FATTY MATERIAL IN BACTERIA AND FUNGI REVEALED BY STAINING DRIED, FIXED SLIDE PREPARATIONS[1]

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Following Hartman, who first suggested the use of Sudan black B, in place of red Sudans, as a bacterial fat stain (Hartman, 1940), Burdon, Stokes, and Kimbrough (1942*a*) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing, from suspensions of the organisms in alcoholic Sudan black B solution, *dried films* counterstained with safranine. Previously it had been thought that dried, fixed films were entirely unsuitable for fat stains (Lewis, 1941).

These permanent films were regarded as an obvious improvement over the wet preparations used by earlier workers, and they were shown to be of practical aid in the classification of aerobic sporeforming bacilli (Burdon, Stokes, and Kimbrough, 1942*b*), but the staining method still had a number of undesirable features.

Further experimentation has resulted in the development of the much superior procedure to be described here. The new method is not only simpler, requiring no more effort than a gram stain, but it is also far more rewarding, for the films now reveal clearly intracellular lipid matter that previously has not been seen or even suspected. The improved stain has increased differential value. Moreover, its application to various bacteria has resulted in certain general findings of unusual interest.

SUDAN BLACK B FAT STAIN FOR FIXED PREPARATIONS

Technique. 1. Prepare the film, let it dry thoroughly in the air, and fix it by heat in the usual way. (Chemical fixation has no special advantages and may result in some loss of demonstrable lipid.) 2. Flood the entire slide with Sudan black solution (0.3 g of the powdered stain[2] in 100 ml of 70 per cent ethyl aclohol), and allow the slide to remain undisturbed at room temperature for 5 to 15 minutes. (A staining period of less than 5 minutes will often suffice, but the intra-

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[2] The original, imported Sudan black B dye is not available commercially at the time of writing. But recently the National Aniline Division, Allied Chemical and Dye Corporation, 40 Rector Street, New York 6, N. Y., has developed a duplicate of this stain, and the company promises to have a supply of the American-made Sudan black B on the market shortly. In my hands this new product has given results equal to those obtained with the imported dye. The writer is indebted to Dr. H. J. Conn, president of the Biological Stain Commission, for his kindness in making various dye samples available for comparative tests.

cellular lipid is colored somewhat more intensely when the staining is continued for 5 minutes or longer. No further staining apparently occurs after the solution precipitates and turns a greenish or brownish color, but no harm is done if the stain is allowed to dry completely over the film. 3. Drain off excess stain and blot the slide thoroughly dry. 4. Clear the slide with cp xylol by dipping it in and out of the solvent in a Coplin jar or by adding xylol from a dropping bottle. Blot the cleared slide dry. 5. Counterstain with safranine (0.5 per cent aqueous solution) for 5 to 10 seconds (for ordinary bacteria or fungi), or with dilute carbol fuchsin (Ziehl's carbol fuchsin diluted 1:10 with distilled water) for 1 to 3 minutes (for acid-fast organisms). (Overstaining with the counterstain must be avoided). 6. Wash in water, blot, and dry the slide.

Comment. After the bulk of the dye has been dissolved, the Sudan black B solution should be thoroughly shaken at intervals, then allowed to stand overnight before use. It remains good for several months at room temperature, provided it is kept in a well-stoppered, chemically clean container.

The entire slide is flooded with the staining solution to prevent the too rapid evaporation that otherwise occurs. (For reasons not entirely understood, the staining is generally unsatisfactory when slides are immersed in the Sudan black B solution in a Coplin jar.) Since the cellular lipid in most organisms takes up the characteristic blue-black color almost at once, it is possible to complete the whole fat-staining procedure within a minute or two if desired. On the other hand, if the technician is occupied with other tasks, the stain may simply be allowed to dry on the slide; then the clearing with xylol and counterstaining may be carried out later at a more convenient time. If the Sudan black B solution is allowed to stand on the slide for about 15 minutes and is then set afire by applying the Bunsen flame to the fluid, followed by blotting and xylol-clearing as usual, the intracellular fat in some organisms (e.g., gonococci) is brought out more clearly. Ordinarily, this step is unnecessary.

Examination of the cleared preparation without counterstaining is sometimes interesting and revealing. Care should be taken to avoid obscuring very tiny fat droplets by too strong a counterstain. Films must be examined with the oil immersion lens under critical illumination. To discern the smallest lipoid particles the observer must have a good sense for the color distinction between the blue-black or blue-gray of the fat droplets and the pink of the counterstain.

