INFRA-RED SPECTROSCOPY AND ITS APPLICATION TO MICROBIOLOGY

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(With 2 Figures in the Text)

Colorimeters and non-recording spectrophotometers for visible and ultra-violet light have been used for many years and are to be found in most microbiological laboratories. With the need for greater speed of operation, recording instruments are now coming into more general use. During the last two decades recording infra-red spectrophotometers have been developed and these have enabled the absorption measurements on micro-organisms to be extended into the infra-red region of the spectrum. Two factors have tended to retard the use of infra-red spectrophotometry. One is the high initial cost of the equipment and the other is the large absorption of infra-red radiation by water. Despite these difficulties, a considerable amount of work has now been done and it seems profitable to review the varied applications to which infra-red spectroscopy has already been put and to indicate the results which have been obtained by its use.

Infra-red radiation is part of the electromagnetic spectrum. The lower wavelength limit is set by the long wavelength limit of perception by the human eye and the upper limit is arbitrarily fixed by the lower limit of present microwave techniques. The range of infra-red wavelengths is thus between $0 \cdot 75$ micron (μ) and 500μ. The measurements so far reported on biological materials have been confined to the region 1–16μ. The infra-red absorption spectra of most materials consist of a large number of absorption bands. These bands are due to the vibrational and rotational motions of the molecules which are excited by the absorption of infra-red radiation. The fundamental frequency of vibration of a particular bond or group of atoms in a molecule involves the motions of all the atoms in the molecule. For large molecules, however, it is found that a certain group of atoms or a chemical bond gives rise to an absorption band at a particular frequency which is almost independent of the remainder of the molecule. A spectrum comprises several of these absorption bands of different strengths and different frequencies, sometimes overlapping, sometimes simple and sometimes complex. No two different molecules, with the exception of optical enantiomorphs, possess the same bonds and groups of atoms and therefore no two different molecules have the same infra-red absorption spectrum. It is for this reason that infra-red spectroscopy is one of the most powerful analytical tools available to the organic chemist.

The fundamental frequency of the stretching vibration of a carbon-hydrogen bond is approximately 10^{14} cyc./sec. The frequencies involved are so high that it is more convenient to characterize radiation by its wavelength or wave-number. The latter unit may be defined as the number of waves per centimetre. Radiation

of wavelength 1μ (10^{-4} cm.) is equivalent to 10^4 wave-numbers or reciprocal centimetres (cm.$^{-1}$). The wave-number unit (cm.$^{-1}$) is to be preferred because it is directly proportional to the true frequency which can be obtained by multiplying the wave-number of the radiation by the velocity of light c.

Stair & Coblentz (1935) were perhaps the first to record the spectra (1–15μ) of biological materials such as onion skins, the membrane of fish bladder, bat's wings, egg albumin, egg membranes, gelatin and pith of feathers. They did not attempt to interpret the spectra but they commented upon their similarity. In fact, it has since been found that all tissues have very similar spectra. The methods then available for producing spectra were not adequate to allow extensive use of the technique and it is largely improvements in the instruments, particularly the introduction of double-beam recording spectrophotometers, that have enabled infra-red spectroscopy to be more widely and profitably applied. Modern instruments equipped with a rock-salt prism can record the spectrum between 2 and 15μ in a matter of minutes. If greater resolution is required other prism materials or diffraction gratings can be used but the rock-salt prism covers a wide range of wavelengths at moderate resolution. The spectra are usually recorded as percentage transmission versus wavelength (μ) or percentage transmission versus reciprocal centimetres (cm.$^{-1}$).

With a microscope attachment a satisfactory spectrum can be obtained from samples weighing only 10μg., although it is more usual to use about 1 mg. Nearly all the work done so far on micro-organisms has been on samples of 1 mg. or greater.

Before reviewing the applications of infra-red spectroscopy to microbiology it is desirable to consider the interpretation of spectra, particularly the spectra of molecules of biological importance.

Absorption bands between 2 and 7μ can usually be associated with the fundamental vibration of a particular group or bond in the molecule. For instance, the stretching vibration of a nitrogen-hydrogen bond absorbs radiation of wavelength 3μ and the stretching vibration of a carbon-oxygen double bond absorbs near 6μ. At wavelengths greater than 7μ absorption bands arise usually from skeletal vibrations of the molecule and can rarely be associated with the presence of a particular group of atoms. Absorption bands between 7 and 15μ tend to be characteristic of the whole molecule and have often been likened to a molecular 'fingerprint'. Charts and bibliographies giving the absorption frequencies of many types of bond and group have been compiled by Randall, Fowler, Fuson & Dangl (1949), Colthup (1950), Bellamy (1954) and Jones & Sandorfy (1956). These correlations between structure and absorption frequency may be used, with caution, to identify groups and bonds in an unknown substance.

The spectra of many molecules of biological importance have already been recorded by Thompson, Nicholson & Short (1950) and it is found that as the molecules increase in complexity the isolated absorption bands found for simple molecules tend to merge into broad diffuse absorption bands. Despite the difficulty of working with substances having large molecules, such as proteins, nucleic acids and carbohydrates, much work has been done on such substances. Sutherland

(1952) and Fraser (1953) have reviewed the applications of infra-red spectroscopy to molecules of biological importance and Levine (1953) has compiled a very helpful bibliography entitled 'The Applications of Infrared Spectrophotometry in the Biological Sciences', which lists over 260 references. May & Grenell (1957) published a comprehensive review dealing only with infra-red absorption of tissues.

