Photoregulation of Biological Activity by Photochromic Reagents. Inactivators of Acetylcholinesterase†

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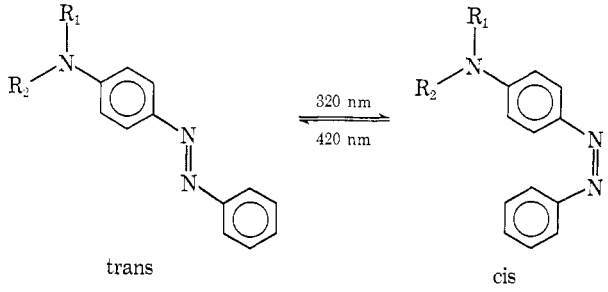
ABSTRACT: Three photochromic reagents were synthesized and examined for their ability to inactivate acetylcholinesterase. They are: *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl chloride (PAPCl), *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl fluoride (PAPF), and *N*-*p*-phenylazophenyl-*N*-methylcarbamyl chloride (PAMCCl). These photochromic compounds can exist as cis and trans isomers that are interconvertible under the influence of light of selected wavelengths. In the trans configuration, the azobenzene portion of the molecule is planar; this is not so for the cis isomers. As a result, the two isomeric forms of each of the reagents acylated the enzyme acetylcholinesterase at different rates; deacylation rates also differed. In all cases, the cis isomers acylated the enzyme at faster rates than the trans isomers. The reverse was true for the deacylation reaction. The most active reagent was PAMCCl. Kinetic constants were evaluated and some observations were made on the utility of photochromic reagents as probes of the topography of biological systems having the property of specificity.

We have shown that the inactivation (*i.e.* irreversible inhibition) of α-chymotrypsin by *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl chloride (PAPCl),[1] a compound that can exist as a cis or trans isomer, depended upon the stereochemical configuration of the inactivator (Kaufman *et al.*, 1968). The relative concentrations of the two isomers in solution were shown to be influenced by light of specific wavelengths in the following way



trans cis

A similar behavior was found for PAPF, an inhibitor of acetylcholinesterase (Bieth *et al.*, 1969). Both inactivation processes therefore could be regulated by light and were put forward as models for photoregulated processes that occur in nature (Bieth *et al.*, 1970; Deal *et al.*, 1969). Other photochromic reagents were used to photoregulate the electrogenic membrane of *Electrophorus electricus* (Bartels *et al.*, 1971) and to probe the topography of the active center of trypsin (Wainberg and Erlanger, 1971).

If space-filling models of *cis*- and *trans*-azobenzene are examined, it is easy to understand why they interact differently with enzymes or other biological systems possessing specificity (Figure 1). The trans isomer is planar with extended conjugation occurring throughout the molecule. This is not true for the cis isomers, in which the two benzene rings are not in the same plane but are in planes at an angle of about 56° to each other (Hampson and Robertson, 1941). These differences are certainly adequate to account for the observed differences in reactivity.

In this paper, we will present kinetic studies of the interaction of three photochromic carbamyl halides with acetylcholinesterase from *Electrophorus electricus*. All are *p*-phenylazo derivatives of reagents known to inactivate acetylcholinesterase, *viz.*, DPCCl, DPCF, and MPCCl (Metzger and Wilson, 1964).

Experimental Section

Preparation of PAPCl. PAPCl was prepared by the reaction of *p*-phenylazodiphenylamine with phosgene, as described previously (Kaufman *et al.*, 1968), and had an absorption maximum at 332 nm with an extinction coefficient of 23,400.

The synthesis of PAPF is described by Kaufman (1968). The method involves an exchange of fluoride for chloride by means of excess SbF_3 and is a modification of a procedure described by Metzger and Wilson (1964) for the conversion of diphenylcarbamyl chloride to the corresponding fluoride. For convenience, we present the procedure here.

