Proceedings of the National Academy of Sciences
Vol. 66, No. 3, pp. 850–854, July 1970

Photoregulation of Biological Activity by Photochromic Reagents, IV. A Model for Diurnal Variation of Enzymic Activity*

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Communicated by David Nachmansohn, April 23, 1970

Abstract. Levels of acetylcholinesterase activity can be made to vary in response to the presence or absence of sunlight in a system that can be considered as a model for photoperiodic processes found in nature. The enzyme is rendered photosensitive by the presence of a photochromic inhibitor, *N*-*p*-phenylazophenylcarbamyl choline, which changes from a *trans* to a *cis* isomer under the influence of the light of the sun and reverts back to the *trans* isomer in the dark. The two isomers differ in their ability to inhibit acetylcholinesterase, thus rendering the enzyme system responsive to sunlight. The relationship of this system to photoresponsive processes in nature is discussed, and a possible role in photoregulation is suggested for naturally occurring carotenoids.

We have recently shown how systems normally insensitive to light (the enzymes chymotrypsin[1] and acetylcholinesterase[2] and the electroplax of the electric eel)[3] can be photoregulated by means of photochromic effector molecules. Those molecules share a common *p*-phenylazophenyl group which, under the influence of light, undergoes a reversible configurational change to yield a *cis* or *trans* isomer (Fig. 1). Their ability to induce photoregulation derives from differences in the biochemical activities of the two isomers. For example, carbamylcholine-induced depolarization of the excitable membrane of the electroplax was inhibited unequally by the *cis* and *trans* isomers of *p*-phenylazophenyltrimethylammonium chloride (in Fig. 1, substitute $N(CH_3)_3{}^+Cl^-$ for $R_1—N—R_2$). The extent of depolarization, therefore, depended upon the relative concentrations of the two isomers in solution, which in turn was influenced by light of specific wavelengths. The potential difference across the excitable membrane, therefore, was regulated by light.

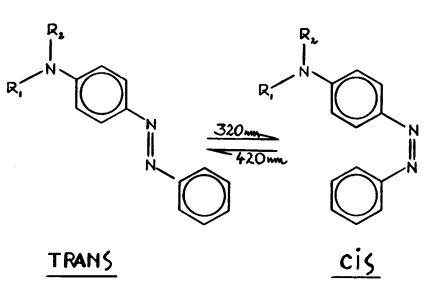

FIG. 1.—Photostimulated *cis-trans* isomerism.

Although the systems studied can serve as models for photoregulated processes found in nature, generation of the *cis* and *trans* isomers (and hence regulation)

required bright sources of ultraviolet and visible light, respectively. Ideally, one would prefer a system in which the *cis* isomer could be produced by sunlight, with reversion to the *trans* isomer occurring in the dark. We now report this type of model, in which levels of acetylcholinesterase activity can be made to respond to the presence or absence of sunlight. The photochromic molecule responsible for the properties of the system is *N*-*p*-phenylazophenylcarbamyl choline iodide (I). (In Fig. 1, R_1 = H; R_2 = $C(=O)-O(CH_2)_2-N^+(CH_3)_3I^-$.)

Materials and Methods. Preparation of *trans*-I via *N,N*-dimethylaminoethyl ester of *N*-*p*-phenylazophenylcarbamate: *p*-Phenylazophenyl isocyanate was prepared by reacting 5 g (0.025 mol) of *p*-phenylazoaniline (Eastman), suspended in a mixture of 5.3 g (0.053 mol) *N*-methylmorpholine and 50 ml dry benzene, with 55 ml of a 12.5% solution of phosgene in benzene. The mixture was stirred for 1 hr at 5°C and then for 3 hr at room temperature. Excess phosgene was removed *in vacuo* and 200 ml of dry benzene was added. After methylmorpholine hydrochloride was removed by filtration, the benzene solution was distilled to dryness *in vacuo* at 40°C leaving the crude isocyanate as a yellow-brown solid (mp 95–96°C). It was dissolved in a mixture of 15 ml of dimethylaminoethanol and 50 ml of acetone and refluxed on a steam bath for 1 hr, with protection from moisture. Acetone was removed *in vacuo;* the residue was dissolved in 200 ml of benzene and washed three times with 200 ml volumes of water. After the solution had been dried over anhydrous Na_2SO_4, benzene was removed by distillation, leaving the crude *N,N*-dimethylaminoethyl ester of *N*-*p*-phenylazophenylcarbamate, which was recrystallized from 30 ml of 66% methanol; yield, 4.8 g, mp 88–90°C.

