INFRARED MICROSPECTROSCOPY IN BIOLOGICAL RESEARCH

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Introduction

At present, infrared microspectrophotometry can be defined as the study of infrared absorption intensities for samples smaller than the mechanical slit of the spectrometer. This definition arises from the fact that the smallest sample that can be studied by a spectrophotometer without auxiliary optics is that which just covers the entrance or exit slit of the monochromator. Ordinarily the slit[1] will be no shorter than 10 mm., and may vary from 0.01 mm. to 1 mm. in width, depending on the particular region of the spectrum under consideration. If an appropriate sample thickness is about 0.025 mm., then, with no occlusion of the radiation beam incident on the slit, the minimum sample volume would be 2.5×10^{-4} cm.^{3}, or a quarter of a milligram for substances of unit density. This is, therefore, the maximum sample size for microspectrophotometry, or the minimum for macrospectrophotometry, according to the present definition. For various reasons this minimum is not reached in actual practice, but the limit may be set in this arbitrary way for the present discussion.

For smaller samples a microilluminator of some sort must be used. The most elementary microilluminator consists[2] of a pair of infrared-transmitting lenses, one producing a reduced image of the source in the sampling space, and the other restoring the radiation to its former path with an image of the source on the entrance slit. In the original work with this device, the reduction in the minimum sample size was about 3 times, and the final minimum sample size was about 0.75×6 mm., since the slit was rather larger than 1×10 mm. Application of the arrangement to any spectrometer may reduce the sample size by a similar factor, and for the case under consideration would give a minimum of 0.33×3.3 mm., or 2.5×10^{-6} cm.^{3}

One drawback of such a system arises from the errors of the refracting elements involved, especially the chromatic aberrations. The wave-length range usually investigated covers the wide interval from $2.5\,\mu$ to $15\,\mu$, a factor of 6 in wave length compared to the factor of 2 at most required to cover the visible spectrum. Thus, chromatic aberration may be considerable, especially since not many transparent materials are available for the construction of compound lenses for the infrared. The fairly large aperture required of such a system also causes the aberrations other than chromatic to be considerable for simple lenses.

The solution to many of the optical problems connected with microilluminator design has come with the development of totally reflecting micro-optics.[3-5] In fact, the first report of infrared microspectroscopy as such[6] came shortly after the description of the Schwarzschild-type totally reflecting microscope objectives

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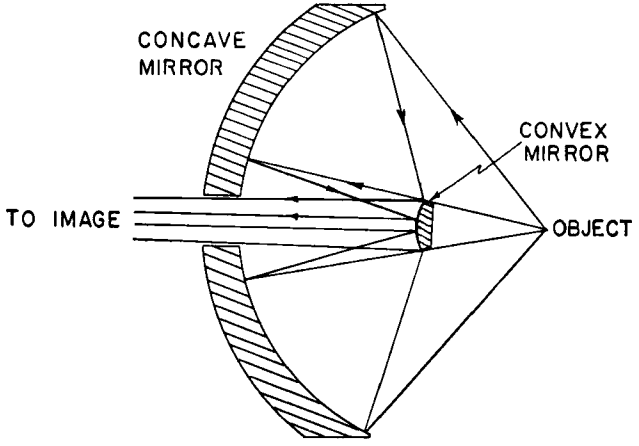

FIGURE 1. Schwarzschild-type totally reflecting microscope objective.

by Burch.[3] At the present time most infrared microspectrometers use this type of optics for the microilluminator.

Microspectrometer Optics

The reflecting microscope objective may consist of two mirror surfaces, either aspherical[3] or spherical,[5] one convex and one concave, arranged as shown in FIGURE 1. Radiation from the object is reflected from the larger concave mirror onto the small convex mirror, and then passes through the hole cut in the larger concave mirror to the image of the object. The small convex mirror obscures some of the incident radiation, and the use of aspherical mirrors is favored by some workers in order to minimize the resultant loss of energy. In a microspectrometer two of these units are arranged like an ordinary microscope, with one unit for the condenser and one for the objective, as shown in FIGURE 2. Radiation from the exit slit of the monochromator is collected by the condenser unit and is brought to a focus on the sample, forming an image of the slit at the sample. The radiation passing through the sample is then collected by the objective and passed on to the detecting system. There usually is an arrangement for visual observation of the object and a masking device in the focal plane of the visual system so that the appropriate sample areas may be selected.

