STUDY OF BIOLOGICAL PIGMENTS BY SINGLE SPECIMEN DERIVATIVE SPECTROPHOTOMETRY

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ABSTRACT The single specimen derivative (SSD) method provides an absolute absorption spectrum of a substance in the absence of a suitable reference. Both a reference and a measuring monochromatic beam pass through a single sample, and the specimen itself acts as its own reference. The two monochromatic beams maintain a fixed wavelength difference upon scanning, and the difference in absorbance of the two beams is determined. Thus, the resulting spectrum represents the first derivative of the conventional type absorption spectrum. Tissues and cell fractions have been examined at room and liquid N_2 temperature and chromophoric molecules such as the mitochondrial cytochromes and blood pigments have been detectable in low concentrations. In the case of isolated cellular components, the observed effects of substrates and inhibitors confirm similar studies by conventional spectrophotometry. The extension of the SSD concept to the microscopic level has permitted the study of the tissue compartmentalization and function of cytochromes and other pigments within layered tissue.

INTRODUCTION

Spectroscopy, the study of selective absorption or emission of electromagnetic radiation by matter, is used in the analysis of materials and as a sensitive and discriminating research method. As is the case for many physical methods, light absorption is more easily quantitated by comparison with a standard than by absolute measurement. Traditionally, this has been done by measuring in sequence the intensity of a monochromatic light beam in the absence and presence of absorbing substances. The benefits of comparing the absorption differences that occur when two light beams of slightly different wavelength are subjected to the same atomic or molecular absorbing milieu have also been found useful. Investigators, such as Chance (1) and others, have successfully studied kinetic changes in the mitochondrial cytochromes by monitoring absorption at fixed wavelength pairs. Dual wavelength radiation comparison methods involving spectrum scanning, however, have been

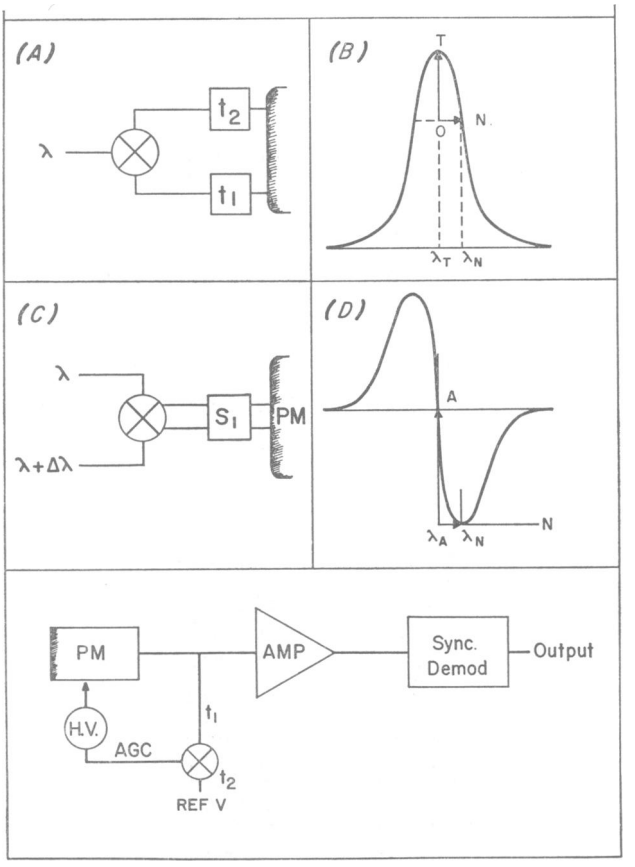

FIGURE 1 A comparison of the split beam and the SSD spectrophotometer. In the split beam method, light of wavelength λ is time shared at t_1 and t_2 through two separate samples and then imaged on a common detector. The SSD technique time-shares light of wavelength λ at t_1 and wavelength λ + Δλ at t_2 through a common sample S_1. It produces a derivative peak (D) corresponding to a conventional split beam absorption peak such as (B). In both methods, the ratio measurement results from the automatic gain control (AGC), which is achieved by variation of the high voltage (HV) on the photomultiplier detector (PM) during the referencing time period t_2. The recorded output signal is either the ratio of light through the two samples in the case of split beam or the ratio of the intensities of the two wavelength differing beams through one sample in SSD. It is obtained by synchronously demodulating the amplified photomultiplier signal.

utilized infrequently. This report considers the theory and practice of dual wavelength spectroscopy for the spectral examination of nonhomogeneous biological materials and presents some examples of its usefulness.

Yang and Legallis (2) extended the use of absorption spectroscopy to *in situ* studies in mitochondrial cytochromes by devising apparatus (Fig. 1 A and B) which eliminated the need of prior clarification of turbid materials. A comparison of three methods of obtaining cytochrome spectra is presented in Fig. 2. The upper

