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SPECTROPHOTOMETERS FOR THE STUDY OF PHYTOCHROME *IN VIVO*

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1. INTRODUCTION

Phytochrome is a pigment, found in many plants (1). It is considered to be the photoreceptor for a wide variety of photomorphogenetic and related physiological responses, characterized by the antagonistic effects of red (around 660 nm) and far red (around 730 nm) light. The pioneering work of Butler and coworkers (2) has demonstrated the feasibility of direct spectroscopic identification and estimation of the pigment in plant parts. This is based upon the photoreversible changes in the absorption spectrum after red or far red irradiation, which can be observed in the presence of relatively large amounts of other, non photoreversible, pigments.

Since about 1959, we have been using a number of differential spectrophotometers, constructed in our laboratory, for the study of phytochrome and its transformations in isolated plant parts, as well as in cell free extracts and purified pigment solutions (3, 4, 5, 6). Like several instruments, used for similar or related studies, our machines are based on principles, originally outlined by Chance (7), Duysens (8) and others. Such instruments are now available commercially from a number of sources, and at least one of them is in regular use for phytochrome measurements (e.g. 9).

Although our instruments are not, therefore, fundamentally new, they appear to have a sensitivity considerably surpassing that of those, described so far. Since we have had numerous requests for particulars about their working principle and details of their construction, it appeared desirable to publish a brief description together with a discussion of some practical problems attending to phytochrome measurements *in vivo*.

2. Differential spectrophotometry of highly scattering media

It may be useful to present some elementary considerations concerning the theory of such measurements. There is an abundant literature on the measurement of small absorbancy changes in light-scattering media, especially in relation to photosynthesis (e.g. 10, 11). Due to the very low concentration of phytochrome in most plant material, the instrumental problem is somewhat different here. In order to obtain a sufficiently large change in optical density upon phototransformation of the pigment, it is usually necessary to use thick and, therefore, highly scattering, samples (2). Notwithstanding the small contribution of true absorption to the total optical density, in most samples the apparent optical density, AOD:

$$AOD = \log \frac{I_i}{I_m}$$

where I_i is the light flux of the measuring beam incident upon the front of the sample and I_m the light flux collected by the photodetector, is high. As an example, in the majority of our measurements, the AOD of the samples was between 2 and 3. Occasionally, samples with AOD of 4 or higher had to be measured.

In this type of measurement, the smallest optical density changes that can be observed are limited by the signal-to-noise ratio. Under the conditions of relatively high intensity of the measuring beam, the noise in a well designed photodetector-amplifier system is determined exclusively by shot noise originating in the photocathode, and is proportional to the square root of the light flux falling on the detector. Since, for a particular sample, the signal is proportional to the first power of the same light flux, the signal-to-noise ratio, σ, is proportional to the square root of the intensity:

$$\sigma \sim \sqrt{I_m}$$

It would appear, therefore, that in order to obtain the highest possible spectrophotometric sensitivity, it would be desirable to increase the intensity of the measuring beam as much as practically possible. While this is unquestionably true, there is an important limitation in the type of measurement under consideration. For, whatever the intensity of the measuring beam, it tends to drive the photoconversion of the photoreversible pigment in the sample to some extent during the observation period. A requirement is, therefore, that the intensity of the measuring beam is kept sufficiently low so that it will not provoke appreciable photoconversion during this period (in our experiments usually $\frac{1}{2}$–1 minute). These conflicting requirements set a certain limit to the intensity of the measuring beam and a compromise is to be found for each type of sample separately.

Due to light scatter, the effective light path in the sample is a complicated function of sample thickness. Increasing sample depth usually will increase the effective optical density, at the same time decreasing I_m. Theoretically, for each

particular type of sample there is a definite thickness giving optimum signal-to-noise ratio, following from

$$\sigma \sim f(c).g(d) \sqrt{I_i}$$

where c is the average pigment concentration and d the sample depth. Generally, f(c) and g(d) are unknown and inaccessible to measurement. As the sample material is extremely inhomogeneous with respect to light scatter and possibly also with respect to microscopic pigment distribution, there is no practical way to determine optimum sample depth, except by trial, and this will have to be done for each new material to be measured. In most cases, a sample depth of a few millimeters was found adequate, but in some cases, layers up to 10 mm thick have been used.

There is one obvious, but unfortunately not very effective, way to increase σ viz. increase in the surface area of the sample (and, concomitantly, the cross section of the measuring beam). At constant flux density of the measuring beam, σ increases as the square root of the illuminated area O, giving:

$$\sigma \sim f(c) \, . \, g(d) \sqrt{OI_i}$$

Due to technical limitations, no very impressive gains in σ can be achieved this way, but it is evidently advisable to keep the sample area as large as feasible. In our machines O usually was about 75 mm^2.

Finally, σ will increase proportional to the square root of the light flux, collected by the photodetector. A large area of the photodetector and the minimum possible distance between photocathode and sample are, therefore, important, fig. 1. For this reason, end-on photomultipliers should be used. Since the light flux is relatively high, no very sensitive specimens are needed, but it is desirable to select a type with a sufficiently high quantum efficiency at the longest wavelength used. We have had very satisfactory results with the photomultiplier type EMI 9558 B.

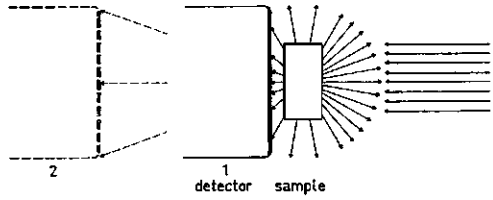

FIG. 1. In order to collect a maximum of scattered light, the distance between sample and photodetector should be as small as possible.