***N*-*p*-phenylazophenylcarbamyl choline iodide (I):** 3.12 g (0.01 mol) of the above product was stirred with 20 ml of methyl iodide at 10°C in a 100-ml flask with an efficient condenser. A reaction started almost immediately, causing the separation of a thick yellow precipitate. The temperature rose to the boiling point of methyl iodide. After 30 min, 50 ml of dry methanol was added and the precipitated solid was dissolved by heating the mixture to 40°C. After 16 hr at room temperature, the methanolic solution was poured into 400 ml of dry ethyl ether with stirring, resulting in the precipitation of I, which, after 5 hr in the freezer, was recovered by filtration, washed with dry ether, and air-dried at 37°C; yield, 3.6 g of orange crystals, mp 217–218.5°C. Recrystallization twice from 3 vol of methanol raised the melting point to 224–225°C. Calcd. for $C_{18}H_{23}N_4O_2I$ (454.32): C, 47.59; H, 5.16; N, 12.33; I, 27.93. Found: C, 47.39; H, 5.18; N, 12.27; I, 28.04.

Ultraviolet light source: A Spectroline B-100 high-pressure mercury lamp was used. It was fitted with a 2F/91 filter with a peak transmission at 366 nm.

Results. The absorption spectrum of *trans* I has a maximum at 348 nm (ϵ = 19,500) at pH 7.0 in the phosphate buffer used in our assay system (see below). The spectrum of the *cis* isomer could not be recorded accurately because of its fast dark relaxation to the *trans* isomer. However, the 348-nm peak was much smaller in the mixtures obtained and undoubtedly does not exist in the *cis* isomer. The *trans* to *cis* conversion occurred very rapidly upon exposure to long-wavelength ultraviolet light: a 5×10^{-5} M solution in the phosphate assay buffer, pH 7.0, in a quartz cuvette placed 30 cm from the ultraviolet source was converted almost completely to the *cis* isomer in less than 60 sec. (A lucite container of water was placed between the lamp and the solution to prevent overheating.) No exact estimate of the extent of conversion could be made because relaxation to the *trans* isomer commenced immediately upon putting the solution into the compartment of the spectrophotometer. The dark relaxation

could be followed by observation of the appearance of the 348-nm peak, as shown in Figure 2. Maximal reversion to *trans* occurred in about 600 sec, the half time being about 120 sec. Reversion to the *trans* isomer could also be effected by exposure to light of a photoflood. In this case, complete reversion required only about 60 sec. The reversion rate was sensitive to pH, taking place more rapidly at pH 1.8 than at pH 10.7. More careful examination of the photochemical properties of I is now being made and will be reported in a subsequent publication.

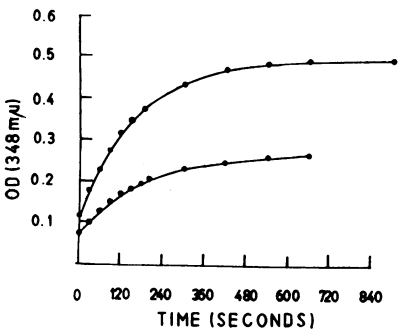

FIG. 2.—Rate of dark relaxation of *cis*-I at pH 7.0 (in "assay" buffer, see text), 25°C, as measured by the appearance of 348-nm peak. Upper curve represents concentration of 2.5×10^{-5} M; the lower curve represents 1.25×10^{-6} M.

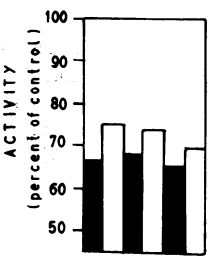

FIG. 3.—Effect of sunlight and darkness on acetylcholinesterase levels. (Experimental conditions in text.) White bars represent activity after 15 min in the sunlight; dark bars represent activity after 15 min in darkness.

Exposure to sunshine at midday in August in New York City caused an approximate 50% conversion of the *trans* to the *cis* isomer at pH 7.0. Almost complete reversion to the *trans* isomer occurred upon returning the solution to the laboratory, which was illuminated by fluorescent ceiling lights.