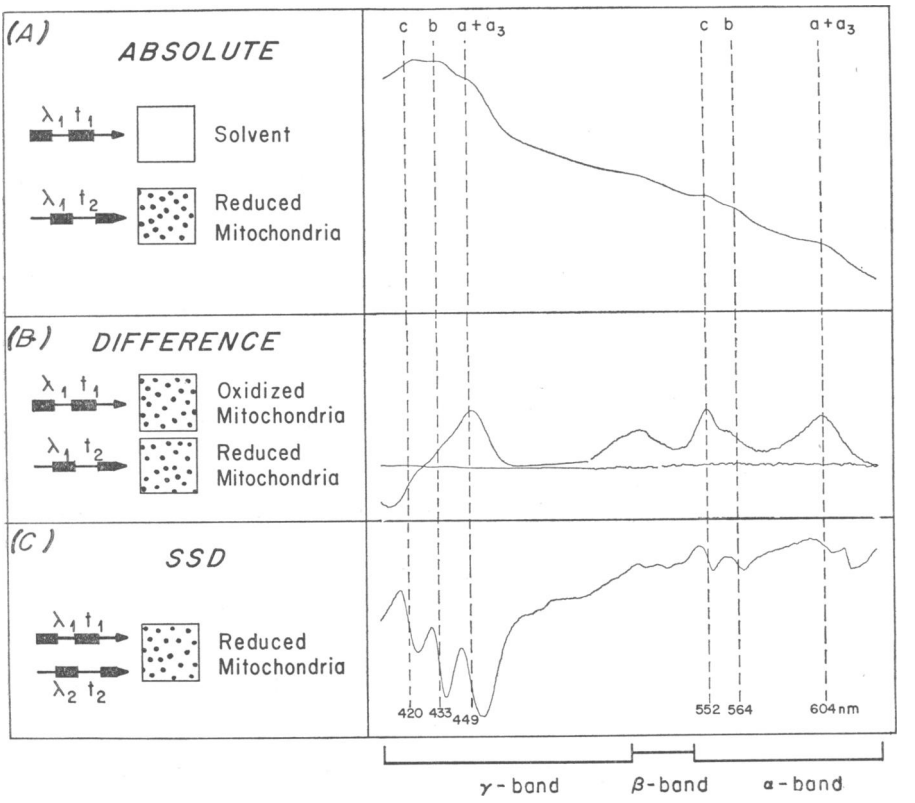

FIGURE 2 Three methods of obtaining spectral information. The sample was a dilute suspension of isolated heart mitochondria contained in a standard 1 cm cuvette.

trace (Fig. 2 A) presents an absolute spectrum of reduced cytochromes in mitochondria, using buffer in the reference cuvette. The uncompensated turbidity of the sample causes the steeply ascending absorption trace (right to left) which obscures the pigment absorption. In Fig. 2 B the turbidity is compensated for in the Yang-Legallis spectrophotometer by having the photodetector view essentially equivalent scattering material in each cell, permitting the absorption maxima of the reduced cytochromes to become clearly apparent. The spectrum in Fig. 2 C also shows good resolution of the cytochrome peaks and was obtained by the method to be described. Since double beam spectrophotometers compensate for instrument instability and component characteristics by sample comparison, nonhomogeneous materials that lack a suitable reference for this comparison become problematical by this method. For example, it is difficult to control the thickness and homogeneity required to obtain an equivalent sample and reference slice of fresh animal tissue. Liquid sample comparisons also can be invalidated by artifacts which affect the contents of the cuvettes in a disproportionate manner. These artifacts may include

the settling of particles at different rates in the two cuvettes, the different light-scattering properties of the two suspensions being compared and the dissimilar fissuring in paired samples being frozen at low temperature.

In contrast to the usual comparison of two spatially separated samples by two beams (dual channel), an alternative method of beam comparison depends upon the passage of two beams of slightly different wavelength through the single sample (single channel) as is shown in Fig. 1 C and D. This method, herein designated as single specimen derivative spectrophotometry (SSD), differs from conventional spectrophotometry by the following characteristics. (*a*) Although only a single sample is examined, it is not a single beam system because two time-shared beams that differ by a small constant wavelength offset (λ_1 at t_1 and λ_2 at t_2) alternately pass through the sample. (*b*). Spectra produced by the method demonstrate absorption properties due to all the chromophoric molecules present in the sample. This method differs from the dual channel procedure which cancels out chromophores common to both samples. (*c*) The method produces a first derivative curve of the absorbance dependence on wavelength rather than the usual type of absorption curve.

BACKGROUND OF SSD TECHNIQUE

Studies by C. S. French and coworkers at Carnegie Institution have shown that the resolution of overlapping absorption peaks is improved by presenting spectra as the first derivative of absorbance as a function of wavelength (3). They employed a dual channel instrument in which a vibrating exit slit on the instrument's monochromator caused both channels to receive time-shared radiation that was modulated about a constant spectral interval. Thus, the instrument of French, like conventional spectrophotometers, is designed to compare two samples. By having only one modulated beam traversing a single channel, we can achieve a simpler system which is optically analogous to the method of imposing a small rapidly alternating magnetic field upon a large constant magnetic field as is done in electron spin resonance. Although there are descriptions of such single channel derivative spectroscopy apparatus in the literature (4), few accounts of the applications of the method have appeared. At the Center for Population Studies, Harvard School of Public Health, we have used a commercially available instrument (Dual Wavelength Scanning Spectrophotometer Cat. No. PMD 1010 [Phoenix Precision Inst. Co., Philadelphia, Pa.]) for the application of the single channel derivative method to biological systems.

