Betz, G. (1968), Ph.D. Thesis, University of Kansas Medical School, Kansas City, Kansas.
Chin, C.-C., and Warren, J. C. (1968), *J. Biol. Chem. 243*, 5056.
Chin, C.-C., and Warren, J. C. (1970), *Biochemistry 9*, 1917.
Colins, C. J., and Bowman, N. S. (1970), Isotope Effects in Chemical Reactions, New York, N. Y., Van Nostrand-Reinhold Co., pp 160–211.
Ellis, R. W., and Warren, J. C. (1971), *Steroids 17*, 331.
Fieser, L. F., and Fieser, M. (1967), Reagents for Organic Synthesis, New York, N. Y., John Wiley & Sons.
Fieser, L. F., and Fieser, M. (1969), Reagents for Organic Synthesis, Vol. 2, New York, N. Y., Wiley-Interscience.
Fieser, L. F., and Romero, M. A. (1953), *J. Amer. Chem. Soc. 75*, 4716.
Ganguly, M., and Warren, J. C. (1971), *J. Biol. Chem. 246*, 3646.
Gut, M. (1956), *J. Org. Chem. 21*, 1327.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Melander, L. (1960), Isotope Effects on Reaction Rates, New York, N. Y., Ronald Press, pp 87–97.
Muldoon, T. G., and Warren, J. C. (1968), *J. Biol. Chem. 244*, 5430.
Rao, P. N., Gollberg, H. R., and Axelrod, L. R. (1963), *J. Org. Chem. 28*, 270.
Ringold, H. J., Mancera, O., Rosenkranz, G., and Edwards, J. (1962), U. S. Patent 3,019,239.
Sondheimer, F., Kaufman, S., Romo, J., Martinez, H., and Rosenkranz, G. (1953), *J. Amer. Chem. Soc. 75*, 4712.

Specific Modification of Methionine-192 of α-Chymotrypsin by an Affinity Label Exploiting the Orienting Properties of the Linear Acetylenic Group†

J. Bryan Jones* and David W. Hysert‡

ABSTRACT: The practicability of incorporating acetylenic bonds into affinity labels in order to exploit their rodlike properties for improving the orientation of an alkylating function toward a selected protein nucleophile has been demonstrated by the facile irreversible inhibition of α-chymotrypsin by 6-bromo-1-phenylhex-4-yn-3-one. 6-Bromo-1-phenylhex-4-yn-3-one was designed to achieve optimum orientation of its propargylic bromide alkylating function toward both histidine-57 and methionine-192. However, the latter function was alkylated selectively owing to its 100-fold greater reactivity with propargylic bromides. Rate of inhibition, pH–rate profile, competitive inhibition, effect on K_m(app) and k_3, amino acid analytical, and diagonal peptide-mapping studies established that 6-bromo-1-phenylhex-4-yn-3-one was a methionine-192-specific, active-site-directed, irreversible inhibitor. The calculated values of its K_i (10 mM) and rate of inhibition constant (2.8×10^{-3} sec^{-1}) show it to be one of the best methionine-192 of α-chymotrypsin directed affinity labels yet evaluated. The data demonstrate that the linear geometry of the acetylenic bond can be used to good advantage in improving the degree of specificity achievable in a desired protein modification and that propargylic (and by analogy allylic) bromide functions represent a valuable addition to the list of alkylating groups normally employed for protein modification.

Use of affinity labels (active-site-directed reagents, site-specific reagents) to effect specific modifications of enzymes and immunoglobulins has already proven of great value (Baker, 1967; Singer, 1967; Glazer, 1970; Shaw, 1970). Although the spectrum of modifications which can now be achieved using affinity labels is quite broad, considerable scope still remains for improving the functional group and stereospecificity (or -selectivity) of such reagents and in this regard we had become intrigued by the possibility of exploiting the unique geometries of cis and trans ethylenic and linear acetylenic functions (either alone or in appropriate combinations) to achieve more precise orientation of an alkylating function toward a selected protein nucleophile. The practicability of exploiting the rodlike properties of acetylenic bonds to improve orientation in affinity labels has now been demonstrated by the irreversible inhibition of CT[1] by the dihydrocinnamylpropargylic bromide BPH (**5**, see Scheme I). This compound was designed to alkylate methionine-192 specifically and was found to be very effective in this regard. The data obtained also confirm that propargylic (and *ipso facto* allylic) bromide functions represent useful additions to the list of convenient alkylating agents employed in protein modification.

† From the Lash Miller Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto 181, Canada. *Received March 17, 1972*. This work, which is based on part of the Ph.D. thesis of D. W. H. (University of Toronto, 1971), was supported by grants from the National Cancer Institute of Canada and the Science Scholarship Fund of the National Research Council of Canada.

* To whom correspondence should be addressed.
‡ National Research Council of Canada Science Scholar, 1967–1971.

[1] Abbreviations used are: CT, α-chymotrypsin; BPH, 6-bromo-1-phenylhex-4-yn-3-one; BPH-CT, BPH-inactivated α-chymotrypsin; hve, high voltage electrophoresis.

Materials and Methods

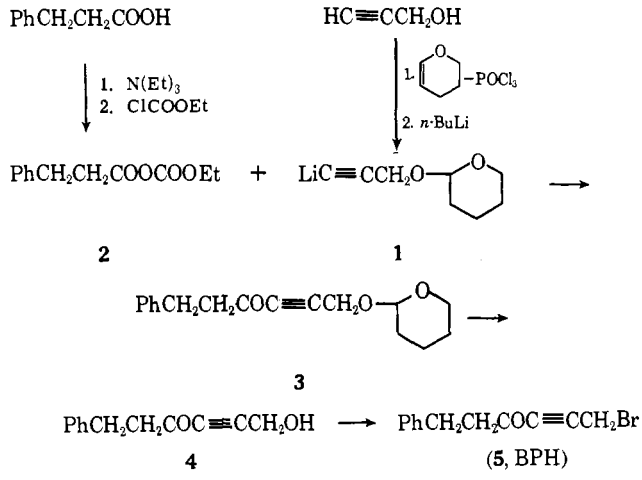
Synthesis of 6-Bromo-1-phenylhex-4-yn-3-one (**5**, *BPH*). Infrared (ir) and ultraviolet (uv) spectra were recorded on Perkin-Elmer 237B and Unicam SP800A spectrophotometers, respectively. Nuclear magnetic resonance (nmr) measurements were performed on Varian T-60 or HA-100 instruments with Me_4Si as internal standard. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. All starting materials and products were purified until no impurities could be detected by gas–liquid and thin-layer chromatographic and spectroscopic analyses. Solvents were either ACS certified grade or were redistilled before use. Solvent evaporations were all effected by rotary evaporation under reduced pressure at 21°. *n*-Butyllithium in *n*-hexane solution was obtained from the Foote Mineral Co.

