A Simplified Microspectrophotometer

Cadmium selenide cells are well suited for absorption microspectroscopy of pigments in living systems.

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The use of sensitive high-speed spectrophotometry for the investigation of the pigment system within living plant and animal cells has only begun to be investigated. Recent progress in this research has been summarized in several reviews (*1*, *2*). The methods of precision spectrophotometry still require fairly complicated apparatus, and all employ a photomultiplier tube as the light-sensitive photocell.

A microspectrophotometer using solid-state electronic components has been constructed in our laboratory and is being used to investigate pigmented structures in living cells. The light-sensitive element is a cadmium selenide photoconductive cell. The photocell output is amplified by a transistorized d-c amplifier and displayed on an oscilloscope used as a d-c voltmeter. For some time, photoconductive cells have been used for spectral measurements in the infrared region. More recently they have been used for x-ray spectroscopy (*3*). However, the present application of these cells to visible and ultraviolet microspectrophotometry is novel.

In Fig. 1 are shown a diagram of the instrumental setup and a photograph of the assembled apparatus in use. Light from source *L* enters a grating monochromator *G*, as shown by the dotted line. The exit slit of the monochromator is focused by a quartz lens on the condenser of microscope *M*. After passing through the specimen, the light beam hits the photosensitive cell *C*, which is accurately positioned relative to the image and located 25 centimeters above the eyepiece of the microscope. The electrical signal from the photocell is amplified by the d-c amplifier *A* and displayed on a Dumont model 403 oscilloscope *D*. The oscilloscope is used here because of its high d-c amplification capabilities; a high-impedance voltmeter would be just as satisfactory.

The microspectrophotometer uses either a xenon arc or a tungsten ribbon filament light source, a Bausch & Lomb 250-mm grating monochromator, and reflecting microscope optics (50 ×, with numerical aperture of 0.5) purchased from the American Optical Co. With these components, the instrument is usable over the wavelength range from 200 to 990 mμ (millimicrons) in a single sweep for specimen areas of 16 μ^2. For specimen areas of the order of 2 μ^2, the useful range is 270 to 990 mμ. With a tungsten ribbon light source, the useful range is 370 to 900 mμ.

The actual construction of the instrument was greatly facilitated by taking advantage of a photomicroscopy setup, as shown in Fig. 1. The lower end of a rubber, light-tight bellows fits over the eyepiece of the microscope. The upper end of the bellows fits into a light-tight tube. At the other end of the tube may be placed either the ground-glass viewer or the photocell holder, by operating a sliding track. The entire upper assembly is swung into place by means of a vertical bar arrangement mounted on a base plate. The height of the photocell can be adjusted by telescoping the tube supporting it.

The photoconductive cells being used at present are general-purpose cells made by the Clairex Corp. of New York. Two sizes are available, with sensitive areas of 0.5 by 1 and 1.6 by 4.7 millimeters. For a magnification of 500, these sizes represent specimen areas of about 2 and 30 μ^2, respectively. The spectral response of the cadmium selenide cells extends all the way from the far ultraviolet to the near infrared, with a rather sharp cutoff at about 1 μ. The peak response is in the visible region of the spectrum. At high light intensity, the time constant of the cell is of the order of milliseconds. At extremely low light levels, the time constant is of the order of seconds. The sensitivity of the photocells per unit area is comparable to photomultiplier tube performance.

In practice, the photocell acts as a light-sensitive resistor. In the dark, its resistance is greater than 100 megohms. In strong light its resistance decreases to the order of 1 megohm or less. The photocell used here is connected in series with a bias battery (70 to 100 volts) and a load resistor of 1 megohm. The load resistor serves as the input to the photocell amplifier, which is chopper-stabilized and battery-powered. The circuit employs two transistors in the common emitter configuration. Further d-c amplification is furnished by the oscilloscope used for output readings.

