sudan black B-safranin a series of parallel smears was prepared from glucose and glycerol agar slant cultures of *Bacillus cereus* and of *Bacillus subtilis* (Michigan type), after incubation for different periods ranging from six hours to seven or more days.

In the *B. cereus* cultures on glucose agar at 6 hours the majority of the organisms showed fat-staining material in the form of very small, scattered, bluish granules, often eight or ten to a cell. A few of the bacteria contained larger, dark-staining fat droplets, one or two to a cell (fig. 1, A). The six-hour glycerol agar cultures showed a similar picture, except that the fat particles were all so tiny that they might easily have been overlooked. The accumulation of fat evidently continued rapidly, for after 24-hours' incubation, in both the glycerol and glucose agar cultures, conspicuous fat granules, most of them large and apparently formed by coalescence of the smaller droplets, were present in all of the organisms. Many of the cells were so crowded with fat that no pink-staining cytoplasm could be made out (fig. 1, B; fig. 2).

At the same time methylene-blue-stained smears showed that the bacteria contained numerous vacuoles. The majority of
these vacuoles evidently represented the spaces occupied by fat, while a few could be distinguished as developing spores. It was not possible to make this distinction definitely in many instances,

however, and without the accompanying fat-stained slides it might have been concluded from these methylene blue smears that virtually all of the cells were sporulating. The Sudan black

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**Fig. 1. Drawings of B. cereus, Made with the Aid of a Camera Lucida, from Smears Stained with Sudan Black B-safranin**

*A,* bacilli from a six-hour growth on glucose infusion agar, showing small, scattered fat droplets only. *B,* the same culture after twenty-four hours' incubation.
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B-safranin preparations thus served to explain otherwise misleading or puzzling morphologic features seen in ordinary stained smears.

In older cultures the fat particles persisted, though they often stained less intensely, and sizable droplets were noticed in individual cells undergoing active sporulation. "Ghost forms" of the bacilli always contained numerous fat granules.

In the B. subtilis (Michigan type) cultures, on both media, most of the cells were free of fat after only six-hours' incubation. A few organisms, however, contained at this time clusters of tiny blue-black bodies, or showed areas colored a very faint grayish-blue.

At 24 hours practically all of the cells showed definite fat droplets, though these were somewhat less numerous and less intensely stained than in B. cereus. Corresponding vacuolations were evident in parallel methylene-blue-stained smears. The fat persisted during further incubation, though diminishing in amount after several days, and did not appear to interfere with sporulation.

DIFFERENTIAL VALUE OF FAT STAINING

Examination of numerous cultures by the Sudan black B-safranin method has shown that the capacity, or lack of capacity, to store fat is a constant attribute of a particular species. We
have applied the stain repeatedly, with entirely consistent results, to 35 stock strains of aerobic spore-bearers, representing 10 of the most common species, and have found that the organisms fall into two classes, as follows:

*Fat positive*—the large-celled varieties, namely, *Bacillus megatherium*, *B. mycoides*, *B. cereus*, *B. subtilis* Michigan, and *B. anthracis*; also *B. brevis* and *B. circulans*.

*Fat negative*—the small-celled varieties, namely, *Bacillus mesentericus*, *B. subtilis* Ford, *B. subtilis* Marburg, and *B. vulgaris*.

![Fig. 3. Drawing of B. megatherium, made with the aid of a camera lucida, from a smear stained with Sudan black B-safranin.](image)

Culture grown forty-eight hours on glycerol infusion agar. The fat granules are relatively small, and many bacilli have rounded areas which are colored only a faint, bluish-gray by the Sudan black.

Among the fat-positive types there are certain constant and characteristic differences in the amount and distribution of the fat droplets at their maximum development. The fat is present abundantly, principally in large granules, in *B. cereus* and *B. mycoides*, and to about the same degree in the Michigan strains of *B. subtilis* (fig. 1, B), but in *B. megatherium* most of the cells contain small scattered granules only, or rounded areas staining a fainter bluish-gray, and the total fat present is decidedly less (fig. 3). In *B. brevis* and *B. circulans* the amount of fat stored is still smaller, and it may not be demonstrable in every glucose
or glycerol agar transplant. These are the only species that are not easily classified by fat staining. Fortunately both are readily distinguishable morphologically, especially in their sporulating forms, from all the other varieties; the bulging, central spores of *B. brevis* and the oval, terminal spores of *B. circulans* are both distinctive.

The fat-negative organisms are not entirely free of lipid matter, for degenerating "ghost forms" of these bacilli contain small fat droplets, and very rarely the Sudan black B-safranin stain will reveal a minute fat granule in an active vegetative rod. Small dark particles of precipitate are invariably caught within clumps of bacilli, and close observation may be required in some cases to be certain that these are not intracellular fat granules. These circumstances do not offer any practical difficulty, however, in distinguishing the fat-negative bacteria from the fat-positive group.

The Sudan black B-safranin stain is thus a reliable differential test. We have found, moreover, that by its use the fat-staining reaction acquires even greater practical usefulness than was indicated by Smith and Clark. We have successfully applied the fat stain as a primary step toward the rapid identification of more than 100 unknown strains of aerobic spore-bearers freshly isolated from dust and other sources. The species within the fat-positive and fat-negative groups respectively may be readily differentiated by carrying out only a few simple procedures. These further identifying tests will be described in later papers in this series.

**SUMMARY**

Sudan black B is a superior stain for demonstrating fat in bacterial cells. When a loopful of a suspension of fat-storing organisms in a 70 per cent alcoholic solution of the dye is dried upon a slide, and counterstained with 1 per cent aqueous safranin, a permanent preparation is obtained in which even the most minute fat droplets are brilliantly revealed as bluish-black or bluish-gray granules in a pink-stained cytoplasm. These dried stained smears have obvious advantages over the wet preparations formerly employed.
Vacuolations in bacilli which in ordinary stained smears might be mistaken for spores, or other special structures, are shown by this Sudan black B-safranin stain to be spaces occupied by fat. By use of this fat-staining procedure on smears from 24- to 48-hour-old cultures on glucose or glycerol agar slants the common aerobic spore-forming bacilli may be classified readily into a fat-negative and a fat-positive group. Moreover, individual species among the latter class show characteristic differences in the amount and distribution of the stored fat. The application of the stain to cultures of unknown aerobic spore-bearing bacilli as a primary differential test is of material aid toward their rapid identification.

REFERENCES