1-(2′-Tetrahydropyranyloxy)-2-propyne was obtained in 70% yield from propargyl alcohol (8.4 g, 0.15 mole), 2,3-dihydropyran (12.6 g, 0.15 mole), and phosphoryl chloride (100 mg) as described by Henbest *et al.* (1950): bp 74–75° (9 Torr) (lit. bp 78° (25 Torr)).

Lithium 1-(2′-Tetrahydropyranyloxy)-2-propynylide (**1**). To 1-(2′-tetrahydropyranyloxy)-2-propyne (2.8 g, 20 mmoles) in dry tetrahydrofuran–ether (4:1, 20 ml) at −50° under dry nitrogen was added dropwise with stirring 11.3 ml (20 mmoles) of a 1.77 M solution of *n*-butyllithium in *n*-hexane (standardized by the procedure of Gilman and Haubein (1944)). The ≃0.1 M solution of the lithium propynylide so obtained was stored at −50° under nitrogen until required in the sequence described below. (Analysis of an aliquot of the solution by shaking with 0.1 N DCl and examination of the relative intensities of the ≡C—H (3322 cm^{-1}) and ≡C—D (2604 cm^{-1}) ir bands indicated that formation of the desired lithium salt had occurred virtually quantitatively (Dessy *et al.*, 1962).

1-Phenyl-6-(2′-tetrahydropyranyloxy)hex-4-yn-3-one (**3**). Ethyl chloroformate (2.17 g, 20 mmoles) in petroleum ether (bp 30–60°, 10 ml) was added dropwise with stirring to a cold (−20°) solution of dihydrocinnamic acid (3 g, 20 mmoles) and triethylamine (2.02 g, 20 mmoles) in dry toluene (75 ml)–petroleum ether (bp 30–60°, 30 ml). The reaction mixture was stirred for a further 2 hr at −20°, and the voluminous white precipitate of triethylamine hydrochloride was then removed at −20° in a dry nitrogen atmosphere by pressure-suction filtration. The ethoxycarbonic dihydrocinnamic anhydride (**2**)[2]-containing filtrate was cooled to −50° under dry nitrogen and the cold (−50°) solution of lithium 1-(2′-tetrahydropyranyloxy)-2-propynylide (**1**, 20 mmoles) prepared above was added dropwise with stirring during 20 min. After being kept at −50° for 30 min and then at 20° for a further 6 hr, the reaction mixture was washed successively as quickly as possible with ice-cold solutions (200 ml each) of 0.5 N sulfuric acid, saturated sodium bicarbonate, and saturated sodium chloride. The organic layer was then dried ($MgSO_4$) and evaporated and the yellow oil obtained was chromatographed directly on Florisil (500 g). Benzene–ether (20:1) elution gave 1-phenyl-6-(2′-tetrahydropyranyloxy)-hex-4-yn-3-one (2.3 g): bp 131° (0.01 Torr); uv max (CH_3OH) 305 (ε 42), 269 (243), 265 (304), 259 (424), and 226 nm (6900); ir (film) 2208 and 1672 cm^{-1}; nmr (CCl_4) δ 1.4–1.9 (6 H, m, O(O)CH(CH_2)$_3$), 2.8–2.92 (4 H, A_2B_2 m, $PhCH_2CH_2CO$), 3.3–3.9 (2 H, m, CH_2CH_2O), 4.3 (2 H, s, ≡CCH_2O), 4.72 (1 H, t, J = 1 Hz, O(O)C*H*CH_2), and 7.17 ppm (5 H, s, Ph). *Anal.* Calcd for $C_{17}H_{20}O_2$: C, 74.97; H, 7.40. Found: C, 75.02; H, 7.38.

Scheme I



6-Bromo-1-phenylhex-4-yn-3-one (**5**, *BPH*). 1-Phenyl-6-(2′-tetrahydropyranyloxy)hex-4-yn-3-one (**3**, 2.14 g, 7.8 mmoles) and 0.5 N hydrochloric acid (100 ml, 50% aqueous ethanol) were stirred vigorously at 20° for 1.5 hr. The homogeneous solution obtained was then diluted with water (300 ml) and extracted with ether (three times, 50-ml portions). The ether extracts were washed with water (two times, 100-ml portions), then with saturated aqueous sodium chloride, dried ($MgSO_4$), and evaporated to give 1-hydroxy-6-phenylhex-2-yn-4-one (**4**, 1.32 g): ir (CCl_4) 3636, 3460, 2222, and 1675 cm^{-1}; nmr (CCl_4) δ 2.83–2.88 (4 H, m, $PhCH_2CH_2CO$), 4.29 (2 H, s, ≡CCH_2O), 4.6 (1 H, s, D_2O exchangeable, OH), and 7.16 ppm (5 H, s, Ph).

Tri-*n*-octylphosphine (5.18 g, 14.0 mmoles) was added dropwise during 10 min to a stirred solution of the above alcohol (1.32 g, 7.0 mmoles) and carbon tetrabromide (4.64 g, 14.0 mmoles) in dry ether (45 ml) with the temperature being maintained at 10°. After keeping for a further 6 hr at 20° the solution was concentrated under reduced pressure and the residual oil was chromatographed on Florisil (300 g). Benzene elution yielded 6-bromo-1-phenylhex-4-yn-3-one (0.67 g) as a pale yellow liquid. Molecular distillation gave a colorless analytical sample: bp 135–140° (block) (0.01 Torr); uv max (CH_3OH) 268 sh (ε 837), 258 sh (1360), 224 sh (7510), and 210 nm (13,200); ir (film) 2217 and 1672 cm^{-1}; nmr (CCl_4) δ 2.80–2.93 (4 H, m, $PhCH_2CH_2CO$), 3.94 (2 H, s, ≡CCH_2Br), and 7.1–7.3 (5 H, m, Ph). *Anal.* Calcd for $C_{12}H_{11}BrO$: C, 57.39; H, 4.42; Br, 31.82. Found: C, 57.41; H, 4.46; Br, 32.21.