With the exception of arrangements similar to the one used by Denton (*4*), microspectrophotometers are generally rather elaborate instruments (*1*, *5*, *6*). They employ either split- or dual-beam optical systems with mechanical beam-chopping devices, voltage-regulated photomultiplier tubes with feedback mechanisms, and automatic-recording outputs. The final result of this type of instrumentation is a quantitative measurement of the optical density of a given specimen with an instrumental error of less than 5 percent. Measurements from the ultraviolet through the visible range are recorded in a few minutes, and response times of the instruments are usually of the order of a few milliseconds. In order to record spectra from samples of very small area, relatively high magnification is usually necessary (*5*). At present, the microspectrophotometer described here is not to be compared with these. Our instrument is portable in the sense that it can be easily assembled and used in the field. The maximum magnification employed to date is 500. At this magnification, spectra from areas of 2 μ^2 are easily obtained. Optical alignment is not critical, and the effects of stray light have been found to be negligible. No optical beam chopping is used, mainly because of the relatively long time constant of the photocell. Since the entire photosurface of the cell is exposed to the light beam, no effects due to varia-

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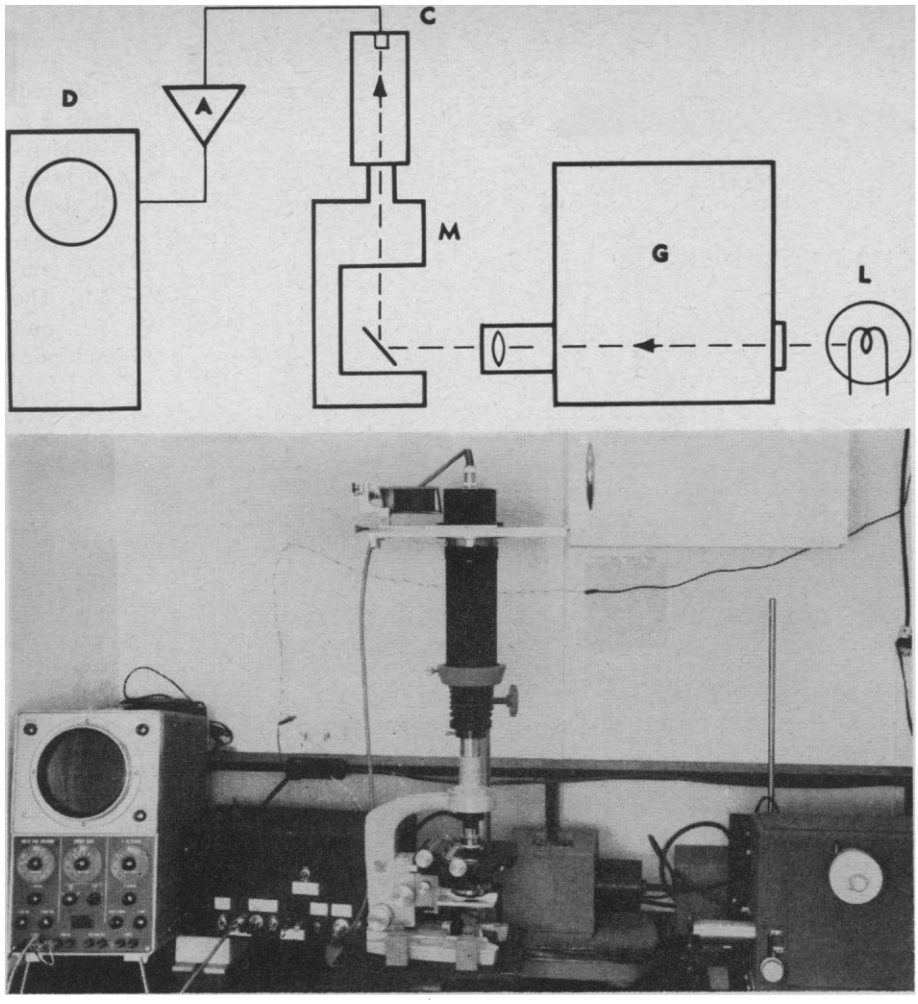