An idealized absorption peak, for example the one shown in Fig. 1 B, would appear by the derivative method as in Fig. 1 D. The latter curve reflects the characteristics of the sample in terms of the rate of change of the slope of the absorption intensity with wavelength rather than intensity itself. Thus, an additional dimension is incorporated into the spectra and is reflected in the marked accentuation of nar-

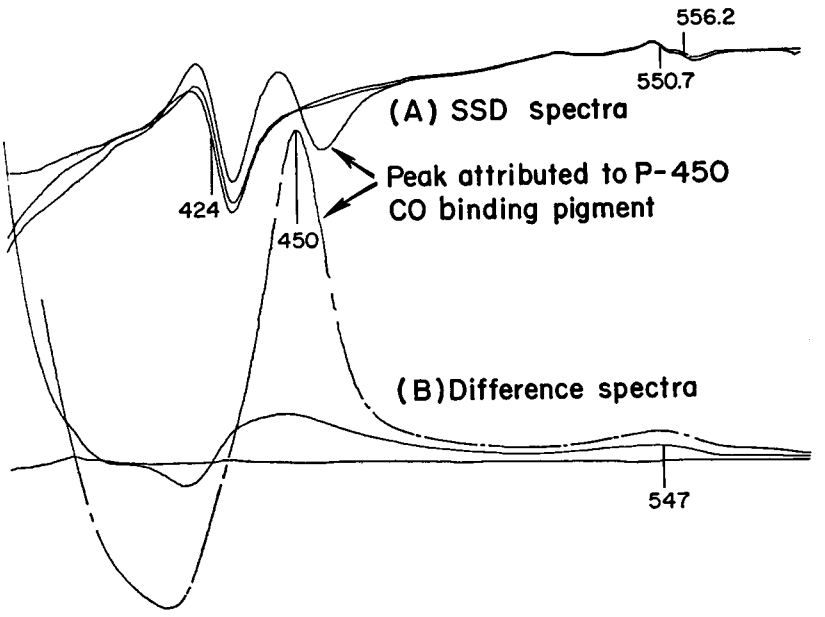

FIGURE 3 Spectral study of hemeprotein P-450 contained in rabbit liver microsomes. The spectra shown in the upper (A) traces are SSD and are obtained by reagent addition to a common sample of isolated liver microsomes. Enzymatic reduction (NADH) to the whole sample produced a curve with no derivative inflection in the region of 450 nm. This sample was then halved into two separate fractions and chemical reduction (dithionite) of one produced an SSD spectrum with a small inflection near 450 nm and a distinct displacement of the 424 nm peak. At this point, both samples were examined by conventional difference spectrophotometry, first producing the curve with weak absorption peaks at 547 and 435 nm, and then, after bubbling the cuvette containing dithionite with CO_2, producing the curve with the intense 450 nm peak. The cuvette with the CO_2 was then run in the SSD mode to obtain the derivative spectrum of the 450 nm absorption.

row absorption peaks compared to mildly varying spectrally dependent phenomena such as light scattering. The bias applied by the method to narrow absorption bands is seen in Fig. 2 C. Here, the reduced cytochromes of the mitochondrial sample that gave diffuse absorption bands by the conventional spectra in Fig. 2 A are now seen to have clearly resolved derivative curves of the spectral absorptions. The light-scattering effects have been significantly reduced due to the relative broad spectral dependence of the scattering phenomena.

Both the double beam and SSD mode offer distinct but different advantages and may be jointly employed to obtain complementary information. For example, in Fig. 3 A, the SSD spectra of two identical samples of rabbit liver microsomes (a centrifuged fraction of subcellular particles derived mainly from endoplasmic reticulum) were obtained. By monitoring the intensity of the SSD absorption due to enzymatically reduced cytochrome b_5, the concentrations in each of two cuvettes were adjusted to be identical. The instrument was then reset to record conventional

difference spectra, whereupon the sequential addition of sodium dithionite and carbon monoxide to one cuvette produced the difference spectra (Fig. 3 B) of the carbon monoxide binding pigment P-450 (See reference 5 for a discussion of this subject). The complementary use of the SSD method in this case allowed exact balancing of a mutually occurring pigment (cytochrome b_5) as well as providing a complete picture of the events occurring in both cuvettes.

PRINCIPLES

In the SSD technique the recorded derivative curve is different from that obtained by conventional spectrophotometers. Two closely spaced wavelengths are alternately passed through the sample. To record spectra, one wavelength is set so as to lag behind the other by a fixed amount during the wavelength scanning process. A hypothetical derivative curve is shown in Fig. 1 D for comparison with an equivalent one obtained by conventional methods in Fig. 1 B. Three points on the derivative curve are of special significance. The point where the curve intercepts the zero line corresponds to the wavelength of the conventional peak's maximum (cf. Fig. 1 B and D, points T and A). The negative portion of the derivative curve has a minimum value, and the positive has a maximum value. The horizontal distance between the two is a measure of peak bandwidth and the vertical distance from the maximum to the minimum relates to the amplitude of the conventional spectral peak and is dependent on chromophore concentration. The absorbance difference between the maximum and minimum, however, can only be a fraction of the value of the total absorbance at the wavelength maximum as measured by conventional means. Although the method itself, therefore, can have a less favorable signal-to-noise ratio than highly optimized conventional spectrophotometry, the area of application of the SSD technique is one where this factor does not generally govern the value of the information sought.

The fundamental photometric relationships between concentration (c), pathlength (l), transmission (T), absorbance (A), and molar extinction coefficient (e), as expressed by the Beer-Lambert law, are essentially similar for the SSD method as they are for conventional spectrophotometry provided that appropriate modifications are made to accommodate the differential aspects of the measurements.