The two isomers differed in their ability to inhibit acetylcholinesterase. In preliminary experiments, in which competitive inhibition was assumed, K_I was found to be 1.6×10^{-6} M and 3.6×10^{-6} M for the *trans* and *cis*, respectively, as determined in the assay system of Metzger and Wilson.[4]

The influence of sunlight on the acetylcholinesterase system was determined between 11:45 a.m. and 4:00 p.m. in August in the following way: A solution containing I (2.5×10^{-4} M) and acetylcholinesterase (2.3×10^{-8} M)[5] in pH 7.0 assay buffer (0.02 M sodium phosphate, 0.1 M NaCl, 0.01 M $MgCl_2$, 5×10^{-5} M EDTA, and 0.01% gelatin) was exposed to sunlight for 15 min at 30°C. An aliquot (0.1 ml) was withdrawn and was added to 0.9 ml of 3×10^{-3} M acetylcholine in the same buffer. Unhydrolyzed ester was determined after 50 sec incubation at 30°C. (This method is essentially that of Metzger and Wilson.[4]) All assays were done in duplicate and performed so that the vessels were always exposed to sunlight. The tubes containing I and enzyme were then placed in the dark for 15 min and aliquots assayed in duplicate in the dark. Controls of enzyme without inhibitor were treated and assayed similarly. Three light-dark cycles were performed.

The results are given in Figure 3. They reflect the ability of sunlight to convert *trans* isomer to *cis* isomer with a concurrent decrease in the inhibition of acetylcholinesterase activity, i.e., an increase in the level of activity. The system is reversible; placing it in the dark restores it to its former level.[6] It should be noted (see above) that under the conditions of the experiment, only a 50% conversion of the *trans* to *cis* isomer occurred. Changes in activity levels of as much as 30% can be induced when complete conversion to the *cis* isomer is effected by means of the Spectroline B-100 ultraviolet lamp instead of New York City sunlight.

A more active compound has recently been synthesized: *m*-bromophenylazophenylcarbamyl choline iodide (substitute *m*-bromophenyl for lower benzene ring of compound I). Acetylcholinesterase levels can be changed as much as 50% using ultraviolet light. Experiments with sunlight will have to await better climatic conditions.

The system described in this paper is exactly analogous to that of Venkatacheri and Muralikrishana,[7] who found diurnal variations in levels of acetylcholinesterase in the ventral cord of the scorpion, *Heterometus fulvipes*. Moreover, it can serve as a model for other photoregulated processes which exhibit diurnal or circadian rhythmic patterns, if one assumes that changing enzyme levels are involved. Even phenomena which apparently entail the "measurement" of the amount of daylight or darkness in a 24-hr day (seasonal flowering of plants, migration of birds, etc.) can be explained by assuming that variations in enzyme levels can induce changes in the level of biologically active molecules (hormones?) that influence the metabolism or behavior of the organism.[8]

Regulation of enzyme activity by light-sensitive reversible effector molecules is an economical process, since there is no need for the organism to continually synthesize large quantities of new enzyme. Can we find evidence of such effector molecules in nature? One example might be abscisin,[9] which induces dormancy in plants. It is known to be photochromic and only the *cis* isomer is biologically active. Another more complex substance is phytochrome, which is known to function by a photochromic mechanism in the regulation of many plant processes.[10] Perhaps their mechanisms involve regulation of enzyme levels by direct interaction with specific enzymes. The widespread occurrence in plants and animals of carotenoid substances, many of which can undergo photochromic alterations, suggests that their function may very well be intimately concerned with photoregulation. In a sense, we know that this is true for retinal, the carotene-related photochromic compound of higher animals, which makes the organism aware of the presence of light. We have already shown how this process can be mimicked in the electroplax of the electric eel.[3] A systematic study of the interaction of naturally occurring carotenoids with various enzyme systems might provide information useful for an understanding of photoregulated processes found in nature.

We gratefully acknowledge a gift from the Raymond and Beverly Sackler Foundation. Dr. Bieth (Chargé de Recherche of Institut National de la Sante et de la Recherche Médicale France) was a Fulbright Scholar and was supported in part by a generous grant from the Irene Heinz Given and John LaPorte Given Foundation, Inc. His present address is: Hospices Civil de Strasbourg, Hôpital Civil, 1 place de l'Hôpital, 67 Strasbourg, France.

* This work has been supported by NIH grant GM-11805-10 and NSF grant GB-4861.

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[4] Metzger, H. P., and I. B. Wilson, *Biochemistry*, **3**, 926 (1964).

[5] See ref. 2 for manner in which enzyme concentration was determined.

[6] It was noted that slight, progressive inactivation occurred during the experiment. This secondary reaction is noticeable in Fig. 3 and resulted from the ability of I to carbamylate acetylcholinesterase slowly.

[7] Venkatacheri, S. A. T., and P. Muralikrishana, *Life Sci.*, **7**, 617 (1968).

[8] Actually, these phenomena cannot always be explained on the basis of one mechanism only, because frequently more than one type of photoreceptor is involved. However, product accumulation has been suggested as playing a part. For reviews on the subject, see Giese, A. C., *Photophysiology*. (New York: Academic Press, 1964), vol. 2, p. 1.

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