RESULTS OF APPLYING THE STAIN TO VARIOUS SPECIES OF BACTERIA AND FUNGI

Methods. The stain has now been applied to films from cultures of virtually all the chief species of bacteria, and of many fungi. In order to obtain a general picture of the occurrence of demonstrable intracellular lipid in these organisms and to permit a comparison between species in this respect, films were made from all cultures at approximately the same stages of growth, i.e., in early maturity (12 to 24 hours old). For the purpose of this preliminary survey, the organisms were grown on the media customarily employed for routine cultures of the species concerned, such as plain extract or infusion agar, potato slants, blood or ascitic

fluid agar (for pneumococci, streptococci, gonococci, etc.), Loeffler's serum slants (for the *Corynebacterium* group), blood agar slants or thioglycolate media (for anaerobes), coagulated egg media and glycerine agar (for acid-fast bacilli), and Sabouraud's medium (for fungi). The majority of the films were made from stock cultures, under conditions permitting accurate comparisons. A smaller number of observations were made on preparations from primary (mixed) cultures, derived from the dust or from the throat, skin, etc., of human beings, and on direct films from the human mouth or from sputum, pus, and the like.

General findings. The films showed that fatty material staining with Sudan black B is present, often in conspicuous amounts, in the great majority of microorganisms, whether aerobic or anaerobic, saprophytic or parasitic, pathogenic or nonpathogenic, and that it is to be seen in a considerable number of bacteria—such as the diphtheria bacilli, anaerobic sporeforming bacilli, and the common species of cocci—which have been described in the earlier literature as lacking any microscopically demonstrable fat (Lewis, 1941). The preparations revealed in a striking way the abundant lipoid matter in fungi of all kinds.

Intracellular lipid was observed in organisms in primary cultures, as well as in pure stock cultures, and some bacteria and fungi in films made directly from body surfaces or excretions were found to contain typical fatty inclusions.

A high proportion of gram-positive bacteria were discovered to be fat storers. Many familiar varieties of gram-negative bacteria, on the other hand, were shown to be free of stainable lipid when in active growth on common media. A tendency for saprophytic varieties to contain more fat than the parasitic species of the same genera was noted in certain cases, notably in *Mycobacterium* and *Corynebacterium*.

It was found that the appearance of distinct intracellular fat droplets, or other Sudan black B staining matter, in bacteria is not influenced directly by the presence or absence of glycerol, or other fermentable carbohydrate, in the medium. Accumulation of the intracellular lipid is affected, however, in any medium by the rapidity of growth, and if cell division is retarded, the relative amount of demonstrable fat is usually increased. In the case of both the aerobic and anaerobic sporeforming bacilli (genera *Bacillus* and *Clostridium*), the fatty material was observed to be reduced somewhat just before active spore formation began. A considerable amount remained, however, and often sizable fat droplets were seen in the tags of protoplasm around incompletely free spores. If sporulation was for any reason delayed, fatty substances continued to accumulate within the bacilli, and this material persisted indefinitely *in situ*, even after the stainable cytoplasm had disintegrated.

The Sudan black B not only stained all cytoplasmic inclusions of lipoid nature, but also colored parts of the cell structure (apparently the cytoplasmic membrane) in the case of certain bacteria and fungi.

The regularity with which fatty inclusions appeared, and the general pattern exhibited by all the fat-staining material within the cells, were found to be remarkably constant for any one kind of organism. The fat-storing habits of a particular species were not appreciably different in the numerous variant strains

observed in this study, whether the variants were naturally encountered or deliberately produced.

Most impressive was the finding that, with only occasional exceptions, the closer the relationship between varieties of bacteria in other respects, the more nearly alike was their content of stainable lipid.

Occurrence of Sudan black B staining material in particular species. The various organisms observed in pure culture may be divided into three groups according to the results of this preliminary survey of their fat-staining propensities (table 1).

In one group (I) stainable intracellular lipid was present regularly in considerable amounts in nearly all the mature cells whenever microscopic examination was made of the growth on the usual culture media. Included here are the larger, common species of *Bacillus;* all representatives of the genera *Clostridium, Corynebacterium,* and *Mycobacterium; Actinomyces* species and the fungi; and some of the more saprophytic gram-positive cocci. Also among the organisms regularly containing conspicuous fatty inclusions are a relatively few species of gram-negative bacteria, including the nitrogen-fixing organisms (*Azotobacter* and *Rhizobium*), and, unexpectedly, such saprophytic species as *Acetobacter aceti, Alkaligenes fecalis,* and *Spirillum rubrum.*

In the case of other bacteria (group II) intracellular fat-staining material was usually present, but the organisms in certain cultures on common media failed to show any fat. These organisms contained relatively small amounts of stainable lipid at most. A clear distinction between these bacteria of group II and those of group I may not be borne out by further investigations. It is convenient, however, to place in this second group, for the present purpose, such organisms as the common, smaller-celled species of the genus *Bacillus,* the human and bovine tubercle bacilli, the familiar varieties of staphylococci, streptococci, and pneumococci, and the gram-negative diplococci, which sometimes grow on the customary media without development of stainable lipid.