Thus, in the case of a single wavelength λ_1

$$A = -\log T = ecl. \tag{1}$$

Differentiating for wavelength difference, $\Delta\lambda$ (obtained by maintaining a constant incremental difference between wavelengths λ_1 and λ_2), we obtain

$$\Delta A = K\Delta T/T = \Delta ecl. \tag{2}$$

By use of feedback circuitry the voltage V_{λ_2} of the photomultiplier signal at λ_2 is

maintained at constant voltage by applying to it an amplification factor (g):

$$V_{\lambda_2} = V_{\text{reference}} = k. \tag{3}$$

The amplification factor (g) is maintained constant during the period in which the intensity difference existing between the measuring and reference wavelengths is measured. Since the voltage output of the photomultiplier is linearly proportional to the transmission being measured,

$$\Delta V/V_{\lambda_2} \approx \Delta T/T_{\lambda_2}. \tag{4}$$

Equations 3 and 4 can be combined and give a voltage analogue of the transmission changes occurring during the measurement process.

$$\Delta V = k'\Delta T/T_{\lambda_2}. \tag{5}$$

Substitution of equation 5 in equation 2 provides the fundamental relationship of the SSD method.

$$\lim_{\Delta\lambda \to 0} \Delta V/\Delta\lambda = dV/d\lambda = k'(dA/d\lambda) = k'(de/d\lambda)\cdot c\cdot l. \tag{6}$$

The function of $dA/d\lambda$ is monitored during wavelength scanning by the SSD instrument, and is a measure of slope change during the spectral observation of progressive portions of scanned absorption bands. The most interesting result of the derivative process is that the final measurement is made directly in *absorbance* units rather than *transmission*. Since absorbance is generally linear in respect to the concentration of the absorbing species, the usual nonlinear function conversion ($T = -\log_{10}A$) of conventional spectrophotometry is avoided.

In conventional spectrophotometry an absorption peak is usually measured from the base line to the peak's apex. For quantitation by the derivative technique, the intensity of absorbance may be measured by the vertical distance between its maximum and minimum. The absorption peak shown in Fig. 1 B has two inflection points, one on each side of the apex (point T) where there is a reversal of slope direction. These are designated as P and N (only N is shown) and correspond to the maximum and minimum, respectively, of the derivative curve. Adherence to the Beer-Lambert relationship implies that all points on the absorbance spectrum of a single chromophore vary linearly with its concentration. Therefore, these inflection points should denote concentration as reliably as the more conventional point, T. An examination of the segment OT on the absorbance curve (Fig. 1 B) and AN on the derivative curve (Fig. 1 D) indicates not only the proportionality of the two, but also that the concentration-dependent parameter of the derivative curve actually involves a triangulation technique since the one measurement involves three points on the absorption peak. The theoretically predicted quantitative capability of the

method has been experimentally verified in the case of serially diluted solutions of a cytochrome *c* standard.[1]

THE SSD SPECTROPHOTOMETER

The SSD studies reported here were performed on a Dual Wavelength Scanning Spectrophotometer manufactured by Phoenix Precision Instr. Co. Fig. 4 depicts the essential elements of this system and the five discrete instrumental processes used in obtaining SSD spectra. The optical portion of the instrument incorporates a duochromator; this device,[2] unlike a conventional monochromator, provides *two* selectable separate wavelengths from a common exit slit (process 1). The light that will eventually comprise the two beams of slightly differing wavelengths is first spatially separated into two separate channels each with its own dispersing grating. After dispersion, these beams are combined into a single, time-shared beam that alternates between the two wavelengths 60 times each second. The leading wavelength can be swept through a spectral range of 350–750 nm in a time of 15 sec–15 min (process 2); the second wavelength will then lag behind the first by a small amount (typically 2–4 nm) which is set by micrometer M. The latter controls the angle between two separate gratings in mount G.

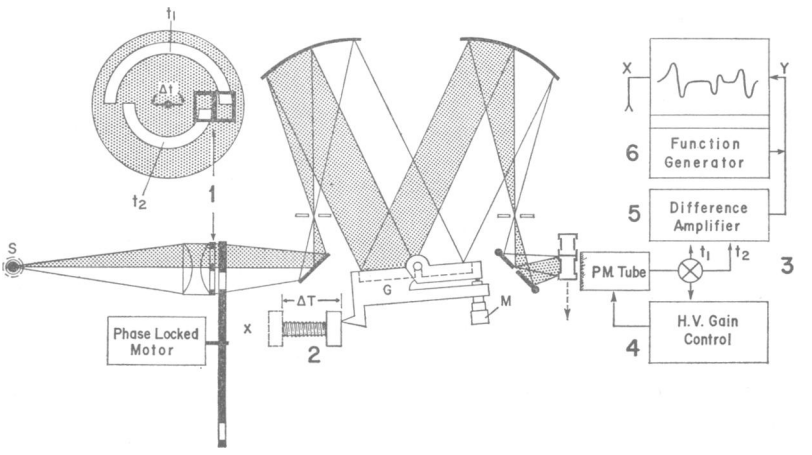

FIGURE 4 The derivative spectrophotometer. Numbers relate to essential processes in obtaining SSD spectra. These include the following: the generation of two time-shared monochromatic beams with a small wavelength offset between them (No. 1), movement of two separate but mechanically linked gratings so as to provide spectral scanning (No. 2), detection by a photomultiplier tube (PM) of the resulting time-shared light beam and electrical time-sorting of the signals from each wavelength (No. 3), the use of automatic gain control on the reference beam (by control of the high voltage supply, HV) in order to achieve constant sensitivity at all wavelengths (No. 4), measurement of the intensity difference between the two wavelengths (No. 5), and the display of this signal as a function of wavelength (No. 6).

