

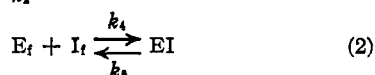
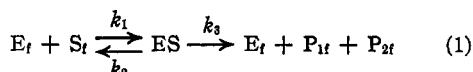
[CONTRIBUTION NO. 1469 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Enzyme-Inhibitor Dissociation Constants of α -Chymotrypsin and Several Competitive Inhibitors of the Unnatural or D-Configuration¹

BY H. T. HUANG AND CARL NIEMANN²

An investigation of the inhibition of the α -chymotrypsin-catalyzed hydrolysis of acetyl-L-tryptophanamide, nicotinyl-L-tryptophanamide, acetyl-L-tyrosinamide or nicotinyl-L-tyrosinamide at 25° and pH 7.9 by acetyl-D-tryptophanamide, nicotinyl-D-tyrosinamide or nicotinyl-D-phenylalaninamide has shown that the inhibition is competitive in nature, all of the above substrates and competitive inhibitors react *via* combination with the enzyme at the same reactive site and that for competitive inhibitors of the acylated α -amino acid amide type possessing the D-configuration, the affinity of the enzyme for the inhibitor, in respect to the side chains of the latter, is in the order β -indolylmethyl \gg *p*-hydroxybenzyl > benzyl.

For the system³



where $k_2 + k_3/k_1 = K_I$ and $k_5/k_4 = K_I$ it follows that competitive inhibition by the inhibitor *I* is established when (a) a family of lines obtained by a plot of the reciprocal of the initial velocity ($1/v_0$) versus the reciprocal of the initial substrate concentration ($1/[S]_0$) for various values of $[I]$ are found to have a common intercept and differ only in respect to slope, the latter increasing, from the condition where $[I] = 0$ by the amount $K_S/K_I [I]$, with increasing values of $[I]$ ⁴; and (b) the value of K_I obtained for any given inhibitor is independent of the nature of the substrate used for its evaluation. Although it is usually assumed that the first criterion is sufficient to establish competitive inhibition by the inhibitor *I*, in order to increase the probability that the reactions given in equations 1 and 2 are for all practical purposes an accurate representation of the *initial* stages of an α -chymotrypsin catalyzed hydrolysis of a substrate in the presence of an added competitive inhibitor⁵ the enzyme-inhibitor dissociation constants of three inhibitors of α -chymotrypsin were evaluated, one with three different substrates and the remaining two with two different substrates. In addition, it was recognized that these experiments would provide useful information in respect to the accuracy of the various K_S and K_I values in the event that K_S was found to be an independent variable in the evaluation of K_I . Further, because of the nature of the substrates and inhibitors employed it would be possible, if the inhibition was found to be competitive in nature, not only to demonstrate that substrates derived from L-tryptophanamide and L-tyrosinamide and competitive inhibitors derived from D-tryptophanamide, D-tyrosinamide and D-phenylalaninamide react with α -chymotrypsin *via* combination at the

same reactive site but also to quantitatively compare the affinity of the enzyme for the respective α -amino acid side chains when present in an acylated α -amino acid amide possessing the D-configuration.

From the data given in Figs. 1-4,⁶ and in Table I and with the knowledge that all constants were evaluated under conditions that placed the systems in zone A of Straus and Goldstein^{7,8} it is evident that acetyl-D-tryptophanamide, nicotinyl-D-tyrosinamide and nicotinyl-D-phenylalaninamide are competitive inhibitors of α -chymotrypsin and that their respective enzyme-inhibitor dissociation constants are independent of the nature of the substrates used for their evaluation. It also follows from these data that the values of K_S and K_I reported in this communication and based in part upon earlier investigations^{3,9} are accurate to within the limits previously specified,³ *i.e.*, approximately $\pm 5\%$.

