DIFFERENTIATION OF MICROORGANISMS BY MEANS OF THE INFRARED SPECTRA OF THEIR ACETONE EXTRACTS

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Recent investigations give an optimistic view that infrared absorption spectra can be very useful in the rapid identification and differentiation of microorganisms. However, it is admitted that such identifications will have to be based on very small differences, often not much above the reproducibility with which the spectra can be measured.

Blout and Lenormant (1953) compared the spectra of Lactobacillus arabinosus in D₂₀ suspension with the spectra of proteins, nucleic acid, and nucleoproteins and pointed out the possibilities of identification by means of the infrared absorption spectra of whole organisms. Stevenson and Bolduan (1952) investigated the infrared spectra of whole bacteria for identification purposes and demonstrated that, while exhibiting quantitative differences because of variations in the thickness and physical homogeneity of the films, the procedure would produce spectra from replicate cultures which were qualitatively very similar. Although "the differences were somewhat subtle in some cases," they concluded that "the use of infrared absorption spectra as an aid to identification appears promising." Stevenson and Levine (1952) investigated eight types of pneumococcal polysaccharides and showed that, while the spectra of some types are quite distinctive and easily identified, the spectra of others are very similar.

Experiments designed to identify specific constituents in the microorganisms have indicated that considerably greater differentiation can be obtained by use of a series of extracts. Levine et al. (1953a, b, c) resorted to chemical fractionation to make correlations between absorption bands appearing in the spectra of intact bacterial cells with vibrating groups which give rise to them. Working with enteric bacteria Levine et al. (1953a) showed that hot dilute acetic acid or cold trichloracetic acid extraction followed by alcohol precipitation yielded a polysaccharide fraction characterized by a deep band from 8.6 to 10.0 μ. Phenol extraction removes proteins, characterized by their peptide linkage bands at 6.05 and 6.45 μ, leaving a polysaccharide residue with a spectrum very similar to the hot acetic acid or cold trichloracetic acid fractions. Extraction with cold trichloracetic acid, to remove polysaccharides, caused an attenuation of the 8.6-μ band. Following this, extraction with hot trichloracetic acid, which removes nucleic acids, resulted in a marked attenuation of the 8.0- to 8.1-μ band and complete loss of the remaining 8.6- to 10.0-μ band. This result was interpreted to mean that the 8.0- to 8.1-μ band arises from a vibration of the nucleic acid, while both polysaccharides and nucleic acids contribute to the intensity of the 8.6- to 10.0-μ band. After cell disintegration by sonic vibration and after removal of the protein by precipitation, first with 95 per cent alcohol and then, to effect complete removal, by shaking with chloroform and isoamyl alcohol, aqueous extract of Aerobacter aerogenes yielded spectra identical to those of commercial glycogen (Levine et al., 1953b, c).

Schneider and McLaughlin (1955) concluded that leptospiral fractions comprise independent antigen systems and that their infrared spectra are dissimilar and reproducible. This permitted an identification of the components and served to supplement serological typing and differentiation of the serogroups.

Randall and Smith (1953) measured the infrared spectra of methanol-chloroform extracts of total lipides from unruptured cells of tubercle bacilli and found spectroscopic differences between strains which were biologically similar. Bovine tubercle bacilli were shown to differ spectroscopically, and therefore chemically, from human tubercle bacilli.
Randall et al. (1951, 1952) correlated the biological properties of mycobacteria with their infrared spectra. Benedict (1955) attempted a group classification of virus preparations by infrared spectroscopy. Simon and Hedrick (1955) observed Saccharomyces cerevisiae cellulose and Hansenula anomala cellulose to exhibit infrared spectra similar to type II rather than type I cellulose which is present in bacteria. Bailey et al. (1953) used infrared absorption curves to investigate the effect of antibacterial agents on organisms and found such agents affect selectively the infrared absorption spectra and may offer a useful tool for studying mechanisms of activity. Cawley et al. (1954) examined the infrared spectra of fungi.

