References and Notes

1. E. Thomsen, *Acta Oto-Laryngol. Suppl.* **224**, 442 (1967).
2. B. Kellerhals, H. Engström, H. W. Ades, *ibid.* **226**, 50 (1967).
3. F. L. Horsfall and I. Tamm, *Viral and Rickettsial Infections of Man* (Lippencott, ed. 4, Philadelphia 1965).
4. E. Reynolds, *J. Cell Biol.* **17**, 208 (1963).
5. J. R. Hunt, *J. Nerv. Ment. Dis.* **34**, 73 (1907).
6. J. Lindsay, *Arch. Otolaryngol.* **69**, 13 (1959).
7. H. A. E. van Dishoeck and Th. A. Bierman, *Ann. Otol. Rhinol. Laryngol.* **66**, 963 (1957); J. Sataloff and L. Vassallo, *Arch. Otolaryngol.* **87**, 56 (1968).
8. H. F. Schuknecht, M. Igarashi, R. R. Gacek, *Acta Oto-Laryngol.* **59**, 154 (1965).
9. K. O. Smith and L. Rasmussen, *J. Bacteriol.* **85**, 1319 (1963).
10. C. A. Carton and E. D. Kirbourne, *N. Engl. J. Med.* **246**, 172 (1952); P. Becker, J. L. Melnick, H. D. Mayer, *Exp. Mol. Pathol.* **4**, 11 (1965).
11. M. H. McGavran and M. G. Smith, *ibid.*, p. 1.

12. Supported by grants from the Deafness Research Foundation and the National Institute of Health, NINDB 3995. We express our appreciation to our associates, Mrs. R. A. Schulz and Dr. A. Sugiura.

10 October 1968

Photoregulation of an Enzymic Process by Means of a Light-Sensitive Ligand

Abstract. *A specific inactivator of chymotrypsin,* p-*azophenyldiphenylcarbamyl chloride, exists as two geometric isomers,* cis *and* trans, *which are interconvertible by means of light. The* cis-*isomer is five times more reactive than the more stable* trans-*isomer, and is obtained by exposure of the latter to light of 320 nanometer wavelength. The* trans-*isomer can be regained by exposure of the* cis-*isomer to light of 420 nanometer wavelength. This interconversion can be made to occur in aqueous solution in the presence of the enzyme under conditions in which the* trans-*isomer reacts relatively slowly with chymotrypsin. Thus, it is possible to regulate the rate of inactivation of chymotrypsin by using light of the appropriate wavelength. This system is presented as a model for some of the light-sensitive metabolic systems present in living organisms.*

Photosensitive and photoregulated processes, such as photoperiodicity, photoreactivation, and phototaxis, function in many living systems (*1*). Although it is assumed that the effect of light is ultimately on some enzymic processes, the mechanism by which the radiant energy is utilized in the chemical response is largely obscure. There is agreement that pigments, in particular certain carotenoids, may be involved as mediating agents, but the manner in which they might influence or regulate an enzymic process is not known. We now describe a system which might serve as a model. In this system, an enzymic process, in itself insensitive to light, can be made subject to photoregulation by means of a small, light-sensitive effector molecule.

Diphenylcarbamyl chloride (DPCC) is a specific inactivator of chymotrypsin (*2*); inactivation occurs because of a reaction between DPCC and an essential serine residue at the active site of the enzyme. In an attempt to prepare a chromophoric analogue of DPCC, we synthesized the reagent *p*-azophenyldiphenylcarbamyl chloride (PADPCC) (*3*), which as a potent inactivator of chymotrypsin, is even more active than DPCC. Recently, it was noted that the absorption spectrum of PADPCC and its reaction with chymotrypsin were influenced by light. It soon became apparent that PADPCC was photochromic (*4*), undergoing a reversible change in structure under the influence of light. In the case of azo derivatives such as PADPCC, the change caused by light involves interconversion of *cis-trans* geometric isomers, that is, rearrangements around the N=N bond. Although unequivocal identification of the isomer is made by dipole moment measurements, it is generally true that the *trans*-isomers are more stable and have more marked absorption spectra with higher extinction coefficients (*5*).