It is sometimes convenient to have the microilluminator located in the undispersed radiation instead of in the exit beam from the monochromator. There is some objection to this arrangement because of the heating effect of the undispersed radiation when it is concentrated to a very small area at the sample. However, Cole and Jones[7] have made quantitative estimates of the heating effect and have concluded that the temperature rise in the sample can be kept under control. They found that an unsupported crystal with a melting point of about 70° C. did not melt at the focus of the beam, although crystals melting somewhat below this temperature did melt. If supported

by a single NaCl plate, a crystal was stable if its melting point was above approximately 40° C. When a crystal of melting point near 30° was supported between two NaCl plates, melting was not observed. Thus, the thermal conductivity of the supporting medium plays a large part in the temperature rise at the sample.

Microspectrometer Sample Sizes

In the visible region of the spectrum, absorption microspectrophotometry has been used since an early date. In this region the minimum sample size approaches that of the smallest resolvable object, and such an object would have a linear dimension equal to 0.6 λ/N.A. where λ is the wave length of the light, and N.A. is the numerical aperture of the microscope objective viewing the sample. This is less than $\frac{1}{2}$ μ for visible light. In the infrared region such desirably small samples are impossible to obtain for several reasons.

First of all, the wave length is larger in the infrared, since the most interesting region lies between 2.5 and 15 μ. The diameter of the smallest object distinguishable from its neighbor at a wave length of 15 μ will be about 6 μ,[8] assum-

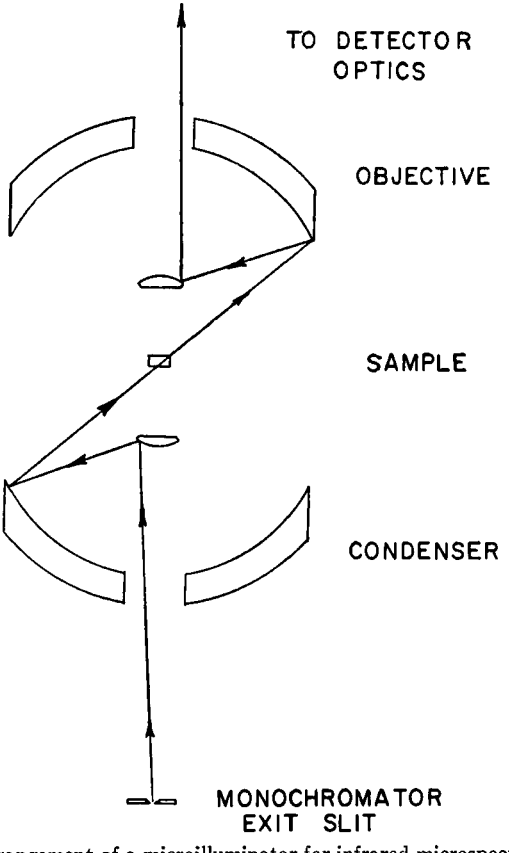

FIGURE 2. Arrangement of a microilluminator for infrared microspectrophotometry.

ing the microscope objective to have N.A. = 1.5. This is about 30 times the diameter of the minimum resolvable limit in the visible. This limit to sample size is imposed by diffraction and will be referred to as the diffraction limit.

The second factor limiting the minimum sample size in the infrared arises from the limited intensity of available sources and the limited sensitivity of available detectors. The limited amount of energy in a given spectral range for a given monochromator focal ratio sets the limit for the slit width, and this in turn sets the sample size limit in the following way.

The maximum solid angle of radiant energy that the monochromator can use is that which just fills the collimator mirror. The numerical aperture of the monochromator $(N.A.)_s$ must be related to the numerical aperture of the microilluminator $(N.A.)_m$ by the relation

$$\frac{(N.A.)_m}{(N.A.)_s} = M$$

where M is the magnification of the sample on the entrance slit of the monochromator. If the monochromator has $(N.A.)_s = 0.1$ and the microilluminator has N.A. < 1.0, then the maximum useful magnification is 10 times and the sample must have dimensions equal to $\frac{1}{10}$ those of the slit in order to realize the full spectral resolving power of the monochromator. Again using the maximum slit dimensions of 1 mm. $\times$ 10 mm., the minimum microsample size is 0.1 mm. $\times$ 1 mm., one order of magnitude of reduction in linear sample dimension, or two orders in cross-sectional area.