Maximum differentiation might be expected by selection, from a series of extracts, of the particular one whose infrared absorption spectrum exhibits greatest differences. To keep the infrared absorption technique simple and rapid, a procedure based on a single reproducible extraction is desirable. The purpose of this paper is to describe such a procedure, based on comparisons of the spectra of acetone extracts rather than on the spectra of whole bacteria.

**EXPERIMENTAL METHODS**

**Control of growth medium.** The infrared spectrum of a mixture of chemicals as are present in a microorganism merely represents the summation of the spectrum of each of the individual chemical molecules. Hence, factors which affect the chemical composition of bacteria must modify its infrared absorption spectrum. Consequently, the medium upon which the organism is cultured, as well as the age and temperature of the culture, must be carefully controlled if reproducible spectra are to be obtained. Several examples of the effect of change in growth medium have been observed in the present investigations. The infrared spectrum of Streptococcus faecalis grown on nutrient agar differs considerably from that grown on microassay agar. The infrared spectrum of Bacillus cereus grown on nutrient agar is very similar to the spectrum of Escherichia coli grown on this medium, but the spectrum of B. cereus grown on fortified tryptose agar is considerably different. Levine et al. (1953a) report similar results with Salmonella paratyphi and Salmonella typhosa. Randall and Smith (1952), Stevenson and Bolduan (1952), and Levine et al. (1953c) studied the effect of composition of medium and of age and temperature of the culture in some detail and found that all these factors grossly affect the infrared absorption spectra.

To compare the infrared spectra of an “unknown” microorganism with a set of infrared spectra of known “standard” organisms, it will be necessary to transfer the unknown to the medium in which the “standards” were grown and control the conditions of culture. For comparisons, the microorganisms were serially transferred to fortified tryptose agar five times. All cultures were exactly 24 hr old, and the cultures were grown at 26 ± 2°C.

**Cell disintegration.** Experiments with a variety of solvents led to the realization that no solvent extraction would be successful unless extraction is made on bacteria after cell rupture. Levine et al. (1953a, b, c) reported inability to extract glycogen from A. aerogenes until the cells had been disintegrated by sonic vibrations. Several methods of disintegration were tested. In order to keep the procedure as simple as possible, cell

*Figure 1. Modified test tube homogenizer*
rupture by mechanical grinding in a modified test tube homogenizer was adopted.

The homogenizer (figure 1) consists of two concentric conical ground-glass pyrex surfaces. The inner cone is rotated by means of a laboratory motor-driven stirrer while the outer cone is held stationary. The grinding surface is 7.5 cm in length and the cone is about 2 cm in diameter at the top, tapering to a rounded tip. Above the grinding surface, and surrounding the inner cone, is a small well 3 cm in depth and 3 cm in diameter with a side arm opening near the top to permit addition of sample during grinding. The sample may be placed in the homogenizer either through this opening or through the top before the inner cone is inserted. The outer cone may be surrounded by a water jacket if it is found desirable to grind at reduced or elevated temperatures. Over the shaft of the rotating cone a glass cover is fitted to prevent spattering. To prevent aerosol from escaping, the opening between the shaft and the cover may be protected with cotton wrapped in gauze. This makes the grinder particularly adaptable for use with pathogenic bacteria. The grinder can accommodate up to 1-g samples. In most cases a 300-mg sample was convenient.

The sample was ground with four times its weight of finely ground pyrex glass (40 to 60 mesh) and twice its weight of water (Dockstader and Halvorson, 1950). During the grinding process it was necessary to lower and raise the outer cone periodically, thus exchanging the material between the grinding surfaces and the well. A grinding time of about 15 min was required for a 300-mg sample to insure complete lysis.

During use the side wall of the homogenizer wears down faster than the tip and the inner cone will eventually fit too loosely. An advantage of a conical type homogenizer is that the tip of the pestle can be easily ground down to once again attain a tight fit.