The principal absorption bands in the spectra of proteins, nucleic acids and carbohydrates are shown in Fig. 1. For comparison the spectra of several species of micro-organisms are given in Fig. 2. The spectra of the organisms contain bands due to the three main cellular components protein, nucleic acid and polysaccharide, but an analysis of the spectrum in terms of the chemical composition is very difficult. The interpretation of spectra is further complicated by the fact that the spectra of most substances are influenced by the physical state of the specimen. This has been demonstrated for crystals by Kendall (1953) and for sugars by Goulden & White (1958) and Norris & Greenstreet (1958a). A warning about the difficulty of interpreting bacterial spectra was given by Ellinghausen (1958). Despite the warning and the difficulties, there have been numerous attempts to use the spectra of bacteria to derive information about the chemical composition of bacterial cells; this work will be discussed separately below.

The natural environment of micro-organisms is an aqueous medium and most organisms contain a large proportion of water. Unfortunately even a thin layer of water absorbs radiation of wavelength about 3 and 6μ very strongly and for this reason very few spectra have been recorded of aqueous suspensions of live bacteria. Heavy water absorbs at about 4 and 8μ. By recording the spectrum of specimens in water and in heavy water it is possible to obtain the spectrum of specimens in the interesting 6μ region. This technique has been exploited by Blout & Lenormant (1953), Lenormant (1953) and Blout (1957) to examine proteins, nucleic acids, polypeptides, yeasts and bacteria in suspension in both water and heavy water. In this way they have been able to obtain the spectra of these specimens in a natural environment. Many hydrogen atoms in large molecules may exchange with deuterium atoms and this possibility must be taken into account in a discussion of the results. Ehrlich & Sutherland (1954) studied the peptide absorption bands of proteins in D_2O, and in DCl and NaOD solutions. In this way they were able to separate the effects of ionization and of deuteration of the proteins on their spectra.

Other methods of preparing organisms for examination involve drying them in some way or other. Most workers have smeared either a colony or a suspension of bacteria on to a sheet of silver chloride and allowed the film to dry in air (Stevenson & Bolduan, 1952; Levine, Stevenson, Chambers & Kenner, 1953; Thomas & Greenstreet, 1954). The introduction of the alkali halide pressed-disk technique made it possible to prepare quantitatively specimens of bacteria for examination in the infra-red. In this method, which has been described by Stimson & O'Donnell (1952) and Ford & Wilkinson (1954), freeze-dried material is mixed intimately with ground potassium chloride or bromide and the whole is then pressed to produce a transparent disk containing the sample. Successful results using bacteria have
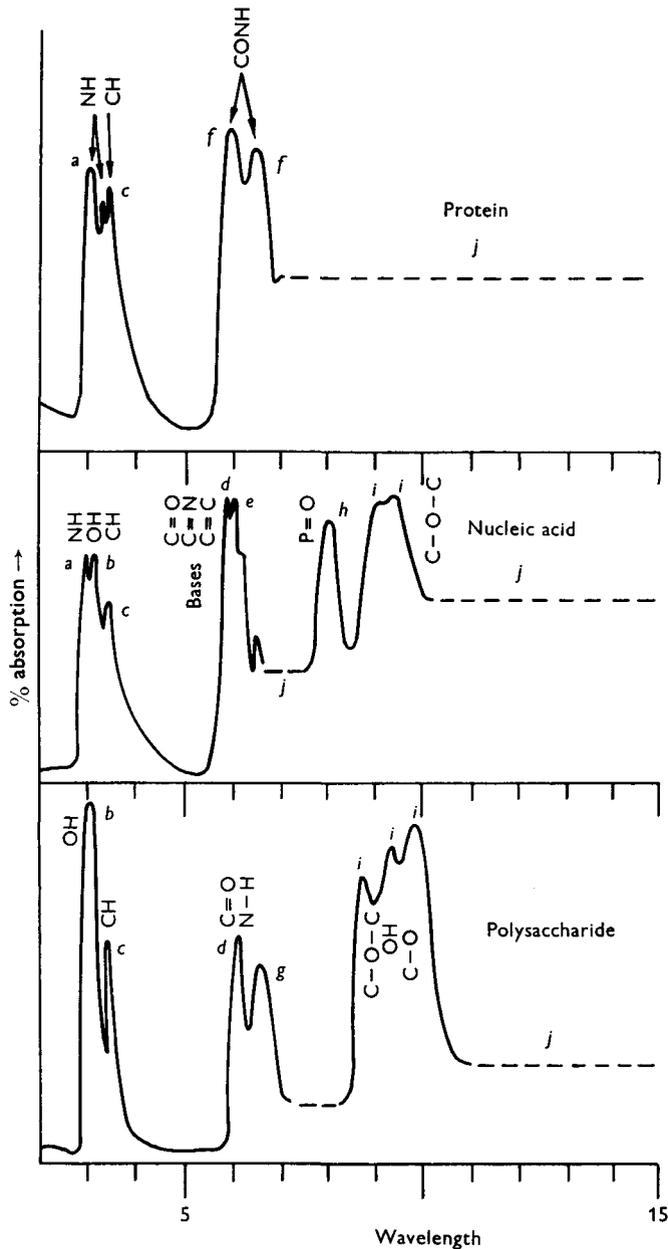
Fig. 1. Principal absorption bands in the spectra of protein, nucleic acid and polysaccharide. *a*, N–H stretching; *b*, O–H stretching; *c*, C–H stretching; *d*, C=O stretching; *e*, C=N and C=C of purine and pyrimidine bases; *f*, CONH vibrations of peptide link; *g*, N–H deformation in amino sugars; *h*, phosphorus vibrations; *i*, C–O–C vibrational modes, C–O stretching vibrations and OH deformation; *j*, regions of the spectrum which tend to be characteristic of the particular substance.

been reported by Levi, Matheson & Thatcher (1956), Adams (1957), Norris & Greenstreet (1958b) and Goulden & Sharpe (1958).