There is, however, a great disparity between this energy limit of the whole microspectrophotometer and the diffraction limit of the microilluminator. This disparity can be reduced by masking the sample and thus sacrificing energy at the detector and subsequently the spectral resolving power or response time. Alternatively, one can reduce the disparity by using sources or detectors that are less convenient or cover a smaller spectral range.

In many cases the spectra of solids or liquids have infrared absorption bands that are wide compared with the available spectral slit width. Thus, relatively little loss of detail in the spectrum will result when the sample size is reduced by masking and the energy reaching the detector is decreased. The sacrifice of spectral resolving power to reduce the sample size often can be tolerated.

If the temperature of the source is raised, the energy output in a given spectral interval will increase, and this may be used to pay for a decrease in sample size. The laboratory source having the highest energy output so far described is the carbon arc,[9] which gives an output about four times that of the conventional Nernst filament or silicon carbide Globar. Other sources such as the tungsten glower[10] and the zirconium concentrated arc[11] have greater output than the Nernst filament and Globar, although not as much as the carbon arc.

There are several detectors that have considerably higher sensitivity than the conventional thermocouple and therefore could be used to pay for a reduction in sample size. The most important of these are the photoconductors such as lead sulfide[12] or lead telluride.[13] The sensitivity of this type of detector

is as much as two orders of magnitude greater than that of a thermocouple, but its spectral range is much smaller. Thus, at the present time only the hydrogenic stretching frequencies can be reached with the photoconducting detectors, and the much more revealing absorption bands in the fingerprint region are inaccessible.

The actual limits of sample size to be expected when these innovations are used depend to a certain extent on the shape of the object to be observed; that is, fibers are better suited for microspectroscopy than spherical or circular objects because of the shape of the monochromator slits. In the case of fibers, when the sampling area is masked at the expense of spectral resolving power, it has been found[14] that spectra recorded from fibers 17 μ in diameter and 650 μ long are so sufficiently well resolved that detailed studies of the fibers can be made over the whole wave-length range from 2.5 μ to 15 μ. This represents the recording of useful spectra from about 10^{-7} gm. of material. If the carbon arc source were used instead of the standard source, this could be reduced by a factor of 2, and a small amount of further reduction could be obtained if the signal-to-noise ratio of the detecting system were increased by reducing its band pass, although this would require longer recording times. The use of a photoconducting detector and the carbon arc source would allow spectra in the hydrogenic stretching region (near 3 μ) to be recorded for fibers of diameters equal to the diffraction limit at those wave lengths.

For samples whose geometry does not resemble that of the monochromator slits, the minimum sample size must be obtained effectively by reducing the slit length. Coates, Offner, and Siegler[14] give a tabular guide to minimum sample area in which they assume a sample length of 100 μ. For sample widths ranging from 6 μ to 100 μ, this yields quite reasonable spectral slit widths and operating conditions between the wave lengths of 2 μ and 14 μ. Thus, when a sample is to be run throughout this range a well-resolved spectrum will result from a sample 100 μ square, or 2.5×10^{-7} cm.^{3} in volume, if a proper thickness of 25 μ is assumed. With a carbon arc source this can be reduced by a factor of 4 in area, giving 50 $\mu \times 50$ μ, and a sample volume near 6×10^{-8} cm.^{3} Although this work has not been published, at least one microspectrometer using a carbon arc source and conventional detector has been capable of measuring useful spectra from samples 30 μ in diameter. For convenience the relative sample dimensions discussed so far are summarized in FIGURE 3.

Samples of 30 μ diameter represent the closest experimental approach to the diffraction limit for this type of specimen and, although the method involves somewhat inconvenient apparatus, this size is a quite practical limit. It is unfortunate that so many tissue cells are smaller than 30 μ and therefore will not be amenable to individual study in the infrared. Large cells and tissues are, however, promising material for microspectroscopy.

The Effects of Convergence in Microspectrophotometry

Because of the large numerical aperture of the microilluminator system of a microspectrophotometer the object is viewed with highly convergent radiation.

This may cause some difficulties in the interpretation of data since it is usually assumed that the radiation traversing the sample is parallel.