No systematic attempt was made to discover the precise circumstances required for the regular formation of intracellular fat by these group II organisms. Incidental observations indicated, however, that at least the majority of them will form characteristic fatty inclusions regularly when a suitable special medium is provided. For example, *Bacillus subtilis* (Marburg) and its close relatives among the aerobic sporeformers rarely show more than traces of fat when cultivated on plain or glucose nutrient agar media. Because of this the writer originally classified these bacilli as "fat-negative" (Burdon, Stokes, and Kimbrough, 1942*a*). But intracellular lipid does appear in characteristic amount when cultures of the same strains are made on potato slants or on glucose starch agar. Similarly, *Lactobacillus acidophilus* apparently stores no fat in milk cultures, but the cells contained moderate amounts when they were grown on tomato juice agar slants.

Finally, the remaining species form a third group made up of bacteria that apparently do not store demonstrable fatty material at all, as a rule, except that one or two tiny droplets are sometimes to be seen in occasional cells (group III). Here are to be found virtually all the chief varieties of gram-negative bacilli,

TABLE 1

An arbitrary grouping of bacteria and fungi according to their content of demonstrable intracellular lipid

(Based on preliminary observations of dried, fixed films stained with Sudan black B and counterstains)

GROUP I. FATTY MATERIAL REGULARLY PRESENT IN CONSPICUOUS AMOUNTS IN NEARLY ALL THE MATURE CELLS

Gram-positive bacteria

- *Actinomyces bovis* (1)*
- *Actinomyces* sp. (saprophytic) (3)
- *Bacillus alvei* (1)
- *Bacillus anthracis* (7)
- *Bacillus cereus* (65)
- *Bacillus circulans* (7)
- *Bacillus megatherium* (15)
- *Bacillus mycoides* (10)
- *Clostridium botulinum* (1)
- *Clostridium histolyticum* (2)
- *Clostridium perfringens* (2)
- *Clostridium septicum* (1)
- *Clostridium sporogenes* (4)
- *Clostridium tetani* (3)
- *Corynebacterium diphtheriae* (17)
- *Corynebacterium pseudodiphthericum* (hoffmanni) (3)
- *Corynebacterium xerose* (7)
- *Gaffky tetragena* (3)
- *Mycobacterium "leprae"* (11)
- *Mycobacterium phlei* (3)
- *Mycobacterium smegmatis* (2)
- *Mycobacterium tuberculosis* (avian) (3)
- *Mycobacterium tuberculosis* (cold-blooded type) (2)
- *Mycobacterium* sp. (saprophytic) (8)
- *Sarcina lutea* (3)
- *Staphylococcus citreus* (2)
- *Streptococcus faecalis* (1)

Gram-negative bacteria

- *Acetobacter aceti* (1)
- *Alkaligenes fecalis* (4)
- *Azotobacter beijerinkii* (2)
- *Azotobacter chroococcum* (2)
- *Chromobacterium violaceum* (2)
- *Rhizobium leguminosarum* (2)
- *Spirillum rubrum* (2)

Fungi

- *Aspergillus* sp. (2)
- *Blastomyces dermatitidis* (2)
- *Candida albicans* (2)
- *Coccidioides immitis* (1)
- *Cryptococcus neoformans* (2)
- *Epidermophyton floccosum* (1)
- *Histoplasma capsulatum* (1)
- *Hormodendrum pedrosoi* (1)
- *Microsporum gypseum* (1)
- *Mucor* sp. (2)
- *Penicillium notatum* (1)
- *Penicillium* sp. (3)
- *Phialophora verrucosa* (1)
- *Rhizopus* sp. (2)
- *Saccharomyces cerevisiae* (2)
- *Sporotrichum schencki* (1)
- *Trichophyton mentagrophytes* (1)
- *Rhinosporidium seeberi* (in tissue sections) (1)

GROUP II. FATTY MATERIAL USUALLY PRESENT, BUT SOMETIMES ABSENT IN CULTURES ON COMMON MEDIA

Gram-positive bacteria

- *Bacillus mesentericus* (>75)
- *Bacillus subtilis* (Ford) (25)
- *Bacillus subtilis* (Marburg) (>30)
- *Diplococcus pneumoniae* (6)
- *Lactobacillus acidophilus* (2)
- *Mycobacterium tuberculosis* (bovine) (3)
- *Mycobacterium tuberculosis* (human) (8)
- *Staphylococcus albus* (3)
- *Staphylococcus aureus* (3)
- *Streptococcus pyogenes* (3)
- *Streptococcus salivarius* (4)