The classical method of mulling a solid with mineral oil to reduce the scattered light has been used only by Randall, Smith & Nungester (1951) to prepare whole cells for examination. It has of course been used by others to prepare specimens of cell extracts.

The infra-red absorption spectra have been recorded of specimens of bacteria, viruses, yeasts, fungi, moulds and algae and the uses of the infra-red method can, for convenience, be classified under the following headings:

(1) To identify or differentiate strains of micro-organisms.

(2) To interpret the differences between the spectra of organisms in terms of the chemical compositions of the cells.

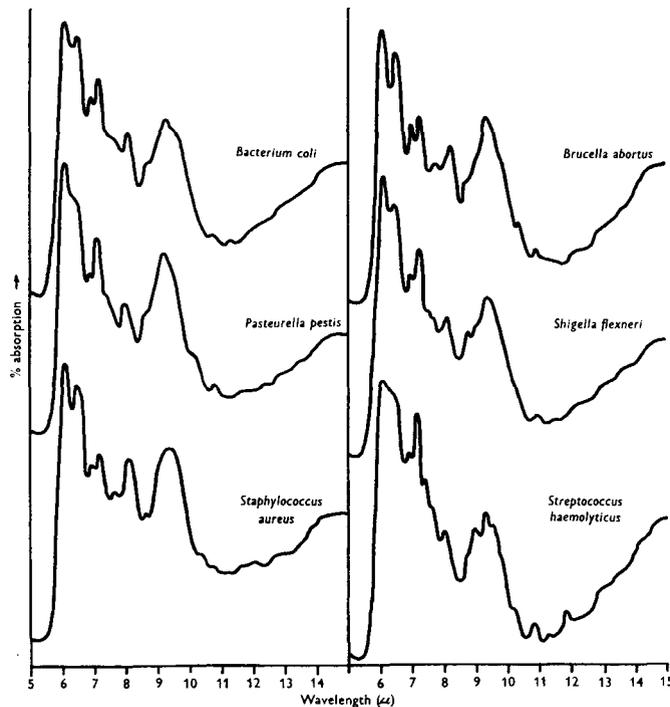(3) To characterize extracts and metabolites of cells.

Fig. 2. Spectra typical of certain species of bacteria.
(The spectra are displaced vertically.)

This arrangement does not enable the chronological development of the subject to be followed, but where necessary reference is made to important new contributions.

The identification and differentiation of micro-organisms

The desirability and practical value of a rapid method of identifying organisms has prompted many workers to explore the possibilities of using infra-red absorption spectra for this purpose. At present there is no agreement about the usefulness of the infra-red technique or whether whole cells or cell fractions should be used.

There is agreement, however, that the spectrum of organisms depends upon the composition of the culture medium, the condition and size of the inoculum, the duration and temperature of incubation, the method of preparing the specimen for examination and the way in which the spectrophotometer is used (Levine, Stevenson, Chambers & Kenner, 1953; Thomas & Greenstreet, 1954; Greenstreet & Norris, 1957; Norris & Greenstreet, 1958*b*). Kenner, Riddle, Rockwood & Bordner (1958) further recommend that the pH of the medium should be kept within ± 0·1 pH of the optimum for each strain. In spite of these limitations a critical review of the literature shows that reproducible spectra of bacteria can be obtained when due care is paid to standardizing all the above variables. A considerable amount of work has been done with viruses but the major difficulty has been to separate the virus from the host material. This has prevented an investigation of many other factors which might influence the spectra. Simon & Hedrick (1955) found that the spectrum of yeast cells depended upon the duration of incubation. So far insufficient work has been done with moulds, fungi and algae but it seems probable that the spectrum of these materials depends upon many of the parameters mentioned above.

Examination of whole cells

Randall *et al.* (1951) were the first to record the spectra of whole cells of bacteria. Since then they have made great progress in differentiating strains of *Mycobacterium* by means of the spectra of lipids extracted from the cells. They showed that the spectra of simple extracts were more easily distinguished than were the spectra of intact cells of *Bacillus subtilis, Saccharomyces cerevisiae, Staphylococcus aureus, Staph. citreus, Rhodotorula glutinis, Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, Chromobacterium prodigiosum* and *Klebsiella pneumoniae*. A careful examination of the spectra (2–14·5μ) of the intact cells reveals the kinds of difference which Stevenson & Bolduan (1952) thought were adequate to effect differentiation of many of the species used by Randall. Stevenson & Bolduan grew the bacteria under standard conditions and smeared the resulting growth on to silver chloride plates. They showed that there were apparent differences between the spectra (6–12μ) of whole cells of *Esch. coli, Pseudomonas aeruginosa, Staph. aureus, Micrococcus rosaceus, Aerobacter cloacae, B. subtilis, B. megaterium, Sarcina lutea, Chr. prodigiosum* and *Brucella tularense*. Two months after the publication of this paper the *Lancet* (1952) found it 'difficult to picture any practical application of this work but Stevenson & Bolduan have at any rate added another cell to the body of abstract knowledge'. This pessimism is a little hard to share in view of the successful applications of the method which are discussed below.