The first effect of convergence is the change in effective thickness of the sample when viewed with the microilluminator. The central ray of the cone of radiation traverses the true thickness t of the sample. The marginal ray of the cone, however, traverses a thickness $t/\cos\theta$ where θ is the angle between the cone axis and the marginal ray. This means that the effective thickness that would give the true extinction coefficient in the Beer-Lambert law is something between t and $t/\cos\theta$. Blout, Bird, and Grey[8] have calculated the magnitude of this effect and give graphical data from which the appropriate correction may be made. It should be noted that in unfavorable circumstances this effect could adversely influence the contrast in a spectrum in the following way. The central bundle of rays passes through a smaller sample thickness and suffers less absorption than the marginal rays. If the marginal rays for large numerical apertures suffer nearly complete absorption at the peak of an absorption band, the higher transmission of the axial rays will apparently reduce the absorption maximum. Likewise, the minimum of absorption from the central ray bundle will be raised by the absorption of the marginal rays. Thus, a decrease in the disparity between maxima and minima in the spectrum will be observed and reduced contrast will result. This is similar to the situation observed with a strongly absorbing sample having holes through which unabsorbed radiation may pass. The contrast in such a sample is low. Although it has not been evaluated quantitatively, this effect for convergent radiation is probably small.

The second effect of convergence arises in oriented samples where dichroic ratios are being investigated. Here the absorption of marginal rays for plane-polarized radiation will vary with azimuth as well as with elevation. An oblique ray incident in the plane containing an absorbing transition moment (FIGURE 4a) will be absorbed less than an oblique ray incident in a plane per-

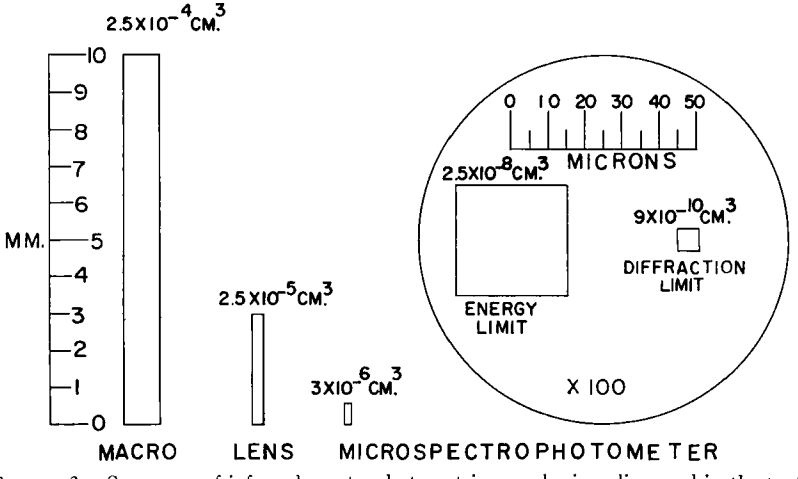

FIGURE 3. Summary of infrared spectrophotometric sample sizes discussed in the text.

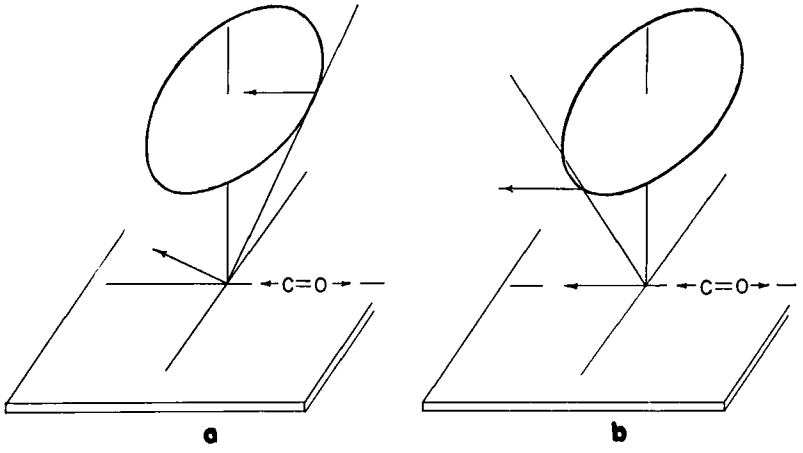

FIGURE 4. Absorption of oblique rays in convergent radiation: (a) azimuth showing decreased absorption; (b) azimuth showing absorption equal to that for normal incidence.

pendicular to the absorbing moment (FIGURE 4b). This is because in the former case the electric vector is not parallel to the moment while, in the latter case, it is parallel. Thus, the intensities for convergent radiation will differ somewhat from those observed in parallel radiation, and erroneous dichroic ratios will result. This effect has been evaluated by Fraser,[15] who has found that a small correction is sufficient.