3. DOUBLE BEAM *versus* TWO-WAVELENGTH SPECTROPHOTOMETERS

In the type of measurement under consideration, the change in light flux falling on the photodetector, caused by the photoconversion of the pigment is a small fraction of the total flux. In order to obtain high sensitivity, some form of compensation for the inactive part of the total transmitted flux has to be applied. This can be done in two ways.

a. Double beam operation.

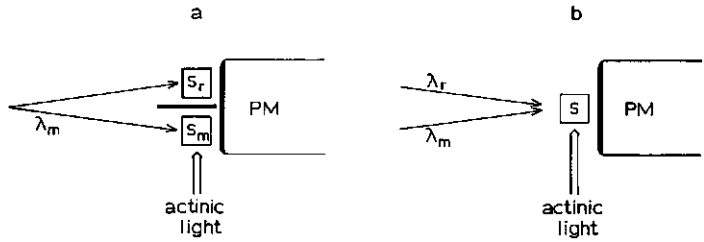

Fig. 2. Differential spectrophotometry. a. Double beam mode. Light of the required wavelength λ_m alternatively falls on two identical samples. The reference sample S_r is protected from actinic irradiation; in the test sample S_m, phototransformation can be induced by the actinic beam. b. Two-wavelength mode. Light of a selected wavelength λ_m, falling on the sample S is alternated with light of the reference wavelength λ_r. The latter is chosen so that the absorbancy changes at this wavelength due to the actinic irradiation are negligible. In the case of phytochrome measurements, λ_r was at 800 or 806 nm.

In fig. 2a, the measuring beam, wavelength λ_m, usually close to an absorption maximum of the component to be measured, oscillates between two 'identical' samples, S_r and S_m. Since at the start the light flux, transmitted by both samples is identical, the illumination of the photodetector is constant and the photocurrent contains no alternating component. Phototransformation of a component in one of the samples (S_m) by actinic irradiation, will change slightly the optical density of the sample and the photodetector current carries an alternating component, proportional to the change in transmitted flux.

b. Two-wavelength operation.

In fig. 2b, the sample is illuminated alternately by two beams. The measuring beam, λ_m is chosen at or near an absorption maximum of the component to be measured. The reference beam, λ_r is chosen either at an isosbestic point or at a wavelength, sufficiently far removed from the absorption band under study so that the absorption changes at the reference wavelength can be neglected. For reasons to be outlined below, in the routine estimation of phytochrome we have chosen 730 nm for λ_m and 800 or 806 nm for λ_r.

The choice between the two systems is governed by a number of practical considerations. As will be discussed further on, the limit of detectability during an actual phytochrome estimation often is not set by photodetector noise, but by spontaneous fluctuations in sample transmission. Apparent optical density of the samples is almost exclusively due to light scatter, and differential changes in the two samples will appear at the output of the amplifier as a 'signal'. Now, in living material, the degree of light scatter will vary with time owing to a number of causes like drying, water migration, spontaneous movements or those, triggered by the actinic irradiation, and the like. In the double beam arrangement, with two samples, such spontaneous density fluctuations will occur independently and add up to a certain error signal. In the two-wavelength arrangement, the density fluctuations will be equal in first approximation for λ_m and λ_r, provided these wavelengths are not too far apart. Noise and drift are, therefore, much less in a one-sample two-wavelength arrangement

than in a two-sample double beam arrangement under otherwise similar conditions. For measurement of low phytochrome levels, therefore, the two-wavelength arrangement is the method of choice.

On the other hand, if one wishes to scan through a spectrum, the double beam arrangement is usually to be preferred since, theoretically, no time consuming resetting of the relative intensity of the two beams is required in going from one wavelength to the next. At high sensitivity, this advantage is, however, offset to a large extent by unavoidable differences in the transmission of the two samples and, more fundamentally, by inhomogeneous sensitivity of the surface of the detector photocathode. This will be discussed further under section 6.

In the following sections, we will describe the design of some instruments. Only those will be discussed with which we have obtained extensive experience.

4. A DUAL-PURPOSE SPECTROPHOTOMETER

a. Optical part

The measuring and reference beams are chopped by a rotating glass prism, mounted on the shaft of a 3000 rpm synchronous motor, fig. 3.

The parallel deviation of the measuring beam, λ_m, in this prism causes the image of the monochromator exit slit to travel over the sharp edge of a second stationary prism that has the dimensions, required to give the necessary spatial separation between the two beams of λ_m. In the double beam mode of operation, two samples are placed in front of the photomultiplier and the reference beam λ_r is shut off. In the two-wavelength mode on the other hand, one of the sample cells (left) is replaced by an opaque block and the reference beam is opened. It alternates with the measuring beam by a second application of the same chopping principle, using the rotating prism in a direction at right angle to the one, used for the measuring beam. A series of additional prisms (P_3, P_4) returns the reference beam to the prism P_2. It then falls on the sample, forming a small angle with the measuring beam. This is of no consequence in view of the strongly light-scattering properties of the sample. During the actinic irradiation, a shutter protects the photomultiplier from damage by the high light intensity. During measurement, a second shutter closes the aperture for the actinic beam, in order to minimize stray light.

b. Signal detection

We will discuss some points that are important for a successful operation of the instrument, described above. Fig. 4 shows a diagram, illustrating the events during the rotation of prism P_1 in the two-wavelength mode of operation. The intensity of the beams λ_m and λ_r can be adjusted by means of the spectrometer slits and an iris diaphragm in the reference beam, until the photocurrents, set up by the two are equal, a. Pigment transformation, due to actinic irradiation, will cause the transmission of the sample for one of the beams to change slightly, b. An alternating current component of 200 Hz consequently appears in the photomultiplier output, c. This current includes noise, i.e. components of a