Gram-negative bacteria

- *Bacillus brevis* (16)
- *Neisseria catarrhalis* (2)
- *Neisseria gonorrhoeae* (4)
- *Neisseria meningitidis* (2)
- *Neisseria pharyngis* (3)

TABLE 1—*Continued*

GROUP III. FATTY MATERIAL USUALLY ABSENT, OR PRESENT IN TRACES ONLY IN A FEW CELLS	
Gram-negative bacteria	
Aerobacter aerogenes (2)	*Pasteurella tularensis* (1)
Brucella abortus (2)	*Proteus vulgaris* (OX19) (2)
Brucella melitensis (2)	*Proteus* sp. (12)
Brucella suis (2)	*Pseudomonas aeruginosa* (3)
Eberthella typhosa (6)	*Pseudomonas fluorescens* (2)
Escherichia coli (2)	*Salmonella enteritidis* (2)
Escherichia communior (2)	*Salmonella schottmülleri* (2)
Hemophilus influenzae (3)	*Salmonella* sp. (4)
Hemophilus pertussis (2)	*Serratia marcescens* (3)
Klebsiella mutabile (1)	*Shigella dysenteriae* (2)
Klebsiella pneumoniae (2)	*Shigella flexneri* (26)
Listerella monocytogenes (1)	*Shigella sonnei* (9)
Pasteurella avi (1)	*Shigella* sp. (35)
Pasteurella equiseptica (1)	*Vibrio cholerae* (3)
Pasteurella pestis (2)	

* The figure in parenthesis indicates the number of separate, pure strains observed by the writer to date.

including all members of the genera *Brucella*, *Hemophilus*, and *Pasteurella*, as well as the several genera of enteric bacilli and the cholera vibrio.

It must be emphasized that this division of bacteria into three groups in relation to intracellular fat is entirely arbitrary and provisional. It is based on films from cultures in routine media only. As the Sudan black B fat stain is eventually utilized by different investigators in connection with intensive studies of various groups of bacteria under different conditions, new information will be forthcoming that may well require revision of these listings.

Characteristic intracellular distribution of the fatty material in important genera and species. The accompanying drawings (figures 1, 2, and 3) give some idea of the usual picture when organisms of various kinds are fat-stained. The sketches can convey only an inadequate conception of the striking appearances actually seen under the microscope, since they lack completeness and full precision in details, and especially since there is an absence of color.

It seems evident that Sudan black B stains more than neutral fat. It not only imparts a dark blue-black color to distinctly outlined intracellular fat droplets, but also gives a more or less intense bluish-gray tint to relatively large, ill-defined areas of cytoplasm in various bacteria and fungi. Moreover, in certain bacteria and most fungi, it stains intensely a thin, irregular, peripheral line, which is clearly part of the cellular structure, and presumably is the cytoplasmic membrane.

Detailed descriptions will not be attempted here, but certain features of general interest will be pointed out.

Bacillus—All members of this genus that have been studied, including virulent strains of *B. anthracis*, store fat (figure 1). Certain small-celled varieties, how-

ever, do not do so in every culture, and, moreover, even when stainable lipid is present in these bacilli, it is often difficult to discern. Most commonly the fat occurs in these species at the ends of the rods.

In the larger spore bearers, however, fat forms regularly and early as tiny droplets along the periphery, which soon coalesce into larger drops and move into the middle portions of the cell. The species that are otherwise closely similar (*B. anthracis*, *B. cereus*, and *B. mycoides*) all show large central droplets. *B. megatherium*, on the other hand, presents a distinctly different picture. In this case relatively enormous amounts of lipid are present, even in cultures only a few hours old, and the deeply stained fatty material occurs almost entirely in the form of numerous relatively small droplets, which crowd the cytoplasm but show little tendency to coalesce. The distinctive appearance makes this species easily recognizable.

Clostridium—Considerable intracellular lipid is present in all the anaerobic spore

