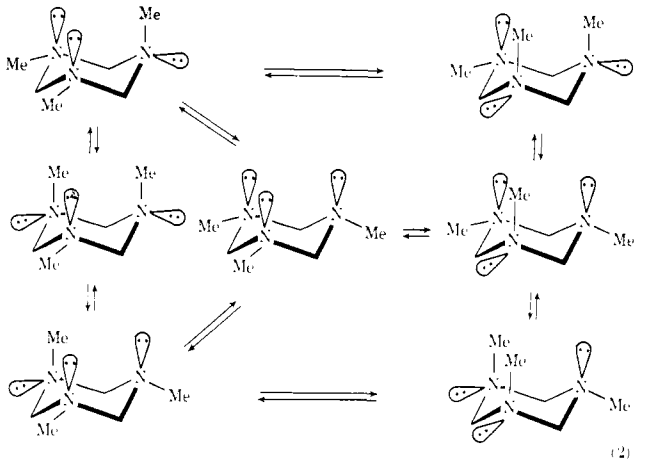
or triequatorial intermediates which then invert at *one* nitrogen to give stable monoaxial forms (eq 2). Simul-



(2)

taneous inversion at two or three nitrogens is not statistically likely. Indeed, to invert *two* nitrogens simultaneously would require an increase in potential energy due to rehybridization at least twice the barrier to nitrogen inversion for a simple unconstrained acyclic trialkylamine (*e.g.*, dibenzylmethylamine, $\Delta H^{\pm} = 7.2 \pm 0.4$ kcal/mol)[8] or about 14 kcal/mol *in addition to* the angle strain associated with the six-ring formation in the transition state. Such a high barrier is clearly not consistent with the nitrogen inversion data presented here.

Although ring reversal had been observed previously,[9] the same general type of dnmr spectral changes as seen in **1** were observed for the CH_2 resonance of *N,N',N''*-tri-*tert*-butyl-1,3,5-triazane (**6**, 5% v/v in CH_2CHCl) at substantially lower temperatures (−140 to −160°) than for **1** consistent with slowing nitrogen inversion and a significant population of axial *N-tert*-butyl.[1c] The *N-tert*-butyl resonance of **6** also changed in a complex fashion consistent with slowing both nitrogen inversion and *tert*-butyl rotation. The broad dnmr lines observed for **6** at low temperatures and the apparent inability to reach slow exchange conditions precluded an accurate measure of axial *N-tert*-butyl population. Other effective solvent systems are being sought.

Acknowledgment. We are grateful to the National Science Foundation (Grant No. GP-18197) for support.

(8) C. H. Bushweller, J. W. O'Neil, and H. S. Bilofsky, *Tetrahedron*, **28**, 2697 (1972).
(9) J. M. Lehn, F. G. Riddell, B. J. Price, and I. O. Sutherland, *J. Chem. Soc. B*, 387 (1967).
(10) Alfred P. Sloan Research Fellow, 1971–1974; Camille and Henry Dreyfus Teacher–Scholar, 1972–present.
(11) NDEA Title IV Fellow, 1971–1974.

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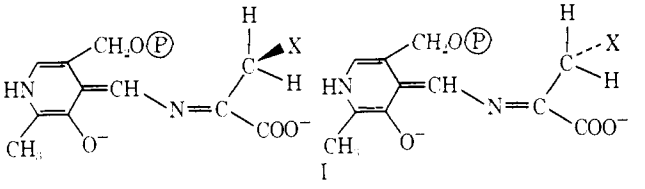
Stereochemistry of the Tryptophan Synthetase Reaction

Sir:

We wish to report on the determination of the steric course of the tryptophan synthetase-catalyzed substitution reaction at C-3 of serine. Tryptophan synthetase (E.C. 4.2.1.20)[1] belongs to a group of pyridoxal phosphate-containing enzymes, which catalyze nucleophilic β-substitution reactions and/or α,β-elimination reactions of certain amino acids. These reactions are thought[2] to proceed *via* a ketimine intermediate (I) which then undergoes elimination of the electronegative β-substituent to give an enzyme-bound α-aminoacrylate–pyridoxal phosphate Schiff base. Addition of a new nucleophilic substituent at the β-carbon followed by reversal of the process constitutes the enzymatic substitution reaction, whereas hydrolysis of the Schiff base ultimtely leads to pyruvate and ammonia (α,β-elimination reaction). In the removal and addition of the β-substituent, orientation of both these ligands perpendicular to the plane of the π system is required in order to optimize interaction of the electron pair of the σ-bond to be broken or formed with the electrons of the extended π system. Thus, reaction should only be possible in the two conformations shown.