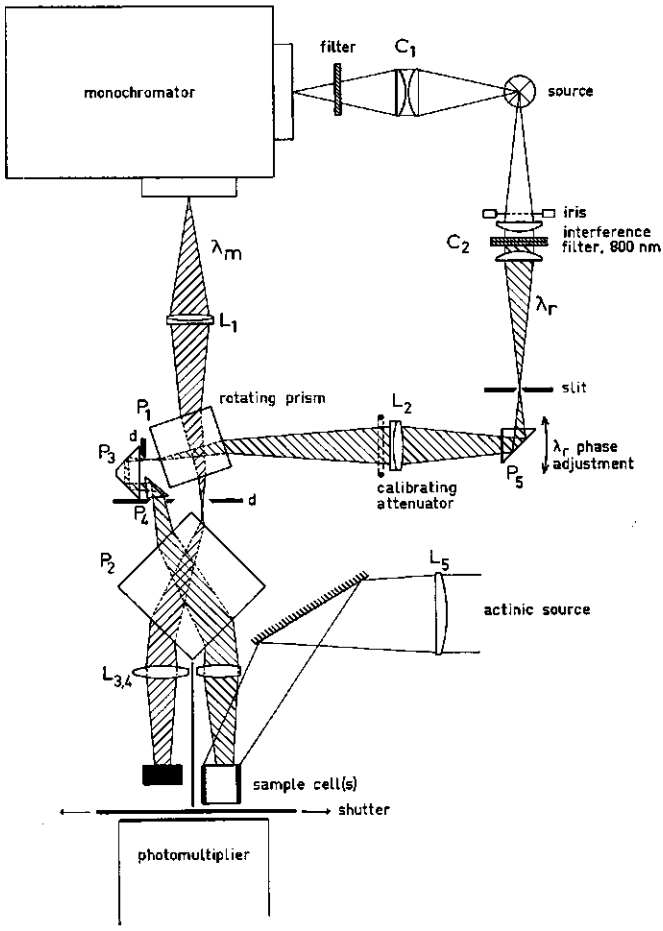

FIG. 3. Double purpose spectrophotometer. Light from a single filament lamp (source) is focussed upon the entrance slit of a monochromator (Bausch and Lomb grating monochromator with 100 × 100 mm grating, 1200 grooves/mm, blaze wavelength 500 nm) to give the measuring beam of wavelength λ_m. A second system of lenses, iris diaphragm and interference filter focusses the same filament upon another slit to give the reference beam of λ_r. The rotation of prism P_1, mounted on the shaft of a 3000 rpm synchronous motor, causes the two beams to fall alternatively on the sample. In the double beam mode of operation, beam λ_r is closed and a second sample is placed in the left sample holder. The stationary prism P_2 serves to obtain the required distance between the two beams. In the two-wavelength mode of operation, the left sample position is occupied by an opaque block, the reference beam is opened and the prism arrangement P_3 P_4 returns the reference beam towards the sample. A wire screen attenuator in the reference beam is used to calibrate instrument sensitivity. The actinic source consists of a 500 Watt slide projector with suitable interference filters.

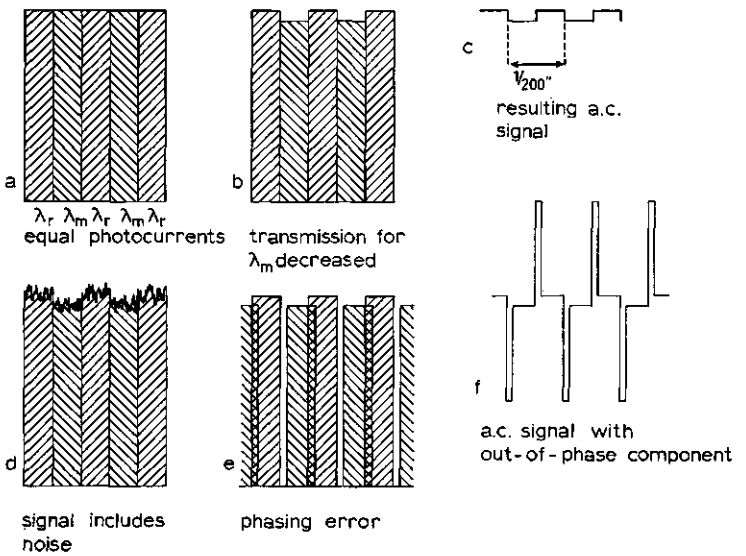

FIG. 4. Formation of a.c. signal in the two-wavelength spectrophotometer. Before the first actinic irradiation, the intensities of the two beams, λ_1 and λ_2 are adjusted to give equal photocurrents in the detector. A change in transmission, due to phototransformation of a component in the sample by the actinic irradiation, gives rise to an alternating current component of 200 Hz, proportional to the transmission change. Practically, the signal is mixed with noise, the 200 Hz component of which contributes to the output. Errors due to incorrect alternation of the two beams will show up as a strong 200 Hz signal which, however, is 90° out of phase with the main signal, and is excluded by the phase-locked amplifier. It should, however, be kept as small as possible, which can be done by adjusting prism P_5 of fig. 3.

multitude of frequencies, d. The detection system, in order to separate the desired 200 Hz component from unwanted signals, will have to be of the phase and frequency locked type. If there is no strict alternation between the two beams, a strong 200 Hz signal appears in the output, e and f, that, however, is about 90° out of phase with the main signal. This out-of-phase signal may result from the optical inhomogeneity of the sample, leading to a different apparent optical density distribution for the two wavelengths over the front surface of the sample. Although the phase-locked amplifier should be insensitive to this 90° signal, it may contribute to noise when excessive, and should be reduced to a minimum before starting a measurement. To this end, the direction of the reference beam towards prism P_1 can be altered slightly by rotating an auxiliary prism P_5 around a vertical axis, going through the center of lens L_2, fig. 3. In this way, it is possible to shift the moment when beam λ_r appears over a fraction of the period of one rotation. The position for minimum out-of-phase signal can be monitored continuously on an oscilloscope.

