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SENSITIVE QUASI-CONTINUOUS MEASUREMENT OF PHOTO-INDUCED TRANSMISSION CHANGES

C. J. P. SPRUIT

*Laboratory of Plant Physiological Research,
Agricultural University, Wageningen, The Netherlands,
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INTRODUCTION

In many cases of photoreactions of pigments in materials of biological origin, the most effective wavelengths for excitation are close to, or identical with those, absorbed by the reacting pigments. Obviously, this poses a problem when measuring the associated transmission changes, especially if the latter have relatively short lifetimes in the dark. In such a case, it would be desirable to measure sample transmission simultaneous with the actinic irradiation. However, the exciting light, usually of an intensity greatly surpassing that of the spectrometer beam, is either transmitted or scattered by the sample, or may be reemitted as fluorescence and cannot easily be prevented from entering the photodetector. Usually, one attempts to overcome this problem by some sort of separation of exciting and measuring light, either in time (1) or with respect to wavelength (2, 3). Spatial separation, such as used in flow-techniques for the study of delayed light emission (e.g. 4) should also have potential application in differential spectrophotometry. It is sometimes possible to separate the undesirable component of the light, entering the detector, from the one to be measured by some form of coding, e.g. by periodically interrupting the measuring light while the exciting light is continuous. The electronic part of the equipment sorts out the coded part from the total signal. With this technique, the problems arise from increased noise during actinic irradiation, and from transients during switchover from darkness to actinic irradiation and *vice versa*. The literature concerning photoreactions of photosynthetic pigments contains numerous examples of instruments functioning according to one or more of these principles. For a review see DUYSENS (5). We have previously described (6) instruments for the measurement of phytochrome in plants, in which the method of time separation between actinic and measuring light was employed. This is

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made possible by the property of this pigment to occur in two forms, interconvertible by appropriate illumination, that are stable in the dark over periods of time, long compared with the time, required for measurement of transmission. The method is slow in the sense that there is always a time lag of at least some seconds between the end of the actinic irradiation and the beginning of transmission measurement. Transmission changes occurring during the actinic irradiation cannot be observed easily. Short lived intermediates in the pigment photoconversion, if any, would escape observation. For several reasons it appeared desirable to have available an instrument of high photometric sensitivity, but capable of much better time resolution than that which can be obtained with the instruments, described earlier (6).

DETAILS OF CONSTRUCTION

The inherent advantages of the dual-wavelength principle induced us to use it again in the present design. The method of temporal separation between actinic and measuring light has been employed, fig. 1. It is an adaptation of a design, described earlier (see ref. 6, section 5). An image of the filament of lamp La_1 is formed twice in the plane of a rotating sector disc BM ('butterfly mirror'). Interference filters F_1 and F_2 , placed in parallel sections of the beams isolate the desired wavelengths. The front surface of the disc is highly reflecting

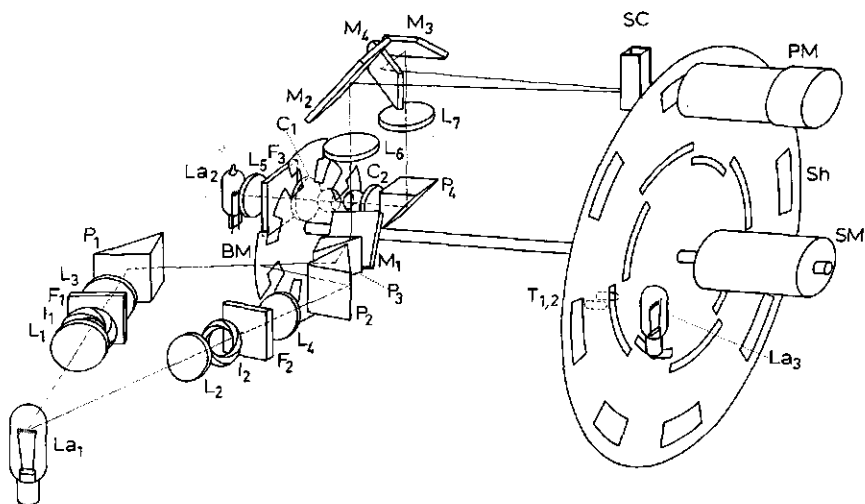
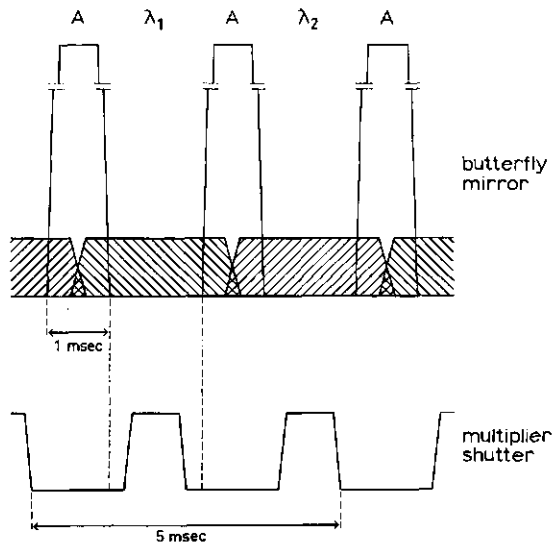