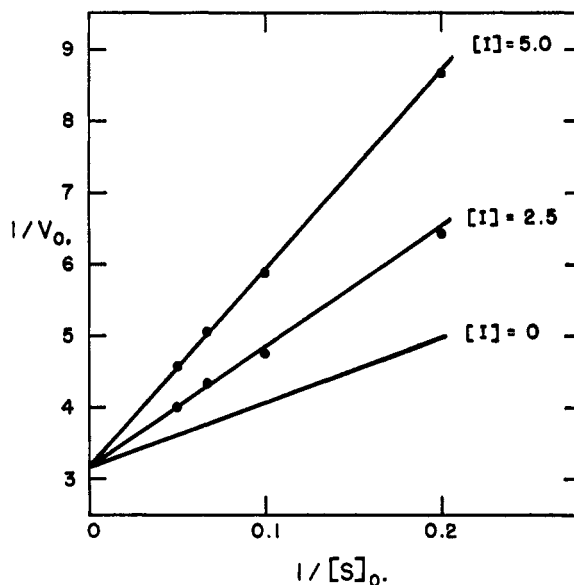


Fig. 1.—Substrate, nicotinyl-L-tryptophanamide; inhibitor, acetyl-D-tryptophanamide; $[E]$, 0.208 mg. of protein-nitrogen per ml.; $[S]$, $[I]$ in units of 10^{-3} M.

With the demonstration that acetyl- or nicotinyl-, D-tryptophanamide, D-tyrosinamide and

(1) Supported in part by a grant from Eli Lilly and Co.
 (2) To whom inquiries regarding this article should be sent.
 (3) Cf., H. T. Huang and C. Niemann, *THIS JOURNAL*, **73**, 1541 (1951), for definitions of symbols used in this communication.
 (4) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).
 (5) The fact that one of the hydrolysis products, *i.e.*, the acylated α -amino acid is known to be a competitive inhibitor of the hydrolytic reaction³ has no practical significance because it is experimentally impossible, at least with present methods, to detect such inhibition in the initial stages of the hydrolytic reaction.

(6) For data relative to the system, α -chymotrypsin, acetyl-L-tryptophanamide, acetyl-D-tryptophanamide, see ref. 3, Fig. 6.
 (7) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1948).
 (8) A. Goldstein, *ibid.*, **27**, 529 (1944).
 (9) D. W. Thomas, R. V. MacAllister and C. Niemann, *THIS JOURNAL*, **73**, 1548 (1951).

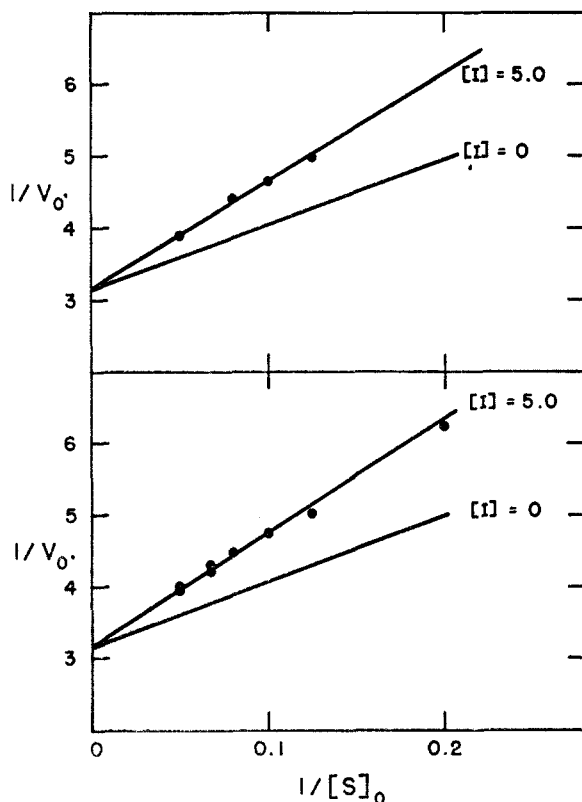


Fig. 2.—Both curves, substrate, nicotinyl-L-tryptophanamide; [E] 0.208 mg. of protein-nitrogen per ml.; [S], [I] in units of 10^{-3} M; upper curve, inhibitor, nicotinyl-D-phenylalaninamide; lower curve, inhibitor, nicotinyl-D-tyrosinamide.