c. Amplification and demodulation

The 200 Hz signal, proportional to the change in light transmission of the sample is converted to direct current and fed into a potentiometer recorder. Fig. 5 gives a block diagram of the phase-locked amplifier-demodulator. The system requires a 200 Hz reference signal for the demodulation. This can be obtained in a number of ways. Since phase jitter in this signal may contribute significantly to noise, absolute short- as well as long term synchronicity between beam chopping and generation of the reference signal is essential. After experimenting with a number of methods, we have found the simplest arrangement to be the most satisfactory. To this end, a four-pole bicycle dynamo is mounted permanently on the lower end of the chopper motor shaft and its 200 Hz output is used directly to lock the ring demodulator. In order to compensate for phase shift in the amplifier, the pole pieces of the dynamo can be rotated around the motor axis, until maximum recorder excursion is obtained for the calibration signal (see below). Once set, this adjustment remains correct. This method works very well. Equally good results can be expected, however, from a system, using a sector disc mounted on the motor shaft, periodically obscuring one or more photodiodes that either take care of the demodulation or generate the reference signal. This method lends itself more easily to the rotating-sector type of beam chopper, to be described under section 5.

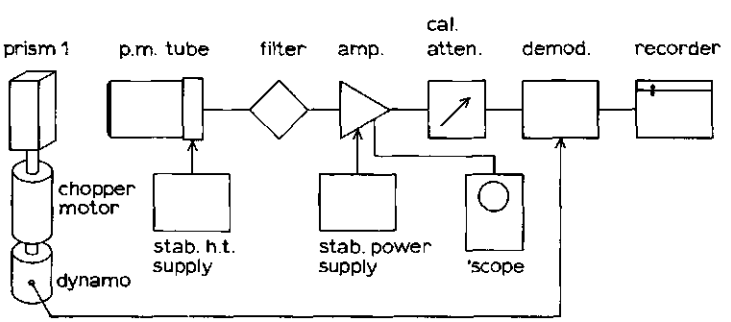

FIG. 5. Block diagram of amplifier-demodulator. Prism 1 is P_1 of fig. 3.

Fig. 6 gives a circuit diagram of the amplifier-demodulator that has been in use, with minor modifications, since 1957. A transistorized version is under construction.

Under section 8, we will give some examples of actual measurements, made with this instrument.

d. Calibration

The recorder deflection, produced by a given change in transmission of the sample depends upon a number of factors, such as overall amplification, light intensity of the spectrophotometer beams, photomultiplier sensitivity etc. It is, therefore, mandatory to have a means of calibration. This is provided by an op-

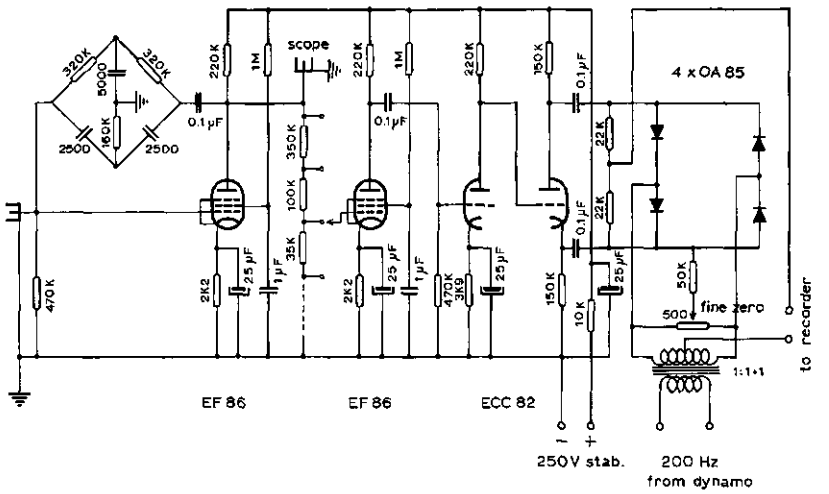

FIG. 6. Circuit diagram of the amplifier with 200 Hz filter section and ring demodulator. A symmetrical low pass filter is inserted between the amplifier output and the potentiometer to remove any 200 Hz that may appear at the output terminals.

tical attenuator that can be inserted into the reference beam, fig. 3. It introduces an optical density increment of 0.004. Examples can be seen in figures 14, 15 and 16. The attenuator is made from black nylon wire of 25 micron diameter, wound with appropriate spacing on a metal frame.

e. Stabilization of spectrometer light source

In a two-wavelength spectrophotometer of high sensitivity, it is important that the temperature of the filament of the light source is kept very constant. A relatively small temperature change results in a differential change in emission at λ_m and λ_r, which is translated by the instrument into an apparent change of the optical density. The spectrometer lamp is, therefore, operated from a well stabilized power supply.

5. A TWO-WAVELENGTH SPECTROPHOTOMETER WITH INTERFERENCE FILTERS

The instrument, described under section 4 is very convenient if one wishes to do measurements at arbitrary wavelengths of the measuring beam. If the main aim is to make measurements of relative phytochrome concentrations, a considerably cheaper instrument can be constructed by replacing the monochromator by a second interference filter. Fig. 7 gives a drawing of such an instrument. Signal detection is the same as described under section 4b. We have also applied the method of beam chopping of this machine to an instrument in which one of the interference filters was replaced by a glass prism monochromator. The principles of the method can, of course, be applied to suit any particular requirement of the user.