At about the same time Bolduan, Muth & Orlando (1952) claimed that reproducible spectra can be obtained from bacteria. As a safety precaution when they were working with pathogens the film of bacteria was sealed between two silver chloride plates. Similar methods were used by Levine, Stevenson & Chambers (1952) who showed that changes in the chemical composition of bacteria due to heating or storage in isotonic solutions were easily detected by means of a change in spectrum. On the other hand, any effects produced by ultrasonic

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disintegration, centrifugation or washing could not be detected by differences between spectra.

In the same year an ambitious project was started by Pollard, Engley, Redmond, Chinn & Mitchell (1952); they attempted to differentiate between animal viruses and found that the spectra of the viruses of mumps and of Newcastle disease were distinctly different from those of the viruses causing meningopneumonitis and ornithosis. From the description of the preparation of the viruses it is not certain whether the spectra were of virus or of modified host material. This aspect and the number of cycles of differential centrifugation necessary to get specimens suitable for differentiating the viruses has been studied in several reports by Benedict (1953), Benedict & Pollard (1953) and Benedict, Pollard & Engley (1954).

Siegel, Ng, Freeman & Bostick (1957) found particles of two sizes in mouse brain infected with a virus which had been isolated from mice having granulomata of the lymph nodes, whereas normal mouse brain material contained particles of only one size. Despite this difference the infra-red absorption spectra $(2-11\mu)$ did not permit them to differentiate between normal and infected brain material. Virus preparations made by butanol extraction had a different absorption spectrum from those prepared by differential centrifugation. This is attributed to the removal of lipid by the butanol. The virulence of the infective material obtained by both methods of preparation was essentially the same and it seems likely that each preparation was still grossly contaminated with host material. This could easily account for their inability to detect differences between the spectra of normal and infected material.

Kull & Grimm (1956a) had more success, however, with six serologically different 'M' bacteriophages of *Bacillus megaterium*. The six phages could be distinguished by their infra-red spectra and they were found to be different from the host cell, tobacco mosaic virus and the T_4 phage of *Esch. coli* Kull & Grimm concluded that 'the application of the infra-red spectrum for viral taxonomic differentiation is possible only under certain well defined conditions, the specificity of which limits the practicability of the method'.

Benedict (1955, 1957) was able to separate the viruses he studied into four groups; the related viruses within each group, however, could not be differentiated by infra-red spectrophotometry $(2-16\mu)$. The four groups were as follows: Group A, normal host component, influenza strains A, A' and B, and the virus of mumps; Group B, Newcastle disease virus; Group C, the viruses of psittacosis, meningopneumonitis, lymphogranuloma venereum, feline pneumonitis and mouse pneumonitis; Group D, vaccinia and fowl-pox virus. Benedict admitted that many of the preparations were not pure but he showed that useful data could be got from the spectra provided the limitations were understood.

The similarity of the intestinal Gram-negative bacilli prompted Levine, Stevenson, Chambers & Kenner (1953) to examine the spectra of strains of *Esch. coli*, *Aerobacter aerogenes*, *Salmonella montevideo*, *Salm. paratyphi*, *Salm. typhi* and *Shigella dysenteriae*. The spectra $(7-12\mu)$ enabled most of the organisms to be differentiated. Growth of strains of *Salmonella* in the presence of fermentable carbohydrate gave cultures with a spectrum different from those of

cultures grown in the absence of the carbohydrate. A paper by Kabler, Riddle & Kenner (1956) gave the results of an examination of 142 strains of coliform organisms. Standardized spectra (5·6–12·0μ) were obtained by procedures providing reproducibility of a very high order. These spectra were coded and punched on to International Business Machines (I.B.M.) cards. Their conclusions were that 'every species and serological group in this study could be differentiated from each of the others and standard infra-red absorption spectra of these coliform and related bacteria are of value in their differentiation'. This work was extended by Riddle, Kabler, Kenner, Bordner, Rockwood & Stevenson (1956) who gave more details of their experimental techniques. The films of bacteria were made wedge-shaped by tilting the silver chloride as the suspension dried. The transmission of the film at 8·05μ was adjusted to 50 % by moving the film in the beam of light. If the transmission at 5·0μ was less than 78 % the film was rejected. In this way poor films which had a high apparent absorption due to excessive scattering were eliminated. To incorporate some tolerance to allow for small differences in transmission between strains of the same species and to facilitate the coding of the spectra, the spectra were divided into seven sections starting at the following wavelengths: 5·6, 6·0, 7·0, 8·0, 9·0, 10·0 and 11·0μ. Each section was adjusted upwards or downwards to a predetermined fixed transmission. The transmissions, having been adjusted, were measured at intervals of 0·1μ and were coded and punched on to I.B.M. cards. Summary cards of all the strains of one species were prepared and comparisons between the summary cards (or species) were made only at wavelengths at which all spectra of all strains of each species agreed within 2 %. These methods were applied to 650 strains of 201 species and the results showed that many species of bacteria have characteristic differences between their infra-red absorption spectra. It was found that certain bacteria, which they do not specify, cannot be differentiated by this type of analysis.

The production of a summary card averages the differences between the spectra of strains of organisms belonging to one species. It is interesting therefore to compare these results with the work of Thomas & Greenstreet (1954) who compared the spectra of closely related strains of *Pasteurella pestis*, of *Esch. coli* and of *Salm. typhi*.