D-phenylalaninamide are competitive inhibitors of the α -chymotrypsin catalyzed hydrolysis of acetyl- or nicotinyl-, L-tryptophanamide and L-tyrosinamide (cf. Table I) it follows that substrates and competitive inhibitors of the general formula $RCONHCHR_1COR_2$, where R = methyl and β -pyridyl, $R_1 = \beta$ -indolylmethyl, p -hydroxybenzyl and benzyl, and $R_2 = -NH_2$, react with α -

TABLE I
ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF SEVERAL COMPETITIVE INHIBITORS OF α -CHYMOTRYPSIN^a

Substrates	Inhibitors					
	Acetyl-D-tryptophanamide [I] ^b	K_I ^b	Nicotinyl-D-tyrosinamide [I] ^b	K_I ^b	Nicotinyl-D-phenylalaninamide [I] ^b	K_I ^b
Acetyl-L-tryptophanamide ^c	2.5	2.7 ^c				
	5.0	2.9				
	7.5	2.4				
	10.0	2.3 ^d				
Nicotinyl-L-tryptophanamide ^e	2.5	2.5	5.0	6.0	5.0	6.8
	5.0	2.9				
Acetyl-L-tyrosinamide ^f	2.5	2.7				
Nicotinyl-L-tyrosinamide ^g			5.0	6.3	5.0	7.1
		2.7 ^h		6.2 ^h		7.0 ^h

^a At 25° and pH 7.9. ^b $\times 10^3$ M. ^c Duplicate determination, both values 2.7. ^d Not included in average. ^e [E] = 0.208 mg. protein-nitrogen per ml. ^f [E] = 0.139 mg. protein-nitrogen per ml. ^g 0.047 mg. protein nitrogen per ml. ^h $\pm 5\%$.

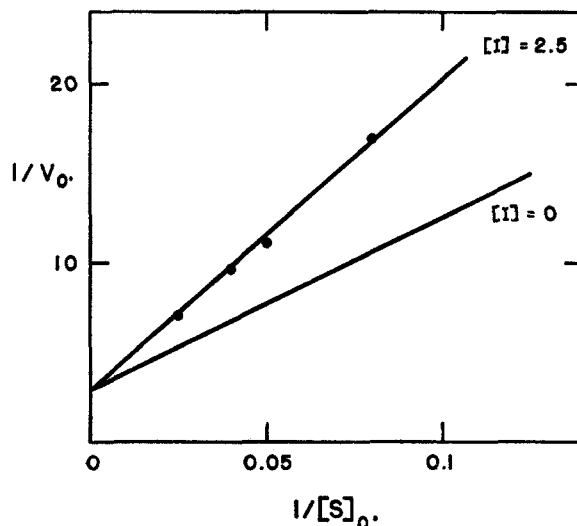


Fig. 3.—Substrate, acetyl-L-tyrosinamide; inhibitor, acetyl-D-tryptophanamide; [E] 0.139 mg. of protein-nitrogen per ml.; [S], [I] in units of 10^{-3} M.