Inhibition of CT by BPH. MATERIALS, SOLUTIONS AND APPARATUS. CT (three times crystallized, Worthington), *N*-Ac-L-TyrOEt (Mann Research Corp.), and dihydrocinnamic acid (Aldrich) were used as purchased. All other chemicals were of analytical reagent grade and were used without further purification.

Carbon dioxide free, glass-distilled water was used for all aqueous solutions. Sodium phosphate buffers (0.1 M) were made up as described by Gomori (1955). Stock solutions of CT (≃10 mg/ml) were prepared in 0.001 N HCl–0.1 M NaCl and were stored in ice. The actual enzyme concentrations were determined according to the procedure of Sigman *et al.*

[2] When isolated, **2** was a colorless oil: ir (film) 1821 and 1757 cm^{-1}; nmr (CCl_4) δ 1.28 (3 H, t, J = 7 Hz), 2.6–3.0 (4 H, m), 4.22 (2 H, q, J = 7 Hz), and 7.15 ppm (5 H, s).

(1969) on the basis of a molecular weight of 25,000. *N*-Ac-L-TyrOEt stock solutions (≃2 mM) were made up directly in pH 7 phosphate buffer and the exact substrate concentrations were ascertained spectrophotometrically (Schwert and Takenaka, 1955; Kumar and Hein, 1969).

Eppendorf pipets with disposable tips were used for dispensing standard aliquots. Spectrophotometric measurements (kinetic and otherwise) were determined on a nitrogen-purged Cary 16 spectrophotometer equipped with a Model 1626 recorder interface and Varicord 43 recorder using standard 3.0-ml, 1-cm path-length, quartz cells (Hellma) thermostatted at 29.3° (bath temperature 30°). High-voltage electrophoresis (hve) was performed with a Savant Instruments hve apparatus using Isopar (Esso) coolant and an appropriate buffer of pH 6.5, 3.5, or 1.9 (Bennett, 1967). Amino acid analyses were carried out on a Beckmann 120B automatic amino acid analyzer. A Radiometer pH-Stat was used for determining the rate of hydrolysis of BPH.

Kinetics of BPH Inactivation of CT. The rates of inactivation of CT by 0.1–0.4 mM concentrations of BPH were studied by the following method. A mixture of pH 7.2 phosphate buffer (8.0 ml), Me_2SO (1.0 ml), and CT stock solution (1.0 ml) was incubated for 10 min at 30° in a constant-temperature bath. After withdrawal of an aliquot (100 μl) for use as a control, the appropriate amount of a 52.8 mM solution of BPH in Me_2SO was added to the mixture and both control and inhibitor-containing enzyme solutions were incubated at 30°. Aliquots of 20 μl were withdrawn from these solutions at convenient times and assayed for residual chymotryptic activity by the method of Kumar and Hein (1969). Apparent first-order rate constants for the inactivation of CT by BPH were then obtained from plots of log (initial velocity) *vs.* time. These plots gradually deviated from straight lines after 3–4 hr due to the spontaneous hydrolysis of BPH. The rate of the latter reaction under the reaction conditions was measured independently using a standard pH-Stat procedure (Hysert, 1971) and the CT inactivation rate constants were then calculated from data obtained during the first 2–3 hr (at least 1 half-life) of reaction during which time the effect of BPH hydrolysis was not significant. The loss of enzymic activity in the control solutions during this period was negligible. The effect of the competitive inhibitor dihydrocinnamic acid (2.5 mM) was determined under identical conditions with a BPH concentration of 0.215 mM.

Effect of pH on the Rate of CT Inactivation by BPH. Values for the first-order rate constants of inactivation of CT by BPH were determined by the above method using phosphate buffers of pH 6.15, 6.62, 7.20, 7.52, and 7.78. The inactivation runs at each pH were carried out with 37.6 μM CT–0.21 mM BPH solutions in 10% aqueous Me_2SO at 30°.

Preparation of BPH-CT. CT (100 mg, 4 μmoles) was dissolved in phosphate buffer (pH 7.0, 90 ml)–Me_2SO (9 ml) and 1.0 ml of this solution was removed as a control. BPH (20.1 mg, 80 μmoles) in Me_2SO (1 ml) was then added slowly and the resulting solution was stirred at 25° for 25 hr at which time the residual activity of the inhibited CT had become constant (3% of the control). The reaction mixture was then acidified to pH 3.0 with 12 N HCl, shaken gently with ether (100 ml) to remove the excess of BPH, centrifuged at 10,000*g* for 10 min to remove the fine yellowish precipitate, and finally dialyzed at 0° for 24 hr against four 3.5-l. changes of 1 mM HCl. Lyophilization of the dialyzed solution afforded the inactivated enzyme as a fluffy, almost colorless, powder.

Amino Acid Analysis. Samples (*ca.* 1 mg) of BPH-CT were hydrolyzed at 110° for 21 hr with 1.0 ml of 6 N HCl in sealed, evacuated tubes. The cooled tubes were then opened, evaporated to dryness at 40°, dissolved in 0.1 M citrate buffer, and subjected to amino acid analysis by the method of Spackman *et al.* (1958). Methionine sulfone analyses were carried out using the procedure developed by Moore (1963). All analyses were carried out in duplicate.

Hve Diagonal Peptide Map Analyses. Diagonal paper ionograms of native and inactivated CT were prepared according to the method of Brown and Hartley (1966). Typically, native CT or BPH-CT (10 mg) was digested with pepsin (1 mg) in 5% formic acid (2.5 ml) for 17 hr at 37°. The peptic digest was then applied in a band (1 mg/cm, 0.5 cm wide) to the center of a sheet of Whatman No. 3MM paper and subjected to hve at 2000 V at pH 6.5 for 1.5 hr. A 3-cm wide strip was then cut from the edge of the dried sheet and exposed for 2 hr at 20° to performic acid vapor. After drying, the oxidized strip was sewn across the center of another sheet of Whatman No. 3MM paper and subjected again to hve at pH 6.5 at 2000 V for 1.5 hr. The peptides were detected with the cadmium–ninhydrin reagent (Heilman *et al.*, 1957) and those containing histidine with the Pauly reagent (Dent, 1947).

