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A Simple and Efficient Method for Photometric Estimation of the State of Pigment Aggregation in Fish Melanophores

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ABSTRACT—The existing photometric methods for the assessment of the state of pigment aggregation in fish scale melanophores are not well suited for pharmacological or related investigations when an appreciable number of scales are studied simultaneously. We have therefore developed a method which make use of a new photometric apparatus, a scale photometer, for the assessment of the state of pigment aggregation; this apparatus allows a pharmacological methodology that is simple and time-efficient, but also allows investigations of time dependent processes, initialized by drugs or electrical nerve stimulation. In this article we describe the method and the novel scale photometer. The method is evaluated by means of the aggregating responses elicited either by noradrenaline and medetomidine or by electrical field stimulation. The method is discussed in relation to previously applied methods and some potential applications are suggested.

INTRODUCTION

The study of pigment cells with translocatable pigment granules, chromatophores, has for a long time attracted large interest [1] and a number of methods for assessing the state of pigment aggregation in chromatophores have been employed.

We have recently attempted a pharmacological characterization of the pigment aggregating adrenoceptors of fish melanophores using isolated scales [2, 3]. In our opinion the most efficient method for estimation of the degree of pigment aggregation in response to various pharmacological agents has been an ocular method [2], even if a photometric method in many aspects would be preferable. The existing photometric methods, however, typically include a microscope stage in the measuring setup and only one preparation can be studied at a time [4–6], this make these methods both expensive and impractical for pharmacological or related applications.

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We have therefore developed a method which make use of a new photometric apparatus for the assessment of the state of pigment aggregation; this apparatus allows a pharmacological methodology that is simple and time-efficient, but also allows investigations of time dependent processes, initialized by drugs or electrical nerve stimulation, since it is easy to connect the apparatus to a chart recorder.

In this article we describe our method and the novel scale photometer. The method is discussed in relation to previously applied methods and some potential applications are suggested.

MATERIAL

Isolated fish scales were obtained from the dark areas of the dermis of the Cuckoo Wrasse (*Labrus ossifagus* L.) as previously described [2]. The isolated scales were suspended in a saline buffer solution of the following composition (concentrations in mM): NaCl 150.0, KCl 5.2, $CaCl_2 \cdot 2H_2O$ 2.9, $MgSO_4 \cdot 7H_2O$ 1.8, $Na_2PO_4 \cdot 2H_2O$ 2.4, $NaHCO_3$ 17.9 and glucose 5.6 [7]. The solution was equilibrated with 5% CO_2 in O_2 and kept at

20° and pH 7.3.

The following drugs were used: l-noradrenaline bitartrate (Sigma Chemical Company, St. Louis, Mo., USA), medetomidine hydrochloride (Farmos Group Ltd, Turku, Finland) and yohimbine hydrochloride (Sigma Chemical Co.). The drugs were dissolved in saline buffer solution.

METHODS

Ocular estimation of aggregation

The scales were placed on glass microscope slides with the dermal side down and immersed in 50 μl of buffer. The scales were viewed in a microscope (Leitz SM Lux) and the state of aggregation was evaluated according to a modified melanophore index [2, 8]. This index estimates the degree of pigment aggregation by the use of a scale ranging from 1 to 5 (with half-step resolution); 1 denotes complete aggregation and 5 denotes complete dispersion of the pigment.

During the course of the experiments the microscopist was unaware of what kind of agent, if any, that was applied to a scale.

The scale photometer

In combination with a well trained observer the ocular method is simple, sensitive and useful. The result of the method however is highly dependent on the ability of the observer. In order to minimize the influence of the observer, increase the resolution, sensitivity and reproducibility and to make the observing more convenient we have developed a simple apparatus, the scale photometer.

In the state of complete pigment dispersion the melanosomes are spread out and all pigment particles are completely exposed to the incoming light. In the opposite state, when maximal aggregation occurs, the melanosomes cluster and pigment particles are only partially exposed to the incoming light. It is then expected that the amount of transmitted light through the fish scale is dependent on the state of pigment dispersion. Consequently the basic principle of an instrument would be to detect transmitted visible light through the pigmented area of a fish scale.