I

The incoming and the outgoing substituent can orient either on opposite faces of the double bond plane, resulting in reaction with inversion of configuration at C-3 of the amino acid, or on the same face, leading to retention of configuration. In the latter case the reaction must either proceed by a ping-pong mechanism[3] or it must involve a significant conformational change of the enzyme as part of the catalytic process. As a variation of this mechanism, Braunstein[4] has suggested that the replacement of the hydroxyl group of serine by indole in the tryptophan synthetase reaction involves an SN2 process, which of necessity would result in inversion of configuration.

To study this question (2*S*,3*R*)- and (2*S*,3*S*)-3-phosphoglyceric-*3-t* acid (specific activity >100 μCi/μmol), available from earlier work,[5] were converted into phosphoserines using an enzyme preparation from *E. coli* and essentially the conditions given by Pizer.[6] The phosphoserines were hydrolyzed with alkaline phosphatase directly in the reaction mixture and the serine samples were isolated by adsorption on Dowex 50 H^+ and elution with 1.5 *M* NH_4OH and purified by paper chromatography in *n*-butyl alcohol:acetic acid:water (35:10:25) (yields 30–50%). They were then mixed with L-serine-*U*-^{14}C to give T/^{14}C ratios of 3.70 and 3.31 (3*R* and 3*S* isomer, respectively) and incubated with purified native tryptophan synthetase from *N. crassa*.[7] Indole was used as the second substrate,

(1) C. Yanofsky and I. P. Crawford in "The Enzymes," Vol. VII, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1972, pp 1–31
(2) L. Davis and D. E. Metzler, ref 1, pp 33–74.
(3) W. W. Cleland, *Biochim. Biophys. Acta*, **67**, 104 (1963).
(4) A. E. Braunstein in "The Enzymes," Vol. II, 2nd ed, P. D. Boyer, H. Lardy, and K. Myrbäck, Ed., Academic Press, New York, N. Y., 1960, pp 113–184.
(5) H. G. Floss, D. K. Onderka, and M. Carroll, *J. Biol. Chem.*, **247**, 736 (1972).
(6) L. I. Pizer, *J. Biol. Chem.*, **238**, 3934 (1963).
(7) R. G. Meyer, J. Germershausen, and S. R. Suskind, *Methods Enzymol.*, **17**, 406 (1970); C. Yanofsky, *ibid.*, **2**, 233 (1955).

Table I[a]

Entry no.	Product analyzed	Tryptophan obtained enzymatically from (3R)-Serine-3-t T/^{14}C	(3S)-Serine-3-t T/^{14}C	Synthetic (3R)-Tryptophan-3-t T/^{14}C	Synthetic (3S)-Tryptophan-3-t T/^{14}C
1	Starting tryptophan	2.10	2.06	*c*	*c*
2	Aspartate	1.86	1.63	4.05	5.00
3	Malate (enzymatically from 2)	2.28	2.17	4.58	4.22
4	Fumarate + malate (enzymatically from 2 in presence of fumarase)	0.33	1.68	3.55	0.34
5	Malate (from 2 with HNO_2)	2.06	1.96	4.28	4.31
6	Fumarate (from 5 with fumarase)			3.58	0.30
7	Fumarate + malate (from 5 with fumarase)	0.91[b]	1.91[b]		

[a] Degradation of tryptophan stereospecifically tritiated at C-3 of the side chain to determine the configuration at the labeled carbon atom. [b] Insufficient material to isolate fumarate from the mixture. The results are as expected if the malate from the diazotation reaction is racemic at C-2. [c] Not available.

and the conditions were optimized for essentially quantitative conversion of serine to tryptophan. The two samples of the latter were isolated and purified by paper chromatography (*n*-butyl alcohol:acetic acid: H_2O 35:10:25 and isopropyl alcohol:concentrated NH_4OH:H_2O 80:2:18) and their T/^{14}C ratios were determined (3.68 and 3.38, corresponding to 99.5 and 102% T retention, respectively). To check on the degree of stereospecific labeling at C-3, both samples were converted into the antibiotic indolmycin by feeding them to cultures of *Streptomyces griseus* ATCC 12648.[8] In this biosynthetic conversion, one of the two methylene hydrogens of tryptophan is replaced by a methyl group[9] and the experiments did show that, as expected, one of the tryptophan samples retained all of its tritium (98.5, 104.0%) whereas the other lost all of its tritum (7.7, 3.1% retention). Thus, both the synthesis of the serines and their conversion into tryptophan had proceeded completely stereospecifically and, at least in the latter case, without loss of tritium.