Dry powdered PAPCl (1.0 g; 0.003 mol) was mixed with 0.80 g (0.0045 mol) of dry powdered SbF_3 in a dry tube protected from external moisture. The mixture was melted and maintained as a liquid for 2 hr in a silicone bath at 136–140° with occasional stirring. After cooling, the dark violet material was thoroughly agitated with 30 ml of anhydrous ethyl ether for about 15 min. After filtration, the orange colored filtrate was distilled to dryness *in vacuo*. The residue was

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[1] Abbreviations used are: PAPCl, *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl chloride; PAPF, *N*-*p*-phenylazophenyl-*N*-phenylcarbamyl fluoride; DPCCl, diphenylcarbamyl chloride; DPCF, diphenylcarbamyl fluoride; MPCCl, methylphenylcarbamyl chloride; PAMCCl, *N*-*p*-phenylazophenyl-*N*-methylcarbamyl chloride.

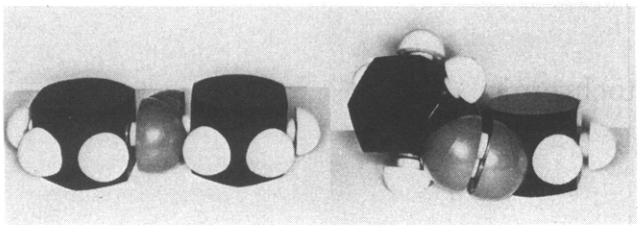

FIGURE 1: Courtauld space-filling models of (left) *trans*-azobenzene and (right) *cis*-azobenzene.

dissolved in 25 ml of acetone, refluxed with Norit A for a short time, and then filtered through Celite. Water was added to the filtrate in order to induce crystallization.

The orange needlelike crystals were recrystallized from acetone–water: yield 0.385 g of pure product (41%); mp 135°. *Anal.* Calcd for $C_{19}H_{14}ON_3F$ (mol wt 319.3): C, 71.5; H, 4.4; N, 13.2; F, 5.9. Found: C, 71.0; H, 4.4; N, 12.9; F, 5.7.

The absorbance maximum in methanol is 331 nm; the molar extinction coefficient is 19,700. Its photochromic characteristics are identical with those of PAPCl (Kaufman *et al.*, 1968).

Preparation of trans-PAMCCl. A suspension of 10.6 g (0.05 mol) of *p*-phenylazo-*N*-methylaniline (Miller and Miller, 1948) in 50 ml of dry benzene containing 5.31 g (0.0525 mol) of *N*-methylmorpholine was stirred and cooled to 5°. To this was added 55 ml of a 12.5% phosgene solution in benzene (0.064 mol). The mixture was stirred at 5° for 2 hr and then at room temperature for 3 hr. The excess phosgene and benzene were removed by distillation *in vacuo*. The residue was suspended with stirring in a mixture of 100 ml of dry benzene and 200 ml of dry acetone. Insoluble *N*-methylmorpholine hydrochloride was removed by filtration and washed with three 50-ml portions of acetone. The filtrate was distillled to dryness and the residue was dissolved, on a steam bath, in a mixture of 300 ml of acetone and 150 ml of water. After standing in the freezer overnight, the crystalline carbamyl chloride was recovered by filtration, mp 94–95°. One recrystallization from 200 ml of acetone and 75 ml of water yielded 10.3 g (75.2%) of pure product, mp 95–96.5°. *Anal.* Calcd for $C_{14}H_{12}ON_3Cl$ (mol wt 273.7): C, 61.4; H, 4.4; N, 15.4; Cl, 13.0. Found: C, 61.6; H, 4.4; N, 15.5; Cl, 13.0.

The absorbance maximum in methanol is 322–323 nm; the molar extinction coefficient is 22,400. The compound was photochromic (Figure 2).