Purification of Histidine-Containing Peptides. The procedure followed was based on that described by Stevenson and Smillie (1968). Peptic digests of native CT or BPH-CT were spotted (1 mg/cm) in the centers of sheets of Whatman No. 3MM paper and subjected to hve at pH 6.5 at 2000 V for 1.5 hr. In each experiment a cystine peptide containing the two histidine residues was located by staining side strips with the Pauly and cadmium–ninhydrin reagents. At pH 6.5 the Pauly-positive (histidine-containing) band was found to run with the neutral band for both native and alkylated CT. The cystine peptide containing the two histidine residues was cut out as a band, sewn on to a second sheet of Whatman No. 3MM paper 10 cm from the anode and further purified by hve at pH 1.9 and 3000 V for 1 hr. The basic Pauly-positive region was again cut out and, after oxidation with performic acid vapor, was brought into a single band with pH 3.5 buffer. The strip was then sewn on to a sheet of Whatman No. 1 paper 10 cm from the anode and following hve at pH 3.5 and 2000 V for 1.3 hr complete separation of the histidine-40- and histidine-57-containing peptides was effected.

Results

Synthesis of BPH. BPH was synthesized *via* the Schmidt and Schwochau (1964) mixed-anhydride method as outlined in Scheme I. Treatment of propargyl alcohol with 2,3-dihydropyran in the presence of phosphoryl chloride afforded 70% of the corresponding tetrahydropyranyl ether derivative which on treatment at −50° with 1 equiv of *n*-butyllithium gave lithium 1-(2′-tetrahydropyranyloxy)-2-propynylide (**1**) in quantitative yield. Ethoxycarbonic dihydrocinnamic anhydride (**2**) was prepared by treatment of the triethylamine salt of dihydrocinnamic acid with ethyl chloroformate (Albertson, 1962) and was reacted with **1** to give a 42% yield of the desired tetrahydropyranyl acetylenic ketone **3**. Removal of the tetrahydropyranyl protecting group was readily accomplished with aqueous ethanolic hydrochloric acid and 1-hydroxy-6-phenylhex-2-yn-4-one (**4**) was obtained in 95% yield.

Treatment of **4** with phosphorus tribromide–pyridine under the conditions usually successful in effecting bromination of acetylenic alcohols (Jacob and Petty, 1963) gave an intractable tar. However, use of the milder brominating conditions provided by tri-*n*-octylphosphine and carbon tetra-

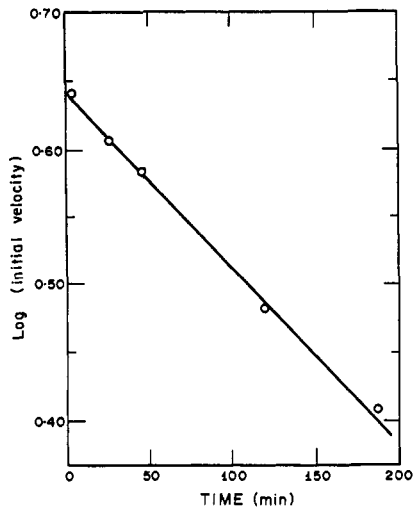


FIGURE 1: Representative pseudo-first-order kinetic plot for the inactivation of CT (37.6 μM) by BPH (0.215 mM) in 10% Me_2SO–water (pH 7.2) (phosphate buffer) at 30°.

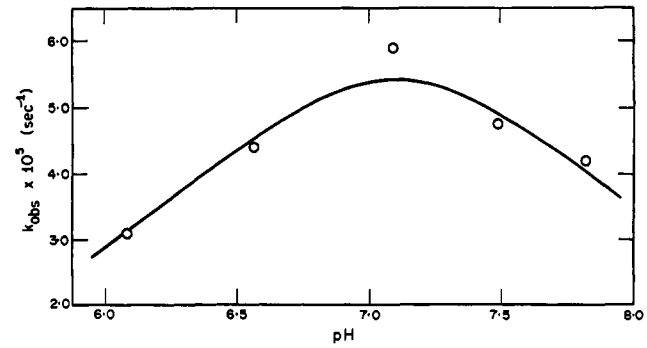


FIGURE 2: The effect of pH on the rate of inhibition of CT by BPH. Conditions as outlined for Figure 1 using phosphate buffer solutions.

bromide (Hooz and Gilani, 1968) effected the desired conversion of **4** into BPH (**5**) in 40% yield. When purified, BPH was a colorless liquid which was stable indefinitely at −20°.

Kinetic Studies. A preliminary study established that BPH inactivated CT rapidly to a maximum extent of 97% and that exhaustive dialysis restored none of the lost activity. In order to avoid the need to correct for BPH hydrolysis during the CT-inactivation reactions, a minimum BPH excess of fivefold was used in each kinetic run and only the data obtained during the first 2–4 hr (≃1 half-life) were used. The rapid *N*-Ac-L-TyrOEt hydrolysis assay method developed by Kumar and Hein (1969) was employed to follow the kinetics of the irreversible inhibition reaction. The straight-line log (initial velocity) *vs.* time plots obtained (Figure 1) demonstrated a first-order dependence on enzyme concentration and the pseudo-first-order rate constants (k_{obsd}) observed for differing BPH concentrations are recorded in Table I. That the reaction was also first order with respect to BPH concentration was shown by the linear correlation of the Table I k_{obsd} and [BPH] values and the second-order rate constant k_{obsd}/[BPH] was found to be 0.28 ± 0.01 M^{-1} sec^{-1}. The relatively small effect of pH on the apparent first-order rate constants for BPH inactivation of CT is summarized in Figure 2.

The addition of the competitive inhibitor (I) dihydrocinnamic acid (2.5 mM) reduced the rate constant of CT (37.6 μM) inactivation by 0.215 mM BPH from 5.90×10^{-5} to 5.00×10^{-5} sec^{-1}. Kézdy *et al.* (1967a) have shown that when irreversible inactivation occurs by an active-site-directed mechanism, the ratio of uninhibited to competitively inhibited rate constants (here 1.18) should be equal to $1 + [I]/K_i(I)$. Calculation of the K_i for dihydrocinnamate on this basis gives a value of 14 mM. This represents a three- to fourfold increase over the K_i values previously recorded for this inhibitor (Neurath and Schwert, 1950; Canady and Laidler, 1958). However, in view of the different conditions used in the current study, this is not considered significant.