The PADPCC was prepared by the reaction of *p*-phenylazodiphenylamine with phosgene. The material isolated and purified by crystallization from methanol had an absorption maximum at 332 nm with an extinction coefficient of 23,400. Exposure to ultraviolet light having a maximum intensity at 320 to 330 nm (Spectroline model B-100) caused disappearance of 332 nm peak and the appearance of a smaller shoulder at about 290 nm. Short exposure of the new compound to light of about 420 nm (*6*) resulted in the rapid reappearance of the original spectrum. From these data, we can assume that the more stable isomer—that is, the one originally isolated—is *trans* PADPCC. The *cis*-isomer could be isolated in pure form after exposure of the *trans*-isomer in cyclohexane to ultraviolet light (320 nm) followed by evaporation of the solvent in subdued light and chromatography on silica gel, with cyclohexane as the developing solvent. In this system, the two isomers migrated at different rates, the R_F for the *cis*-isomer being 0.04 and that for the *trans*-form being 0.18. The *cis*-isomer was stable in solution if kept in the dark; storage for as long as 2 days in the refrigerator caused no observable change in absorption spectrum. However, exposure to light of the wavelength about 420 nm caused rapid conversion to the more stable *trans*-isomer. The absorption spectra of the two isomers in methanol are shown in Fig. 1.

The reaction of the isomers with chymotrypsin was examined at 15°C in 0.05*M* tris(hydroxymethyl)amino methane-chloride buffer, *p*H 7.0, containing 0.5 percent methanol. The concentration of chymotrypsin was $1 \times 10^{-6}M$; that of the inactivators was $2 \times 10^{-6}M$. The second-order rate constants of inactivation were for the *cis*-form, $5300M^{-1}$ sec^{-1}, and for *trans*, $1150M^{-1}$ sec^{-1}. Thus, the *cis*-isomer was about five times more reactive than the *trans*-isomer.

Since the two isomers have different activities, and because their relative concentrations in solution can be controlled by light, it should be possible to use light to regulate the rate of inactivation of chymotrypsin (Fig. 2). *Cis-p*-azophenyldiphenylcarbamyl chloride (PADPCC) (1×10^{-6} mole/liter) was allowed to react with chymotrypsin (5×10^{-7} mole/liter) in 0.05*M tris*-chloride buffer (*p*H 7.0) containing 0.5 percent methanol, at 0°C in a darkened laboratory. Samples were withdrawn at suitable intervals and assayed for chymotrypsin activity (*2*). At 280 seconds,

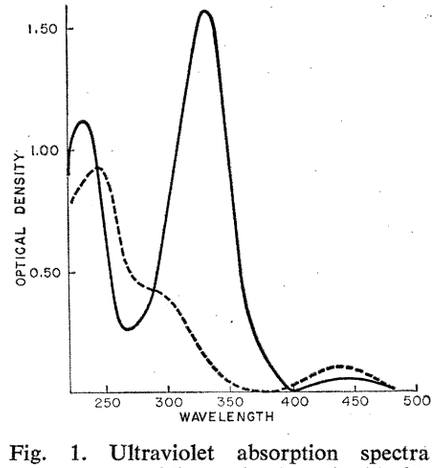

Fig. 1. Ultraviolet absorption spectra of *p*-azophenyldiphenylcarbamyl chloride (PADPCC) isomers, 6.31×10^{-5} mole/liter in methanol. *Trans*-isomer, solid line; *cis*-isomer, broken line.