To determine the configuration at the labeled center, samples of tryptophan obtained from (3*R*)- and (3*S*)-serine-*3-t* were mixed with 5 mg of unlabeled *L*-tryptophan and about 2 μCi of *L*-tryptophan-(*alanine-3-*^{14}*C*). These tryptophan samples were reduced with lithium in liquid ammonia to give 4,7-dihydrotryptophan,[10] which was ozonized immediately without purification and then oxidized with H_2O_2 under controlled conditions. Workup by chromatography on Dowex 50 H^+, tlc on silica gel (*n*-propyl alcohol:water 7:3) and crystallization with carrier material gave aspartic acid in yields of 1–3%.[11] Aliquots of these aspartate samples were incubated with α-keto glutarate and NADH and the enzymes glutamic–oxalacetic acid transaminase, malate dehydrogenase (large excess), and fumarase to accomplish the reaction sequence apartate → oxalacetate → malate → fumarate. The reaction was followed spectrophotometrically at 340 nm and the incubation was continued for five times the time required for complete conversion of the aspartate. The reaction mixtures were then washed through columns of Dowex 50 H^+ and the effluents were evaporated to dryness. The residues were counted to determine the T/^{14}C ratios of the mixtures of fumarate plus malate after equilibration with fumarase. Parallel incubations from which fumarase had been omitted were carried out to give malate. The results of this degradation were confirmed by an alternate procedure in which the aspartate was converted to malate by chemical diazotation[12] and the latter was isolated, purified by tlc, and subjected to the fumarase reaction, and also by correlating samples of (3*R*)- and (3*S*)-*D,L*-tryptophan-*3-t* prepared by chemical synthesis in Professor Kirby's laboratory with our enzymatically prepared samples *via* the indolmycin system. In addition, the latter two tryptophan samples were subjected to the same degradation procedures as the ones obtained enzymatically from serine.

From the experimental results, which are summarized in Table I and the known stereochemistry of the fumarase reaction[13] (loss of the *pro*-3*R* hydrogen from 2*S*-malate), it follows that the tryptophan from (2*S*,3*R*)-serine-*3-t* has 3*S* and that from (2*S*,3*S*)-serine-*3-t* has 3*R* configuration. Thus the tryptophan synthetase reaction using the *Neurospora* enzyme proceeds with retention of configuration at C-3 of the amino acid substrate[14] (Scheme I). The same steric course was observed for the native enzyme from *E. coli* using either indole or indoleglycerol phosphate as substrate and for the tryptophan formation by *E. coli* tryptophan synthetase β_2 subunit in the presence of excess indole. Thus it appears that tryptophan synthetase might fit the emerging concept[15] that the reactions of pyridoxal phosphate enzymes take place on only one face of the amino acid–pyridoxal phosphate complex.

Acknowledgments. We are indebted to Professor

(8) We thank Miss Linda Zee for carrying out these feeding experiments.

(9) U. Hornemann, L. H. Hurley, M. K. Speedie, and H. G. Floss, *J. Amer. Chem. Soc.*, **93**, 3028 (1971).

(10) O. Yonemitsu, P. Cerutti, and B. Witkop, *J. Amer. Chem. Soc.*, **88**, 3941 (1966).

(11) During the oxidative degradation, a keto group is presumably generated adjacent to the labeled methylene group, leading to some exchange of tritium by enolization and concomitant scrambling of tritium between the two heterotopic positions. This exchange eliminated an earlier version of the degradation procedure, ozonolysis and oxidation of *N*-acetyltryptophan, which gave good yields of aspartate (30–40%) but with loss of about 85% of the tritium. The ozonolysis in this case did presumably not cleave the benzene ring, generating a carbonyl group adjacent to the ring and the methylene group. To minimize this exchange problem, the Li–NH_3 reduction step was introduced which, however, did not allow the use of the acetyl protecting group. This is the cause of the low yield of aspartate; about 85–90% of the radioactivity is not retained by Dowex 50.