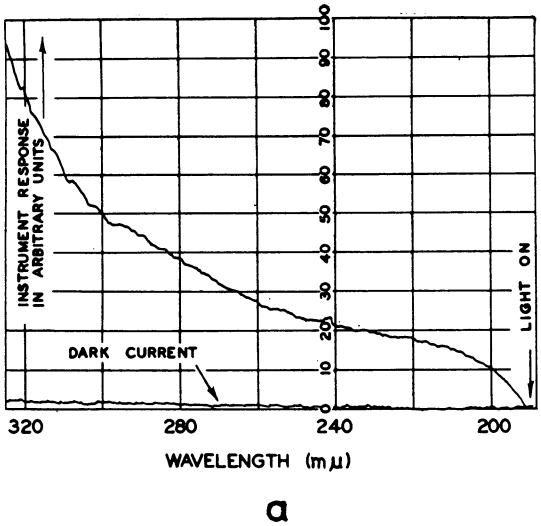

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Fig. 1 (Above). Microspectrophotometer showing light source *(L)*, monochromator *(G)*, microscope *(M)*, photoconductive cell *(C)*, d-c amplifier *(A)*, and oscilloscope (D). (Bottom) The microspectrophotometer in use.

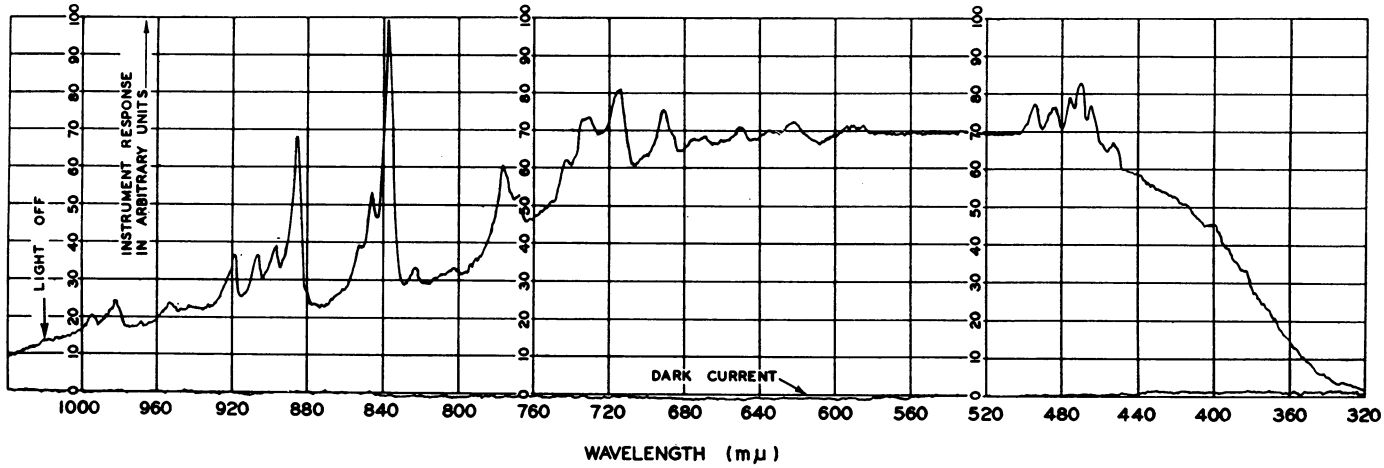
Fig. 2 (At left and below). Microspectrophotometer response as a function of the wavelength of the incident light (*a*) from 200 to 320 mμ and (*b*) from 320 to 1000 mμ, with reduced intensity of incident light.

tion in the sensitivity of the photosurface are observed. A simple resistance-capacitance filter is employed at the amplifier output, resulting in an instrumental time constant of 2 seconds.