Assay for Acetylcholinesterase Activity. Enzyme activity was measured by the decrease in acetylcholine chloride concentration, determined as the hydroxamate, by a procedure which was essentially that described by Metzger and Wilson (1964). The buffer consisted of 0.02 M sodium phosphate, 0.1 M NaCl, 0.01 M $MgCl_2$, 5×10^{-5} M EDTA, and 0.01% gelatin, pH 7.0. The enzyme was incubated with 2.7 mM acetylcholine (Sigma) at 25° in a final volume of 1 ml. The time of the enzymatic hydrolysis depended upon the initial concentration of acetylcholinesterase; assay time and enzyme concentration were chosen to give 50% hydrolysis or less. Under these conditions, a linear relationship between enzyme concentration and substrate hydrolysis was observed. The precision of the assay was about ±3%.

Acetylcholinesterase (*Electrophorus electricus*, EC 3.1.1.7) was a highly purified preparation obtained from Worthington (code, ECHP). A solution in the above buffer was stable for at least 2 weeks at 4°. The molarity of acetylcholinesterase was

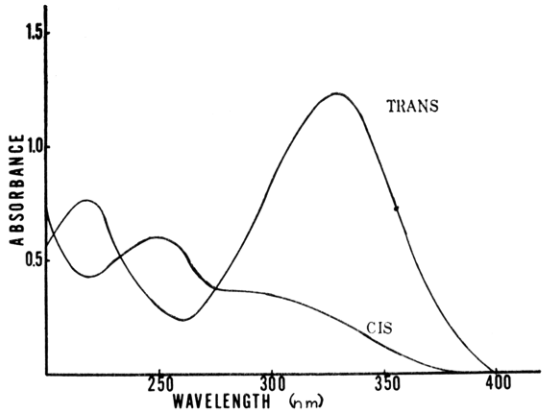

FIGURE 2: Ultraviolet spectra of *cis*- and *trans*-PAMCCl; concentration, 5×10^{-5} M in methanol.

estimated using the turnover number of 7.4×10^5 min^{-1} determined by Wilson and Harrison (1961) under assay conditions identical with those described here.

Inactivation Experiments. The inhibitors were incubated with enzyme and the inactivation was followed by withdrawing samples at suitable time intervals and diluting them into the substrate solution. Two conditions prevailed in these experiments: (1) the inhibitor was in excess over the enzyme and (2) the assay time chosen was short enough to minimize recovery of free enzyme during the assay.

One millimolar stock solutions of the inactivators were made up in methanol (PAPCl and PAPF) or in dioxane (PAMCCl) and diluted with methanol to the required concentration. The trans compounds, obtained by synthesis, were converted into their cis isomers by exposure of the diluted methanolic solutions to ultraviolet light for 10–15 min (Spectroline B 100, long-wavelength ultraviolet lamp, Spectronics Co., Westbury, N. Y.). Since the cis isomers were stable in the dark, their study was performed in a darkened laboratory. The spectra of the reagents were always checked before a series of assays, as the preparation contained 10–15% trans isomer.

For PAPCl and PAPF, 0.025 ml of a diluted inactivator solution was added to 1.25 ml of the buffered enzyme solution (*ca.* 1.4×10^{-8} M) and the mixture was incubated at 25°. Samples (0.1 ml) were withdrawn and assayed as described. The assay time (80 or 100 sec) was short enough to avoid appreciable recovery of the inactivated enzyme.

Different conditions had to be applied in the case of PAMCCl which was the most potent inactivator and also yielded the most unstable carbamyl enzyme. For the cis isomer, 0.050 ml of the methanolic solution was added to 2.5 ml of a 2.2×10^{-9} M acetylcholinesterase solution at 25°. The inactivation was followed by withdrawing 0.25-ml samples. During the assay time (200 sec), a recovery of activity occurred (2.5%) which was within the experimental error of the total procedure. For the trans isomer, which was less active than the cis but led to a more unstable carbamyl enzyme, a higher enzyme concentration (8×10^{-9} M), and, hence, a shorter assay time (60 sec) was used. During this time, the reappearance of free enzyme, corresponding to about 5%, was taken into account for the calculation of the inactivation rate constant.

The inactivation was studied at several inactivator concentrations, except for *trans*-PAPCl, for which the highest soluble concentration gave a relatively slow inactivation rate. Enzyme

controls were run under similar conditions. The enzyme was stable during the time required to follow the inactivation.