To avoid difficulties with pathogenic organisms they worked with heat-killed suspensions. The spectra of killed organisms are slightly different from those of live ones, but they found the spectra of killed organisms sufficiently reproducible to permit differentiation of the strains. The ratios of the strengths of certain strong absorption bands were used to help in the differentiation; the weaker bands were assigned numbers by comparison with an arbitrary scale. Further evidence that infra-red spectrophotometry can be used as an aid in differentiating bacteria came from Norris (1953) and Greenstreet & Norris (1957). The spectra of 136 strains of bacteria from twenty-nine species were compared by an objective method which eliminated personal judgement as far as possible. Rank correlation coefficients were used to compare the transmissions of pairs of spectra. A statistical analysis of the results showed that the differences between spectra of strains representing different genera were significantly greater than those between the spectra of

strains of the same species. To demonstrate the possibilities of strain differentiation, two strains of an organism were each grown in quadruplicate at the same time and under the same conditions. The differences between the four spectra of each strain alone were smaller than those between spectra of each of the two strains. It was thus possible to separate the eight spectra into two groups of four corresponding to the two strains, but this was not possible if the samples were not grown at the same time.

Goulden & Sharpe (1958) examined seventy-six strains of Lactobacilli representing eleven different species. Freeze-dried bacteria were incorporated into pressed potassium bromide disks. With few exceptions the spectra could be grouped into five distinct types each corresponding to a *Lactobacillus* species or a group of related species. They concluded that 'the classification of Lactobacilli according to their infra-red spectra is related to changes in the chemical composition of the organisms and is in agreement with the differentiation of species by other methods'.

Kull & Grimm (1954, 1956*b*) compared the spectra of parent bacterial strains with those of substrains resistant to antibiotics. There were no differences between the spectra of a penicillin-resistant and of a penicillin-sensitive strain of *Staph. aureus* or between those of a streptomycin-resistant strain of *Esch. coli* and of the parent strain. The morphology of Type 1 pneumococcal cells is affected by growth in the presence of choline chloride but there was no corresponding change in spectrum. The spectra of strains of *B. megaterium* resistant to bacitracin and 1-(*p*-butoxyphenyl)-3-(*p*-diethylamino-*p*-phenetyl)-2-thiourea monohydrochloride were different from the sensitive strain. There was also a difference between strains of *Mycobacterium tuberculosis* resistant to isoniazid and 1-(*p*-butoxyphenyl)-3-(*p*-dimethylaminophenyl)-2-thiourea. The spectral differences could not be related quantitatively to the degree of *in vitro* resistance. The observations on strains of *B. megaterium* can perhaps be explained by small differences between the growth rates of sensitive and resistant strains (Norris & Greenstreet, 1958*b*).

Kull & Grimm (1956*c*) later expressed the opinion that 'infra-red as a qualitative differentiating tool is limited if crude extracts, whole or intact cells are used'. Their principal objection to the method seemed to be that all the spectra had similar major and, in many cases, minor absorption bands. This objection might be overcome by extracting from the cell the component responsible for the differences between the spectra, or by subtracting from the spectra the spectrum of the common component which contributes to the major absorption bands. This may be done by difference spectroscopy. In this technique a sample of bacteria is placed in the sample beam of the spectrophotometer and a protein film or a standard film of bacteria in the reference beam. The instrument then records only the difference between the two beams. There are many practical difficulties to be overcome before difference spectroscopy can be applied to the differentiation of bacteria. So far insufficient work has been done to assess the usefulness of the method but the preliminary results of Norris & Greenstreet (1958*b*) are encouraging.

Even though different bacteria have similar absorption bands, the small differences which exist between bacteria are reproducible and the results which have

been described in this section seem to have justified the use of the spectra of whole cells. Riddle *et al.* (1956) have demonstrated that machines capable of comparing standardized spectra are of use. More advanced methods of comparing spectra such as multicomponent analysis (Perkin-Elmer Instrument News, 1954) and automatic analysis (Rogoff, 1957) are available but so far they have not been applied to the spectra of micro-organisms.

Perhaps one of the greatest difficulties in assessing the value of infra-red spectroscopy applied to the identification of bacteria is to distinguish between identification and differentiation. Identification in Natural History can be defined as 'the action of referring a specimen to its proper species'. In view of the inconsistencies, from genus to genus, of the grounds upon which specific rank is accorded it is not surprising that with a single technique, such as infra-red spectro-photometry, it is difficult to identify a species of bacteria. It is our experience, however, that if two different bacteria are grown as far as possible in exactly the same way at the same time and compared by infra-red spectrophotometry, it is possible to distinguish between them. Whilst this information may be of only limited value in a scheme of identification it can be of immense help in practical microbiology. As experience accumulates it may be possible to close the gap between differentiation and identification. At the present time identification of an organism would involve growing it together with a wide range of organisms with which it could be compared. As this is such a wasteful procedure it cannot be regarded as a practical scheme for identification.

Examination of cell fractions

Randall and his school have been primarily responsible for showing how useful infra-red spectrophotometry can be as a method of studying the differences between closely related strains of bacteria. It has already been shown that the spectra of the lipids extracted from strains of *Mycobacterium* were more easily distinguishable than the spectra of the intact cells. To do this a simple extract was made by treating the cells with a mixture of equal parts of methanol and chloroform. The extract was concentrated by evaporating the solvent under vacuum after it had been cleared of cell residue by filtration. The extract prepared in this way was further fractionated by chromatography. A series of papers since 1951 has described this work (Randall, Smith, Colm & Nungester, 1951; Smith, 1951; Randall, Smith & Nungester, 1952; Randall & Smith, 1953; Smith, Harrell & Randall, 1954; Harrell, 1954; Kubica, Randall & Smith, 1956; Smith, Randall, Gastambide-Odier & Koevoet, 1957). They showed that:

(1) The spectra of lipids extracted from a virulent and an avirulent strain are different.