Inactivation of an enzyme by an affinity label proceeds as represented in eq 1. Since the rates of inactivation are dependent on the concentration of E·I, it is desirable for the inhibition constant to be determined, particularly if valid comparisons with inhibition rates of other affinity labels are to be made (Baker, 1967). A plot of $1/k_{obsd}$ *vs.* 1/[BPH] from the data in Table I gave a straight line passing through the origin, thereby indicating that the concentrations of BPH used in the kinetic experiments were considerably less than K_i (Kumar and Hein, 1970) and thus the K_i for the CT·BPH complex could not be determined directly. However, although it was also subject to the above concentration limitations, application of the method of Bittner and Gerig (1970), with K_m for *N*-Ac-L-TyrOEt taken as 0.85 mM (Figure 3), enabled an approximate K_i value of 10 ± 4 mM to be calculated. Since [BPH] is $\ll K_i$, k_{obsd}/[BPH] is equal to k_2/K_i (Kumar and Hein, 1970) and the calculated value of the alkylation rate constant, k_2, is 2.8×10^{-3} sec^{-1}.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} EI \qquad (1)$$

Affinity labeling of an enzyme usually results in changes in either, or both, the apparent Michaelis–Menten ($K_{m,app}$) or catalytic constants. Application of the integrated Michaelis–Menten rate equation (Schwert, 1969; Kumar and Hein, 1969) to the rapid *N*-Ac-L-TyrOEt assays showed (Figure 3) that the values of $K_{m,app}$ increased and those of k_3 decreased progressively as the BPH inhibition of CT proceeded.

Amino Acid Analyses. The amino acid analysis of BPH-CT is presented in Table II. The data indicated modification of 0.7 residue of methionine but that no alkylation of histidine had occurred. Further evidence for methionine modification in BPH-CT was provided by the detection of homoserine lactone (Gundlach *et al.*, 1959), in the acid hydrolysates. Methionine sulfone analysis (Neumann *et al.*, 1962) of BPH-CT was also carried out and the results obtained (Table III) showed that 0.9 of the two methionine residues had been alkylated by BPH.

TABLE I: Rate Constants for Inactivation of CT by BPH.[a]

[BPH] (mM)	$k_{obsd} \times 10^5$ (sec^{-1})	k_{obsd}/[BPH] (M^{-1} sec^{-1})
0.175	4.86	0.28
0.215	5.90	0.27
0.320	9.50	0.29
0.350	9.30	0.27

[a] Kinetic runs were carried out using 37.6 μM solutions of CT in 10% Me_2SO–water at 30°, pH 7.2 (phosphate buffer).

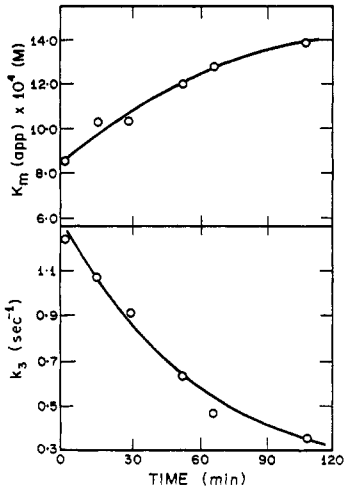


FIGURE 3: Effect of inactivation of CT by BPH on $K_{m,app}$ and k_3(deacylation). The representative data shown were obtained by applying the integrated Michaelis–Menten equation to single *N*-Ac-L-TyrOEt assays carried out at pH 7.0 at 29.3° on aliquots of a BPH (1.1 mM)–CT (37.6 μM) solution in pH 7.2 phosphate buffer containing 10% Me_2SO at 30°.

Diagonal Peptide Mapping. The above data indicated that inhibition of CT by BPH was occurring exclusively by alkylation of the active-site methionine-192 residue. Confirmation of this was provided by the diagonal peptide-mapping procedure developed by Brown and Hartley (1966). The methods followed and peptide terminology used are as described by Stevenson and Smillie (1968, 1970). The maps obtained following hve of pepsin digests of native CT and modified CT are reproduced in Figure 4 and their close similarity to those recorded previously for histidine-57- and methionine-192-alkylated CT (Brown and Hartley, 1966; Stevenson and Smillie, 1968, 1970; Hartley, 1970) enabled the peptides of interest to be readily identified. The Pauly-positive (and thus not N-alkylated) histidine-40- and histidine-57-containing peptides, A1 and A2, respectively, were identical in both maps. This evidence supported the conclusion drawn from the amino acid analyses that no histidine modification had occurred in BPH-CT. Further corroboration that the A1 or A2 peptides were unaffected by BPH was provided by their isolation and further purification by hve at pH 6.5, 1.9, and 3.5. The identity of the purified histidine-40 and histidine-57 peptides from native CT and alkylated CT is demonstrated in Figure 5. In view of exact correspondence of the electrophoretic and color-reaction properties of these histidyl peptides with those completely characterized previously by Hartley and Smillie and their coworkers, determinations of their amino acid sequences were not carried out. The similarly well-documented methionine-180-containing peptide bands were also identical in both maps.

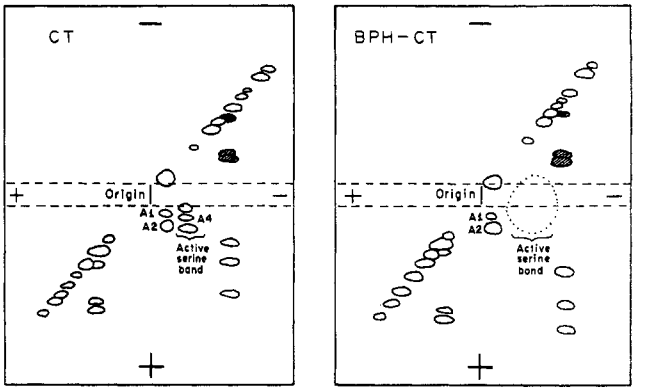


FIGURE 4: The diagonal peptide maps of CT and BPH-CT. Hve was carried out at pH 6.5, 2000 V for 1.5 hr in both dimensions. His-40- and His-57-containing peptides (A1 and A2, respectively, Stevenson and Smillie, 1968) were Pauly positive. A4 (orange color with cadmium–ninhydrin) includes Met-192; the Met-180-containing peptides (Brown and Hartley, 1966) are hatched.

In contrast, very marked differences were observed in the active serine band regions. As Figure 4 shows, in the map from native CT, this band was composed of three spots of which the middle one (A4) had the characteristic mobility and orange cadmium–ninhydrin color of the methionine-192-containing peptide shown to correspond to residues 190–207 by Brown and Hartley (1966) (*cf.* also Stevenson and Smillie, 1970). On the other hand, in the BPH-CT map the appearance of this region was very different showing that substantial methionine-192 modification had taken place. Unfortunately,

TABLE II: Amino Acid Analysis of BPH-CT.

