PHOTOREGULATION OF BIOLOGICAL ACTIVITY BY PHOTOCROMIC REAGENTS, II. INHIBITORS OF ACETYLCHOLINESTERASE†*

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Communicated by David Nachmansohn, August 8, 1969

Abstract.—The enzymic activity of acetylcholinesterase can be photoregulated through the mediation of photochromic inhibitors of the enzyme. *N-p*-phenylazophenyl-*N*-phenylcarbamyl fluoride, an irreversible inhibitor of acetylcholinesterase, exists as two geometric isomers which are interconvertible through the action of light. The *cis* isomer, which predominates after exposure to light of 320 nm, is more active than the *trans* isomer, which results from exposure to light of 420 nm. It was possible, therefore, to use light energy to regulate the inactivation of the enzyme. Similarly, levels of acetylcholinesterase activity could be photo-regulated in a completely reversible manner by means of the photochromic reversible inhibitor *p*-phenylazophenyltrimethylammonium chloride. These experiments can serve as models for similar phenomena observed in nature, particularly in photoperiodic rhythms of higher animals.

A system was recently described in which an enzymic process, in itself insensitive to light, could be made subject to photoregulation through the mediation of a light-sensitive effector molecule.[1] The photosensitive compound, *N-p*-phenylazophenyl-*N*-phenylcarbamyl chloride[2] (PAPC), is a specific inactivator of chymotrypsin.[3] PAPC is a photochromic (or phototropic) molecule[4] which, under the influence of light, can undergo a reversible configurational change involving the N=N bond, to yield either a *cis* or a *trans* isomer. The change in structure is influenced by the wavelength of light as follows:

$$trans \underset{420\ nm}{\overset{320\ nm}{\rightleftharpoons}} cis$$

Although both isomers could inactivate chymotrypsin, the *cis* isomer was found to be about five times more active. Conditions were found in which the rate of inactivation by *trans* PAPC was very slow. Thus, it was possible to "turn off" (i.e., inactivate) the enzyme by exposing a solution of enzyme in the presence of *trans* PAPC to light of 320 nm. Similarly, experiments in which the inactivation process could be halted by light were also possible by starting with the *cis* isomer. It was suggested that these experiments could serve as models for certain photosensitive processes found in nature, e.g., phototaxis.[5]

Our investigations have now been extended to the enzyme acetylcholinesterase (AcCh-esterase). Its activity can be regulated in the same way as the activity of chymotrypsin. Moreover, by using a photochromic reversible inhibitor, it was possible to regulate the level of AcCh-esterase activity reversibly, by the action of light.

Diphenylcarbamyl fluoride is an acid transferring inactivator of AcCh-esterase,[6] which combines with the active center of this enzyme to yield an inactive carbamyl derivative. The photochromic analog, *N-p*-phenylazophenyl-*N*-phenylcarbamyl fluoride (PAPF) was prepared by the reaction of PAPC with SbF_3.[6] It was also a potent inactivator of AcCh-esterase. Its spectral characteristics and its photochromic behavior were identical to those of PAPC.[1] The second-order rate constant of inactivation of acetylcholinesterase by the *trans* and *cis* isomers were 450 and 1270 M^{-1} sec^{-1}, respectively. The reaction of PAPF with AcCh-esterase differed in one important way from the reaction of PAPC with chymotrypsin: deacylation occurred at a measurable rate (k_{trans} = 2.9×10^{-4} sec^{-1}, $k_{cis} = 0.79 \times 10^{-4}$). Thus, a steady state which could last for many hours was reached when the rate of reactivation equaled the rate of inactivation. The level of enzymic activity during the steady state depended upon the initial concentration of inactivator.[7]

Because of the difference in activity between the *cis* and *trans* isomers of PAPF, as manifested in the inactivation and reactivation steps, it was possible to design experiments in which the inactivation of acetylcholinesterase could be controlled by light. *Cis*-PAPF (0.9×10^{-6} *M*) was incubated with AcCh-esterase (*Electrophorus electricus*, Worthington) (3.7×10^{-8} *M*)[8] at pH 7.0 and 25° in a buffered medium (0.02 *M* sodium phosphate, 0.1 *M* NaCl, 0.01 *M* $MgCl_2$, 5×10^{-5} *M* EDTA, 0.01% gelatin). At suitable intervals, 0.1-ml samples were withdrawn and tested for activity against acetylcholine, essentially by the procedure of Metzger and Wilson.[6] At 1200 seconds, one-half of the solution was exposed to visible light (photoflood) for 300 seconds and samples were withdrawn from both solutions and assayed. The results, which are shown in Figure 1*a*, indicate that it was possible to stop the inactivation process almost immediately by exposure to light. By starting with *trans* PAPF, ultraviolet light with a maximum intensity of 360 nm (Spectroline B-100) could be used to increase the rate of inactivation and ultimately to lower the level of enzymic activity at the steady state (Fig. 1*b*).