Steady-state experiments were performed by allowing a large excess of inactivator to react with the enzyme during a specified period of time and then measuring the enzymic activity during another period of time. For PAPCl, acetylcholinesterase (1.2×10^{-8} M) was incubated with inactivator (1×10^{-5} M for trans; 1×10^{-6} M for cis) for 3 hr at 25°; the steady-state activity was then measured at regular intervals of time during the next 2 hr. For PAPF, the enzyme (same concentration) was incubated with inactivator (2×10^{-6} M for trans; 5×10^{-7} M for cis) for 2 hr at 25°; the steady-state activity was again measured during the next 2 hr. For PAMCCl, the enzyme (2.2×10^{-9} M) was incubated with inactivator (4×10^{-7} M for trans; 1×10^{-7} M for cis) for 1500 sec (trans) or 700 sec (cis); then enzymic activity was measured at regular intervals of time during the next 2000 sec. Enzyme controls containing the same amount of methanol were run under the same conditions. Spontaneous recovery during the assay was a problem only in the experiments with PAMCCl, where it amounted to almost 18% of the measured activity.

Recovery Experiments. A sufficient quantity of inactivator was added to a concentrated enzyme solution to give a rapid, nearly complete inactivation of the enzyme. After a period of time, free inactivator was no longer present and enzyme recovery could be measured. At this point, the reaction mixture was diluted with buffer containing 2% methanol and the recovery of enzyme activity was followed. For PAPF and PAMCCl, the initial enzyme concentration was 2.6×10^{-7} M. For measurement of enzyme recovery, the enzyme + inactivator solution was diluted 1:130; 0.9 ml of this dilution was dispensed into several test tubes and the assay was started with 0.1 ml of 27 mM acetylcholine. The assay time was 80 sec. For PAMCCl, corrections for recovery of enzyme during the assay were done by adding 40 sec (one-half of the assay time) to the recovery time. Recovery could not be studied in the case of *trans*-PAPCl because of its low solubility and low activity. For *cis*-PAPCl, the enzyme (1.9×10^{-6} M) was allowed to react with the inhibitor (2.4×10^{-5} M) in the presence of a higher concentration of methanol (4.7%) in order to increase the solubility of the inhibitor. After 90 min at 25°, the solution was diluted 1:200 with buffer containing 2% methanol. The recovery was followed by withdrawing samples (0.1 ml) and assaying them for 100 sec.

For each recovery experiment, an enzyme control was incubated, diluted, and assayed under the same conditions as the test. During the first hour following the dilution, the activity usually decreased and then remained constant. The deterioration was sometimes as high as 20% and had to be taken into account for the calculation of the recovery rate constants. The "end point" of recovery (*i.e.* the activity of the fully recovered enzyme) was measured after more than five half-times of recovery and coincided with the activity of the enzyme control.

Results

The reaction of carbamyl halides with acetylcholinesterase proceeds *via* a stepwise process in which enzymic activity first decreases, then remains constant, and finally returns to the initial level. This pattern has been interpreted kinetically by Wilson *et al.* (1960). The initial decrease in activity is due to the formation of an inactive carbamyl enzyme which hydrolyzes slowly in water. The reaction of enzyme (E) with inactivators (I) can therefore be described by a scheme which resembles the enzymic hydrolysis of a substrate

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E' \xrightarrow[H_2O]{k_4} E$$

where E·I is the adsorption (Michaelis) complex and E′ the inactive carbamyl enzyme.

This scheme indicates that after an initial increase of E′ (or decrease in activity), a steady state will be approached in which the rate of carbamylation will equal the rate of hydrolysis of the carbamyl enzyme. The scheme also emphasizes that for the inactivation rate a "saturation effect" should be observed at high concentration of inactivator, an effect which could not be observed with our reagents because of their low solubility.