Direct solvent extraction of the microorganisms yields little material, and very poor spectra with only weak absorption bands are obtained. After grinding, the same extraction procedure gives spectra exhibiting several sharp, well-defined absorption bands. Evidence for cell lysis is supplied by micrographs of the microorganisms before and after grinding. Photomicrographs indicated evidence of considerable cell lysis. It is shown even more clearly in electron micrographs (figure 2).

**Acetone extraction procedure.** For a simple identification procedure a single solvent and a reproducible procedure are required. Experiments demonstrated that cell rupture is more readily accomplished with water. After considerable comparisons of infrared spectra of a wide variety of solvents, aqueous acetone was selected as the most suitable single solvent. A simple, rapid extraction procedure was then devised.

Approximately 300 mg of a 24-hr culture of the organisms to be ground was removed with a loop from the agar slant, care being taken to avoid carrying agar along with the organism. The sample was placed on a tared watch glass and weighed. It was then mixed with enough finely ground pyrex glass (40 to 60 mesh) to facilitate transfer with a small spatula to the grinder. Additional glass was added to the mixture in the grinder to equal four times the weight of the organism. Distilled water, equal to twice the weight of the sample, was added and the mixture ground for 15 to 20 min. After grinding, the sample was transferred with a small amount of acetone from the grinder to a 50-ml pyrex centrifuge tube. This transfer was aided by the use of a broken-tip pipette. The sample was heated in a cotton-plugged centrifuge tube for 1 hr in a water bath at 50 to 60 C. It was necessary in some cases to add more acetone. After heating, the sample was centrifuged for 30 min at 2,000 rpm and the supernatant fluid was removed by pipette to a clean test tube. The extract was evaporated to near dryness in a water bath and the remaining few drops of liquid were transferred to a silver chloride disc.

**Preparation of silver chloride discs.** Silver chloride discs were prepared as described by Stevenson and Bolduan (1952), using the microadapter of Stevenson and Levine (1952, 1953). Since it is usually impossible to put the entire sample on the disc at one time, an infrared lamp was used to hasten drying of the sample between the addition of portions, care being taken to remove the heat source just before the sample became completely dry. During drying, segregation of some of the constituents may occur causing a nonhomogeneous distribution of the sample. To insure a uniform and homogeneous smear, each sample was reworked with a rubber policeman to resmear it on the disc just before the sample became completely dry.

**Measurement of infrared absorption spectra.** All
Electron micrographs showing cell lysis.

RESULTS

Reproducibility. Before the efficiency of differentiation of microorganisms by infrared absorption curves of acetone extract could be tested and compared to use of curves of the entire organisms, some measure of the reproducibility (repeatability) of the procedure was required. Tests of the entire procedure included: (a) culture of the organisms on fortified tryptose agar under controlled conditions, (b) cell disintegration with the

spectra were measured from 2 to 12 μ with a Perkin-Elmer Model 21 spectrophotometer, using the following instrument settings: resolution 915, response 2, gain 7, speed 4, and suppression 2. All measurements were made with a piece of the same silver chloride sheet used to prepare the sample disc in the balance beam of the instrument. The spectrophotometer was balanced at 100 per cent transmission with silver chloride discs in both beams.
Infrared spectra of acetone extracts: A, Aerobacter aerogenes; B, Azotobacter chroococcum; C, Pseudomonas fluorescens; D, Bacillus pumilus.

Figure 3. Infrared spectra of acetone extracts: A, Aerobacter aerogenes; B, Azotobacter chroococcum; C, Pseudomonas fluorescens; D, Bacillus pumilus.

mechanical grinder, (c) extraction with aqueous acetone, (d) preparation of the silver chloride discs, and (e) measurement of the infrared absorption spectra. In figures 3 and 4 curves of several pairs are used to illustrate the satisfactory reproducibility obtained. Quantitative reproducibility cannot be expected as neither the exact thickness of the smear nor its physical homogeneity can be completely controlled. Variations in the absolute absorbancies of the various bands must be expected. However, ratios of the absorbancies of these bands should be independent of the smear thickness and should be a measure of the degree of reproducibility. A number of such ratios have been calculated from the most prominent absorption bands in the curves of figures 3 and 4 and are tabulated in table 1. All of the ratios agree within about 0.2, most being well within 0.1. These differences are considerably smaller than those which will be used in the proposed method for differentiation. A more complicated procedure to improve reproducibility would not seem to be required, since a simple procedure is best suited for use in different laboratories.