(2) The spectra of whole cells of human and bovine strains show small differences whereas the lipids extracted from the cells have very different spectra.

(3) Lipids of the human strains can be further separated by extraction and chromatography and characterized by infra-red spectrophotometry.

(4) The separated components of the lipids can be characterized by infra-red and used to classify the human strains.

(5) The tubercle bacilli can be classified by the infra-red absorption spectra of the components of the lipid extract.

(6) The spectra of lipids extracted from strains representing 18 genera other than *Mycobacterium* exhibit greater differences than do the spectra of the whole cells.

One of the conclusions reached by Smith *et al.* (1957) is that 'we see little hope for the use of infra-red spectroscopy as a means of identifying bacteria in general, particularly when the method must compete with the highly specific techniques involving the use of fluorescent antibody'.

The technique of examining the infra-red spectra of extracted lipids was applied to fungi by Cawley, Wheeler, Boatwright, Smith, Randall & Lingamfelter (1954). They obtained spectra from three strains of each of *Microsporon lanosum*, *M. fulvum* and *Trichophyton gypseum*. The spectra did not allow positive identification or differentiation but the work was not comprehensive enough for any significant conclusion to be made.

Strains of *Streptococcus pneumoniae* are typed by the serological behaviour of their capsular polysaccharides. The spectra ($2-15\mu$) of fifty-seven samples of pneumococcal polysacchardes were examined by Stevenson & Levine (1952) and Levine, Stevenson & Kabler (1953). They found that all but two of the poly-saccharides could be distinguished by their spectra. Specimens of the purified polysaccharides were prepared for infra-red examination by drying an aqueous solution of the polysaccharide on a silver chloride support. They did not examine whole cells but the purified extracts were sufficiently different to enable the cells to be typed. Furthermore, the spectra enabled the polysaccharides to be divided into four groups depending upon their chemical composition. In the same way strains of *Klebsiella* can be typed serologically by their capsular polysaccharides. Levine *et al.* (1955) prepared crude capsular polysaccharides from cultures in-cubated for 18 hr. at 37° C. on a nutrient agar. The growth from one or two Petri dishes was scraped off and boiled in water for 15-30 min. The cells were spun out, and a few drops of sodium acetate solution were added to the supernatant. The polysaccharide was precipitated from this by the addition of cold 95 % ethanol. The precipitate was removed, washed in alcohol and recentrifuged. The poly-saccharide was dissolved in water and spread on a silver chloride support. The spectra ($5-15\mu$) of such extracts could be used to type the bacteria; the spectral typing largely, but not completely, paralleled the serological typing. One of their opinions is that the availability of infra-red spectrophotometers is the most serious limitation to the use of infra-red spectrophotometry in microbiology.

The principal objection to the use of whole cells is that most bacteria have common major absorption bands. One way of overcoming this is to extract the component responsible for the small differences between the spectra of whole cells. O'Connor, McCall & DuPré (1957) tried several organic solvents for this purpose. The quantity of material extracted from whole cells was too small so the bacteria were ground with the solvent. In this way, with careful standardization, they were able to get acetone extracts of *Acetobacter*, *Achromobacter*, *Azotobacter*, *Bacillus*, *Lactobacillus* and *Pseudomonas* which gave reproducible spectra and which

permitted ready differentiation. It may be that with some bacteria differentiation cannot be achieved with a single solvent so they suggested that a second extracting solvent, if properly chosen, may reasonably be expected to yield the desired differentiation. They do not suggest what the second solvent should be.

In specific research problems the technique of using the spectra of extracts of bacteria is very promising, but the method presupposes some knowledge of the identity of the organisms and therefore appears to be of little use for identifying organisms. Its use for differentiating between organisms seems to be well established. So far little has been done with the extracts of viruses, yeasts or algae.

The interpretation of the spectrum in terms of the chemical composition

The spectra of plant and animal tissues were first recorded by Stair & Coblentz (1935) and since then numerous attempts have been made to interpret the spectra in terms of chemical groups within the tissues. Tissue sections were studied by Blout & Mellors (1949) and Schwarz, Riggs, Glick, Cameron, Beyer, Jaffe & Trombetta (1951). Tissues have structure and are therefore heterogeneous to the light beam; this adds to the difficulty of interpreting the spectra. To overcome this Barer, Cole & Thompson (1949) used a microscope in conjunction with a spectrometer to record the spectra of muscle fibres, nerves and hair. A microscope attachment was also used by Fraser & Chayen (1952) when they recorded the spectra of bean root tips before and after the tips had been treated with enzymes. There were differences between spectra which enabled them to show the presence of ribonucleic acid in the root tips. A similar experiment demonstrated the presence of deoxyribonucleic acid in ram spermatozoa.

The first attempt to interpret the spectra of bacteria was made by Levine, Stevenson, Chambers & Kenner (1953). Carbohydrate was extracted from *Esch. coli* with hot acetic acid or cold trichloroacetic acid; protein was removed with phenol; nucleic acid with hot trichloroacetic acid and nucleoprotein with sodium hydroxide. Cells were treated with the above reagents and the spectra of the cells before and after treatment were compared. The results showed that the absorption bands at $6 \cdot 05$ and $6 \cdot 45 \mu$ are due to the peptide link of the protein. The band at $8 \cdot 0$–$8 \cdot 1 \mu$ is due largely to nucleic acid, the broad band between $8 \cdot 6$ and $10 \cdot 0 \mu$ being attributed to carbohydrates.