The complete kinetic description according to this scheme was given by Wilson *et al.* (1960). The equation for the steady state is as follows

$$\frac{v}{v_0 - v} = \frac{k_4}{k_3}\left(1 + \frac{K_I}{[I]}\right) \tag{1}$$

where v is the reaction velocity at the steady state, v_0 is the initial velocity, *i.e.* at zero time, [I] is the initial inactivator concentration which, because it is in excess, remains essentially constant, and $K_I = k_2/k_1$. Since no saturation effect was observed at the concentrations of inactivator that we were able to achieve, $[I] < K_I$ and eq 1 simplifies to eq 2.

$$\frac{v}{v_0 - v} = \frac{k_4}{k_3}\frac{K_I}{[I]} \tag{2}$$

Substituting k_3' for k_3/K_I we get $v/(v_0 - v) = (k_4/k_3')(1/[I])$. The ratio k_4/k_3' can therefore be determined; values for the different inactivators and their isomers are reported in Table I. As was shown by Metzger and Wilson (1964), k_3' represents the second-order rate constant for the acylation of the enzyme.

The pre-steady-state equations are complex since they contain terms for both the formation and decomposition of the carbamyl enzyme. In particular, they contain the term $v/(v_0 - v)$, for which any error in v will be reflected twice. Our experience shows that the use of the fully pre-steady-state equation may, in some cases, lead to inactivation constants with rather low precision.

It was, therefore, decided to use a more simplified rate equation which did not take into account the reactivation of enzyme. When the concentration of inactivator is in excess over the enzyme concentration, the progressive decrease in enzyme activity may be described by a simple first-order equation

$$\log (v/v_0) = -(k_{app}/2.3)t \tag{3}$$

where v is the enzyme activity at different times and v_0 is the initial enzyme activity; k_{app} is the pseudo-first-order constant for the inactivation of the enzyme and equals $k_3'[I]$. This simplification is valid only if the experimental conditions are carefully controlled. For instance, the inactivation experiments were carried out using concentrations of inactivator high enough to give a high degree of inactivation in a relatively short time, so that no appreciable recovery occurred during the experiment. In the few cases in which these conditions were not met, early points of the logarithm plot were determined by extrapolation.

Figure 3 shows a typical plot in accordance with eq 3 for the

TABLE I: Kinetic Data for Inactivation and Recovery of Acetylcholinesterase with the Trans and Cis Isomers of Three Photochromic Inactivators.

Compd	Isomer	k_4/k_3' (M)	k_3' (M^{-1} sec^{-1})	$\frac{k_3'(cis)}{k_3'(trans)}$	k_4(calcd) (sec^{-1})	k_4(recovery) (sec^{-1})
PAPCl	Trans	1.5×10^{-5}	11		1.7×10^{-4}	*a*
	Cis	1.6×10^{-6}	67	6.1	1.1×10^{-4}	0.68×10^{-4}
PAPF	Trans	1.1×10^{-6}	450		5.0×10^{-4}	2.9×10^{-4}
	Cis	1.4×10^{-7}	1,270	2.8	1.8×10^{-4}	0.79×10^{-4}
PAMCCl	Trans	1.6×10^{-7}	6,700		10.7×10^{-4}	17.3×10^{-4}
	Cis	0.23×10^{-7}	26,600	4.0	6.2×10^{-4}	2.0×10^{-4}

[a] Not carried out. Inactivation was too slow at the required concentration.

initial inactivation of acetylcholinesterase by the cis and trans isomers of PAPF. Values of k_{app} obtained from such plots could then be plotted *vs.* the concentration of inactivators as shown in Figure 4 for PAMCCl. The values of k_3' obtained in this way are reported in Table I.