Fig. 1. Diagram of the instrument (not to scale). La_1 , spectrometer lamp, 32 W straight filament. La_2 , 250 W quartz iodine lamp. La_3 , small lamp for generation of the 200 Hz lock-in signal. P_1 ; P_2 ; P_3 ; P_4 , 90° prisms. L_1 -4; L_6 , spherical lenses. L_5 , aspheric condensing lens. L_7 , combination of a spherical and a cylindrical lens. F_1 ; F_2 ; F_3 , interference filters. C_1 ; C_2 , microscope condensing lenses. I_1 ; I_2 , iris diaphragms. M_1 ; M_2 ; M_3 ; M_4 , plane mirrors. T_1 ; T_2 , phototransistors. BM, 'butterfly mirror'. Sh, multiplier shutter. PM, photomultiplier tube. SC, sample cell. SM, 3000 rpm synchronous motor. Various baffles, diaphragms, optical cryostat and a water filter cuvette are not shown.

FIG. 2. Illumination sequence. Top, at the sample cell: A, actinic flash, λ_1 ; λ_2 , the two measuring beams. Bottom, at the cathode of the multiplier. The shutter opens only during the central parts of each measuring beam period.



and during rotation, light from the two beams is directed alternatively towards the sample cell SC by means of prism P_3 , mirrors M_1 and M_2 and lens L_6 . The latter forms a slightly unsharp upright image of the filament of La_1 upon the front of the sample cell. The relative intensities of the two wavelengths can be regulated by means of the iris diaphragms I_1 and I_2 . At the opposite side of the butterfly mirror, provision is made for chopping of the actinic beam. Light from a 250 watt quartz-iodine lamp La_2 is focussed in the plane of the butterfly mirror by means of an aspheric lens L_5 and a microscope condensor C_1 . In this way, a greatly reduced image of the filament is formed at this spot, which aids in keeping the time interval, required for obscuring the actinic beam at a minimum. Slit shaped diaphragms, close to the butterfly mirror aid in reducing stray light, due to imperfect optics. A second microscope condensor transforms the beam to parallel light again, which is directed towards the sample by prism P_4 , mirrors M_3 and M_4 and lens L_7 . The latter is a combination of a spherical with a cylindrical lens, which gives a more even illumination of the sample cell. Interference filter F_3 isolates the desired wavelength.

The photomultiplier shutter Sh is mounted on the same axle that bears the butterfly mirror. It rotates in a narrow space between two metal plates that form the division between the photocell compartment and the remainder of the instrument. The width of the openings in these plates as well as those in the shutter are such that there is a suitable dead period between the end of the actinic flash and the beginning of exposure of the multiplier. The duration of this period was determined experimentally in such a way that the multiplier did not 'see' the actinic light. Additionally, the shutter carries a number of openings through which light from lamp La_3 reaches two phototransistors T_1 and T_2 (in the actual instrument, only one is operating). This arrangement provides the

A water filter of about 5 cm depth is interposed between P₄ and L₇ (not shown). It minimizes heating of the sample and facilitates measurement of the intensity of the actinic light with a thermopile.

The sequence of events around the sample cell is as illustrated in fig. 2. Since the motor rotates at 3000 rpm and there are 4 openings in the circumference of the butterfly mirror, the measuring beams repeat with a frequency of 200 Hz. The inner circle, for chopping the actinic light, has 8 openings, corresponding with 8 sectors in the multiplier shutter. Consequently, the actinic flashes repeat with a frequency of 400 Hz. This ensures that phosphorescence or delayed light emission from the sample or the optics also has this frequency, which is rejected effectively by the amplifier-demodulator.

The electronic part of the instrument, a diagram of which is given in fig. 3, needs little comment. Like in our previous instruments, the photomultiplier was an EMI type 9558B operated at low voltage. The operational amplifier 709 has been made selective for 200 Hz by a twin-T filter network. Phase sensitive demodulation is provided by a differential amplifier stage, locked to the chopping frequency by a signal from the phototransistors, mentioned above. Like any sensitive dual wavelength spectrophotometer, the instrument requires a very stable light source for the measuring beams. The lamp La₁ is therefore operated from a highly stabilised power supply.

Requirements for optimising signal to noise ratio have been discussed previously (6). Under comparable conditions of measuring beam intensity, optical density of the sample and absolute magnitude of the signal, the signal to noise ratio in the present instrument is about one third that in the spectrophotometer.