The photocell output as a function of the intensity of the incident light was investigated by comparison with the output of an RCA 926 phototube calibrated in our laboratory. Linearity of photocell output within 2 percent with incident light intensity was observed except for very low light levels, which are not normally encountered with this instrument.

At present, the instrumental noise level is determined by the light source and the amplifier fluctuations rather than by the noise in the photocell. For the Soret band peak of a single human blood cell, the noise level was 2.5×10^{-3} optical density units at a wavelength setting of 420 mμ and an entering bandwidth of 2 mμ. Although the useful optical density range of the instrument depends upon the wavelength setting and the intensity of the incident light, data taken to date indicate an upper limit of 1.5 optical density units before serious inaccuracies occur.

The procedure for obtaining an absorption spectrum with the apparatus in its present form is relatively simple. The area of interest in the specimen is located on a marked ground-glass screen at the top of the apparatus. The photocell is then put in position, and the dark current is balanced out with bias voltage. At each desired wavelength setting, one reading is taken over the specimen and another over a reference area by moving the cell assembly from one area to the other. Before another reading is taken, the dark current level is checked and set to zero. The time required to determine a spectral curve depends upon the number of wavelength settings desired and the time constant of the photocell. For a 10-mμ interval over the range from 400 to 700 mμ, the time required is about 20 minutes.



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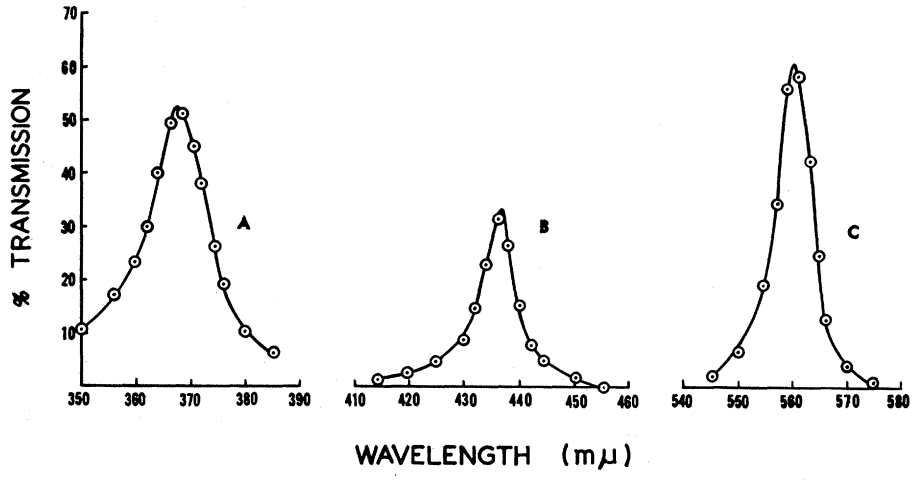


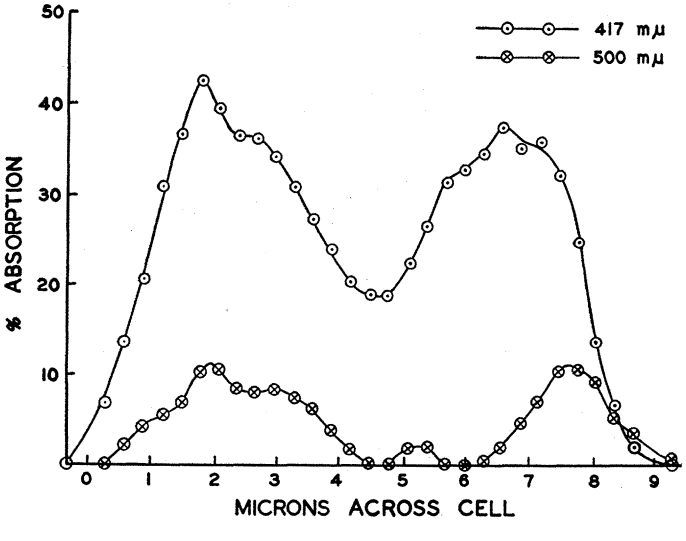
Fig. 3. Spectral response curves for three different interference filters with maxima at 368, 436, and 560 mμ, respectively. Filter *A* is Bausch & Lomb No. 33-78-36; *B* is Bausch & Lomb No. 33-78-43; *C* is Baird Associates No. 7-3470-3. Incident half-bandwidth, 2 mμ.