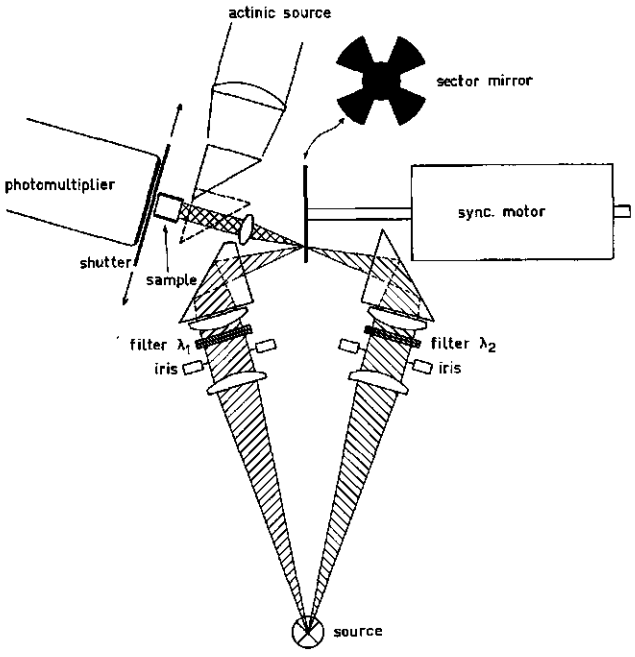

FIG. 7. Two-wavelength interference filter spectrophotometer for phytochrome measurements. The chopper sector is constructed from a piece of highly polished chromium plated brass sheet. It alternatively transmits and reflects one or the other of the beams λ_1 and λ_2. Usually, λ_1 is chosen at 730 nm and λ_2 at 806 nm. During actininc irradiation of the sample, a prism is inserted in the light path in front of the sample, directing the actinic beam from a 500 Watt slide projector with interference filters towards the sample. At the same time, a diaphragm obturates the photomultiplier.

6. A DOUBLE BEAM RECORDING SPECTROPHOTOMETER OF HIGH SENSITIVITY

Using the glass prism monochromator and rotating sector chopper, mentioned under section 5, we have successfully operated a direct recording differential spectrophotometer that will be described briefly here. Fig. 8 gives a simplified diagram. In order to obtain constant photometric sensitivity over the whole wavelength range, the photomultiplier high tension was automatically regulated. To this end, the light was chopped twice: once, at 200 Hz for beam alternation, Ch_2, and additionally at 2000 Hz at the entrance slit of the monochromator, Ch_1. This chopper consisted of 40 wires, soldered into the circumference of a disc mounted on the shaft of a 3000 rpm synchronous motor. During passage of a wire through the light beam, the intensity at the entrance slit decreased by about 10%. The output signal from the photomultiplier PM therefore contained a frequency of 200 Hz and one of 2000 Hz, the magnitude of the latter being a function of spectral light intensity, sample transmission and photomultiplier sensitivity. Filter networks at the amplifier input separate the 200 and 2000 Hz

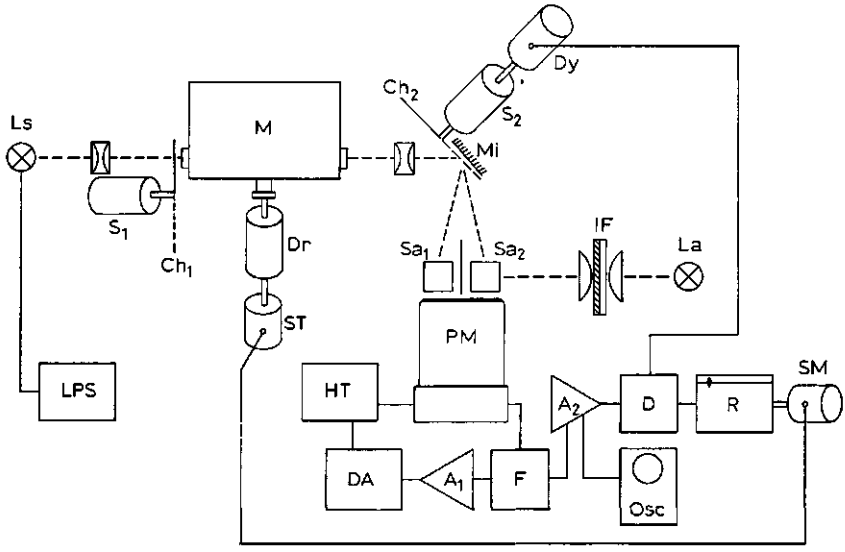

FIG. 8. A double beam recording spectrophotometer of high sensitivity. Before entering the monochromator, the light from a single filament lamp Ls is attenuated about 10% at a rate of 2000 times per second by the chopper Ch_1. Monochromatic light from the exit slit of the glass prism double monochromator M (Kipp and Zonen, Delft) is directed by a 200 Hz chopper Ch_2 towards sample Sa_2. The construction of this chopper is similar to the one, shown in figure 7. Closely behind this chopper is a stationary mirror Mi, rotated over a small angle so as to direct the reflected beam towards sample Sa_1. Sa_2 can be irradiated by the actinic beam from the slide projector lamp La. The signal from the photomultiplier comprises components of 2000 Hz, proportional to the total light flux, and of 200 Hz, proportional both to the total flux and to the transmission difference between Sa_1 and Sa_2. Filter networks F separate the two frequencies. The 2000 Hz is fed into an a.c. amplifier, demodulator and d.c. amplifier. The latter regulates the photomultiplier dynode supply, HT. The 200 Hz signal is processed as described under section 4c. Dr: variable speed motor. ST and SM: selsyn transmitter and receiver for coupling wavelength drive to recorder chart movement. LPS: lamp power supply.

components and the latter, after amplification, simple demodulation and suitable d.c. amplification, was used to regulate the high tension for the photomultiplier. By making the open loop gain of the system sufficiently large, any reasonable degree of constancy of the 2000 Hz output signal can be obtained and this in turn provides automatically for constant photometric sensitivity of the 200 Hz system, as long as the optical density changes in the sample, due to pigment conversion by the actinic irradiation, are small.