Efficiency of acetone extracts. Several microorganisms were selected whose spectra of the whole organism are identical or very similar. Acetone extracts of these bacteria were obtained and compared with one another. The spectra of four series of such organisms, representing typical experiments, are shown in figures 5–8. The microorganism in each series can be distinguished from the microorganisms of each of the other series by means of the spectra of the whole organism. A casual glance at the figures reveals that the spectra of the entire organisms within a single series are similar, and differentiation could not be achieved without a statistical study of small quantitative differences from spectra measured under more reproducible conditions. However, further inspection of each figure shows that the spectra of the acetone extracts in all cases reveal
some differentiating features. In most spectra these differences are very marked.

The spectra of Chromobacterium violaceum, Pseudomonas fluorescens, and Bacillus polymyxa are compared in figure 5. At about 8 μ there is a bathochromic shift of the band which appears with a maximum at 8.08 μ in the spectra of the entire bacteria. This shift is greatest in B. polymyxa, which exhibits a strong band with maximum at 8.40 μ. P. fluorescens exhibits a weaker band with a smaller shift to 8.28 μ. The band for C. violaceum is quite weak at 8.36 μ. The spectrum of the acetone extract of B. polymyxa reveals a hypsochromic shift of the 9.2 μ band, seen in the spectra of the entire microorganism, to 9.0–9.1 μ. In the spectra C. violaceum and P. fluorescens there is a corresponding bathochromic shift, the band in the spectra of these two organisms appearing at 9.3 μ. B. polymyxa can readily be differentiated from either C. violaceum or from P. fluorescens by the appearance of distinct bands at 10.84 and 11.32 μ. Neither of these bands is observed in the spectra of the other two organisms.

The spectra of three organisms of a somewhat different spectral type are shown in figure 6. At 6.0 μ there are characteristic differences in the band which appears in the spectra of the three entire organisms as a broad absorption from 6.0 to 6.1 μ. In the spectra of the acetone extract of Bacillus subtilis the position of this band is almost unchanged, whereas in Acetobacter turbidans it remains, but a second C=O stretching vibration is observed with maximum at 5.79 μ. In the acetone extract spectrum of A. aerogenes there is no maximum above 6.0 μ, the only C=O stretching being observed at 5.96 μ, thus affording a differentiation. At about 7.0 μ, the 7.10-μ band becomes noticeably weaker in going from the acetone extract spectra of A. turbidans to B. subtilis and still weaker for A. aerogenes, as it appears only as a weak shoulder. The broad ab-
Aerobacter aerogenes

Azotobacter chroococcum

Pseudomonas fluorescens

Bacillus pumilus

Lactobacillus fermenti

Bacillus subtilis

Acetobacter capsulatum

Acetobacter turidans

* Wave length ratio, μ.
† Duplicate absorbance ratios.

Absorption band at 9.0 to 9.8 μ in the spectra of the three entire organisms is unchanged in the spectrum of the acetone extract of A. turidans. In the spectra of the extracts of B. subtilis and of A. aerogenes, definitely resolved bands appear with maxima at 9.05 and 9.76 μ. The 9.76-μ band has been attributed to deoxyribonucleic acid (Blout and Lenormant, 1953). The strong band with maximum at 10.8 μ in the acetone extracts of A. aerogenes and B. subtilis easily differentiates these two organisms from A. turidans, whose acetone extract spectrum shows no indication of a band at this wavelength.