Because of the simplicity of the microspectrophotometer design, the demands made upon the operator are greater than those made by an automatically controlled instrument. For each specimen under investigation one must choose optimum values for the monochromator slit widths, which govern both the purity and the intensity of the light entering the system. In addition, one has a choice between critical or Köhler (even) illumination of the specimen. For critical illumination the image of the exit slit of the monochromator appears in the object plane, and the light intensity is greater than it is when the specimen is evenly illuminated. Both of the foregoing factors depend upon the choice of magnifying power.

Applications

This instrument was designed primarily to make relatively short *in situ* spectral analyses on pigment granules and photoreceptor structures—that is, chloroplasts, chromatophores, and the retinal rods and cones of the eye. Use of these structures, which in general contain pigments in relatively high concentration, reduces the sensitivity requirements of the instrument. In addition to determining spectral absorption, the instrument is ideally suited for scanning single cells (*7*) or small organisms at fixed wavelengths in order to geometrically locate cell constituents which have characteristic absorption peaks.

The complete spectral range of the instrument is illustrated graphically in Fig. 2, which shows the recorded output of the photocell over the range 200 to 1000 mμ. The recordings were obtained with a Varian G-10 recorder connected to the amplified photocell output, set for 50 millivolts full scale. The wavelength drum of the monochromator was turned by hand throughout the spectral ranges indicated. In Fig. 2*a*, the entrance half-bandwidth was 2.6 mμ and the incident light was used at full intensity. In Fig. 2*b*, the half-bandwidth was 2 mμ, and the intensity of the incident light was greatly reduced to keep the recorder pen on scale at the peaks. For wavelengths greater than 600 mμ, the second-order grating spectrum was filtered out. The series of response peaks around 460 mμ and in the region 800 to 1000 mμ is due to line spectra of the xenon arc light source. For both of the