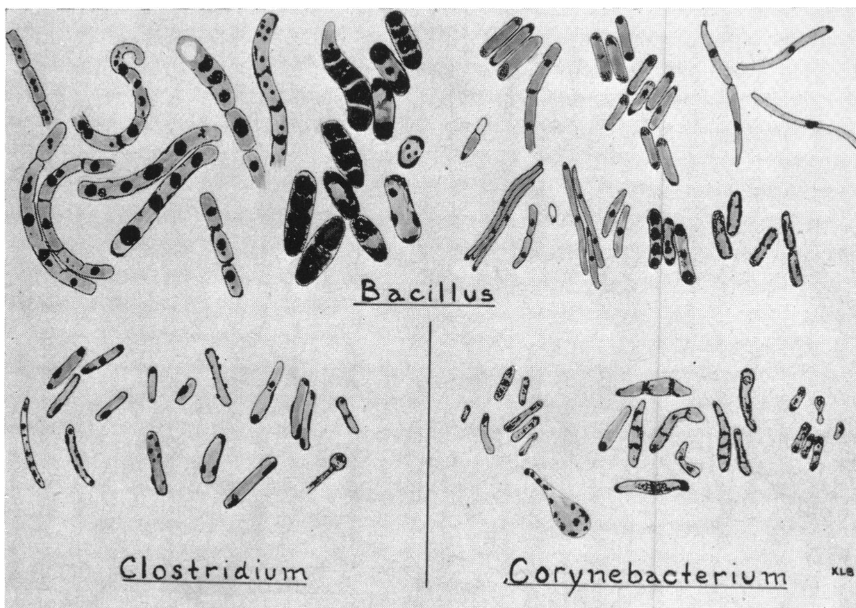

FIG. 1

bearers (figure 1). Characteristically, fat droplets occur at the extreme periphery of the counterstained rod, or even appear to be just outside the cells.

Corynebacterium—Abundant fatty material is revealed in all the diphtheria bacillus group; it is especially conspicuous in *C. xerose* (figure 1). In this genus the fat stain outlines with bluish black the cell periphery and the cross septa so characteristic of the barred types of these bacilli. Lipid matter also appears as diffuse masses at the ends or edges of the cells.

Mycobacterium—The general picture in the case of all varieties of the acid-fast bacilli is essentially the same There are differences, however, in the number and size of distinctly staining, intracellular fat droplets, and in the regularity with which these appear, among different varieties of these organisms (figure 3). A characteristic feature of the whole group is the tendency of the cells to take a light bluish-gray color throughout. This is in addition to the appearance of distinct, dark-blue droplets of fat within many of the rods.

The human and bovine tubercle bacilli often contain many fat granules, but since

we have observed a number of cultures showing only traces of fat, we have listed these organisms in group II (table 1). Unfortunately, no features have been de-