The measuring cuvettes of the scale photometer (see Fig. 1A) are arranged five in a row in a sliding multi sample holder which can be slid into one out of five measuring positions. The multi sample holder is removable and can easily be exchanged with other identical multi sample holders. The bulk material of the multi sample holder is black acrylic plastic. The use of black material minimizes the influence of ambient light. The cuvettes are milled in the bulk material and covered by glass on two sides (see Fig. 1A). The top of the cuvettes are open to allow mounting of the scales and the addition of solutions. A scale is slid into a U-shaped groove on one of the sides of the cuvette (see Fig. 1A) and is then clamped into position by inserting a plastic strip into the upper part of the groove. The epidermal side of the scale is oriented

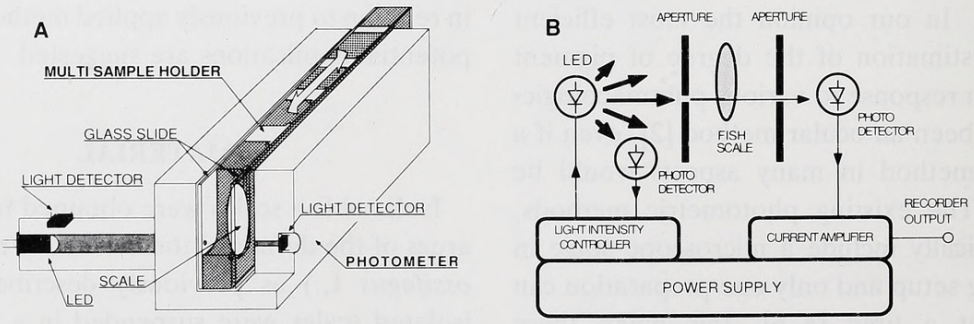

FIG. 1. Schematic representations showing the essential parts of the scale photometer (A) and the principles of operation (B). The light source is positioned to the left in the figures and consists of a light emitting diode (LED) and a reference light detector (that is part of an intensity controller); the main light detector is found to the right in the figures. In between the light source and detector are the cuvette, containing the scale, and two apertures (12 mm apart). All parts shown in (A) with the exception of glass slides and electronic components are made of black acrylic plastic.

towards the interior of the cuvette.

As light source a light emitting diode (LED) is used. This LED (H–2000, Stanley Electric Co., Ltd., Japan) has a high luminous intensity of about 2000 millicandela. The peak wavelength is 660 nm with a spectral half bandwidth of 25 nm. The light from the LED is detected by a reference photodetector and a detector for the transmitted light (both detectors are PIN silicon photo diodes, BPW34, Siemens, W. Germany). The signal from the reference photodetector is fed to a light intensity controller (see Fig. 1B) and compared to a preset value. The current through the LED is controlled by the light intensity controller resulting in a very stable light intensity. The emitted light passes through an aperture (diameter 1.5 mm), the cuvette containing the fish scale, another aperture (diameter 1.5 mm) and finally the remaining light is detected by the photodetector. The signal from the detector is converted to a voltage output in the current amplifier and the voltage level is directly proportional to the light transmission through the scale.

Reducing the light beam through the apertures makes it possible to choose a very small detected area of the scale. The investigated area on the scale is 1.8 mm^2. The radiant sensitivity of the PIN silicon photo diode is about 0.4 A/W at 660 nm and the active area is 7.3 mm^2. The maximum light power exposed to the scale is less than 10 μW which minimizes heating of the scale and buffer medium. Furthermore the power unit is separated from the rest of the device to avoid excess heating of the scales when the multi sample holders are positioned in the scale photometer.

In the prototype version of the scale photometer an external digital voltmeter was used and a two-channel recorder was connected.

Photometric estimation of aggregation

The scales were mounted in the cuvettes and immersed in 50 μl of the saline buffer. The transmission range was defined by the intensities measured when the light beam was either totally interrupted (0%) or allowed to pass through a buffer-filled cuvette without a scale in position (100%).

Stimulation of the melanophores

The melanophores were stimulated to aggregate their pigment granules, either by the addition of a pharmacological agent or by electrical field stimulation of intrinsic nerves. The electrical field stimulation was performed by means of two silver wire electrodes (0.2 mm in diameter) that was mounted, 4 mm apart, in a cuvette. A Grass S88 stimulator equipped with an isolation unit (Grass SIU 5) was used to deliver trains of varying duration at a frequency of 20 Hz (1 msec biphasic square pulses, 60 V nominally out from the stimulator).