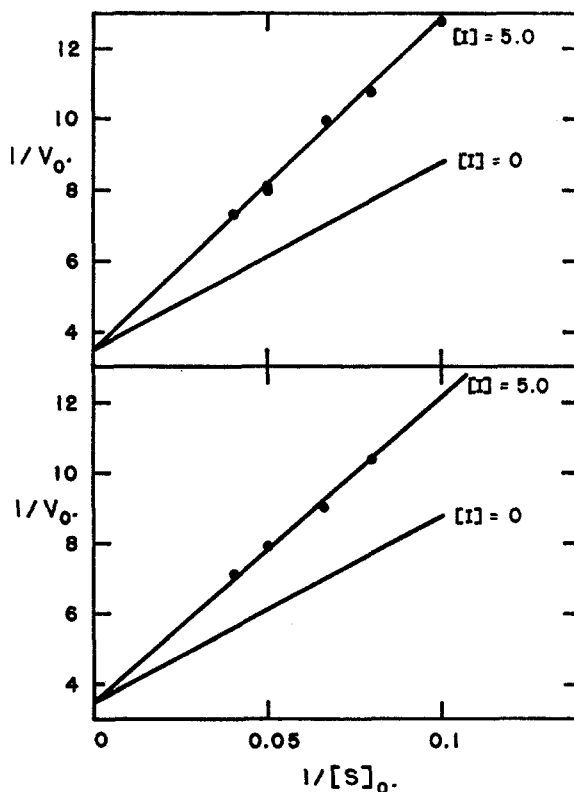


Fig. 4.—Both curves, substrate, nicotinyl-L-tyrosinamide; [E] 0.047 mg. of protein-nitrogen per ml.; [S], [I] in units of 10^{-3} M; upper curve, inhibitor, nicotinyl-D-tyrosinamide; lower curve, inhibitor, nicotinyl-D-phenylalaninamide.

chymotrypsin *via* combination with the enzyme at the same reactive site irrespective of the configuration about the asymmetric carbon atom of the above substrates and competitive inhibitors. On the basis of the observed reaction kinetics of the α -chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide in the presence of two competitive inhibitors, *i.e.*, nicotinyl-L-tryptophan

and nicotinyl-D-tryptophanamide,⁸ it is clear that the above generalization can be extended to the case where $R_2 = -O^-$, and from the kinetics of the competitive inhibition of the α -chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide by acetyl-D-tyrosine ethyl ester,⁹ to the case where $R_2 = -OC_2H_5$. It will be noted that the above conclusion is in accord with independent evidence which indicates that α -chymotrypsin contains but one catalytically active site per molecule, in respect to esterase and proteinase activities,^{10,11} and with the demonstration, based upon competitive hydrolyses, that acetyl-L-tryptophanamide and acetyl-L-tyrosinamide are hydrolyzed in the presence of α -chymotrypsin *via* combination with the enzyme at the same reactive site.¹² In view of the above evidence it is surprising that with substrates such as acetyl- and nicotinyl-L-tryptophanamide and acetyl- and nicotinyl-L-tyrosinamide α -chymotrypsin exhibits such strikingly different pH-activity relationships.^{3,9}

From the values given in Table II for K_I , the enzyme-inhibitor dissociation constant, and for ΔF° , the standard free energy change for the equilibrium reaction described by equation 2, it can be concluded without any ambiguity that for acylated α -amino acid amides possessing the D-configuration the affinity of α -chymotrypsin for the respective α -amino acid side chains is in the order β -indolylmethyl \gg *p*-hydroxybenzyl $>$ benzyl. Kaufman and Neurath^{13,14} have proposed the order *p*-hydroxybenzyl $>$ benzyl $>$ β -indolylmethyl $>$ β -thiomethylethyl principally on the basis of K_S values. Because of the uncertainty as to the degree to which $K_S \approx k_2/k_1$ in each particular case it is clear that the proposal of these authors cannot be accepted on the basis of present evidence.

TABLE II

STANDARD FREE ENERGY RELATIONSHIPS OF SEVERAL COMPETITIVE INHIBITORS OF α -CHYMOTRYPSIN^a

Competitive inhibitor	K_I^b	$-\Delta F^\circ^c$
Acetyl-D-tryptophanamide	2.7	3500
Acetyl-D-tyrosinamide ^d	12.0	2620
Nicotinyl-D-tryptophanamide ^e	1.4	3890
Nicotinyl-D-tyrosinamide	6.2	3010
Nicotinyl-D-phenylalaninamide	7.0	2930

^a At 25° and pH 7.9. ^b $\times 10^3 M$. ^c In calories per mole to the nearest 10 calories. ^d Cf. ref. 9. ^e Cf. ref. 3.

In the absence of a more reliable index the ΔF° values given in Table II may be provisionally accepted as a measure of the bonding energy of enzyme and inhibitor.¹⁵ Comparing the ΔF° values for acetyl-D-tryptophanamide and acetyl-D-tyrosinamide and for nicotinyl-D-tryptophanamide and nicotinyl-D-tyrosinamide it is seen that replacement of a *p*-hydroxybenzyl group by a β -

indolylmethyl group under the conditions specified is accompanied by an increase in $-\Delta F^\circ$ of 880 calories. In contrast, replacement of a benzyl group by a *p*-hydroxybenzyl group in the corresponding nicotinyl-D- α -amino acid amide is accompanied by an increase in $-\Delta F^\circ$ of only 80 calories. These comparisons clearly illustrate the marked affinity of α -chymotrypsin for compounds possessing a β -indolylmethyl side chain.