Amino Acid	CT[a]	BPH-CT[b]
Lysine	14	13.6
Histidine	2	2.0
Arginine	3	2.9
Aspartic acid	22	22.7
Threonine	22	22.3
Serine	27	27.6
Glutamic acid	15	16.2
Proline	9	9.0
Glycine	23	23.0
Alanine	22	22.0[c]
Half-cystine	10	7.5
Valine	23	19.2
Methionine	2	1.3[d]
Isoleucine	10	7.7
Leucine	19	18.6
Tyrosine	4	4.0
Phenylalanine	6	6.2
Homoserine lactone		+

[a] From sequence data of Hartley (1964); Blow *et al.* (1969). [b] Average of two 21-hr amino acid analyses. [c] Arbitrarily taken as 22.0 residues. [d] Sum of methionine and methionine sulfoxides.

TABLE III: Methionine Sulfone Analysis of BPH-CT.

Amino Acid	CT[a]	BPH-CT
Cysteic acid	10	9.7[b]
Methionine sulfone	2	1.1
Glutamic acid	15	15.5
Glycine	23	22.1
Alanine	22	22.0[c]

[a] From sequence data (*cf.* Table II). [b] Corrected for 94% recovery (Moore, 1963). [c] Arbitrarily taken as 22.0.

hve conditions could not be found which would resolve the individual peptides comprising this broad and diffuse active serine band and thus the further corroboration that sequencing of the modified A4 peptide would have provided could not be obtained. However, the total evidence available leaves little doubt that alkylation of CT by BPH occurs exclusively at methionine-192.

Discussion

In view of the wealth of structural (Blow *et al.*, 1969; Steitz *et al.*, 1969; Henderson, 1970), kinetic and specificity (Cunningham, 1965), and affinity labeling (Shaw, 1970) data available, and the ease with which modified active-site residues can be identified (Brown and Hartley, 1966), CT was a natural choice for the evaluation of a new type of active-site-directed reagent. As the majority of CT-specific reagents have resulted in histidine-57 or methionine-192 alkylation, attention was concentrated on the design of an affinity label in which incorporation of an acetylenic group should improve the orientation of the alkylating function toward one or other of these functions.

An affinity label was desired which would interact with the aromatic binding locus (Henderson, 1970) of the active site. Ordinarily this would have been ensured using an aromatic amino acid derivative but in view of the synthetic difficulties envisaged, less sensitive nonspecific substrate groups were considered initially. From the total data available dihydrocinnamyl derivatives appeared to provide the best compromise of the conflicting synthetic and specificity aspects. On the basis of the X-ray and affinity-labeling studies on various aromatic derivatives (Steitz *et al.*, 1969; Henderson, 1970; Shaw, 1970) it was felt that the dihydrocinnamyl group, for which no hydrogen bonding with the acylamido site is possible (Henderson, 1970; Stevenson and Smillie, 1970), would probably bind in the aromatic pocket (Baker, 1967) with the carbonyl group adjacent to serine-195. Models showed that with the dihydrocinnamyl group in such a position the dimensions of BPH provided for optimum juxtaposition of a propargylic bromide alkylating group with either target nucleophile. In one preferred conformation, SN2 displacement of bromide by the methylthio group of methionine-192 was favored while in another the situation was equally advantageous for bromide displacement by N-3 of histidine-57. However, since methionine derivatives are alkylated ~100 times faster by propargyl bromide than are the analogous histidine compounds (Hysert, 1971), it was hoped that virtually exclusive modification of methionine-192 by BPH would occur.

The synthesis outlined in Scheme I provided a convenient route to BPH. All the reaction conditions applied are mild and the approach should be generally applicable, with minor modifications, to the introduction of properglyic bromides into a variety of affinity labels. The kinetic studies showed that irreversible inhibition of CT by BPH occurred rapidly and that the reaction was first order with respect to both the CT and BPH concentrations. In addition, the competitive inhibitor dihydrocinnamic acid was found to reduce the rate of CT inactivation and the K_i values (14 and ~10 mM, respectively) for the structurally similar dihydrocinnamic acid and BPH inhibitors were encouragingly close. These data, together with the changes observed in $K_{m,app}$ and k_3 (Figure 3) as the irreversible inhibition proceeded, indicated that BPH inactivation of CT was occurring *via* an active-site-directed mechanism. It is of interest to note that, as expected, the $K_{m,app}$ values of specific substrates increase for all methionine-192-modified CT's for which kinetic data are available but that the corresponding k_3's may be affected positively or negatively. Although it has been suggested that steric factors are involved, the situation regarding changes in k_3 is not well understood (Schramm and Lawson, 1963; Lawson and Schramm, 1965; Knowles, 1965; Baker, 1967; Kézdy *et al.*, 1967b; Kumar and Hein, 1970).

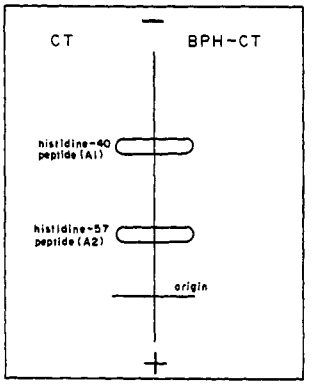


FIGURE 5: Purification of the Pauly-positive His-40 (A1) and His-57 (A2) peptides from native and BPH-modified CT. Hve was effected at pH 3.5 and 2000 V for 1.3 hr. Under these conditions His-57-alkylated peptides should have migrated toward the anode (Stevenson and Smillie, 1968).