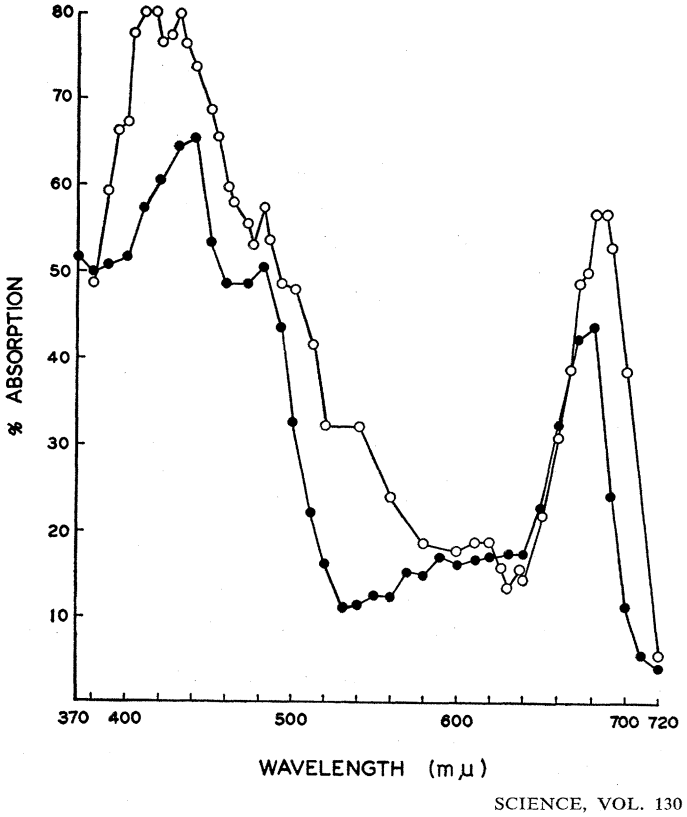


Fig. 4 (Above). Spectral absorption at fixed wavelengths of 417 and 500 mμ as a function of the distance across a single human blood cell. Fig. 5 (Right). Absorption spectrum of a single chloroplast of *Euglena gracilis* (open circles) compared with the spectrum of a single chloroplast of *Chlorella* (solid circles), taken from the data of Swift and Rasch (*1*).

response curves the microscope was focused on a blank quartz slide at a magnification of 175, representing a specimen area of 16 μ^2.

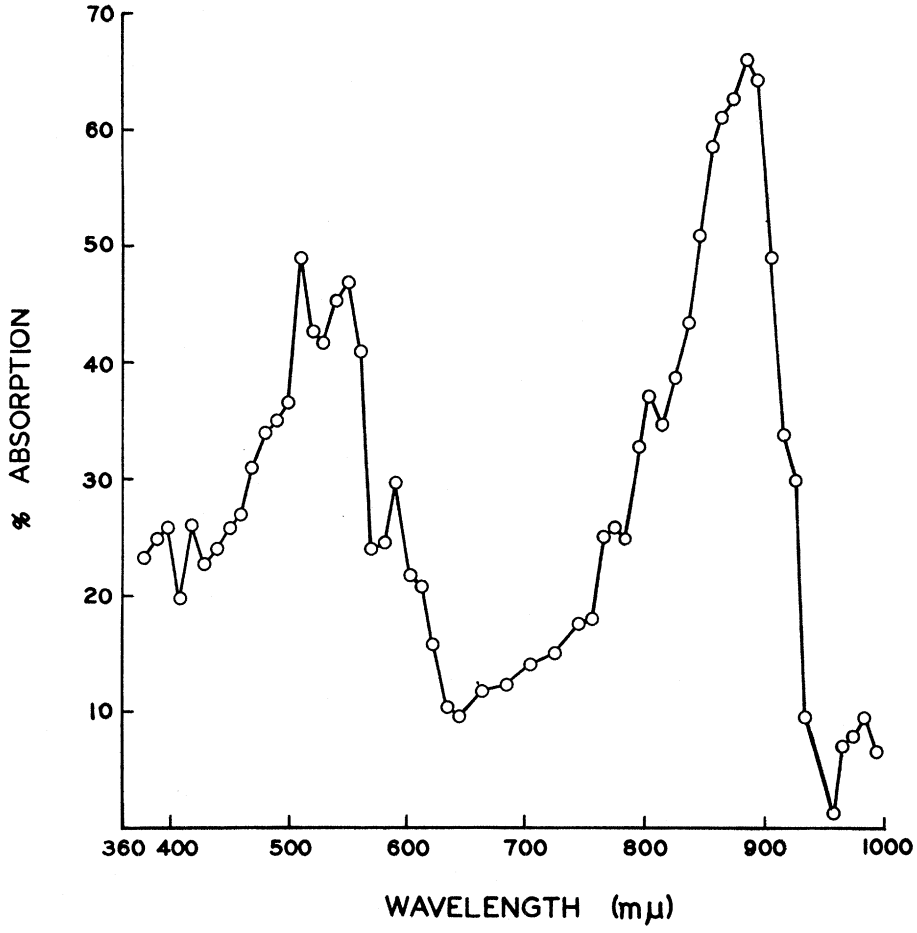
The performance of the microspectrophotometer has been checked with bandpass filters, line spectra, and materials with known absorption peaks. In Fig. 3 are plotted response curves for Bausch & Lomb interference filters Nos. 33-78-36 and 33-78-43 and for a Baird Associates interference filter No. 7-3470-3; the major peaks of these filters are at 368, 436, and 560 mμ, respectively. These absorption curves are in excellent agreement with those obtained with a Beckman model DK recording spectrophotometer for the same filters.