In a previous communication⁸ it was shown that nicotinyl-L-tryptophanamide and nicotinyl-D-tryptophanamide are bonded more firmly to α -chymotrypsin than are acetyl-L-tryptophanamide and acetyl-D-tryptophanamide. From the ΔF° values of acetyl-D-tryptophanamide and nicotinyl-D-tryptophanamide and of acetyl-D-tyrosinamide and nicotinyl-D-tyrosinamide it appears that replacement of a methyl group by a β -pyridyl group, in a competitive inhibitor of the acylated-D- α -amino acid amide type and under the conditions specified, is accompanied by an increase in $-\Delta F^\circ$ of 390 calories.

Experimental^{16,17}

Nicotinyl-DL-tyrosine Ethyl Ester (I).—Acylation of 2.5 g. of DL-tyrosine ethyl ester with 2.0 g. of nicotinyl chloride¹⁸ in ethyl acetate in the presence of dilute aqueous sodium bicarbonate gave 3.3 g. of I, fine needles, m.p. 141°, after three recrystallizations from ethyl acetate.

Anal. Calcd. for $C_{17}H_{18}O_4N_2$ (314): C, 65.0; H, 5.8; N, 8.9. Found: C, 65.0; H, 5.9; N, 8.9.

Nicotinyl-D-tyrosine Ethyl Ester (II).—To a suspension of 2.25 g. of I in 120 ml. of water and 30 ml. of methanol, contained in a beaker thermostated at 30° and fitted with a stirrer and electrodes, was added 20 mg. of α -chymotrypsin and the pH of the reaction mixture maintained at 7.9 by the addition of 0.01 *N* aqueous sodium hydroxide. Although the reaction was apparently completed in 15 minutes the reaction mixture was stirred for an additional 2 hours and allowed to stand overnight at 25°. The solution was evaporated *in vacuo* to one-half of its original volume, the precipitate collected, dried and recrystallized twice from ethyl acetate to give 1.1 g. of II, glistening stunted needles, m.p. 148–149°, $[\alpha]^{25}_D + 42 \pm 1^\circ$ (c, 1% in methanol).

Anal. Calcd. for $C_{17}H_{18}O_4N_2$ (314): C, 65.0; H, 5.8; N, 8.9. Found: C, 65.1; H, 5.8; N, 8.8.

Nicotinyl-L-tyrosine (III).—The aqueous mother liquor remaining after the isolation of II was evaporated to 30 ml., in a current of air, and acidified to pH 5 with 1.0 *N* hydrochloric acid. The gummy precipitate was thoroughly extracted with ethyl acetate (5 \times 30 ml.) and the non-aqueous phase dried over calcium sulfate. Removal of the solvent gave an oily residue which was induced to crystallize by rubbing with a warm ethyl acetate–ether mixture. This product was recrystallized twice from ethyl acetate and thrice from water to give 0.3 g. of III, tiny thin needles, m.p. 192°, $[\alpha]^{25}_D + 5.5 \pm 0.5^\circ$ (c, 1.82% in water containing an equivalent amount of sodium hydroxide), $[\alpha]^{25}_D - 41 \pm 1^\circ$ (c, 1% in methanol).

Anal. Calcd. for $C_{16}H_{14}O_4N_2$ (286): C, 63.0; H, 4.9; N, 9.8. Found: C, 63.3; H, 4.8; N, 9.5.

Nicotinyl-D-tyrosinamide (IV).—Ammonolysis of 0.7 g. of II in methanolic ammonia gave 0.50 g. of IV, short stunted needles, m.p. 226–227°, after recrystallization from water, $[\alpha]^{25}_D + 34.0 \pm 1^\circ$ (c, 0.5% in methanol).

Anal. Calcd. for $C_{16}H_{16}O_3N_2$ (285): C, 63.2; H, 5.3; N, 14.7. Found: C, 63.2; H, 5.4; N, 14.7.