This instrument has been mentioned especially in order to illustrate one of its main shortcomings, viz. the effect of inhomogeneous sensitivity of the photocathode surface of the photomultiplier. Using two samples, it is unavoidable that somewhat different parts of the photocathode are illuminated by each sample. If one wishes to make the instrument really sensitive, e.g. to give full scale deflection for a transmission change of say, 1%, the photocathode has to

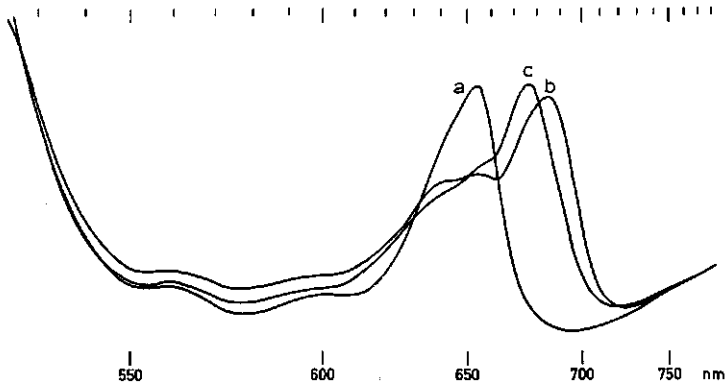

FIG. 9. Example of a recording, made with the instrument, shown in fig. 8, at low sensitivity. Leaves of dark grown maize, mounted between microscope slides. Reference sample was a suitable thickness of filter paper.
a. Before irradiation: absorption spectrum of protochlorophyll.
b. After irradiation for 30 sec. with 643 nm: partial conversion of protochlorophyll to chlorophyll a 682.
c. Recording repeated after 35 min. dark: the absorption peak of chlorophyll a has shifted to 675 nm ('SHIBATA' shift).

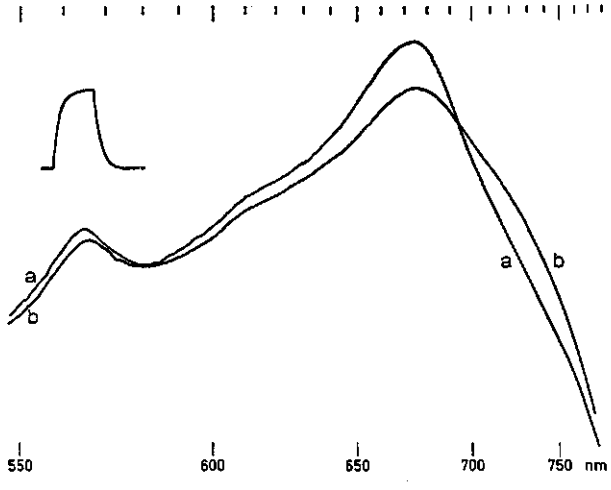

FIG. 10. Same instrument as fig. 9., high sensitivity. A 10 mm layer of maize mesocotyls was measured before (a) and after (b) an exposure to 5 min. red of 643 nm. The difference between the two spectra is due to photoconversion of P_r to P_{fr}. The reference sample was an identical layer of the same material, and was kept in the dark. Insert top left: calibration mark of $\triangle$ O.D. $= 4.10^{-3}$. A difference spectrum for the phytochrome reaction can be computed from these curves by subtracting (b) from (a).

be homogeneously sensitive over its whole area for all wavelengths. Apparently, this is a requirement that cannot be easily met, especially at the extreme ends of the usable wavelength region where photocathode sensitivity drops off rapidly. Fig. 9 shows a measurement of absorption spectra of maize leaves, demonstrating this effect from about 680 nm upwards, at low sensitivity. Fig. 10 is a similar example, at higher sensitivity. Instrumental measures to correct this sort of error are bound to be more complicated than is probably justified by the simplicity of the whole set-up.

7. The Cary-14 spectrophotometer adapted to phytochrome measurements

It may be helpful to know that some materials, especially rich in phytochrome can be measured in the Cary-14 spectrophotometer[1]. We have extensively used this instrument for studies of phytochrome in plumules of dark-grown pea (12, 13). Measurements have also been made with the primary leaves of bean, cotyledons of mustard, and other materials.

Fig. 11 shows the alterations that have been made to the scattered transmission accessory of this instrument in order to make possible the irradiation of the samples from the same direction as the measuring beam, and at low temperature. The irradiation equipment consists of two 90° prisms P_1 and P_2, mounted together on a sliding carriage C that can be operated from the outside. One of the prisms, P_1, can be interposed in the front beam, between the flat mirrors m_2 and m_4 of the scattered transmission accessory. The actinic light falls on the

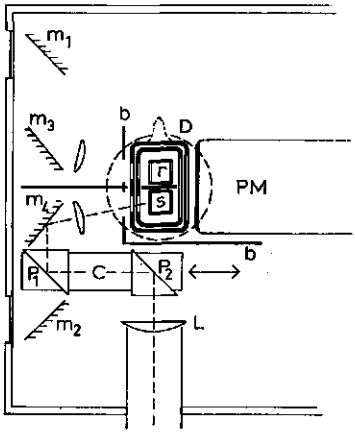

Fig. 11. Adaptation of the scattered transmission accessory of the Cary-14 spectrophotometer to irradiation of sample s inside a cryostat. D: cross section of dewar flask (see fig. 12). The 90° prisms P_1 and P_2 are mounted together on a carriage C. During spectrum recording, this carriage is slid out of the measuring beam. b: Baffles. r: reference sample, receiving no actinic irradiation.