The third effect of convergence is important only in single-crystal studies where there is a transition moment in the crystal parallel to the axis of the convergent radiation in the microilluminator. In this case the absorption is forbidden for parallel radiation, as indicated in FIGURE 5, but the marginal rays of the convergent radiation strike the sample obliquely and are absorbed. Thus, absorption bands normally forbidden for the particular crystal section will be observed in the spectrum recorded with convergent radiation. This effect may have considerable magnitude, although this type of sample will be relatively rare in biology.

In summary, one can say that the effects of convergence are not likely to be especially important in biological work but their existence should be kept in mind.

Applications to Biological Problems

In most investigations of biological problems that have involved infrared microspectrophotometry the technique has been applied to chemical systems for which only small quantities of sample were available. An intact cell is a complex system in which the more interesting components are often present in concentrations far below the modest threshold of infrared absorption. Moreover, because of their small size, it is unlikely that studies of cell parts will yield the sort of useful infromation that ultraviolet microspectrophotometry has given in the case of the cell nucleus.[16]

One favorable example, however, where useful information has been ob-

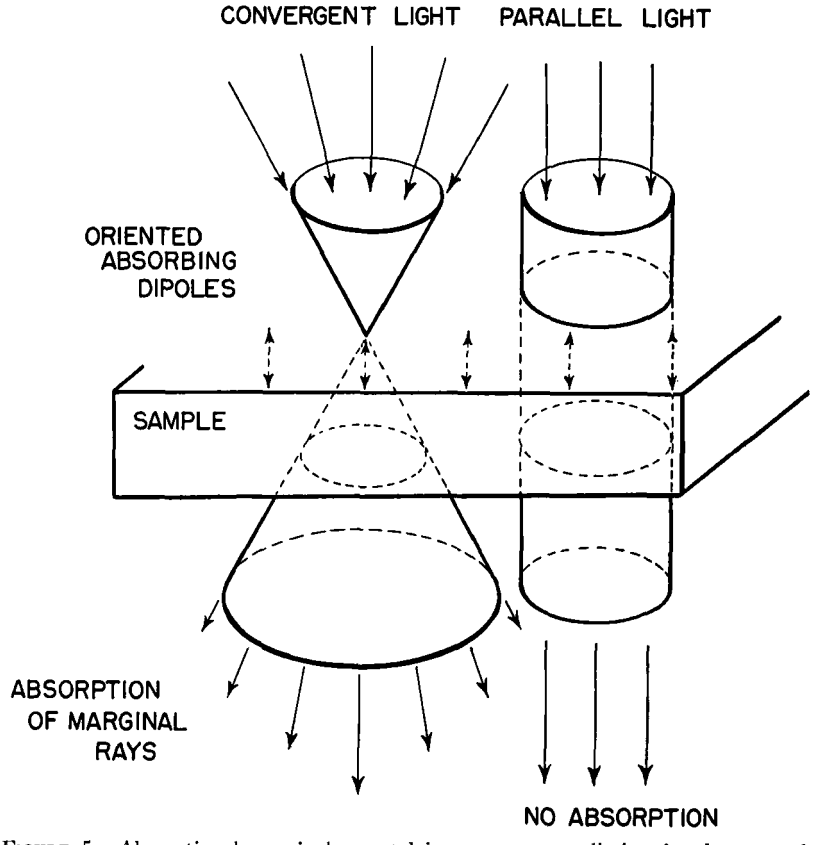

FIGURE 5. Absorption by a single crystal in convergent radiation for the case where this absorption would be forbidden in parallel radiation.

tained from single intact cells is the case of muscle. Since the individual cells are in the form of elongated fibers their geometry is well suited for microspectrophotometry, and spectra of single cells have been reported in the literature.[17] The spectra of the cells strongly resemble those of the principal protein components, myosin and actomyosin, although there are differences in the spectra of cells from different muscles in the same animal and from the same muscle in different species. These differences have never been explained. The fact that dichroism is not observed in the microspectra of intact muscle cells suggests that the contractile mechanism is constructed on a level higher than that of the elementary polypeptide chain. It also implies that the contractile process involves a more complex mechanism than the simple folding of a polypeptide chain.