Nicotinyl-L-tyrosinamide (V).—V, $[\alpha]^{25}_D - 33.5 \pm 1^\circ$ (c, 1% in methanol), was prepared as described previously.¹⁹

(16) All melting points are corrected.

(17) Microanalyses by Dr. A. Elek.

(18) F. Laufranchi, *Atti acad. italia, rend.*, **3**, 103 (1941); *C. A.*, **41**, 1001a (1947).

(19) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, *This Journal*, **72**, 1729 (1950).

(10) E. Jansen, M. D. Fellows-Nutting, P. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(11) E. Jansen, M. D. Fellows-Nutting and A. K. Balls, *ibid.*, **179**, 201 (1949).

(12) R. J. Foster and C. Niemann, *This Journal*, **73**, 1552 (1951).

(13) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).

(14) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 437 (1949).

(15) It is difficult to evaluate the reliability of this supposition because of the lack of data in respect to the relative contributions of ΔH° and ΔS° to ΔF° for each particular case.

Nicotinyl-D-phenylalaninamide (VI).—To a solution of 0.54 g. of nicotinyl-DL-phenylalaninamide¹⁸ in 200 ml. of water containing 5 ml. of 0.5 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer was added 50 mg. of α -chymotrypsin and the reaction mixture maintained at 25° for 2 days. The solution was then evaporated *in vacuo* to 60 ml. and the crystalline precipitate collected to give 0.20 g. of VI, fine long needles, m.p. 187°, after recrystallization from water, $[\alpha]_D^{25} + 37 \pm 1^\circ$ (c, 1% in methanol).

Anal. Calcd. for $C_{15}H_{18}O_2N_3$ (269): C, 66.9; H, 5.6; N, 15.6. Found: C, 66.7; H, 5.7; N, 15.7.

Nicotinyl-L-phenylalanine (VII).—The mother liquor remaining after the isolation of VI was acidified with *N* hydrochloric acid and evaporated to dryness *in vacuo*. The residue was extracted with acetone, the acetone extract evaporated to dryness, the residue induced to crystallize by rubbing with water and the product recrystallized twice from water to give 0.12 g. of VII, stunted needles, m.p. 177–178°, $[\alpha]_D^{25} - 45 \pm 1^\circ$ (c, 0.67% in methanol).

Anal. Calcd. for $C_{15}H_{14}O_2N_2$ (270): C, 66.7; H, 5.2; N, 10.4. Found: C, 66.4; H, 5.3; N, 10.2.

Enzyme Experiments.—The methods used were identical with those described previously.³ All experiments were conducted at 25° and pH 7.9 in aqueous solution 0.02 M in respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. The K_1 values given in Table I are based upon the following K_8 values, acetyl-L-tryptophanamide, 5.3³; nicotinyl-L-tryptophanamide, 2.7³; acetyl-L-tyrosinamide, 30.5³; nicotinyl-L-tyrosinamide, 15.0,³⁰ all $\times 10^{-8}$ M. In the experiments where acetyl-L-tryptophanamide was used as a substrate³ it was shown that the experimental conditions were such as to permit the reaction to proceed under zone A conditions.^{7,8} From the data given in Figs. 1–4 it can be shown that this is also true for all of the experiments reported in this communication. The α -chymotrypsin used in this study was an Armour preparation, lot no. 90402, of bovine origin.

(20) This value is based upon unpublished data obtained in these laboratories by R. V. MacAllister, D. W. Thomas and H. T. Huang. An account of this work will be given in the near future.