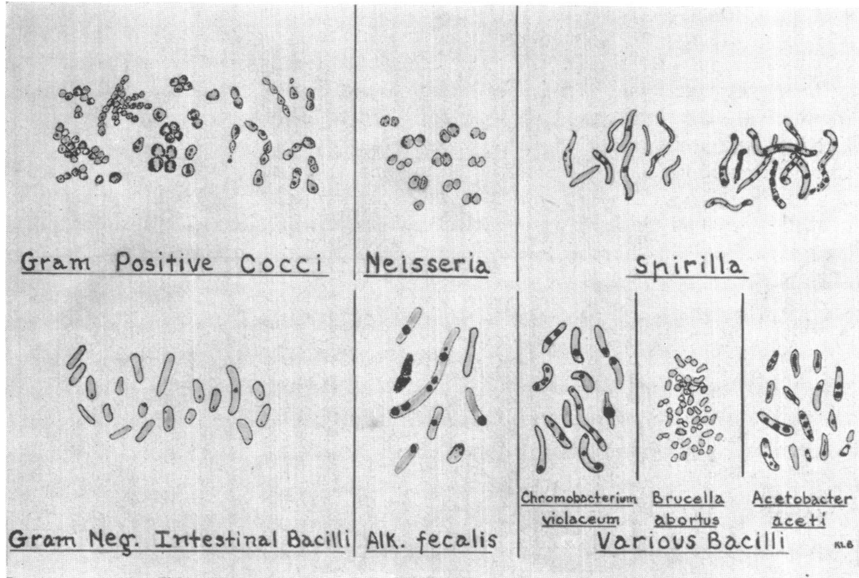

Fig. 2

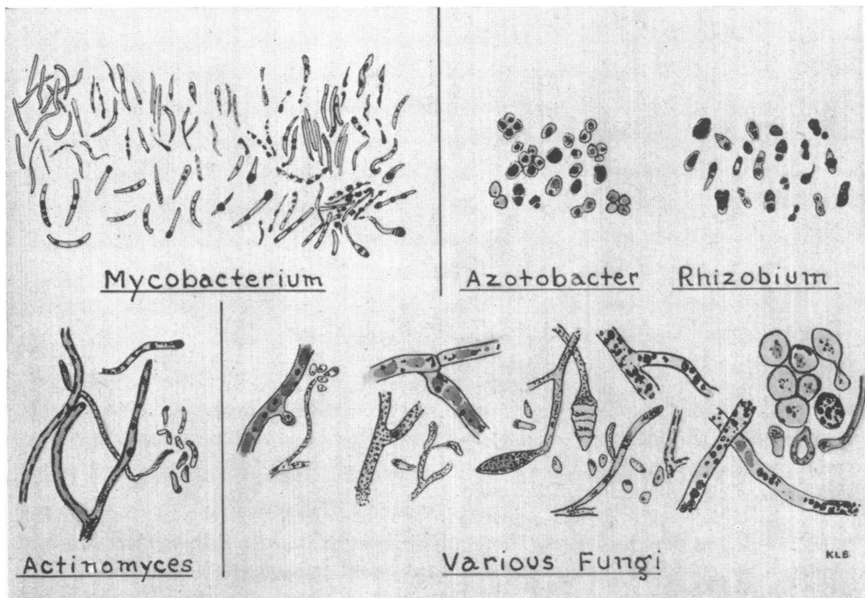

Fig. 3

tected which would assist in differentiation of the human from the bovine type. The fat-containing rods have no relation to the "beaded" forms of tubercle bacilli (not infrequently seen after carbol fuchsin staining), whose significance was so long

debated. The "beads" have recently been shown to be artifacts (Porter and Yegian, 1945; Lamanna, 1946).

The most conspicuous fatty inclusions are seen in the frankly saprophytic acid-fast strains, and in cultures labeled *Mycobacterium leprae*. (The bacilli in direct preparations from leprous lesions apparently contain no stainable fat. This disparity in the appearance of the true Hansen's bacilli and the cultured, so-called "leprosy bacilli" when fat-stained is pointed out in a separate communication (Burdon, 1946).

Azotobacter and *Rhizobium*—The extraordinary amount of lipid in the mature cells of the nitrogen-fixing bacteria is revealed in a striking way (figure 3). All stages in the process of accumulating the fat, from the first appearance of almost invisible droplets at the edges of the cells to the final stage in which the entire organism is solidly filled with fat-staining material, may be followed easily.

Cocci, *Actinomyces*, and Fungi—In cocci the Sudan black B staining material most frequently occurs as a dark bluish mantle around the circumference of the cells (figure 2). Some of it also appears as separate, small, internal droplets.

In *Actinomyces* and the filamentous fungi (figure 3) thin strips of lipoid matter or innumerable tiny fat droplets seem to line the hyphae throughout their length. In addition, sizeable round masses and irregular areas within the cytoplasm take the fat stain.

In the yeastlike fungi much fat is also revealed, although it is apparently not as abundant, as a rule, as it is in the mycelium-forming fungi.

Fat in ghost forms; "fatty degeneration." An observation of general interest is the frequent occurrence of fat globules within the "ghost forms" of bacteria, from which stainable cytoplasm is largely or wholly lost. Often these degenerated organisms are packed full of material that takes a dark, blue-black color. These forms appear not only in those species that regularly store lipid, but also in varieties containing no demonstrable fatty inclusions when in their active, growing state. They have been seen, for example, in cultures of *Pasteurella* and *Klebsiella*.

This appears to be a kind of "fatty degeneration." It is probably a phenomenon basically different from the storage of intracellular fat by actively metabolizing cells. Further study is needed before the mechanisms involved can be understood.

Results of staining films from primary cultures and from body secretions. Microscopic examination of colonies of various kinds developing on agar plates exposed to dust showed that the sporulating bacilli and common fungi in such primary mixed cultures contain fat in a characteristic pattern, and that various other fat-positive organisms are likely to be present. Many, but not all, of the bacteria in ordinary hay infusions, and similar mixed growths, were found to contain fat.

Films made from the growth on blood agar plates inoculated with swabbings from the human throat or nose often showed fatty material present in the usual form in various cocci, and in special abundance in diphtheroids. A few of the tubercle bacilli in films of tuberculous sputum were found to contain stainable lipid, and also fat droplets have been seen in the fusiform bacilli in direct preparations from patients with Vincent's angina. On the other hand, no fat has been detected in gonococci in the several films of gonorrheal pus examined. Capsulated anthrax bacilli in direct preparations from the spleen or blood of

animals dying of natural or experimental infection were observed to contain only a few very tiny fat droplets along their outer edges. In their very first growth on common media, however, these same organisms always develop characteristic large, centrally located fat granules.

EXTRACTION EXPERIMENTS WITH LIPOID SOLVENTS

As a preliminary step toward some understanding of the nature of the material stained, experiments were carried out to test the loss of Sudan black B staining matter when bacteria are exposed to recognized fat solvents.

Methods. A majority of the extraction tests were carried out by exposing ordinary fixed, but unstained, films to the solvents for varying lengths of time. A number of films were prepared at the same time on separate slides from cultures of various bacteria. These films were fixed by heat as usual, but left unstained, except for one slide of each organism, which was fat-stained to serve as a control. The slides having unstained films were then immersed in the various solvents in Coplin jars. After certain time intervals a slide representing each culture was removed from each of the solvents, stained by the routine procedure, and examined for comparison with the controls. The principal solvents used were glacial acetic acid, 95 per cent ethyl alcohol, acetone, chloroform, and carbon tetrachloride.