[1] This work was done with the assistance of Ernest Manders and Barbara Ballow of the Harvard Medical School, whose enthusiastic efforts are gratefully acknowledged.

[2] The commercial development of this device was done at the American Instrument Co., Inc., Silver Spring, Md. The contribution to its successful design by George Lowy, Jerome Kremen, and Donald Watson is gratefully acknowledged.

The electronic portion of the instrument is similar to that of Yang and Legallis (2) (Fig. 1). The photomultiplier detector is placed close to the sample so that it can collect a large fraction of the scattered light from turbid media. Light striking it produces a square-wave modulated electrical signal with alternating portions representing the light attenuated by the sample at each wavelength (process 3). The portion of the electrical signal corresponding to the leading wavelength is time-sorted by electrically switching in synchronism with the light shutter and compared to a reference voltage. An error signal thus generated is used to adjust the sensitivity level of the photomultiplier through control of the amount of high voltage applied to the photomultiplier dynodes (process 4). With the sensitivity of the system momentarily set by the feedback control loop, comparisons are made of the samples' attenuation of light at the leading wavelength as opposed to the tracking wavelength. These comparisons, averaged over a period of time determined by a preset time constant, are displayed as a difference value on the Y axis of an X-Y recorder (process 5). As was indicated in the theoretical portion, the difference in the attenuation of light by the sample corresponds to an *absorbance* difference (rather than transmission) at the two wavelengths being observed, i.e., $\Delta A/\Delta\lambda$. The X axis of the recorder indicates spectral wavelength and is driven by a signal from the scanning mechanism of the duochromator. Data, therefore, are recorded directly in the form of the first derivative of the absorbance of a specimen as a function of the wavelength. A multitapped potentiometer function generator is built into the recorder and provision is available by means of 13 adjustment dials on the front of the X-Y recorder to remove base line curvature of instrument origin. Each of these dials is located directly below a major vertical coordinate line on the chart paper. Adjustment of each appropriate dial controls the pen position when it is on the coordinate line (process 6).

INSTRUMENT PERFORMANCE

The instrument used for the derivative studies reported here is a multipurpose unit with both double beam and SSD capability. A limitation in its use is its restricted wavelength range of 350–750 nm. The method itself is not theoretically limited in range, however, and can extend from the infrared to the vacuum ultraviolet regions. The dynamic absorption range presently measurable by the instrument extends to approximately 3 OD. Within this range, optical density differences as small as 2×10^{-4} can be measured. This represents a signal-to-noise ratio in the order of 10^4. The size of the slit opening is principally determined by the opacity of the speciman. A compromise must be made, such that the slit opening is small enough for adequate resolution but large enough to provide sufficient light energy throughput. For proper operation, the reference beam cannot be attenuated beyond the point where dynode feedback control is inoperative; a case most likely to happen when the apex of an intense peak is scanned. In keeping with this requirement, slit settings between 0.15 and 0.25 mm representing a spectral bandwidth of 1–1.6 nm are frequently used with turbid solutions. Time-constant settings are chosen on the basis of desired instrument response time and the scanning speed to be used. Long time constants are useful to reduce noise, but when spectral scans of transitory phenomena must be made at very fast speed, a very short time constant must be employed in both the measuring circuit which is controlled by the time-constant switch and also the reference feedback circuit. The measured recovery time of the dynode

feedback loop is of the order of 0.05 sec permitting scanning rates as fast as 100 nm in 5 sec. Faster scanning would be very desirable, but would require a faster rate of beam comparison and a storage oscilloscope or similar recording techniques. The dynamic bandwidth, which is defined as the product of the scanning speed (nm/sec) and the response time, is presently of the order of 1 nm.

The incremental difference, $\Delta\lambda$, between the reference and measuring wavelength is set by micrometer adjustment M (Fig. 4). Selection of the wavelength differential increment $\Delta\lambda$ must take into account the sensitivity and the spectral resolution required. The intensity of the derivative of an absorption band increases linearly with $\Delta\lambda$ within limits set by the peak width itself (equation 3). Increasing the increment beyond several nm may cause sensitivity to be gained at the expense of resolution.

ABSOLUTE NATURE OF SPECTRA

Although spectra should describe sample properties only, small predictable nonlinear spectral discrepancies occur in SSD and are caused by the spectral characteristics of the instrument's components[3] differing at the measuring wavelength as compared to the reference wavelength. Therefore, measurements less than 0.1 Δ OD must be corrected for this effect. We assume that this factor as well as turbidity dependence have additive effects on the recorded spectra, or $(dA/d\lambda_{measured}) = (dA/d\lambda_{absolute}) + (dIn/d\lambda) + (dTu/d\lambda)$.[1] The term $(dIn/d\lambda)$ is indicative of contribution of a constant instrument artifact; and $(dTu/d\lambda)$ is the light-scattering contribution. The instrument is normally calibrated by using an appropriate blank, such as water for nonturbid solutions, to eliminate base line curvature. This is done by setting 13 wavelength adjustment points, each occurring 25 nm apart so that the pen will reside on the same horizontal chart line when the instrument is scanned with the blank. This, in effect, generates a function equal but opposite in sign to $(dIn/d\lambda)$. Similarly, the examination of turbid samples can be facilitated by setting the function generator to give a flat base line with a piece of lightly ground glass in the beam. Here the function generated is $(dIn/d\lambda) + (dTu/d\lambda)$.