Biochemical infrared microspectrophotometry has been mainly concerned with the identification of minute quantities of compounds extracted from natural systems. One such investigation[18] involved the identification from ox spleen of propionylcholine which, like acetylcholine, is an active mediator

of excitation across the neuromyelar junction. In the whole study[19] a total of about 300 μg. of the propionyl ester was available. Because of the extreme sensitivity of bioassay methods this quantity was sufficient for a large number of biological experiments, but the whole quantity was not enough for one infrared macrospectrum. The microspectrum, however, was easily recorded from about 200 μg. and the successful identification of the natural extract was easily made.

The minimum sample size in this type of work is determined by the handling procedures rather than by the limitations of the microspectrometer itself. Microgram quantities are usually handled in solution and, in many cases, the solution itself will be satisfactory for a spectrum. The sample, however, must be confined in some sort of cell. Cole and Jones[7] have described a liquid cell that 3×10^{-3} cm.^{3} of solution will fill and have recorded good spectra from 10 μg. of solute in this cell. According to their estimate, only 9×10^{-4} cm.^{3} of solution actually was in the radiation beam, and an appropriate reduction in the cell size to 9×10^{-4} cm.^{3} would have given the same spectra from about 2.5 μg. of solute.

Blout[20] has developed another technique for liquids, in which the sample is contained in a short length of silver chloride capillary tubing having a bore about 100 μ in diameter. The solutions or the pure liquids can be taken up directly in the capillary and the spectrum run without a sample transfer step. With this technique, spectra of a few micrograms of solute in a solution have been obtained.

The Future of Infrared Microspectrophotometry

What has been said thus far about infrared microspectrophotometry deals with apparatus and techniques already described in the literature, and with equipment commercially available and within the financial resources of the majority of research laboratories. The future developments that may come to pass are, of course, only a matter of speculation at this time, although some possibilities may be pointed out.

The type of information that makes infrared spectrophotometry interesting is the specific absorption intensity as a function of wave length or frequency. It is mainly the absorption in the region from 4000 to 650 cm.$^{-1}$ that is of value, although some information is also available[21, 22] in the region from 6500 to 4000 cm.$^{-1}$. Thus, the use of the infrared image converter tube as an adjunct to microscopy[23] seems unlikely to yield pertinent information since its useful range is between the visible and about 7700 cm.$^{-1}$ Photographic plates are also limited to this range outside the more useful infrared wave lengths.

There are, however, two new developments that show promise in rendering an infrared microscope image visible. These are the Evaporagraph[24] and the photographic scanning radiometer.[25] Although based on widely different principles, both of these devices produce photographs, the density or color of which depends on the intensity of the infrared radiation of particular wave lengths present in the image of an object.

Suppose an infrared image of some tissue section, for example, is formed at the focal plane of one of these devices by a reflecting microscope objective.

The blackening of the thermal photomicrograph image would depend on the infrared transmittance of the section. Thus the total absorption of the section for some wave-length interval isolated by a filter could be recorded. Even more interesting information might be obtained by combining thermal photomicrography with nondispersive spectrophotometry.[26] For example, in the scanning system of thermal photography the detector can be sensitized for a specific material such as protein by masking the surface with a protein film. The thermal photomicrograph might then reveal the distribution and concentration of protein in the tissue section. It is entirely possible that a closer approach to the diffraction limit could be obtained by such an apparatus. At the present time, however, these possibilities have not been investigated.

Summary

Infrared microspectrophotometry is defined as infrared spectrophotometry of samples smaller than the slit of the monochromator. It therefore requires a microilluminator in the sample space. Present practice tends toward Schwarzschild-type totally reflecting microscope objectives for the microilluminator, with the sample in the dispersed radiation beam.

The minimum sample size for spectrophotometry from 2.5 to 15 μ is limited by diffraction to 6μ in diameter (about 10^{-9} gm.), although the practical limit due to instrumental limitations is about 30 μ in diameter (about 2×10^{-8} gm.) for extreme conditions. The difficulties in handling and containing such small liquid samples further increase the minimum sample size to about 1 μg., either as a solution or as a pure liquid. For fibers it is possible to use samples having a diameter equal to the diffraction limit.

The effects of the large angle of convergence on the radiation in the microilluminator are usually not important except for single-crystal spectra and for quantitative analysis.

The applications of infrared microspectrophotometry to biological problems are mostly concerned with identification of extracted compounds or studies of isolated chemical systems. Some interesting information can also be obtained from larger single cells and from whole-tissue preparations.

Recent developments in the field of infrared instrumentation suggest a bright future for infrared microspectrophotometry in biology.

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