In figure 7 Acetobacter capsulatum, Acetobacter vini-acetati, and Bacillus pumilus are compared. An interesting band appears at 4.42 to 4.44 μ for the acetone extracts of A. capsulatum and A. vini-acetati. This band probably arises from a vibration of a C=O stretching in a long-chain fatty acid or derivative (Celmar and Solomons, 1953; Khan, 1953).

Its appearance in the spectra of these two organisms distinguishes them from B. pumilus, whose acetone extract spectrum does not exhibit this band. At 6.0 μ there are differentiating features in the spectra of the acetone extracts. A. capsulatum exhibits the broad single C=O stretching band at 6.0 to 6.5 μ, characteristic of the spectrum of the entire organisms. B. pumilus and A. vini-acetati exhibit two bands, one below and one above 6.0 μ, but the wave length positions of these two bands, at 5.76 and 6.12 μ and at 5.82 and 6.00 μ, respectively, serve to differentiate the two bacteria. In the spectra of the entire organisms there is a broad minimum between 8.40 and 8.50 μ. The acetone extract spectra of A. vini-acetati and A. capsulatum have a strong absorption band with maximum at 8.38 μ. This band is not seen in the acetone spectrum of B. pumilus, which readily distinguishes it from the other two organisms. At about 11.0 μ, the acetone extract spectrum of A. capsulatum exhibits two distinct bands with maxima at 10.80 and 11.30 μ, while that for A. vini-acetati acetone extract reveals only one band with maximum at 11.30 μ. The corresponding spectrum of B. pumilus exhibits no characteristic bands in this region.

Figure 8 shows the spectra of a third spectral

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
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<th>B</th>
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<tr>
<td>Aerobacter aerogenes</td>
<td>5.93</td>
<td>0.87</td>
<td>6.84</td>
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<td>7.45</td>
<td>1.61</td>
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<td>1.12</td>
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<td>6.85</td>
<td>0.83</td>
<td>7.48</td>
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<tr>
<td>Pseudomonas fluorescens</td>
<td>5.83</td>
<td>0.89</td>
<td>6.84</td>
<td>0.97</td>
<td>6.96</td>
<td>0.99</td>
<td>7.08</td>
<td>1.21</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>6.12</td>
<td>1.00</td>
<td>6.00</td>
<td>0.60</td>
<td>7.44</td>
<td>1.03</td>
<td>7.76</td>
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<tr>
<td>Lactobacillus fermenti</td>
<td>7.12</td>
<td>1.01</td>
<td>7.47</td>
<td>0.84</td>
<td>8.00</td>
<td>1.87</td>
<td>9.06</td>
<td>0.88</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>6.00</td>
<td>0.96</td>
<td>7.12</td>
<td>0.73</td>
<td>7.47</td>
<td>2.12</td>
<td>8.00</td>
<td>0.80</td>
</tr>
<tr>
<td>Acetobacter capsulatum</td>
<td>6.29</td>
<td>1.23</td>
<td>6.84</td>
<td>0.40</td>
<td>8.38</td>
<td>1.57</td>
<td>10.82</td>
<td>1.67</td>
</tr>
<tr>
<td>Acetobacter turidans</td>
<td>5.80</td>
<td>0.80</td>
<td>6.08</td>
<td>1.40</td>
<td>6.57</td>
<td>0.88</td>
<td>7.09</td>
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<tr>
<td></td>
<td>6.08</td>
<td>0.80</td>
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<td>0.77</td>
<td>7.43</td>
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</table>

Table 1

Reproducibility of extraction procedure
Figure 5. Infrared spectra of entire microorganisms (A, B, and C) and of their acetone extracts (D, E, and F): A and D, Chromobacterium violaceum; B and E, Pseudomonas fluorescens; C and F, Bacillus polymyxa.