The presence of turbidity can distort the spectral absorption characteristics of chromophoric molecules, both qualitatively and quantitatively. Randomly dispersed particles having a different refractive index from the media surrounding them scatter light in a manner that is wavelength dependent; in the case of mitochondria, the intensity of scattered light is inversely proportional by the square of the wavelength of light used (6). SSD spectra are affected less by turbidity than are conventional spectra because the reference and measuring beams are exposed to the same array of scattering particles. This permits the single specimen technique to provide very defin-

[3] The components with spectral contributions used are (*a*) General Electric 15 w, 6 v vertical ribbon filament microscope lamp (General Electric Co., Schenectady, N. Y.), (*b*) Bausch & Lomb diffraction grating, 600 lines/mm 5000 A Blaze (Bausch & Lomb, Inc., Rochester, N.Y.) and (*c*) E. M. I. trialkali (S-20) photomultiplier 9558 C (Varien/E.M.I., Plainsville, N.Y.).

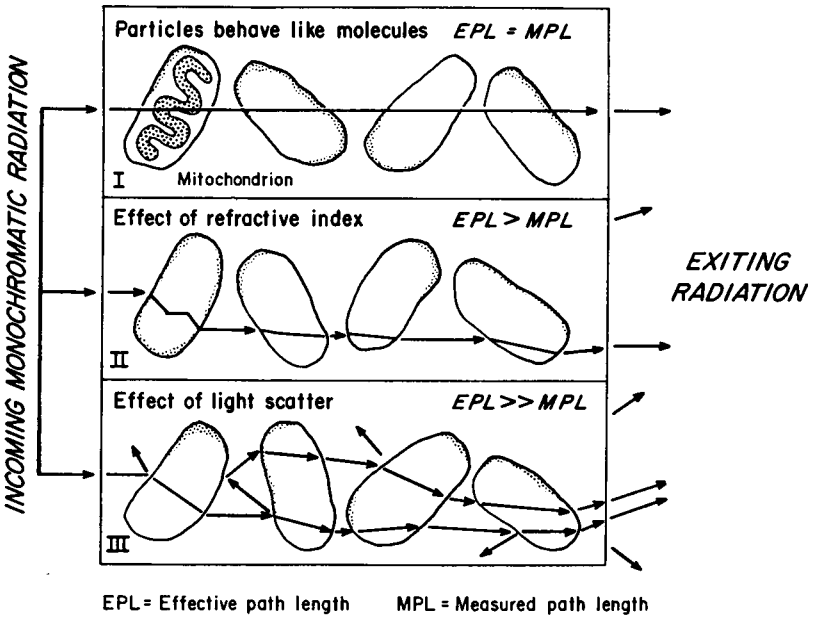

FIGURE 5 Variation in optical path length (cases I, II, and III) as a function of the nature of the interaction between the light and the suspended particles.

itive recognition patterns. Due to the undefined nature of the chromophore path length, however, problems do exist in the quantitation of data from turbid systems. In the case of cellular particles suspended in cytoplasm or buffer, the light beam is transmitted through mobile scattering units of alternate solid and liquid phase. Phase changes occur in the interface between suspending media and particle surface and also at junctures of membranous tissue to cytoplasm and interstitial fluids. Light penetrating this colloidal system probably undergoes multiple reflections and refractions. This circuitous routing allows its exposure to more chromophoric molecules than a straight path would. Since quantitative measurements depend upon the amount of absorption of monochromatic light per unit path length, the effective path length (EPL) or the average distance a light ray travels between entering and leaving a turbid specimen is an important factor.

Three examples of optical behavior relating to the EPL are illustrated in Fig. 5. In case I, light is transmitted through a suspension of chromophore-containing particles in a manner similar to light going through a solution of chromophoric molecules. If the particles and media had a similar refractive index, the EPL would equal the measured path length (MPL) in a manner similar to conventional spectrophotometry. The refractive index of the media could be much like that of homogeneous particles; however, many particles, especially those of biological origin are internally nonhomogeneous. A mitochondrion, for example, has a double-membraned sheath containing numerous inner tubular or platelike lamellae. Its lipoprotein outer sur-

face might be of refractive index 1.60, whereas the "aqueous" phase within the membrane enclosure would be expected to have a refractive index between 1.3 and 1.4. In case II, discontinuities of refractive index cause the EPL to be only slightly larger than the MPL, and not to be significantly dependent upon wavelength. The effect of a ray of light undergoing multiple scatter effects is shown in case III. Here the light traverses a very circuitous route and the EPL could conceivably be many times the MPL.

Since a high degree of reproducibility and precision is offered by the SSD method for the examination of turbid systems, it is possible to achieve quantitative accuracy in these systems. The EPL of mitochondrial systems can be studied experimentally by cytochrome *c* extraction, solubilization, and dye addition. In each of these cases, an equal amount of chromophore can be measured and compared in suspension and in clear solution. From this comparison the enhancement factor β (7) can be calculated from the relationship $\beta = (\Delta AT_{\text{turbid}}/\Delta A_{\text{solution}})$. The examination of cytochrome *c* is conveniently done by first examining the spectra of a suspension of mitochondria or tissue homogenate, then extracting the cytochrome *c* (8) and examining it in solution. For rabbit heart mitochondria in buffer, an enhancement factor of 1.5 is obtained[1] which may indicate that the EPL is 1.5 times the MPL.