By using a photochromic reversible inhibitor of acetylcholinesterase, it was possible to photoregulate the level of enzyme activity in a completely reversible manner. Wilson and Alexander have shown[9] that phenyltrimethylammonium chloride is a moderately potent reversible inhibitor of acetylcholinesterase (K_I = 5.3×10^{-5} *M*). The photochromic analogue, *p*-phenyl-azophenyltrimethylammonium chloride (azo-PTA), was synthesized from *p*-dimethylaminoazobenzene. *Trans* azo-PTA has an absorption spectrum similar to that of *trans* PAPC,[1] except for a shift of the maximum to 316 nm; ϵ, 15,800. Exposure to ultraviolet light (Spectroline B-100) converts it to the *cis* isomer. Although the kinetics of their interaction with enzyme have not been examined carefully, as yet, both isomers were found to be reversible inhibitors of AcCh-esterase, the *trans* isomer being somewhat more effective. Conditions were sought, therefore, in which photoregulation of enzyme levels could be demonstrated. A mixture containing AcCh-esterase (1.1×10^{-7} *M*) and azo-PTA (1.25×10^{-3} *M*) dissolved in the pH 7.0 buffer (above) was alternately exposed for 3-minute periods to ultraviolet and to visible light and assayed after each exposure by a modifica-

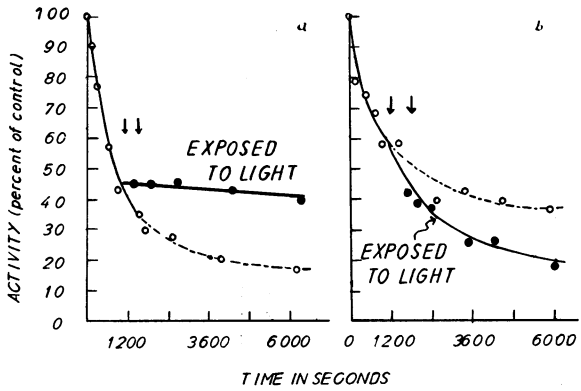

FIG. 1.—Effect of light on reaction of PAPF with AcCh-esterase. (*a*), Reaction with *cis* isomer; (*b*), reaction with *trans* isomer. Vertical arrows indicate interval of exposure to light.

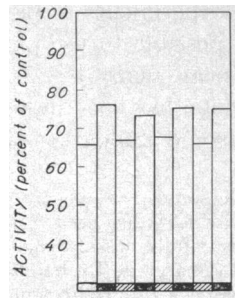

FIG. 2.—Reversible changes in enzyme levels using azo-PTA. □, activity before exposure to light; ■, activity after exposure to ultraviolet; ▨, activity after exposure to visible light.

tion of the method of Kramer and Gamson.[10] Controls were run for the exposed enzyme without inhibitor.[11] The results are shown in Figure 2. Depending upon the radiation (visible or ultraviolet) to which the enzyme-inhibitor mixture was exposed, AcCh-esterase activity levels could be made to rise or fall. Although the changes observed with azo-PTA are not overly dramatic, they are consistent and reversible and demonstrate that enzyme activity levels can be reversible regulated by light *via* the binding of a small light-sensitive phototropic molecule. Similar experiments were attempted with *N-p*-phenylazophenyl-*N*-phenyl-carbamylcholine chloride. However, there was not sufficient difference in the reactivities of its two isomers with AcCh-esterase.