[1] Cary Instruments, Monrovia, California.

front of sample cell s, inside the cryostat D. During spectrum recording, the prism carriage is slid out of the beam. Actinic light is obtained from a 500 W slide projector equiped with interference filters. The light enters the instrument through a hole, made in the front wall. Auxiliary optics focus the front surface of the condensing lens of the projector onto the sample cell. Fig. 12 gives details of the construction of the optical Dewar. A hole in the instrument cover accomodates the cryostat and its mounting. This low temperature accessory was constructed in such a way that it could be used interchangeably on all our spectrometers. Fig. 13 gives an example of a spectrum, measured with this set-up.

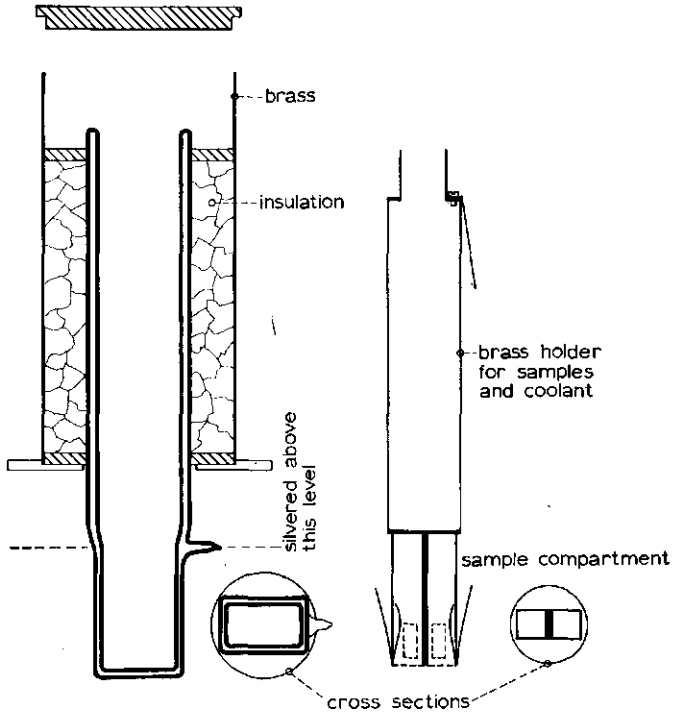

FIG. 12. Liquid nitrogen dewar for measurements at low temperature. In the lower part of the vacuum flask, of rectangular cross section, apertures are left unsilvered for admission of the beams. The coolant is introduced into the brass sample holder (right) and samples are cooled by conduction. There is no coolant in the light path. A thermocouple is soldered to the lower edge of the sample compartment in order to measure the temperature inside the cryostat (not shown).

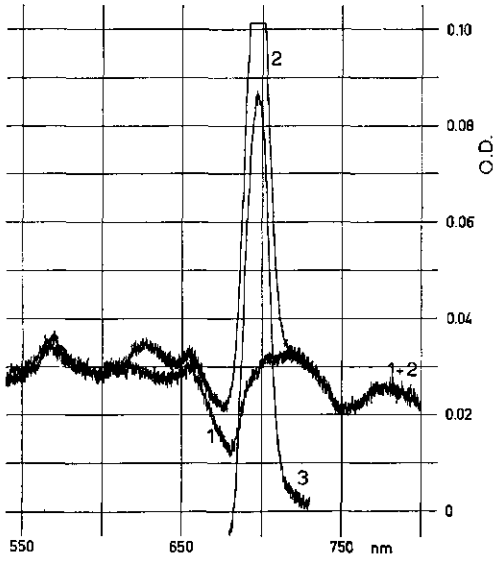

FIG. 13. Example of spectra, measured with the arrangement of fig. 11. Internode sections of dark grown pea, temperature -196°.
1. Before actinic arradiation (base line).
2. After irradiation of front sample (s) with 653 nm for 5 min.
3. Repetition of part of scan 2, after shifting the trace downwards over 0.028 O.D. units.
This experiment demonstrates the formation of the intermediate P_{698}, formed by low temperature irradiation of P_r.

8. PRACTICAL CONSIDERATIONS IN MAKING MEASUREMENTS; SOME SOURCES OF ERROR

Fig. 14 gives two examples of actual phytochrome measurements made with the instrument described under section 4. The samples are relatively low in phytochrome. These experiments illustrate the capability of the instrument to detect phytochrome in sources, hitherto considered unusual (leaves grown in natural daylight, dry ungerminated seeds). In most etiolated plant material, the phytochrome content is very much larger and the instrument sensitivity has to be decreased accordingly.

As has already been stated (section 2), it is desirable to use the highest intensity of the measuring beams, compatible with the photochemical properties of the sample. Fig. 15 illustrates the effect of beam intensity on the signal-to-noise ratio, indicating that the noise was due exclusively to shot noise in the photodetector. In actual practice, there are other sources of irregularities, some of them simulating noise. The most common problem is drift. Fig. 16a shows a form of drift due to heating of the entrance slit of the spectrometer by the image of the lamp filament. It is most marked during the first 10 minutes after switching on the lamp, and virtually disappears after some 30 minutes operation.