Absorption spectra (not shown) have been obtained from single human blood cells. These showed characteristic absorption peaks at 418, 538, and 578 mμ. In Fig. 4 are illustrated two transverse scans across a single human blood cell with wavelength settings of 417 and 500 mμ, respectively. The difference between the two curves is a rough indication of the hemoglobin distribution within the blood cell as a function of cell diameter. The specimen area under investigation was slightly less than 2 μ^2, and the results are in good agreement with published data (*7*, *8*).

Figure 5 shows the absorption spectrum in the visible region of a single chloroplast within the algal flagellate *Euglena gracilis* (open circles) compared with the specrum of a chloroplast from *Chlorella* (solid circles) taken from the data of Swift and Rasch (*1*). The average size of *Euglena gracilis* is 50 by 15 μ, and the green chloroplasts are on the order of 1 by 6 μ (*9*). The magnification was 250, representing a specimen area of 8 μ^2. The reference area was the clear region in the cytoplasm adjacent to the chloroplast. The major absorption peaks at 415 to 430 mμ and at 680 mμ are in agreement with the data for chlorophyll *A* in living algae (*10*), and the secondary peak at 480 mμ is due to the presence of carotenoids, which are known to be present in the chloroplasts (*10*). The absorption spectrum shown in Fig. 5 is also in good agreement with the spectrum for cell suspensions of *Euglena* obtained in our laboratory (*11*).

A suspension from a culture of the photosynthetic bacteria *Rhodospirillum rubrum* was placed on a microscope slide and scanned over the wavelength range 380 to 990 mμ. The bacteria from this culture were too small for individual spectra to be obtained, since they were less than 1 μ in diameter. The absorption spectrum from the microscope slide suspension is shown in Fig. 6. The major peak at 880 mμ and the small peak at 590 mμ are due to bacteriochlorophyll (*12*). The secondary peak in the region 500 to 550 mμ is due mainly to the bacterial carotenoid spirilloxanthin (*12*). The data were taken at a magnification of 250, representing a suspension area of 8 μ^2. The reference area was a clear region under the cover glass adjacent to the bacterial suspension.

The microspectrophotometer has also been used (*13*) to investigate the brightly colored oil globules found in the retina of birds, occurring mainly between the inner and outer segments of the cones (*14*). These colored globules are red, green, yellow, orange, and other, mixed, colors; they range from 3 to 5 μ in diameter, and their exact physiologi-



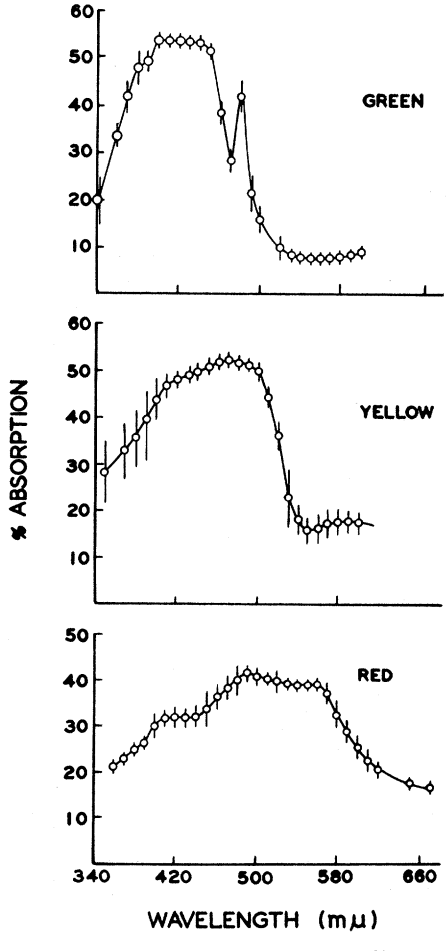


Fig. 6 (Top). Absorption spectrum of a sample of an "old" culture of aerobic *Rhodospirillum rubrum*. Fig. 7 (Bottom). Absorption spectra, measured *in situ* for red, yellow, and green globules within the chicken retina.