A few tests were made by emulsifying the organisms in the solvents directly from cultures. Films were then prepared and stained after varying time intervals.

Results. A summary of the findings in several representative experiments is presented in table 2. It is evident that the Sudan black B staining material was removed in large part from all the organisms by most of the solvents within 72 hours or less. No other change in the microscopic appearance of the bacteria occurred. The rate of extraction varied, however, with different species, as well as with different solvents.

Carbon tetrachloride had scarcely any effect on the fat-staining material in the six varieties of acid-fast bacilli tested, or in *Corynebacterium diphtheriae*, but it removed the lipid matter from five species of aerobic sporeforming bacilli about as readily as the other solvents did. Chloroform also extracted the fatty material from the acid-fast bacilli and from the diphtheria bacillus with relative slowness. The latter organism was the only species not cleared, or nearly cleared, of lipid by acetone within 72 hours. Most rapid extraction was brought about by alcohol, with glacial acetic acid a close second. These two solvents acted only slowly, however, on the closely related, large, chained bacilli *B. anthracis* and *B. cereus*.

The most important influence in determining the extraction rate seemed to be the relative ease with which the solvent was able to enter the bacterial cytoplasm. (It is supposed that this may depend, in part, upon the miscibility of the solvent with water and, in part, upon some peculiarity in the chemical nature of the bacterial cell membranes.) In the case of the slow removal of the lipid from the large bacilli mentioned above, the successive films showed clearly that the delay

TABLE 2

Loss of Sudan black B staining material from bacteria by extraction with fat solvents

ORGANISMS	SOLVENTS														
	Glacial acetic acid			95 % ethyl alcohol			Acetone			Chloroform			Carbon tetrachloride		
	Extraction time (hours)														
	1	24	72	1	24	72	1	24	72	1	24	72	1	24	72
Aerobic sporeforming bacilli															
B. anthracis	−	−	−	−	±	+	−	+	++	+	+++	++++	−	+	++
B. cereus	−	±	+	−	+	++	−	±	+++	−	+	+++	−	+	++
B. megatherium	+	++	+++	+	++	+++	±	+	+++	++	+++	++++	±	++	+++
B. subtilis (Ford)	++++			+++	++++		−	±	+++	±	++++		−	±	+++
B. brevis	+++	++++		+	++++		−	+	+++	−	+	+++	−	+	+++
Acid-fast bacilli															
M. tuberculosis (human, H37)	−	−	++	−	±	++++	−	−	+++	−	−	+	−	−	−
M. tuberculosis (bovine)	−	−	++	−	+	++++	−	+	++++	−	+	++	−	−	−
M. tuberculosis (avian)	−	−	++	±	+	++++	−	+	++++	+	+	++	−	−	±
M. smegmatis	−	−	++	+	++	++++	+	++	++++	−	−	++	−	−	−
M. "leprae"	−	+	+++	−	+	+++	−	−	+++	−	−	++	−	−	−
M. phlei	+	++	+++	+	++	+++	−	±	++	−	±	++	−	−	−
Other bacilli															
A. fecalis	++++			++++			−	+++	++++	+	++++		−	±	+
A. chroococcum	++	++++		−	+++	++++	+	+++	++++	++	++++		−	−	++
C. diphtheriae	+++	++++		−	++++	++++	−	−	±	−	±	+	−	−	−

Key: − = no change; ±, +, ++, +++ = increasing loss of Sudan black B staining material; ++++ = no fat-staining material remaining.

was due chiefly to the slowness with which the solvents were able to penetrate into the cells so as to reach the fatty material. Of course, the relatively large amount of lipid to be dissolved away from these organisms was a factor also.

DISCUSSION

The observations reported here serve chiefly to suggest the potential future usefulness of the staining technique described. More detailed studies with particular groups of organisms will have to be carried out with the aid of the stain before full answers will be obtained to the numerous questions about the intracellular lipid that come to mind.

The origin and fate of fatty inclusions when they occur regularly in actively metabolizing cells, and when they appear in degenerated forms, and the distinction between fat storage as a useful accompaniment of cellular life and the mere accumulation of lipid deposits ("fatty degeneration") are among the matters that should be investigated. From the limited study so far made it seems likely that fat droplets in the cytoplasm originate at the cell periphery, and presumably in some relationship to the cytoplasmic membrane, which itself is apparently colored by Sudan black B in many fungi and in some bacteria. This is in accord with the views of Knaysi (1945, 1946), who has observed the formation of fat droplets from the cytoplasmic membrane in living cells of *Bacillus cereus*. We agree with this investigator that the function (if any) of the fatty inclusions is not clear, and share his opinion that the usual conception of them as droplets of "reserve food material" is inadequate. Although we have noticed indications that some of the intracellular fat is utilized in the late phases of cell growth, most of it (under ordinary cultural conditions, at least) appears to remain unchanged, *in situ*, while the cytoplasm containing it disintegrates.