The fact that BPH-CT retained 3% of the original catalytic activity and the shallow pH–rate profile (Figure 2) of the inhibition reaction provided the first indications that methionine-192 was being modified since histidine-57 alkylation leads to totally inactive enzyme and is generally much more pH sensitive (Stevenson and Smillie, 1970). As the rate of methionine-192 alkylation should not be affected by pH within the range studied, the variation observed is presumably due to minor conformational changes. Amino acid (Table II) and methionine sulfone (Table III) analyses of BPH-CT showed that 0.7–0.9 methionine residue had been modified and that the two histidine residues were unaffected. Confirmation that the alkylation had occurred exclusively at methionine-192 was provided by diagonal peptide hve of pepsin digests of native and BPH-modified enzyme. The maps obtained (Figure 4) differed only in the methionine-192-containing active serine band region which in the case of BPH-CT was broad and diffuse. Stevenson and Smillie (1970) also noted an increased complexity in the active serine bands of methionine-192-modified enzymes. They attributed this to changes in pepsin specificity induced by the presence of the covalent label and to acid-induced decomposition of the sulfonium salt during the lengthy pepsin digest. These factors, especially the latter, would be expected to be even more marked for BPH-CT in which the acid-sensitive $(CH_3)S^+CH_2C{\equiv}CCO$ group is present. Fortunately, for as well characterized an enzyme as CT, identification of methionine-192 as the site of alkylation was possible without isolation and sequencing of the modified peptide. However, in view of the diffuse nature of the active serine band from BPH-CT, the possibility of serine-195 modification having occurred to a slight extent cannot be excluded.

A comparison of the rate of BPH inactivation of CT to those of other methionine-192-specific reagents for which rate data are available (Table IV) shows that BPH is one of the best affinity labels of this type yet evaluated. Although the rate data included in Table IV were not obtained under

TABLE IV: Comparison of Methionine-192-Specific Inhibitors of CT.

Inhibitor	Solvent (pH)	$k_{obsd}/[I]$ (M^{-1} sec^{-1})[a]	Rel Rates
$C_6H_5(CH_2)_2COC{\equiv}CCH_2Br$(BPH)	10% Me_2SO (7.2)	0.28	100
$C_6H_5CH_2Br$	10% EtOH (5.0)	1.2[c]	428
$C_6H_5COCH_2Br$	10% EtOH (5.0)	0.56[c]	200
$C_6H_5NHCOCH_2Br$	10% EtOH (5.0)	0.28[c]	100
$C_6H_5CH_2NHCOCH_2Br$	10% EtOH (5.0)	0.0028[c]	1
$C_6H_5CH_2CH_2NHCOCH_2Br$	10% EtOH (5.0)	0.14[c]	50
$C_6H_5COCH_2Cl$	5% EtOH (7.2)	0.003[d]	1.1
$C_6H_5CH_2COCH_2Cl$[b]	5% EtOH (7.2)	0.013[d]	4.64
$C_6H_5OCH_2COCH_2Cl$[b]	5% EtOH (7.2)	0.012[d]	4.3

[a] Constant only when $[I] \ll K_i$ (Kumar and Hein, 1970). [b] 0.2–0.3 methionine and 0.3–0.4 histidine residues alkylated.[d] [c] Schramm and Lawson (1963). [d] Stevenson and Smillie (1968, 1970).

the same conditions, and the $k_{obsd}/[I]$ values are not necessarily constants (Kumar and Hein, 1970), the relative rates do in most cases approximate the facilities of alkylation. The fact that BPH reacts so rapidly with methionine-192 provides further indirect evidence that the dihydrocinnamyl group does bind in the aromatic locus as assumed in the initial design process.

The improved orientation resulting from incorporation of an acetylenic bond into BPH has encouraged us to feel that the overall concept may be fairly widely applicable and extensions of the approach to achieve alkylations of selected protein residues exo to the active site of CT are currently being considered. Potential also exists for such systems in the design of rigid reagents for use as structural probes (*cf.* Wold, 1967; Latif and Kaiser, 1969).

The ease with which CT modification was achieved with BPH confirms the earlier indication (Jones and Hysert, 1971) that allylic and propargylic bromides represent useful additions to the list of reagents capable of effecting modification of proteins. In many instances they should complement rather than supplant the more commonly used functions since the selectivities of their reactions with cysteine, histidine, methionine, and lysine residues are markedly different from, for example, α-bromomethylcarbonyl groups (Hysert, 1971).

Acknowledgments

We express our appreciation to Dr. T. Hofmann for his invaluable advice and assistance during this study and to Dr. D. M. Blow for making the X-ray coordinates of CT available to us prior to publication.

References

Albertson, N. F. (1962), *Org. React. 12*, 157.
Baker, B. R. (1967), Design of Active Site-Directed Irreversible Enzyme Inhibitors, New York, N. Y., Wiley, pp 130–144.
Bennett, J. C. (1967), *Methods Enzymol. 11*, 330.
Bittner, E. W., and Gerig, J. T. (1970), *J. Amer. Chem. Soc. 92*, 2114.
Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221*, 337.
Brown, J. R., and Hartley, B. S. (1966), *Biochem. J. 101*, 214.
Canady, W. J., and Laidler, K. J. (1958), *Can. J. Chem. 36*, 1289.
Cunningham, L. (1965), *in* Comprehensive Biochemistry, Vol. 16, Florkin, M., and Stotz, E. H., Ed., Elsevier, New York, N. Y., pp 85–138.
Dent, C. E. (1947), *Biochem. J. 41*, 240.
Dessy, R. E., Okuzumi, Y., and Chen, A. (1962), *J. Amer. Chem. Soc. 84*, 2899.
Gilman, H., and Haubein, A. H. (1944), *J. Amer. Chem. Soc. 66*, 1515.
Glazer, A. N. (1970), *Annu. Rev. Biochem. 39*, 101.
Gomori, G. (1955), *Methods Enymol. 1*, 138.
Gundlach, H. G., Moore, S., and Stein, W. H. (1959), *J. Biol. Chem. 234*, 1761.
Hartley, B. S. (1964), *Nature (London) 201*, 1284.
Hartley, B. S. (1970), *Biochem. J. 119*, 805.
Heilman, J., Barollier, J., and Watzke, E. (1957), *Hoppe-Seyler's Z. Physiol. Chem. 309*, 219.
Henbest, H. B., Jones, E. R. H., and Walls, I. M. S. (1950), *J. Chem. Soc.*, 3646.
Henderson, R. (1970), *J. Mol. Biol. 54*, 341.
Hooz, J., and Gilani, S. S. H. (1968), *Can. J. Chem. 46*, 86.
Hysert, D. W. (1971), Ph.D. Thesis, University of Toronto.
Jacob, T. L., and Petty, W. L. (1963), *J. Org. Chem. 28*, 1360.
Jones, J. B., and Hysert, D. W. (1971), *Can. J. Chem. 49*, 3012.
Kézdy, F. J., Feder, J., and Bender, M. L. (1967b), *J. Amer. Chem. Soc. 89*, 1009.
Kézdy, F. J., Thompson, A., and Bender, M. L. (1967a), *J. Amer. Chem. Soc. 89*, 1004.
Knowles, J. R. (1965), *Biochem. J. 95*, 180.
Kumar, S., and Hein, G. E. (1969), *Anal. Biochem. 30*, 203.
Kumar, S., and Hein, G. E. (1970), *Biochim. Biophys. Acta 206*, 404.
Latif, N., and Kaiser, E. T. (1969), *J. Org. Chem. 34*, 3653.
Lawson, W. B., and Schramm, H. J. (1965), *Biochemistry 4*, 377.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1*, 68.
Neurath, H., and Schwert, G. W. (1950), *Chem. Rev. 46*, 69.
Schmidt, U., and Schwochau, M. (1964), *Chem. Ber.*, 1649.
Schramm, H. J., and Lawson, W. B. (1963), *Hoppe-Seyler's Z. Physiol. Chem. 332*, 97.
Schwert, G. W. (1969), *J. Biol. Chem. 244*, 1278.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Shaw, E. (1970), *Physiol. Rev. 50*, 244.