A similar approach was made by Adams (1957) who extended the method to allow estimations of the principal cell components to be made. Adams's methods of interpreting the spectra were: (1) applications of known frequency-structure correlations to the spectra; (2) comparison of the spectra with those of proteins, nucleic acids, carbohydrates and fats; (3) the use of chemical reagents or enzymes which have known specific actions upon the cell; (4) the preparation of pure cell walls and a comparison of the spectra of cell walls and whole cells. Adams attempted to study the effect of drugs upon bacteria by making rough estimates of the proportions of proteins, nucleic acids, carbohydrates and 'fats' in the cells. A summary of this work was given by Rideal & Adams (1957). In a similar manner Ford & Goulden (1959) used the spectra $(5$–$12 \mu)$ of cells of the flagellate *Ochromonas malhamensis* to determine the changes in the chemical composition of cells when

the amount of vitamin B_{12} in the medium was varied. Organisms grown on a vitamin-deficient medium contained more saturated triglyceride fats. Previously Bailey, Martini & Nachod (1953) had found differences between the spectra of bacteria treated with phenol or benzylammonium chloride and of untreated bacteria. No attempt was made to correlate these changes in spectra with chemical changes in the cells.

Lenormant (1953), who has studied the absorption bands between 6·0 and 7·2μ in great detail, showed that the absorption bands of bacteria and yeasts in this region are very similar to those of proteins. It has already been shown that these bands change with denaturation of the protein, the structural configuration of the protein molecules and the ionization of the molecules. More careful work may yield information about the state of the protein in micro-organisms.

Particular attention was paid to the bands near 3μ by Lembke & Kaufman (1954) who attempted to explain changes in these bands in terms of hydrogen bonding, but caution must be exercised in accepting their results as some of their spectra show signs of atmospheric absorption bands.

Goulden & Sharpe (1958) used the absorption band near 1400 cm.$^{-1}$ (7·14μ) to compare the relative number of carboxyl groups in *Lactobacilli* and they were able to confirm their results by paper chromatography. The 960 cm.$^{-1}$ (10·42μ) band was used to estimate nucleic acid and although this is a weak band they showed that organisms designated Type X contained more nucleic acid than Type Y organisms.

When intestinal Gram-negative bacilli are grown on a medium containing glucose they are able to synthesize large quantities of a glycogen-like material. The spectra of cells containing large amounts of glycogen are very different from those containing small amounts. The spectra have been used by Levine, Stevenson & Bordner (1953) and Levine, Stevenson, Tabor, Bordner & Chambers (1953) to estimate semi-quantitatively the amount of glycogen in the cells. Similar results have been obtained by the author (unpublished) with *Aerobacter aerogenes* grown in continuous culture.

A polymer of β-hydroxybutyric acid accumulates in the cells of *Bacillus cereus* and *B. megaterium* under certain conditions of cultivation. Blackwood & Epp (1957) and Forsyth, Hayward & Roberts (1958) used infra-red spectrophotometry to identify this material in the cell. Norris & Greenstreet (1958b) showed that there was a definite correlation between the morphology of cells of *B. megaterium* and the infra-red absorption spectrum. The appearance and disappearance of poly-β-hydroxybutyric acid and its concentration in the cells was followed with ease using the absorption spectra. They used difference spectroscopy to show that during autolysis of the cells, the polymer depolymerizes to β-hydroxybutyric acid. Haynes, Melvin, Locke, Glass & Senti (1958) examined the spectra of 356 organisms and found that forty of these showed absorption bands of poly-β-hydroxybutyric acid. The majority of these organisms are aerobic spore-formers. It was surprising to find that some strains of *B. megaterium* produced the polymer and others did not. All the strains of *B. cereus* examined were polymer producers. The presence of glucose, galactose, fructose or glycerol in the medium increased the amount of

polymer in the cells but the form of the nitrogen in the medium had no influence upon the spectrum of the cells.

When spores of *B. megaterium* are made to germinate, large quantities of calcium dipicolinate (calcium salt of pyridine-2:6-dicarboxylic acid) appear in the germination medium. Infra-red spectroscopy has been used by Norris & Greenstreet (1958 *b*) to show that the dipicolinic acid in the spore was present largely as the calcium salt.

Levi *et al.* (1956) found that an absorption band (1000–1100 cm.⁻¹) in the spectra of *Staph. aureus* was stronger for strains showing enterotoxic activity than it was for preparations that were biologically inactive. The active component, which is probably a polysaccharide, was not isolated or identified.

Polarized infra-red radiation has been widely used to examine proteins and polypeptides. Fraser (1952) applied this technique to study tobacco mosaic virus. The dichroism exhibited by orientated films of the virus nucleoprotein suggested that the protein chains were arranged perpendicular to the axis of the rod-like virus. A little later Astbury & Saha (1953) used infra-red absorption spectra to show that the flagella of *Chlorogonium elongatum* contain a substance similar to *β*-keratin. The flagella appeared to contain more carboxyl groups than *β*-keratin and probably some sugar.

Characterization of extracts and metabolites of cells

Infra-red spectrophotometry has played a part in the analysis of substances isolated from bacteria. The method adopted compares the spectrum of the isolated material with the spectra of substances of known identity. In this section a selection has been made to show the range of possible applications of the infra-red method. No attempt has been made to make the review comprehensive.