The precise chemical nature of all the material stained in dried films by Sudan black B is as yet unknown. The dye evidently colors not merely free fat but also lipoid complexes. The extraction experiments demonstrate that recognized lipid solvents will eventually remove all this material from intact bacterial cells, although the rate of extraction differs with the species as well as with the solvent. Study of successive preparations exposed for increasing lengths of time to fat solvents shows that the Sudan black B staining material is truly within the cell, and is gradually removed as the solvent penetrates the cell body. These observations reinforce the conclusion that the substances that color with Sudan black B are not merely surface deposits of stain or other artifacts, but are true cytoplasmic inclusions or integral parts of genuine cell structures. They afford new evidence of the complexity of the bacterial cell (Dubos, 1945).

Of special interest is the finding of definite fat droplets in anaerobic bacteria, both sporeforming and nonsporeforming; this is contrary to the expectations of some authors (Imšenecke, 1945; Meyer, 1912).

An obvious advantage of a fat-staining technique for ordinary dried films is the unlimited opportunity afforded for direct comparisons, at leisure, with the appearance of parallel films of the same organisms treated with other dyes that leave the intracellular lipid unstained. Such comparisons make very clear the

important role of fatty inclusions in causing "vacuolation," distortion of the stainable cytoplasm pushed aside by the fat, and "irregular staining" generally. (For a recent study of such staining on the part of the glanders organism, see the article by Worley and Young, 1945.) Lewis (1941) and others have pointed out how the bizarre appearances of fat-containing organisms when colored with methylene blue or other simple stains have led in the past to claims for the presence in various bacteria of "nuclei," "gonidia," and other special structures, and to the idea that such organisms go through complex "life cycles." It is surely no accident that such claims have often been concerned with fat-rich forms (e.g., *Azotobacter*, *Rhizobium*, and *Bacillus megatherium*).

SUMMARY

An improved technique for demonstrating intracellular lipid in microorganisms by staining dried, fixed preparations with Sudan black B and counterstains is described.

The application of this staining method to films of the principal species cultivated on common media revealed that stainable fatty material in the form of cytoplasmic inclusions, or such material associated with structural elements of the cells, is present in all fungi and in the great majority of bacteria, whether these are aerobic or anaerobic, saprophytic or parasitic, pathogenic or non-pathogenic.

A list is given in which the organisms studied are divided arbitrarily into three groups according to their content of demonstrable lipid matter when grown on the media customarily employed for each kind of organism.

With certain notable exceptions (e.g., *Alcaligenes fecalis* and *Azotobacter*), fatty material was found to be more abundant and to occur with greater regularity in gram-positive bacteria than in gram-negative bacteria. An apparent tendency for saprophytic species to contain more fat than parasitic species was noted. Acid-fast bacteria were found to take a bluish-gray color throughout in many instances; the rods were seen to contain distinct, deep-staining fat droplets as well.

Intracellular lipid was discovered in organisms in primary (mixed) cultures, as well as in pure stock cultures, and a number of bacteria and fungi in direct smears from body surfaces and excretions were found to contain fatty material.

Although media of special composition are required for the appearance of stainable lipid in the case of certain bacteria, the formation of fatty inclusions was shown not to be dependent upon the presence of glucose, glycerol, or other fermentable carbohydrate in the medium.

Of greatest interest was the unexpected finding that the relative amount of stainable fatty material and its form and location within the cells of bacteria are remarkably constant for any one kind of organism. Definite differences occur among different kinds, however. Thus, the pattern of intracellular lipid exhibited in the stained films is to a considerable degree characteristic for the bacteria of a particular genus, and in some cases for those of a particular species.

That this is not a mere happenstance is indicated by the further fact that,

throughout the whole phylum, bacteria of species or genera known to be closely interrelated show a marked similarity in their appearance when fat-stained. Indeed, with only occasional exceptions, the closer the relationship between varieties of bacteria in other respects, the more nearly alike is their usual content of stainable lipid. The formation of intracellular fatty material in a particular pattern is evidently a fixed habit in many bacteria, and one that must be associated with something fundamental in the enzymatic or structural make-up of the organisms.

The simplicity of the staining method suggests its routine use in the characterization of microorganisms. Its value as an aid in the differentiation of species and genera will be more fully established by further trials. Its possible usefulness in practical diagnostic work is largely unexplored. The stain should prove especially helpful in future investigations of bacterial cytology and metabolism.

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