APPLICATIONS

The SSD method has been used in this laboratory largely for the examination of chromophoric substances occurring in cellular and subcellular materials. Previous spectroscopic examination of the identification, function, and compartmentalization of the molecular constituents of cells have been made by Keilin, Chance, Caspersson, and others (9). *In situ* spectroscopy is, however, still a young science and difficulties due to turbidity, poor resolution, and inadequate quantitation often limit the functional area of investigation to intensely colored materials such as the hemeproteins, the porphyrins, and retinal pigments. Keilin, for example, extensively studied the cytochrome system in living insects with a microspectroscope but the excellence of this work is limited by its qualitative nature. Chance and others, on the other hand, do obtain quantitative kinetic information using sensitive spectrophotometers, but largely work on in vitro fractionated and reconstituted enzyme systems. The confluence of Keilin's observations on cellular respiration in living organisms and our modern theories obtained from in vitro studies of coupled oxidative phosphorylation and energy transfer, hopefully could be established by precise in vivo spectrophotometry. Our use of SSD has pertinence since it helps to obviate chemical and physical extraction artifacts and may permit living cells to be studied at the molecular level within their native environment.

Initial studies by the author in collaboration with Dr. Martin Morrison and Dr. E. Noel McIntosh compared model systems such as pure cytochrome *c* and isolated mitochondria by SSD and conventional double-beam methods. Some of these results

TABLE I

CYTOCHROME PEAK MAXIMA BY SSD AND OTHER SPECTROSCOPIC METHODS

	Alpha peak—room temp (nm)			Alpha peak(s) at −196°C (nm)		
	SSD	Difference	Visual	SSD	Difference	Difference
Compound	Reference	11	12 *a*		12 *c*	12 *b*
Cytochrome *a*	602–4	600	600–5	599	599–600	601
Cytochrome *b*	564	563	564	560	561–562	560
				556	556–559	
				563	564	
Cytochrome *c*	552	550–552	550	547.3 D*	547–548	549 D
				543.8 D		546 D
Cytochrome c_1		553	554	552	552	554
Cytochrome b_5	551.5 D	556		550.7 D	552.5 D	
	559.7 D			556.2 D	557.5 D	
Oxyhemoglobin	576			572		

The SSD spectra were obtained from examination of whole perfused tissue and isolated mitochondria. The approximate limit of detection of intracellular pigments in a volume of 3 mm^3 is 10^{-12} moles at room temperature and 10^{-14} moles at −196°C.

* Letter D denotes doublet.

are summarized in Table I. It is evident that both yield almost identical data on peak position at both room and liquid nitrogen temperature. The SSD spectra, however, are capable of greater resolution and definition of peaks. Extension of studies on model systems to strips of tissue from perfused animal organs demonstrated that intracellular pigments could be instrumentally observed in situ with no loss of definition. These experiments indicated that the isolation of cell organelles by centrifugation methods is sometimes either remarkably inefficient and/or destructive. Low and variable yields of mitochondria (less than 10%) have been obtained from tissues such as rabbit liver and uterus. To determine inefficient steps and trace degradative processes that may be occurring during the isolation procedures, the SSD spectra may be used to quantitatively monitor chromophore markers in their initial native nonhomogeneous matrix and during preparative phases. The balance sheet accounting of their distribution can then be used to improve preparative procedures.

It is well known that many materials, when cooled to very low temperatures, show remarkable alterations of their usual absorption spectra at ambient temperature. Cytochrome spectra show sharpening, splitting, and intensification of peaks at liquid-air temperature. This was observed first by Keilen, and later studied by Estabrook (10). The theoretical basis for these effects can be complex and may involve aspects such as physical state (glass or crystalline), the nature of the electronic transition, solvent interaction, equilibrium states, and changes in molecular configuration. As an experimental technique, low temperature spectroscopy offers many advantages which include (*a*) the possibility of using small samples, (*b*) the increased

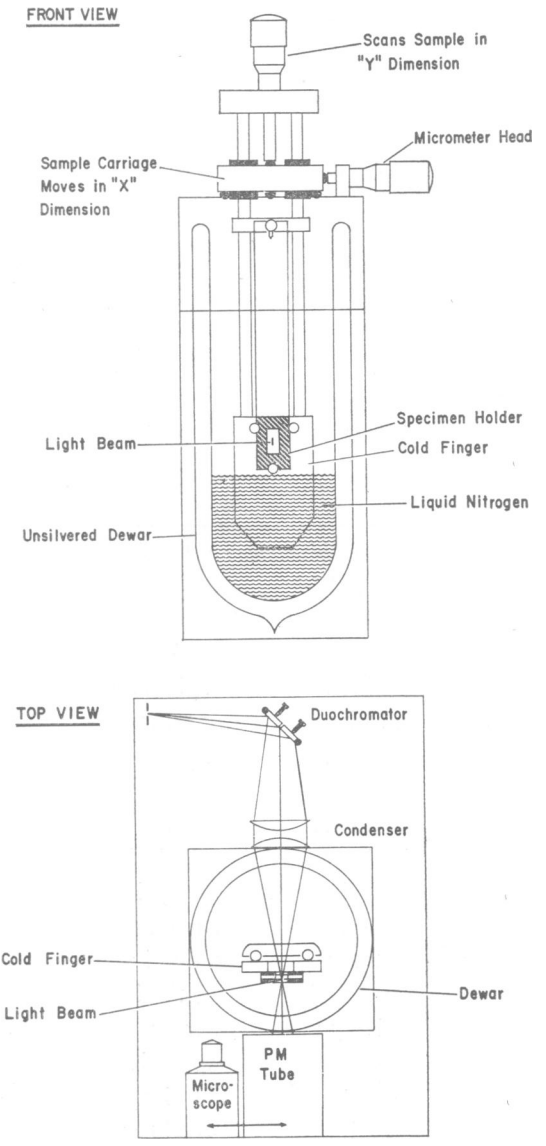

FIGURE 6 The cell compartment used to obtain low temperature spectra.