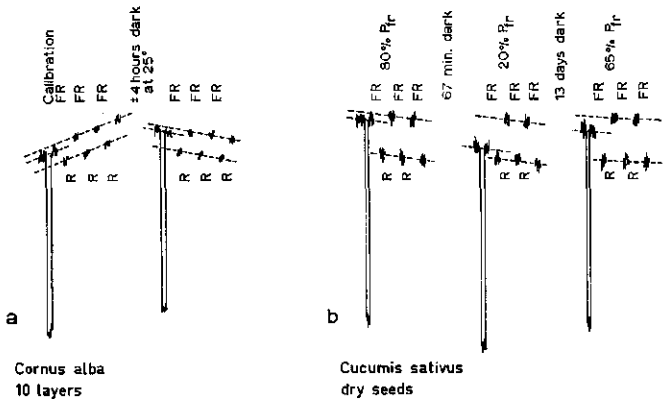

FIG. 14. Examples of phytochrome measurements, made with the dual purpose instrument, described under section 4. Two-wavelength mode, 730–806 nm. After writing a calibration mark, the traces were recorded during 30 sec each, with alternate exposures of the sample to actinic red (R) or far red (FR) light for 45 or 60 sec. Upward movement: increased absorption at 730 nm relative to 806 nm.
a. Ten layers of the white parts of variegated leaves of *Cornus alba* var. argenteomarginata, grown in natural daylight. Before measurement, the detached leaves were kept in the dark for about 3 hours at 25 °. This experiment also shows that in this plant, P_{fr} is not destroyed in the dark at room temperature over a period of several hours and that, during such a period, there is measurable reversion $P_{fr} \rightarrow P_r$.
b. A 4 mm layer of dry seeds of gherkin. In the untreated, dark stored seeds, about 80% of the pigment was in the form P_{fr}. After photoconversion to P_r, the samples were transferred to darkness for various periods. There is a gradual transformation of P_r to P_{fr} in this material. See also ref. 14.

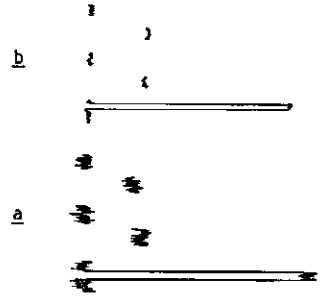

FIG. 15. Effect of beam intensity upon signal to noise ratio. Both recordings with same sample (white paper). In experiment b, the light intensity of the measuring and reference beams was about 10 times that in experiment a, the amplification having been adjusted to give roughly the same signal. This experiment shows that the noise is due to shot noise of the photomultiplier.

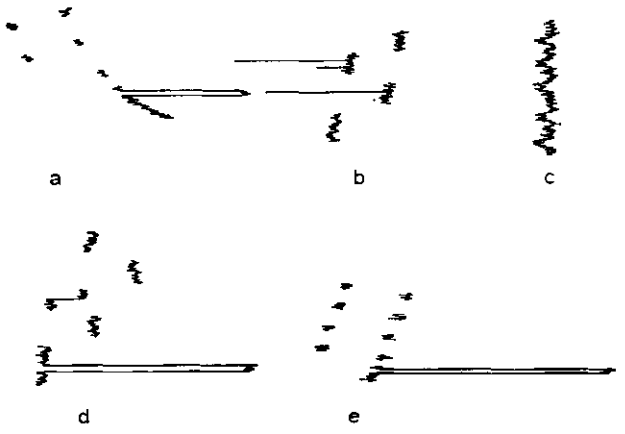

FIG. 16. Forms of non-essential noise and drift.
a. Heating of entrance slit of monochromator.
b. Imminent spectrometer lamp failure.
c. Mechanical vibration of photomultiplier.
d. Movement of liquid in sample.
e. 'Working' of sample.

The effect, shown in fig. 16b (spikes) is a sign of imminent burn-out of the spectrometer lamp filament. Its occurrence is usually followed within a few hours of operation by lamp failure. Replacement before this moment is the remedy.

At one time, a periodically increased 'noise' as shown in fig. 16c proved troublesome. It was traced down to mechanical vibration in one or more of the photomultiplier electrodes, set up by vibrations from the chopper motor. Careful tapping of the multiplier housing, or rotation of the photomultiplier around its axis, usually suffices to eliminate this trouble.

The effect, shown in fig. 16d is usually observed in samples containing adhering liquid. It consists of a sudden displacement of the recorder trace, either to the right or to the left, after which the trace continues with the original slope. Probably it represents the formation or disappearance of a liquid meniscus between plant parts owing to capillary or gravitational forces.

The form of drift, shown in fig. 16e is very common. It is due to mechanical effects in the sample, most likely movements of the material due to release of strain, generated during packing. Other effects, like drying and spontaneous movements undoubtedly also contribute. It tends to disappear after long periods in the dark and for this reason, we keep our samples in the spectrometer in the dark for at least half an hour before starting measurement, whenever possible. It is also much less pronounced at lower temperature, and measurements should preferably be made at 0°.

A comparison of samples, consisting of living plant parts with dummies made from white paper, having roughly the same AOD, indicated that the fluctuations usually were less for the paper samples. This confirms that the fluctuations, ob-

served in samples of living tissue are to a large extent due to spontaneous fluctuations in sample transmission and not to essential photomultiplier noise. For this reason, it appears unlikely that attempts to increase the signal-to-noise ratio by further refinements of the optcial and electronic parts of the instruments would be very effective. The only measure that may have some potential, would be a considerable increase in sample surface area, but as has already been discussed under section 2, this cannot be pushed very far.

9. Summary

Spectrophotometers are described for the estimation of phytochrome in living tissue and for the study of its transformations. Limits of detectability and sources of noise, drift and other fluctuations are discussed as well as aspects of instrument design contributing to their minimization.

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