Sigman, D. S., Torchia, D. A., and Blout, E. R. (1969), *Biochemistry 8*, 4560.
Singer, S. J. (1967), *Advan. Protein Chem. 22*, 1.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46*, 337.
Stevenson, K. J., and Smillie, L. B. (1968), *Can. J. Biochem. 46*, 1357.
Stevenson, K. J., and Smillie, L. B. (1970), *Can. J. Biochem. 48*, 364.
Wold, F. (1967), *Methods Enzymol. 11*, 617.

Kinetic Spectroscopic Studies of Substrate and Subunit Interactions of Tryptophan Synthetase†

Sheldon S. York‡

ABSTRACT: The catalytic properties of the β_2 protein of *Escherichia coli* tryptophan synthetase are markedly altered by the α protein or high concentrations of NH_4^+ ion. The effects of these interactions upon the reaction pathways for the deamination of L-serine and the synthesis of *S*-hydroxyethyl-L-cysteine were investigated by stopped-flow kinetic methods. This study was facilitated by the pyridoxal phosphate prosthetic groups of the β_2 protein, which interact with the substrates to form three spectrally distinct reaction intermediates: the aqua complex fluoresces at 500 nm, the pale species absorbs at 330 nm, and the amber complex absorbs at 470 nm. The results suggest that the aqua, pale, and amber species are sequential intermediates in both the deaminase and synthetase pathways. The α protein and NH_4^+ ion influence the rates of transformations of these species, and thereby alter the catalytic properties of the β_2 protein. Both β_2-catalyzed reactions are rate limited by the disappearance of the aqua species. This step is so greatly accelerated by the $\alpha_2\beta_2$ protein that it no longer limits the rate, and aqua fluorescence appears only transiently before the enzymatic steady state is established. However, the $\alpha_2\beta_2$ protein cannot deaminate L-serine because the pale species that forms with L-serine alone is unreactive. In contrast, the $\alpha_2\beta_2$-catalyzed synthesis of *S*-hydroxyethyl-L-cysteine is rate limited by the disappearance of the amber species. Each of the NH_4^+-β_2-catalyzed reactions is rate limited by two steps. The rate data for these reactions predict turnover numbers which agree with experimental values.

Tryptophan synthetase from *Escherichia coli* is a particularly interesting enzyme because it displays striking effects of subunit interaction upon catalytic activity and specificity (for a review, see Yanofsky and Crawford, 1972). The physiological reaction catalyzed by the fully associated $\alpha_2\beta_2$ enzyme complex (Crawford and Yanofsky, 1958) is the formation of L-tryptophan from indole-3-glycerol phosphate and L-serine (reaction 1, Table I). *In vitro*, the $\alpha_2\beta_2$ enzyme and its dissociated α and β_2 subunits catalyze a variety of reactions (Table I). The $\alpha_2\beta_2$ complex catalyzes reactions 2 and 3 more than tenfold faster than do the isolated subunits (Crawford and Yanofsky, 1958; Miles *et al.*, 1968). Furthermore, the $\alpha_2\beta_2$ complex is a more specific enzyme than the β_2 subunit. The deamination of L-serine (reaction 5) and the transamination of pyridoxal phosphate (reaction 6) by the β_2 subunit are completely inhibited on addition of a stoichiometric amount of α subunit (Crawford and Ito, 1964; Miles *et al.*, 1968).

The elucidation of these subunit interactions is dependent on a detailed knowledge of the reaction pathway. The study of catalytic intermediates is facilitated by the presence of pyridoxal phosphate, which serves as a cofactor in reactions 1 and 3–5 , and as a substrate in reaction 6. The absorption and fluorescence properties of this coenzyme are responsive to substrate and subunit interactions. Three potential intermediates in these enzymatic reactions have been identified in steady-state spectral studies of the pyridoxal phosphate group.

(1) The *aqua* species (Goldberg *et al.*, 1968), which exhibits a strong fluorescence emission at 500 nm, appears when L-serine is added to the β_2 protein. The absorption band of the aqua species is centered at 420 nm.

(2) The 330-nm absorbing species (Miles *et al.*, 1968), which we call the *pale* species, is formed on addition of L-serine to the $\alpha_2\beta_2$ complex.

(3) The *amber* species (Goldberg and Baldwin, 1967), which absorbs maximally at 470 nm, is formed when the $\alpha_2\beta_2$ complex catalyzes synthetase reactions.

Another interesting aspect of this enzyme is that high concentrations of NH_4^+ ion (of the order of 1 M) can substitute for the α subunit in the formation of the amber (Goldberg and Baldwin, 1967) and pale species (York, 1970). NH_4^+ ion also mimics the α subunit in enhancing the synthetase activities of the β_2 subunit (Hatanaka *et al.*, 1962) and in inhibiting the transamination of pyridoxal phosphate (Miles *et al.*, 1968).

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received March 1, 1972.* This work was supported by grants from the National Science Foundation (GB-27408X) and the National Institutes of Health (GM-16708). This work was initiated in the Department of Biochemistry at the Stanford University School of Medicine, and includes portions of a Dissertation submitted by Sheldon S. York in partial fulfillment of the requirements for a Ph.D. degree at Stanford University, August 1970. A preliminary account was presented at the 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 7–12, 1969.

‡ Predoctoral fellow of the National Science Foundation. Present address: Department of Chemistry, University of Denver, Denver, Colo. 80210.