disappears in the spectra of the acetone extracts of Corynebacterium fimii and Acetobacter orleanense and is replaced by two bands below 6.00 μ at 5.78 and 5.82 μ, respectively. In the spectra of the acetone extract of Achromobacter lactiicum the 6.04 μ band is still observed but, in addition, a band with maximum at 5.76 μ appears. At about 8.0 μ the band with maximum at 8.08 μ, observed

type of bacteria characterized by weak absorption bands in the 8.5 μ region and weak, but definite, bands at about 10 μ in the spectra of the entire organisms, all of which are indistinguishable. The acetone extract spectra reveal three regions in which sharp differentiations can be made. The band with maximum at 6.06 μ in the spectra of the entire organisms completely
in the spectra of the three entire bacteria, disappears entirely in the spectrum of the acetone extract of *A. orleanense*. There is a bathochromic shift in the acetone extract spectra of *A. lacticum*, a band appearing at 8.20 μ, while in the spectra of *C. fimi* a hypsochromic shift is observed and the band is seen at 7.80 μ. The weak shoulder at 8.7 to 8.8 μ, which is characteristic of the spectra of these three entire organisms, becomes an indistinguishable part of the broader 7.2- to 9.0-μ region in the spectrum of the acetone extract of *A. orleanense*, becomes still weaker for *A. lacticum*, and disappears entirely in that of *C. fimi*, which exhibits a pronounced transmission band. The acetone extract of *C. fimi* exhibits in acetone a strong band with maximum at 8.94 μ which is
characteristic of this organism as it is not observed at all in the acetone extract spectra of either *A. lacticum* or *A. orleanense*.

**DISCUSSION**

A prerequisite for the identification of any microorganism by means of the infrared spectra of its acetone extract is a complete catalogue of the spectra of such extracts. Such an undertaking is beyond the scope of these preliminary investigations. However, by selecting several organisms which cannot be differentiated by means of the infrared spectra of the complete organisms (except by statistical treatment of very small quantititative differences, which would have to be based on spectra measured under more reproducible conditions than have been attempted in these studies), it has been demonstrated in every case that these same organisms can be readily differentiated by means of the infrared spectra of their acetone extracts.

In some cases, where the acetone extracts do not yield the most desired differentiations between two specific organisms, preliminary studies indicate that a second extraction with another solvent produces differences in spectra. If acetone extracts one or more compounds not common to the two organisms being compared, or if it extracts a substance which is present in one of the two in considerably higher concentrations, then readily observed differences will be obtained. If, however, the acetone is extracting only substances which are common to the two organisms in about equal amounts, obviously the infrared spectra of the two extracts will be nearly identical. In such cases use of a second extracting solvent, if properly chosen, may reasonably be expected to yield the desired differentiation. This conclusion is analogous to that reached by Randall and Smith (1953), who proposed fractionation by means of successive elutions from a chromatographic column.

In regard to the infrared spectra of enteric bacteria, Levine *et al.* (1953a) say: "It is apparent that the absorption of substances present in small concentration can be masked by the absorption of other more plentiful compounds," and they proceed to solvent extraction to identify glycogen. The function of acetone is to remove these "plentiful compounds," mostly proteinaceous,³

³ Blout and Mellors (1949), reporting on an investigation of the absorption spectra of tissues, found that the "main absorbing constituent is protein in nature." Wood (1951) reports similarly that "... the spectra of muscle cells seem to be closely duplicated by those of the protein myosin,"
entire organisms is described. The procedure consists of six independent steps: (a) careful culture of the microorganisms on the same selected growth medium and under standardized growth conditions, (b) cell disintegration with a mechanical grinder, (c) acetone extraction under controlled conditions, (d) preparation of smears of the microorganism extract on silver chloride discs, (e) measurement of the infrared absorption spectra of the organisms on silver chloride discs against a plate containing no extract, and (f) comparison of the spectra of the acetone extract with the spectra of acetone extracts of known bacteria. The details of each step in the proposed procedure, the reproducibility of the entire method, and the advantages of the acetone extraction procedure over spectra of the entire organism are described. The further advantages of successive extraction with a series of a small number of carefully selected solvents are cited.

REFERENCES