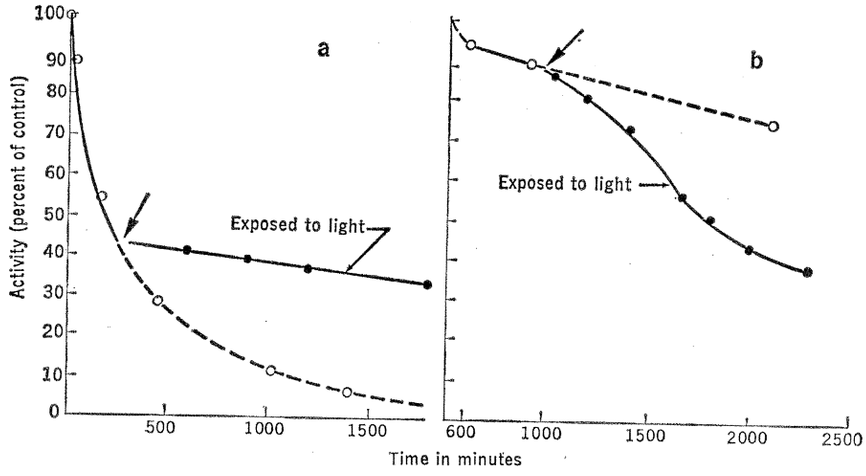

Fig. 2. Effect of light on reaction of *p*-azophenyldiphenylcarbamyl chloride (PADPCC) with chymotrypsin. (a) Effect of light on reaction of *cis*-isomer; (b) effect on reaction of *trans*-isomer.

one-half of the solution was exposed to the light of a photoflood lamp. The other half was protected. Samples were then withdrawn from both solutions and assayed. Exposure to light caused a marked deceleration in the inactivation process, less than a 10 percent loss of activity occurring in the illuminated sample during the same interval in which the activity of the unexposed solution fell to almost zero (Fig. 2a). Presumably illumination had converted the *cis*-isomer to the less reactive *trans*-isomer.

In a second experiment, the initial reagent was the *trans*-isomer. Conditions were identical to those of the previous experiment, except that all containers were of "polycarbonate" plastic or of plexiglass, instead of glass, for a better transmission of ultraviolet light. Initially the inactivation proceeded slowly at a rate expected for the *trans*-isomer and like that shown in Fig. 2a, after exposure to light. At 970 seconds, one-half of the reaction solution was exposed to the ultraviolet source. Samples were withdrawn and assayed as before. Exposure to light of 320 nm caused an acceleration of the inactivation process (Fig. 2b), as expected if the *trans*-isomer were converted to *cis*. From the shape of the curve in Fig. 2b, it would appear that the conversion did not occur immediately upon exposure of the system to 320 nm light. Nevertheless, the increase in reaction rate was marked.

Thus an enzymic process, in itself insensitive to light, can be made subject to photoregulation through the use of a light-sensitive effector molecule. In this case, the effector molecule inactivated the enzyme, but similar regulation might be possible with molecules that are reversibly bound to the enzyme, for example, competitive inhibitors and allosteric reagents. In a living system, regulation by means of an irreversible light-sensitive effector would require that the organism synthesize new enzyme in order to return to its former state. With reversible inhibitors, no new synthesis would be required; the conversion of the light-sensitive effector to its original form would restore enzyme activity. In the case of PADPCC, *cis* to *trans* conversion required light of a wavelength different from that which caused *trans* to *cis* conversion. There are other azo compounds, however, that revert to the *trans*-form in the dark (*7*). The latter type, if present in an organism, could be responsible for a phenomenon such as photoperiodism, in which one type of metabolism takes place in the light and another in the dark.

In *Euglena*, the mechanism of phototaxis is associated with the disappearance of adenosine triphosphate in the region of the flagellum as a result of exposure to light of a photosensitive area at the base of the flagellum (*8*). Our model system would lead to the suggestion that light-sensitive effector molecules present in the organelle might either inhibit an enzyme involved in the synthesis of adenosine triphosphate or activate an adenosine triphosphatase.

A system with characteristics very similar to those of the one we are studying can be found in higher plants. A chromoprotein, phytochrome, is responsible, in part, for the regulation of plant growth (*9*). Phytochrome exists in two forms which are photochemically interconvertible. The physiologically active form has an absorption maximum at 730 nm; that of the inactive form is at 660 nm. The latter is converted into the active form by light of 650 nm and reverts to the inactive form in the dark, or if exposed to light of 730 nm. In the case of phytochrome, the light-sensitive "effector" is apparently covalently bound to the protein, and, in fact, requires the presence of the protein for its photochromic behavior. The exact nature of the photochemical change or of the mechanism by which it regulates growth is not known. With respect to the last point, Hendricks and Borthwick suggest that it regulates cell permeability (*10*), but other mechanisms have also been proposed.

Finally, it should be pointed out that light-regulated *cis-trans* isomerism is a part of the mechanism of vision. The *trans*-form of retinene, which is produced by the action of light on the 11-*cis*-isomer, can no longer bind to opsin. Subsequent dissociation initiates the process of vision. Retinene satisfies our concept of a light-sensitive effector molecule although no enzymic activity has, as yet, been assigned to the protein opsin, or to rhodopsin.