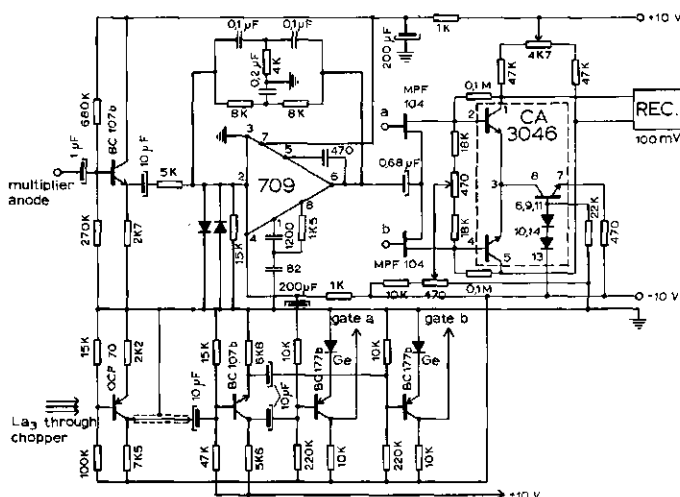


Fig. 3. Circuit diagram of the amplifier-demodulator.

described earlier (ref. 6, section 4). The difference was traced to mechanical vibrations in the butterfly mirror, generated by air turbulence around the disk. For this reason, it is advisable to make the latter as stiff as is compatible with the requirement of a gap between the two microscope condensers of several millimeters. We propose to use fibre optics for the transport of the measuring beams to the sample cell in a future version of this type of instrument, since this should reduce the influence of small variations in the orientation of the mirror surfaces of BM upon illumination of the sample.

LIFETIMES OF PHOTO-INDUCED TRANSMISSION CHANGES

Transmission changes resulting from actinic irradiation may decay in the dark. In the present instrument, the duration of the periods between successive actinic flashes is about 1.5 msec. The 'dead zone' between the end of the flash and the beginning of transmission measurement is about 0.2 msec. It is obvious that effects with lifetimes in excess of 1.5 msec will be but little attenuated, whereas those with lifetimes below 0.2 msec will be measured very inefficiently. This defines the region of dark lifetimes in which the instrument may be useful. Upon turning off the actinic irradiation, sample transmission starts to approach the 'dark' value. Apart from the speed with which the actinic beam can be obscured, the writing speed of the recorder usually will form the lower limit to the decay rates that can be measured reliably. The instrument has its main application, therefore, in the study of 'slow' and moderately fast phenomena.

SOME EXAMPLES OF ACTUAL MEASUREMENTS

Fig. 4 shows the phototransformation of phytochrome in a sample of maize mesocotyls, 10 mm thick upon red (652 nm) and far red (730 nm) actinic irradiation. Fig. 5 gives a similar measurement on a 4 mm layer of plumules of dark-grown pea. Fig. 6 is a recording of some of the photoreactions of pigments in 'white' flower petals of *Viola*. The pigment reactions in the latter material have been studied in some detail. A brief account of this work has been submitted for publication (7) and full particulars will be published elsewhere.

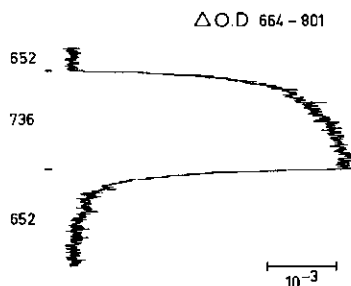


Fig. 4. Phototransformation of phytochrome in a 10 mm sample of mesocotyls of dark grown maize. Time scale: 2 minutes exposure to the wavelengths indicated.

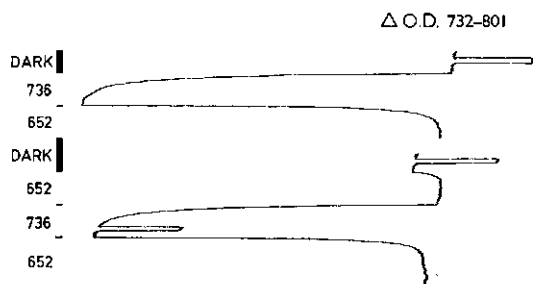


Fig. 5. Phototransformation of phytochrome in a 4 mm sample of plumules of dark grown pea. There are 3 calibration marks shown (rectangular bars), representing a $\Delta O.D.$ of 10^{-2} .

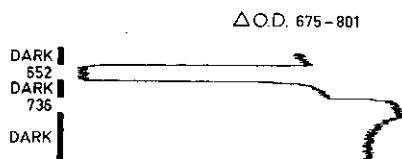


Fig. 6. Photoreactions of a 4 mm layer of 'white' flower petals of *Viola*.

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SUMMARY

Principles of operation and constructional details are given of a sensitive dual-wavelength spectrophotometer for the observation of transmission changes in biological materials, caused by actinic irradiation. The sample is irradiated by actinic flashes of 1 msec duration at a repetition rate of 400 per second. Differential transmission between two wavelengths is measured during the intervals between successive actinic flashes. For phenomena with lifetimes in the dark in excess of 1.5 msec, the method essentially forms a continuous measurement of transmission during sample irradiation. A few examples of measurements illustrate the operation of the instrument.

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