resolution of bands which may be overlapping at room temperature, (*c*) the ability to obtain recorded spectra with absorption peaks accurately located, (*d*) the "freezing in" of labile states (11), and (*e*) the ability to study spin-state interactions because of their temperature dependence. The apparatus shown in Fig. 6 permits the spectral examination of materials at the temperature of liquid nitrogen (−196°C) and the use of the SSD system here avoids the necessity of having to freeze both

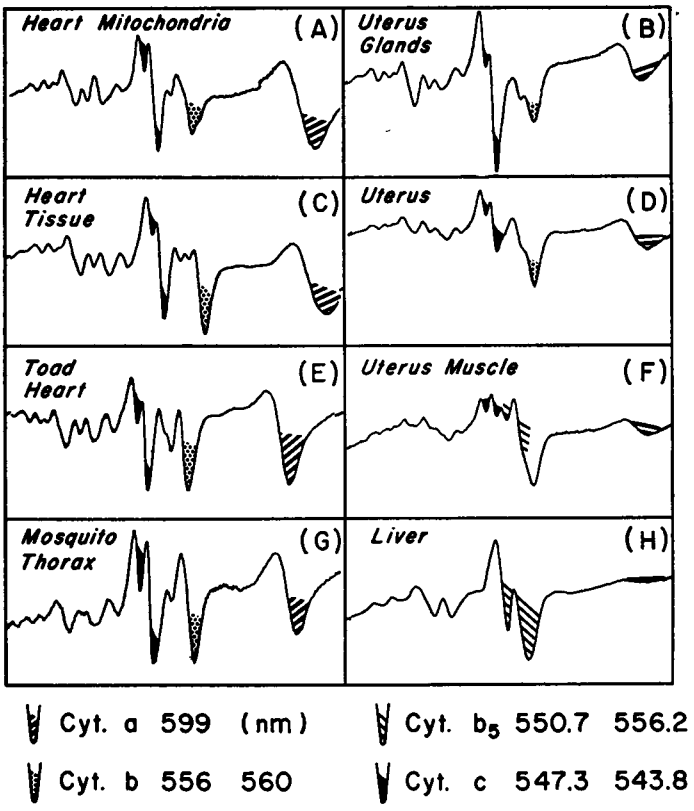

FIGURE 7 Low temperature (−196°C) spectra of animal tissues. Except in the case of E and G, the tissue was obtained from an adult female rabbit. Spectra shown are original and not redrawn.

a sample and reference material and thereby avoids physical inequalities due to fissures, cracks, and nonhomogeneous vitrification.

The low temperature SSD spectra of reduced cytochrome containing systems of animal origin are shown in Fig. 7. The typical cytochrome pattern representing absorptions from cytochromes *a*, a_3, *b*, *c*, and c_1 observed in isolated mitochondria are also clearly seen in whole tissue obtained from different organs and species. When isolated mitochondria are reduced with substrate (Fig. 7 A), unexpected evidence of multiple types of cytochrome *b* was observed, and this aspect is presently being investigated in our laboratory and elsewhere. In addition to cytochromes, heart muscle of most species contains myoglobin as well as remnants of unperfused hemoglobin. For this reason, the clearness of detail in the toad heart tissue spectra (Fig. 7 E) probably reflects the absence of myoglobin in this organ (13) and an unusually efficient perfusion. Since in the derivative method narrowing the peak causes a more intense absorption derivative deflection, the SSD and low temperature tech-

niques work synergistically to increase sensitivity 10–100-fold over room temperature spectra. This sensitivity enhancement is useful in the measurement of samples that are small in size or chromophoric content. For example, when the thorax region of a mosquito was compressed to an area of one square millimeter, it provided the well-defined cytochrome spectra shown in Fig. 7 G. Such microscopic viewing is uniquely compatible with SSD low temperature operation and at greater magnification allows the scanning of a specimen for the spatial distribution of identifiable pigments. In this way examination of a cross-section of a hormonally stimulated rabbit uterus shows compartmentalization of specific pigment patterns in its concentric layers. As shown in Fig. 7 B, the endometrial layer rich in glandular-type cells shows large amounts of mitochondria. The outer muscle layer, on the other hand, is seen in Fig. 7 F to be relatively poor in mitochondria and to contain large amounts of cytochrome b_5. Changing the type and amount of stimulation alters the relative proportions of these cytochromes and provides information consistent with classical histological means of evaluating uterine growth.[4] The spectrum of rabbit liver tissue (Fig. 7 H) is of interest in that it exemplifies a tissue in which cytochrome b_5 pigment is present in much greater quantity than mitochondrial cytochromes.

While cellular pigments are discussed in detail, they represent only one of many areas of application of the method and are emphasized because they reflect the current interests of this laboratory. Similar cases might be presented for the static and dynamic measurement of diverse materials in all states of matter. Specific areas of application in both absorption and emission spectroscopy that suggest themselves include the following: food examination, bacteria culture assay, flame and plasma photometry, and gradient scanning. Each of these areas is potentially rewarding and deserves further investigation.

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