It is possible to calculate k_4 using the values of k_3' and of the ratio k_4/k_3' determined as described above. These values are in the sixth column of Table I. The constant k_4 may also be obtained directly from recovery experiments by using the following relation

$$\log [(v_0 - v)/(v_0 - v')] = (-k_4/2.3)t \qquad (4)$$

where v_0 is the enzyme activity after complete recovery (*i.e.* the activity of an enzyme control), v' is the activity at zero time of the recovery (obtained by extrapolation), and v is the activity at different times of reactivation. A typical plot in accordance with eq 4 for PAMCCl is shown in Figure 5. The values of k_4 obtained by this method are reported in Table I (last column). These values do not quite agree with the results of k_4 calculated from steady-state and inactivation experiments except for *cis*-PAPCl; usually k_4(calculated) is higher than k_4(recovery). Many reasons may account for this discrepancy. (1) There may be a problem with the kinetics. The calculations are based on the reaction scheme proposed by Wilson *et al.* (1960), in which the decomposition of E′, the inactive carbamyl enzyme, is the rate-limiting step for the hydrolysis of the inactivator (*i.e.* the steady-state inhibition level is proportional to the concentration of E′). There may be an additional step between the conversion of E·I into E′, this step being rate limiting so that the actual steady-state inhibition level is not proportional to E′ and, hence, eq 2 is not valid. On the other hand, Wilson *et al.* (1960) found good agreement between k_4 values determined by both methods for dimethylcarbamyl fluoride, dimethylcarbamylcholine, and carbamylcholine. (2) A high value of k_4(calculated) may be due to an incorrect (high) value of the k_4/k_3' ratio, which might be a reflection of the fact that the inactivation process may not follow strictly pseudo-first-order kinetics. Main (1969) has reported on this in detail. (3) Finally, the experimental errors themselves may account, at least partially, for the above discrepancies. The precision of the ratio was estimated to be 10%. From the scatter of the experimental points of Figure 4, it can be seen that the precision on k_3' may not be much better.

Discussion

There is very strong evidence that carbamates and carbamyl halides react at the active center of acetylcholinesterase and that inhibition occurs as a result of carbamylation (*cf.* Metzger and Wilson, 1964; Iverson and Main, 1969). It is likely that the photochromic carbamyl halides do so also. It is interesting to compare the carbamylating activities of the photochromic compounds with the reactivities of their corresponding (parent) carbamates (Table II). It can be seen that the addition of a phenylazo group greatly enhances the

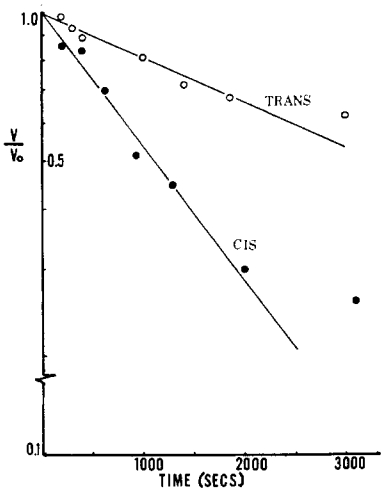

FIGURE 3: A plot of the inactivation of acetylcholinesterase by *cis*- and *trans*-PAPF according to eq 3 (see text). Conditions given in Experimental Section.

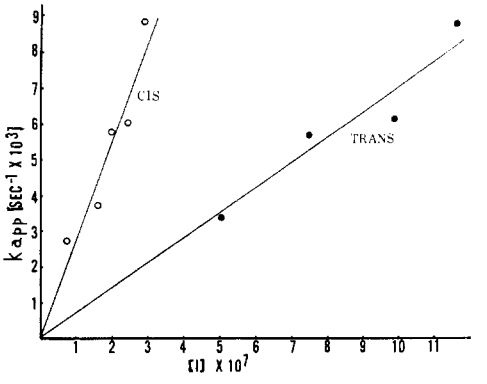

FIGURE 4: A plot of k_{app} of inactivation *vs.* concentration of PAMCCl. See text and Experimental Section for conditions of experiment.

TABLE II: Influence of the *N-p*-Phenylazo Group on the Reactivity with Acetylcholinesterase of Diphenylcarbamyl Chloride, Diphenylcarbamyl Fluoride, and Methylphenylcarbamyl Chloride.