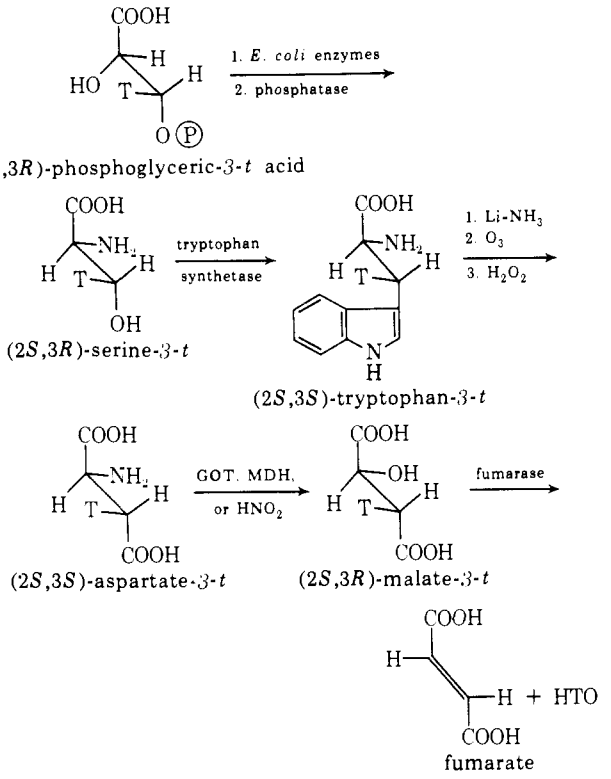
(12) H. Moll, *Chimia*, 20, 426 (1966); P. Besmer, Ph.D. Dissertation No. 4435, ETH Zürich 1970.

(13) O. Gawron and T. P. Fondy, *J. Amer. Chem. Soc.*, **81**, 6333 (1959); F. A. L. Anet, *ibid.*, **82**, 994 (1960).

(14) Note the change in the sequence rule priorities of the substituents in going from serine to tryptophan.

(15) H. C. Duanthan, *Advan. Enzymol.*, **35**, 79 (1971); J. Voet, D. M. Hindenlang, T. J. J. Blanck, R. J. Ulevitch, R. G. Kallen, and H. C. Dunathan, *J. Biol. Chem.*, **248**, 841 (1973), and references therin.

Scheme I



G. W. Kirby, University of Glasgow, for samples of (3*R*)- and (3*S*)-*D*,*L*-tryptophan-3-*t* and to Mr. Detlef Onderka, Purdue University, for the preparation of some of the tritiated phosphoglyceric acid samples. Financial support by the National Institutes of Health (Research Grant GM 18852 and Research Career Development Award GM 42389 to H. G. F.) is gratefully acknowledged.

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Concerning the Carbon-13 Chemical Shifts of Benzocycloalkenes

Sir:

Recently Maciel and coworkers reported[1] carbon-13 chemical shift assignments for a number of benzocycloalkenes. The coherent ^1H spin decoupling technique was employed for assignments in indan, benzocyclobutene, benzocyclopropene, and *o*-di-*tert*-butylbenzene, but this necessitated acceptance of the previously reported ^1H aromatic assignments,[2,3] in no case established.[4] In view of the great interest in the effects of strain on spectroscopic (and other) properties[5] and the use of hydrocarbon data to test theories of nuclear shielding,[6] the need for reliable ^{13}C data is clear. We wish to report such data.

Previously we assigned[7] the aromatic carbons of indane and benzocyclobutene from data for 5-fluoroindane and 4-fluorobenzocyclobutene, readily understood by considerations of ^{13}C–^{19}F couplings and fluorine contributions to carbon screenings in aryl systems.[7,8] These assignments have now been confirmed by tactical introduction of deuterium into the 5 position of indan[9] and the 4 (88%) and 3 (12%) positions of benzocyclobutene[10] by standard transformations of amino or halogeno precursors. 3-Fluorobenzocyclobutene[11] has also been synthesized and yields data completely concordant with our previous assignments.[12] Furthermore, our examination of the ^{13}C satellite patterns in the ^1H spectra of benzocyclobutene and *o*-di-*tert*-butylbenzene[13] demonstrates the previous order of ^1H chemical shifts should be reversed and *ipso facto* any experimentally sound ^{13}C assignments,[14] based on these previous assignments. (The ^1H spectra of the deuterated benzocyclobutene and indane confirm the assignments based on ^{13}C satellite patterns.)