All data are presented as means $\pm$S.E.M.

RESULTS

The melanophores of isolated scales from *Labrus ossifagus* maintain a state of pigment dispersion in saline buffer solution. The dispersed state was also maintained when the scales were positioned for measurement of transmission in the scale photometer. It was noted that the interference from stray light was negligible. In Figure 2A the size and time course of the transmission response after addition of two pharmacological agents are shown. Noradrenaline completely aggregated the melanophores, as confirmed in the microscope, whereas yohimbine (alpha$_2$-adrenoceptor selective) effectively antagonized the aggregation. Repeated experiments, with addition of noradrenaline, on different scales (n=25) showed a typical baseline transmission of $38\pm2\%$ and a stimulated transmission of $57\pm2\%$ after maximal aggregation as confirmed in the microscope.

The function of the scale photometer was also evaluated in concentration-response tests and compared with our ocular method for estimation of pigment aggregation. Accumulated concentration-response curves were obtained using either of the two methods. In both methods the preparations were allowed a 5-min resting period in day light after each addition of drug. Two adrenergic drugs were used: noradrenaline and medetomidine, a potent alpha$_2$-adrenoceptor selective agonist [9]. Following the resting period the state of

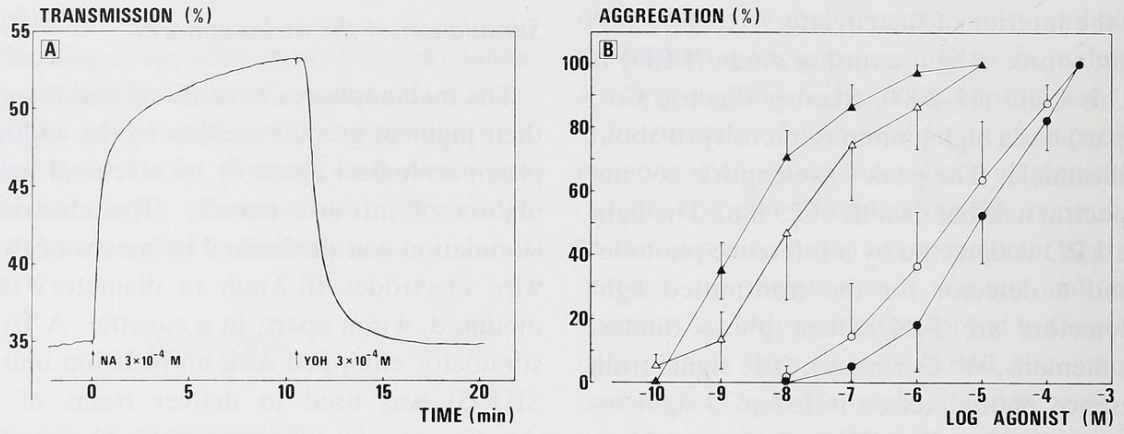

FIG. 2. Effects of drugs on the state of aggregation of melanophores. (A) shows a representative recording of light transmission. The aggregating effect of noradrenaline (NA) and its reversal by the alpha$_2$-adrenoceptor selective antagonist yohimbine (YOH) can be observed. In (B) cumulative concentration-effect curves of aggregation are shown. Filled symbols indicate data evaluated by ocular estimation (for comparison the melanophore index scale was transformed to a percentage scale) and open symbols indicate data evaluated by the photometric method. Two agonistic drugs were used: medetomidine (△, ▲) and noradrenaline (○, ●). The vertical bars indicate S.E.M. (n=5).