In summary, a system in which a photosensitive effector of low molecular weight regulates enzymic activity is proposed as a possible mechanism for some light-sensitive processes found in nature.

H. KAUFMAN*
S. M. VRATSANOS
B. F. ERLANGER
Department of Microbiology,
College of Physicians and Surgeons,
Columbia University, New York 10032

References and Notes

1. For review articles, see A. C. Giese, *Photophysiology* (Academic Press, New York, 1964), vols. 1 and 2.
2. B. F. Erlanger and W. Cohen, *J. Amer. Chem. Soc.* **85**, 348 (1963); B. F. Erlanger, A. G. Cooper, W. Cohen, *Biochemistry* **5**, 190 (1966).
3. H. Kaufman and B. F. Erlanger, *ibid.* **6**, 1597 (1967); S. M. Vratsanos, H. Kaufman, B. F. Erlanger, in preparation.
4. R. Exelby and R. Grinter, *Chem. Rev.* **65**, 247 (1965).
5. H. Zollinger, *Diazo and Azo Chemistry, Aliphatic and Aromatic Compounds* (Interscience, New York, 1961), p. 311.
6. The optimum wavelength has not been precisely determined. In these experiments, a photoflood lamp in a housing fitted with optical filters manufactured by Coleman Instruments Co., Maywood, Ill., was used.
7. W. R. Brode, J. H. Gould, G. M. Wyman, *J. Amer. Chem. Soc.* **74**, 4641 (1952).
8. R. K. Clayton and A. C. Giese, *Photophysiology* (Academic Press, New York, 1964), vol. 2.
9. S. B. Hendricks and H. A. Borthwick, in *Chemistry and Biochemistry of Plant Pig-*

ments, T. W. Goodwin, Ed. (Academic Press, New York, 1965), p. 405.
10. ———, *Proc. Nat. Acad. Sci. U.S.* **58**, 2125 (1967).
11. Supported by PHS grant GM-11805-10, NSF grant GB-4861, and a gift from the Raymond and Beverly Sackler Foundation. We thank Mrs. Anne Cooper for performing a number of the experiments.
* Present address: Sloan-Kettering Institute for Cancer Research, New York 10021.

6 September 1968; revised 30 October 1968

Reactions of Aromatic and Sulfur Amino Acids in Ribonuclease with Hydrogen Atoms in Water Solution

Abstract. *Neutral hydrogen atoms are simple, one-electron-equivalent reagents and their reactions are of fundamental interest for chemical kinetics. Functional correlation between aromatic and divalent-sulfur amino acids in ribonuclease is shown in the reaction of ribonuclease with hydrogen atoms in aqueous solution. Products of the reaction are partially characterized.*

Rather unexpected functional correlation between amino acids which contain divalent sulfur and others which contain aromatic rings was indicated in studies of the interaction of hydrogen atoms—a one-electron equivalent reagent—with enzyme proteins in aqueous solution (*1*). The reactions of atomic hydrogen with inorganic and organic solutes in water may be studied quantitatively; the rate constants and reaction mechanisms can be determined by a procedure developed in recent years (*2*).

As a result of studies of the rates and mechanisms of the reaction of H atoms with simple inorganic solutes (*2, 3*), amino acids (including aromatic and sulfur-containing ones) (*4*), the tripeptide glutathione (which contains divalent sulfur but no aromatic residues) (*5*), and the metal-enzyme cytochrome c (*6*), the action of H atoms on the hydrolytic enzymes chymotrypsin (*7*) and trypsin (*8*) was investigated. Chymotrypsin showed effective specific inactivation, but apparently without the formation of partially damaged, enzymatically still somewhat active molecules. Investigation of the reaction of H atom with trypsin yielded similar results. In addition, changes in the absorption spectrum and fluorescence, as well as the concurrent evolution of H_2S, indicated that tyrosine, tryptophan, and divalent-sulfur-containing amino acids participated in the reaction. Quantitatively, there was a definite relation between the number of enzyme molecules inactivated and the number of aromatic amino acids and amino acids containing divalent sulfur. Ribonuclease was chosen for the next investigation (*9*), because of its well-established tertiary structure (*10*) and because it does not contain tryptophan. Results similar to those obtained for trypsin showed specific inactivation and damage to aromatic and divalent-sulfur-containing amino acids. Total amino acid analysis of the treated sample proved that of all the amino acids present, only cystine and methionine (containing sulfur) and tyrosine and phenylalanine (containing aromatic rings) were appreciably attacked. The results also indicated that the uptake of a few H atoms, possibly only one by an enzyme molecule, might have produced these extensive changes in the protein.