cal role is not known (*5*, *13*). Figure 7 shows absorption spectra for red, yellow, and green globules measured *in situ*. The vertical lines indicate the observed variability for each colored globule. These spectra were measured at a magnification of 500 through a specimen area of 2 μ^2. The reference area was immediately adjacent to the globules and contained a slight amount of retinal material. Spectra taken of globules completely isolated from retinal material indicated no significant error resulting from this procedure over the wavelength range under investigation. These results indicate that the different colored globules within the retina can act as bandpass color filters if they are suitably located in relation to a photosensitive pigment (*5*, *15*).

The spectral curves shown in Fig. 7 indicate the presence of carotenoids, a finding in agreement with the biochemical investigation of Wald and Zussman (*15*) on retinal extracts of chicken. The green globules have a major absorption peak near 420 mμ, indicating the presence of galloxanthin, a carotenoid isolated by Wald from the chicken retina (*16*). The yellow globules have a major absorption peak in the region 470 to 480 mμ. Absorption in this portion of the visible spectrum is typical for both xanthophylls and carotenes. Both lutein and zeaxanthin are present in the chicken retina (*15*), and the yellow globules are believed to be composed mainly of these two carotenoids. The red globules have a broad absorption maximum near 500 mμ, indicating the presence of astaxanthin (*15*). The absorption spectra obtained from the colored globules are not from purified compounds but from mixtures of pigments in the natural state. Therefore, some wavelength shifts toward longer wavelengths are to be expected from spectra reported in the literature. These pigmented globules were also investigated in the ultraviolet region of the spectrum. The presence of lipids was indicated by strong absorption at 310 mμ and below (*13*).

The illustrated spectra described are only an indication of the possible usefulness of the instrument. The microspectrophotometer is well suited for study of the effects of chemical and physical environment on the synthesis and concentration of pigments within a single cell or of organelles within a cell (*17*).

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Electromagnetic Blood Flow Meters

Implantable flow transducers facilitate circulatory studies in conscious and free-moving animals.

Alexander Kolin

The interest in a method for determination of blood flow goes beyond the need for such methods in studies of hemodynamics. The wider scope of the potentialities of an effective method lies in the possibility of using it in a general way as an index of the activity of a great variety of organs whose performance is normally studied by quite diverse methods specifically adapted to the function of the organ in question.

The rate of blood supply to an organ is a determining factor in regulation of the supply of oxygen, hormones, and nutrient materials. It similarly determines the rate of removal of metabolic products. The blood flow through an organ, when correlated with its rate of activity, could thus be used to follow variations in the organ's activity in response to a variety of stimuli and inhibiting factors.

Ideally, the method of blood-flow determination should permit continuous recording of blood flow in intact undisplaced blood vessels of conscious, freely moving animals. The application of the principle of electromagnetic induction to measurement of blood flow (*1–3*) resulted in a method which, in subsequent modifications, made it possible to record blood flow by means of nonirritating implanted measuring devices (*4–7*). The method is based on the induction of an electromotive force in a fluid flowing transversely through a homogeneous magnetic field. In the case of a circular conduit and axially symmetrical flow, the induced electromotive force is a linear function of the average fluid discharge. In the case of a conductive conduit, the electrical signal can be picked up without making contact with the fluid by establishing contact with two points on the outside wall of the conduit situated at the two ends of a diameter perpendicular to the magnetic field. This configuration is illustrated in Fig. 1.

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