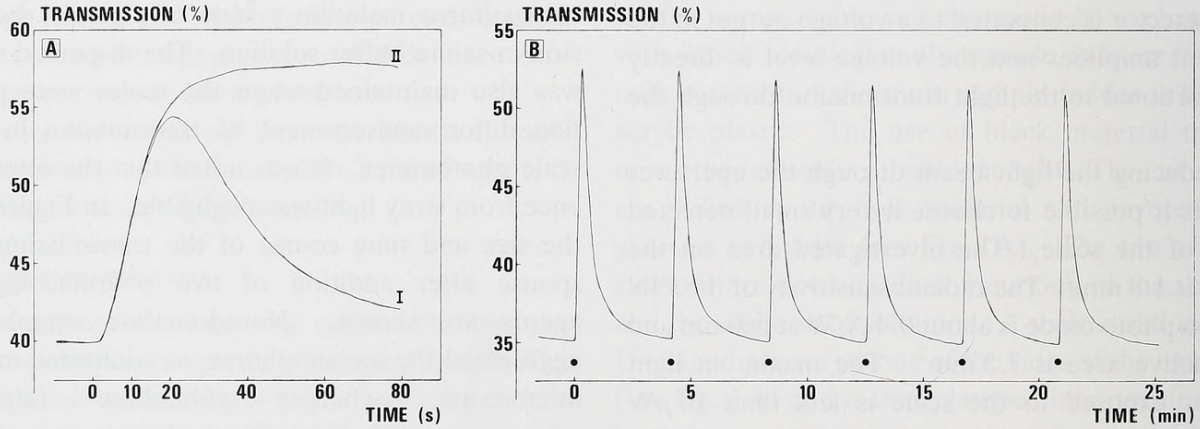

FIG. 3. Typical effects of electrical stimulation on the state of aggregation of melanophores. In (A) the transmission response to a short stimulation (10 s, curve I) and a more prolonged stimulation (60 sec, curve II) are shown. In (B) the reproducibility of repeated short (10 sec) stimulations are shown. The dots indicate the incidence of a stimulation.

aggregation was evaluated using either of the two methods. The results of these experiments are presented in Figure 2B as normalized concentration-aggregation curves.

The size and time course of the transmission response following electrical nerve stimulation of the scales are shown in Figure 3A. The response is, as expected, much faster than after addition of drugs (c.f. Fig. 2A). The reproducibility of repeated electrically induced aggregation was also tested as shown in Figure 3B.

DISCUSSION

In this paper we describe an efficient method for estimation of the state of aggregation of melanophores on fish scales; the method includes a novel apparatus, a scale photometer.

The advantages of using a dedicated apparatus for the estimation of aggregation are several; e.g. there is no need for a microscope as part of the setup and the apparatus can be made very efficient for the evaluation of the state of aggregation when several scales are used in the experiments, which is

the case in pharmacological work.

The apparatus was designed to be simple and reliable. The light source was chosen to be a powerful light emitting diode, emitting in the red. The advantage of using red light is that the interfering effects of other chromatophores than the melanophores, i.e. xantophores and erythrophores, can be virtually eliminated [10].

The recording obtained by the use of the scale photometer (see Figs. 2A and 3A, B) are comparable to the recordings obtained by the use of other methods [e.g. 4, 6]. It can be noted that the change of transmission following maximal stimulation of the melanophores by drugs (Fig. 2A) or by electrical field stimulation (Fig. 3A) is of comparable size. We have previously shown, by the use of tetrodotoxin [4], that the electrical field stimulation applied in this work activates intrinsic nerves only and not the melanophores directly. The aggregating response to repeated electrical stimulation (Fig. 3B) shows a high degree of reproducibility, which may be of importance in experiments when previously induced responses are used as controls to responses elicited after treatment.

The scale photometer is especially useful when concentration response curves are obtained since several multi sample holders (with five cuvettes each) can be used simultaneously. In Figure 2B the aggregating responses elicited by two adrenergic agonists are shown as a function of concentration; the response to each agonist was evaluated either by the ocular or the photometric method. It can be concluded from the figure that the two methods give very similar results, especially if the deviations are taken into consideration. Furthermore, the form of the curve for the two agonists agree regardless of method. It is evident from the curves in Figure 2B that the deviations are substantial, this appears however to be more a characteristic of the scale melanophores themselves since similar deviations appear regardless of the method applied.

We have developed a method which apply a new photometric apparatus for the assessment of the state of pigment aggregation in melanophores. This apparatus, a scale photometer, allows a methodology that is simple and time-efficient and the method could find potential applications especially in the pharmacological field where it could, e.g., complement existing models for characterization of alpha$_2$-selective drugs. The method could also be of potential value as an assay during purification of pertussis toxin [11] or even used as part of a diagnostic method for whooping cough.

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