In order to substantiate these results and to ascertain the mechanism of interaction of H atoms with ribonuclease, we studied the reaction with improved techniques of separation for the analysis of the reaction products. Pancreatic ribonuclease A (Sigma or Worthington)—used in the previous investigation —was purified (*11*) and dissolved in triple distilled water (0.5 mg/ml). The solutions were thus about 3.5×10^{-5} mole/liter for every experiment. The total dose of hydrogen atoms delivered to the solution was determined by the ferricyanide method (*12*). The total dose varied in experiments between about 10 and 100×10^{-5} mole/liter. Ribonuclease competes for H atoms with their recombination reaction.

For a macromolecule such as ribonuclease, it is difficult to determine the exact percentage of H atoms that reacted with it, but it is possible to estimate that not more than 10 percent reacted with the enzyme at the concentrations and doses used. In our experiments at different total doses there were from less than one to about three H atoms available per ribonuclease molecule in the solution. The percentage of inactivation (0 to 50 percent) is roughly proportional to the dose over the range 0 to 80×10^{-5} mole/liter. The extent of experimental scatter, in two discrete series of experiments at eight different total doses, was consistent with the 5 percent error in the dose rate. The enzyme solution was 50 percent inactivated by 80×10^{-5} *M* H (atom). The results are consistent with the assumption that the uptake of only one H atom is sufficient to cause inactivation of the enzyme.

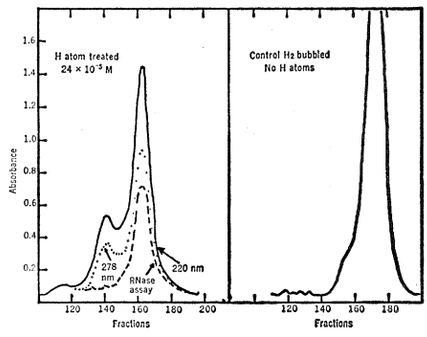

Fig. 1. Separation on Sephadex G 75 in 0.01*N* HCl of ribonuclease treated with H atom and untreated controls. Treated sample: dose $24 \times 10^{-5}M$ H (atoms), $8 \times 10^{-5}M$ per minute for 3 minutes. Untreated sample: bubbled with H_2 gas for 3 minutes. Ribonuclease activity of treated sample was 85 percent of untreated. Column volume 850 ml, length 135 cm, flow rate 24 ml/hr, unit fraction 3.85 ml for treated sample, 3.70 for untreated control. Solid line, absorbance at 220 nm; dotted line, absorbance at 278 nm; and dashed line, ribonuclease activity determined according to Shapira (*11*). Absorbance and enzyme activity was determined on every unit fraction.

The behavior of untreated and treated samples on a Sephadex G75 column is shown in Fig. 1. The untreated sample is uniform and contains no fractions other than the fully active one. After treatment with H atoms the partially inactivated sample separates into two main components: (i) fully active, unaffected enzyme, and (ii) totally inactive, affected one. With increasing dosage of H atom, this latter increases at the expense of the first proportionally, without the appearance of other intermediate or additional fractions.

When the treated sample is permitted to stay at room temperature for several days after H atom treatment, some slight recovery of enzyme activity is observed and three fractions are recovered after Sephadex treatment: unaffected, partially affected, and totally inactive. There is, however, no far-reaching recovery of activity on standing. Separation of the freshly treated material does not yield products of aggregation obtained, for example, in samples inactivated by ionizing radiations (*13*).

Total amino acid analysis was carried out on the totally inactive fraction separated on Sephadex. A Beckman-Spinco model 120C analyzer with resins PA-35 and UR-30 was used in an accelerated system (*14*). The buffer change on the long column was delayed 8 minutes to