More effective photochromic reversible inhibitors are being sought. However, the results in Figure 2 can serve as a model for observations such as that of Venkatacheri and Muralikrishana,[12] who found diurnal changes in AcCh-esterase activity in the ventral nerve cord of a scorpion, *Heterometrus fulvipes*. Our experiments indicate that mediation of a phototropic inhibitor might be responsible for this variation and for other types of circadian rhythms as well. It should be emphasized that, although *cis* azo-PTA requires light energy to return to the *trans* configuration, many phototropic molecules do so in the dark.[4]

The ability to photoregulate the activity of AcCh-esterase is of particular interest because it may be an indication of the manner in which the metabolism of higher animals can respond to light. For example, the level of activity of the melatonin-forming enzyme in the pineal gland of rats[13] and monkeys,[14] hydroxyindole-*O*-methyl-transferase, is different in animals kept in total darkness from the level in animals that are kept in constant light. Unlike the pineal gland of reptiles and amphibia, the gland in higher animals is not directly affected by light but has its own accessory optic system[15] which is joined to the pineal gland by nerve fibers. The ability to demonstrate *in vitro* photoregulation of AcCh-esterase, an enzyme so intimately involved in neurological processes, therefore, may be a clue as to the mechanism of photoperiodic rhythms in higher animals.

In experiments to be reported in a later issue of these PROCEEDINGS,[16] it has been possible to use photochromic reagents to photoregulate the generation of electrical energy by an isolated electroplax.[17] In essence, a photoelectric phenomenon has been induced in a neurological system. These experiments might bear some analogy to the mechanism of the process of vision.

* Reference 1 is paper I of this series.

† This work has been supported by NIH grant GM-11805-10 and NSF grant GB-4861. We also gratefully acknowledge a gift from the Raymond and Beverly Sackler Foundation. Dr. Bieth (Chargé de Recherche à l'Institut National de la Santé et de la Recherche Médicale, France) is a Fulbright Scholar and was supported in part by a generous grant from the Irene Heinz Given and John LaPorte Given Foundation, Inc. The authors wish to thank Dr. R. Kitz for helpful discussions during this work.

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[2] This compound was given the name *p*-azophenyldiphenylcarbamyl chloride (PADPCC) in ref. 1. The more accurate designation is as given in this paper. PAPC *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl chloride; PAPF, *N*-*p*-phenylazoplenyl-*N*-phenylcarbamyl fluoride; azo-PTA, *p*-phenylazophenyltrimethylammonium chloride.

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[4] Exelby, R., and R. Grinter, *Chem. Rev.*, **65**, 247 (1964). Experiments in progress indicate that *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl chloride is a reversible inhibitor that changes into the *cis* isomer when exposed to ultraviolet or sunlight and returns to the *trans* form in the dark.

[5] Clayton, R. K., in *Photophysiology*, ed. A. G. Giese (New York: Academic Press, 1964), vol. 2, p. 51.

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

[7] Wilson, I. B., M. A. Hatch, and S. Ginsburg, *J. Biol. Chem.*, **235**, 2312 (1960).

[8] The molarity of AcCh-esterase (i.e., concentration of active sites) was estimated by the following relationship: Molarity in assay = acetylcholine hydrolyzed (moles/liter/min) $\times$ 1.5×10^{-6}. This formula is based on a turnover number of 7.4×10^{-5} min^{-1} given by Wilson, I. B., and M. A. Harrison, *J. Biol. Chem.*, **236**, 2292 (1961).

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[11] We have found that solutions of AcCh-esterase stored for several weeks in the buffer used in our experiments increased in activity upon exposure to light. Freshly prepared solutions did not behave this way. There is a report that light affects the activity of horse serum cholinesterase similarly Kraupp, O., *Z. Vit. Horm. Fermentforsch.*, **2**, 179 (1949). Kraupp speculates on a disaggregation process stimulated by light. We can offer no explanation but intend to investigate the phenomenon further.

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[13] Axelrod, J., R. J. Wurtman, and S. H. Snyder, *J. Biol. Chem.*, **240**, 949 (1965).

[14] Moore, R. Y., *Nature*, **222**, 781 (1969).

[15] For an extensive description of the structure and function of the pineal gland, see Wurtman, R. J., J. Axelrod, and D. E. Kelly, *The Pineal*, New York: Academic Press (1968).

[16] Deal, W., B. F. Erlager, and D. Nachmansohn, these PROCEEDINGS, in press.

[17] Schoffeniels, E., and D. Nachmansohn, *Biochim. Biophys. Acta*, **26**, 1 (1957); Schoffeniels, E., *Biochim. Biophys. Acta*, **26**, 585 (1957).