Compd	k_3' (M^{-1} sec^{-1})	$\frac{k_3'(\text{azo deriv})}{k_3'(\text{carbamate})}$
DPCCl	2.2[a]	5 (trans); 31 (cis)
PAPCl	11 (trans); 69 (cis)	
DPCF	347[a]	1.3 (trans); 3.6 (cis)
PAPF	450 (trans); 1270 (cis)	
MPCCl	2300[a]	
PAMCCl	6700 (trans); 26,600 (cis)	2.9 (trans); 11.5 (cis)

[a] Data from Metzger and Wilson (1964).

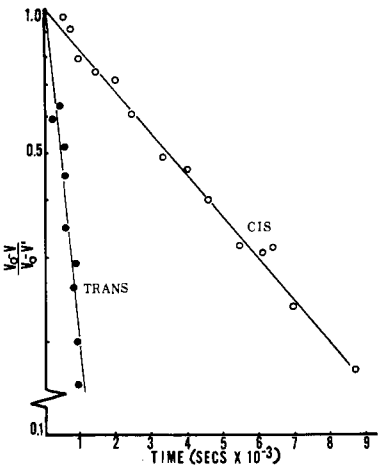

FIGURE 5: A plot of the data obtained from the recovery of PAMCCl-inactivated (cis and trans) acetylcholinesterase (see text).

reactivity of the carbamates, especially of the cis isomers. A similar enhancement was found for the reaction of chymotrypsin and trypsin with the photochromic analog of diphenylcarbamyl chloride (*cf.* Kaufman *et al.*, 1968; Erlanger *et al.*, 1966; Kaufman and Erlanger, 1968). Further inspection of the data of Table II indicates a similarity between the carbamates and their phenylazo derivatives with respect to halide substitution, *i.e.* the fluoride derivatives are more active than the chlorides. As pointed out by Metzger and Wilson (1964), this finding constitutes evidence for an enzyme–substrate interaction, since the intrinsic chemical reactivities of these halides are the reverse. Metzger and Wilson (1964) interpreted this as indicating an electrophilic attack on the carbon–halide bond by a catalytic group of the active center of acetylcholinesterase. Although not shown in Table II, the recovery times for the carbamylated enzyme derivatives are more rapid for the photochromic derivative, *e.g.*, the half-time of reactivation of the diphenylcarbamyl enzyme in water is about 1 day (Metzger and Wilson, 1964); that of the analogous phenylazo derivative is about 40 min for the trans and 145 min for the cis isomer.

Since the inactivation proceeds through a Michaelis complex, there are two important factors determining the effectiveness of an inactivator. First of all, there is the affinity of the inactivator for the active site of acetylcholinesterase and, second, the readiness with which the inactivator once bound can carbamylate the enzyme. Unfortunately, because of the low solubility of the inactivators, these two factors could not be separated in our study. However, evidence that the higher reactivity of *cis*-PAPCl (and PAPF) is not a result of better binding is provided by the observation that *cis-p*-phenyazodiphenylcarbamylcholine, a reversible inhibitor, has a lower affinity for acetylcholinesterase than the trans isomer (Bieth, J., manuscript in preparation).

An interesting finding is that the trans carbamyl-enzymes are hydrolyzed more rapidly than the cis carbamyl-enzymes. This is the reverse of their activities in the carbamylation reaction. We have no data to explain this finding but it may indicate steric interference of the cis isomers with the approach of water in the decarbamylation step. Photochromic reagents may, therefore, prove to be useful new tools for the study of the topography of the active center of enzymes. They already have been used for this purpose in our laboratory (Wainberg and Erlanger, 1971; Bartels *et al.*, 1971) and in others (Martinek *et al.*, 1971; Fournier, M., and Bourdon, J., personal communication; Galley *et al.*, 1973). In experiments with trypsin (Wainberg and Erlanger, 1971), the kinetic characteristics of the planar *trans-p*-phenylazobenzoyl-L-argininehydroxamide, compared to the nonplanar cis isomer, were consistent with the geometry of the side-chain binding site as shown by X-ray crystallography (Stroud *et al.*, 1971).

Additional studies with photochromic quaternary derivatives that act as reversible inhibitors of acetylcholinesterase will be presented in a subsequent paper.

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