Our ^{13}C spectrum of *o*-di-*tert*-butylbenzene, without ^1H decoupling,[12] confirms the order of chemical shifts reported,[1] despite the incorrect ^1H assignments. The fact that the ^{13}C assignments reported for indane[1] are in error, although the ^1H assignments on which they are

(1) E. L. Motell, D. Lauer, and G. E. Maciel, *J. Phys. Chem.*, 77, 1865 (1973).
(2) M. A. Cooper and S. L. Manatt, *J. Amer. Chem. Soc.*, 92, 1605 (1970).
(3) S. Castellano and R. Kostelnik, *Tetrahedron Lett.*, 5211 (1967).
(4) The main thrust of ref 2 and 3 concerned variations in ^1H–^1H coupling with structural factors. Explicit assignment is not possible from an AA'BB' analysis, and in ref 2 the assignments were regarded as tentative, on the assumption of stronger coupling between methylene protons and H_α (*i.e.*, ortho protons).
(5) For leading references see (2) above.
(6) J. B. Stothers, "Carbon-13 N.M.R. Spectroscopy," Academic Press, New York, N. Y., 1972, and ref 3–20 in ref 1.
(7) See S. Q. A. Rizvi, B. D. Gupta, W. Adcock, D. Doddrell, and W. Kitching, *J. Organometal. Chem.*, 63, 67 (1973). Assignments for benzocyclobutene have appeared in the literature (A. J. Jones, P. J. Garratt, and K. P. Vollhardt, *Angew. Chem., Int. Ed. Engl.*, 12, 241 (1973)), apparently based on differential Overhauser effects. They agree with our rigorously established assignments.
(8) F. J. Weigert and J. D. Roberts, *J. Amer. Chem. Soc.*, 93, 2361 (1971).
(9) The sequence was indan → 5-acetylindan → oxime → amide → 5-amino → 5-Br → 5-D. The constitution of the acetylindane has been demonstrated quite conclusively by synthesis to be the 5-isomer. See J. Vaughan, G. J. Welch, and G. J. Wright, *Tetrahedron* 1669 (1965); L. F. Fieser and A. M. Seligman, *J. Amer. Chem. Soc.*, 57, 2174 (1935); 62, 49 (1940). The aryl part of the ^1H nmr spectrum was typical of a 1,2,4-trisubstituted benzene with one proton quite prominent as a broadened "singlet."
(10) The iodination of benzocyclobutene was previously regarded to yield only the 4-iodo compound: L. Horner, P. V. Subramanium, and K. Eiben, *Tetrahedron Lett.*, 247 (1965). Gas chromatography and mass spectral examination revealed two components in the ratio 6.67:1 (both with *m/e* 230), with the major one being the 4-isomer (87%) based on comparison of the physical and spectral properties of the derived acids. The mixture was converted to the deuterium derivative (Grignard) and a small amount of 3-deuteration was apparent also from the ^{13}C spectrum.
(11) Prepared from 3-fluoro-*o*-xylene (from 3-amino-*o*-xylene (Koch-Light) essentially by the procedure of J. A. Oliver and P. A. Ongley, *Chem. Ind. (London)*, 1024 (1965), for benzocyclobutene: bp 74° (100 mm) $n^{19}D$ 1.5121. The synthesis of 4-fluorobenzocyclobutene will be reported in detail (W. Adcock, M. J. S. Dewar, R. Golden, and M. A. Zeb, *J. Amer. Chem. Soc.*, to be submitted).
(12) While this manuscript was in preparation, Professor Günther informed us of his studies of the undecoupled ^{13}C spectra of some symmetric ortho-disubstituted benzenes. The fine structure of the resonances is dissimilar for C_α and C_β. His assignments are in agreement with ours. See H. Günther, H. Schmickler, and G. Jikeli, *J. Magn. Resonance*, 11, 344 (1973). Other data, apparently overlooked by Maciel and coworkers,[1] concerns comparisons of the spectra of indane and 1,3-dimethylindan discussed in ref 7.
(13) The value of this technique for symmetrical ortho-disubstituted benzenes was emphasized by Professor H. Günther (private communication). The overall width of the satellites must be larger for H_β than H_α since $(2J_o + J_m)$ is larger than $(J_o + J_m + J_p)$.
(14) Because $(\delta_{H_\alpha} - \delta_{H_\beta})$ is small in indan (~ 7Hz at 100 MHz),[2] some overlapping of the ^{13}C satellite patterns occurred, but it was clear that the order was $\delta_{H_\alpha} > \delta_{H_\beta}$, *i.e.*, H_β at higher field as suggested by Manatt and Cooper.[2] (Also confirmed by examination of 5-D-indan.)