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[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

An Unusual Twofold Wagner–Meerwein Rearrangement

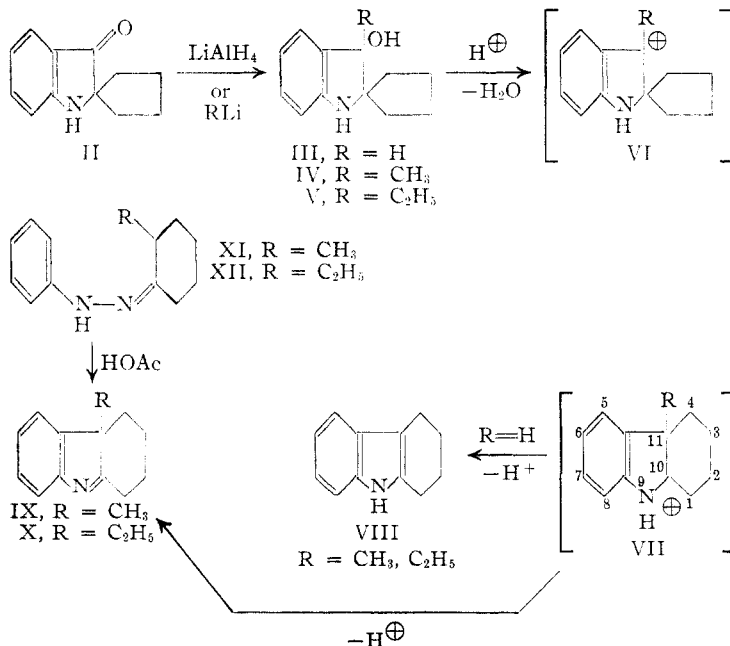
By BERNHARD WITKOP¹ AND J. B. PATRICK^{1,2}

Alkyl lithium compounds react with *spiro*-(cyclopentane-1,2'- ψ -indoxyl) (II) to give tetrahydrocarbazolenines (IX, X) with angular substituents at position 11. The reaction of II with Grignard reagents leads to *spiro*-(cyclopentane-1,3'-indoles) (XXIII, XXIV) with substituents at position 2 via a twofold successive Wagner–Meerwein rearrangement. The intermediate in the latter reaction is 11-hydroxytetrahydrocarbazolenine (I, or the Grignard complex XIX). These differences in the mode of reaction of the two organometallic reagents are discussed in terms of possibly *intramolecular* (lithium) and *intermolecular* (magnesium) rearrangements of the initial coordination complexes (XXX, XXXI).

We have dealt previously with the chemistry³ and kinetics⁴ of the rearrangements of 11-hydroxytetrahydrocarbazolenine under the influence of acids and bases⁵ as well as with its important role as an intermediate in the oxidation of indole compounds.^{6,7} As an extension of these investigations we are describing in this paper the reactions of 11-hydroxytetrahydrocarbazolenine (I) and of *spiro*-(cyclopentane-1,2'-*pseudo* indoxyl) (II) with lithium and magnesium organic reagents. It was found that II reacts with methyl lithium to give the tetrahydrocarbazolenine IX, and with methylmagnesium iodide to yield the indolenine XXIII.

The reaction of the yellow spiran (II) with alkyl lithium would be expected to be analogous to the reduction with lithium aluminum hydride to the colorless alkamine (III)⁸ and should lead to the carbinols IV or V. Owing to steric hindrance of the carbonyl group in II and its conjugation with the imino group it is not surprising that about 80 to 90% of II can be recovered from the reaction mixture. In the case of III acid leads to carbonium intermediates VI and VII (R = H), the

latter losing a proton at position 11 to yield tetrahydrocarbazole. Likewise, the carbinols IV and



V which were not isolated probably underwent reaction through the same intermediates (VI and VII, R = CH₃ and C₂H₅). However, the proton lost from structure VII has to come from the nitrogen atom at position 9. The resulting indolenines, 11-methyl- and 11-ethyltetrahydrocarbazolenines, easily isolable because of their marked basicity, prove to be identical with synthetic

- (1) National Heart Institute, Bethesda 14, Md.
- (2) Research Corporation Fellow, 1950.
- (3) Witkop and Patrick, *Experientia*, **6**, 183 (1950).
- (4) Witkop and Patrick, *This Journal*, **73**, 713 (1951).
- (5) Patrick and Witkop, *ibid.*, **72**, 633 (1950).
- (6) Witkop, *ibid.*, **72**, 1428 (1950).
- (7) Witkop, *ibid.*, **72**, 2311 (1950).
- (8) Witkop, *ibid.*, **72**, 614 (1950).