MacLennan & Davies (1957) extracted sugars from the somatic antigen of *Chr. violaceum* and succeeded in identifying D-glycero-D-galactoheptose among them by infra-red absorption spectroscopy and other techniques. More complex polysaccharides of microbial origin have been widely studied. Hestrin & Schramm (1954) showed that the polysaccharide synthesized from glucose by a cellulose-free suspension of *Acetobacter xylinum* was in fact cellulose. Earlier Shirk & Greathouse (1952) grew *A. xylinum* on media containing various sugars as the carbon source; the structure of the resulting cellulose produced by the organism was shown to be independent of the carbon source. The difference between the infra-red absorption spectra of yeast cellulose and bacterial cellulose was demonstrated by Simon & Hedrick (1954, 1955). The spectrum of whole yeast cells was very similar to that of the extracted cellulose, showing that cellulose contributes substantially to the spectrum of the whole cells.

Dextrans were isolated from ninety-six strains of bacteria and purified by Jeans, Haynes, Wilham, Ramkin, Melvin, Austin, Cheskey, Fisher, Tsuchiya & Rist (1954). Infra-red methods were used to estimate the percentage of '1, 3-like links' in the dextrans. Infra-red, periodate analysis, optical rotation, viscosity and other methods were used to characterize the various dextrans.

The intracellular polysaccharide produced by *Br. suis* was isolated and

examined by Gary, Kupferberg & Graf (1958) and shown to be unlike glycogen, dextran or starch. Infra-red evidence suggests that the glucose units are β-linked.

A new sulphur-containing amino acid was isolated from yeast by Downey & Black (1957) and shown by infra-red and other techniques to be an isomer of β-methyllanthionine. Proteins have been widely studied, but so far few of these have been of microbial origin. Strains of *Bacillus anthracis* produced the poly-peptide poly-D-glutamic acid. The physico-chemical properties of this interesting material have been described in detail by Kent, Record & Wallis (1957). Ambrose (1950) compared the spectrum of synthetic polyglutamic acid with that of polyglutamic acid isolated from *B. anthracis* and found that the type of linkage was probably different in the two materials.

Of the fats and lipids isolated from bacteria those from strains of *Myco. tuberculosis* have been closely examined. Important contributions to the identi-fication of the fatty acids and other substances which constitute the lipids of the tubercle bacillus have been made by Noll & Bloch (1955), Noll (1957), Noll & Jackim (1958) and Noll (1958). The nature of the fatty acids of the plant pathogen *Agrobacterium tumefaciens* has been determined by Hofmann & Tausig (1955) using infra-red spectrophotometry. The acids present were shown to be palmitic, *cis*-vaccenic and lactobacillic acid.

The pigments of bacteria have been studied by Katz & Wassink (1939) and Wassink, Katz & Dorrestein (1939) in the visible and near infra-red region of the spectrum. The bacterial pigment of *Chr. iodinum* was examined by Clemo & Daglish (1950) in the 'fingerprint' region in an attempt to elucidate its structure. More recently Wolf, Jones & Nathan (1958) showed that the fluorescent pigment of *Microsporum canis* is a pteridine.

The qualitative and quantitative applications of infra-red spectroscopy to antibiotics have been reviewed by Price (1955). Earlier the structures of actino-mycin (Dalgleish & Todd, 1949), and cordycepin (Bentley, Cunningham & Spring, 1951) had been examined by infra-red spectrophotometry.

The activity of bacterial enzymes is being widely studied and the identification of metabolites can be aided by using infra-red absorption spectra. Examples of such work have been given by Rabinowitz & Pricer (1956), Wright (1956), Hayaisha, Tabor & Hayaishi (1957) and Webley, Duff & Farmer (1957).

Antigenic fractions from bacteria and viruses are usually obtainable only in small quantities. Under such circumstances infra-red examination is an extremely valuable method for characterizing the fractions. The spectra of the complement-fixing fractions of *Leptospira bataviae* (Schneider & McLaughlin, 1954, 1955) and the psittacosis-lymphogranuloma venereum group of viruses (Benedict & O'Brien, 1956) have yielded some information about the composition of the fractions and so have been of limited help in the investigation of these materials. Bacterial lipopolysaccharides and 'O' somatic antigens usually require treatment with heat or alkali to enhance their adsorption on to erythrocytes. Davies, Crumpton, Macpherson & Hutchison (1958) used infra-red among other techniques to show that removal of O-acetyl residues is associated with the increased adsorption. Stewart, Watchell, Shipman & Yanko (1955) reported that micro-organisms are

capable of synthesizing *cis*-polyisoprene or rubber. A product isolated from fungi of the genera *Lactarius* and *Peziza* was shown by infra-red spectrophotometry and other means to be rubber.

The viability of organisms upon freeze drying can often be preserved by adding certain preservatives to the cultures. A factor which protects the viability of *Br. abortus* cells was characterized by Bergmann, Halleck, Mechalas & Tenney (1957). The active compound was found to be similar to lecithin.

In conclusion it seems that infra-red spectrophotometry can be used to differentiate between closely related strains of organisms. In order to achieve this every procedure must be carefully and rigorously standardized. Whole cells or cell fractions can be used and for differentiation of a small group of closely related organisms it would seem better to use cell fractions.

Bacterial metabolism can in some special cases be studied by infra-red spectrophotometry but so far there is insufficient experience to say how widely the technique may be useful. As a routine analytical tool for the identification of cell metabolites infra-red spectrophotometry can be of immense value and should become more popular and useful as more spectrophotometers become available.

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