shown that the methyl ester of a 10-hydroxyoctadecanoic acid, obtained by the action of a *pseudomonas* species on oleic acid,[5] is optically active.

The methyl esters of the two optically active hydroxy fatty acids were converted to their enantiomorphs by treatment of their tosylates with base.[6] Hydrogenolysis of the tosylates of each of the four optically active hydroxy fatty acid methyl esters (9L, 9D, and 10L, 10D) with tritium-labeled lithium aluminum hydride yielded, after chromic acid oxidation of the resulting octadecanols, the four desired tritium-labeled stearic acids.[7] The chemical purity of the fatty acid samples as determined by v.p.c. was greater than 99%. The radiopurity, as judged by vapor phase chromatographic and isotope dilution techniques, was greater than 90% in all cases. Each of the four labeled stearic acids was mixed with a small amount of stearic acid 1-C^{14} and incubated with growing cultures of a nontoxin-producing strain of *Corynebacterium diphtheriae*.[8] Under the conditions used, up to $^2/_3$ of the stearate was converted to oleate and no significant degradation of fatty acid occurred.[9] The total cellular fatty acids were then isolated as the methyl esters and separated into saturated and unsaturated esters by treatment with mercuric acetate followed by chromatography on silicic acid columns.[10] Methyl oleate[11] and methyl stearate were isolated by v.p.c. Simultaneous assay of tritium and C^{14} was carried out in a liquid scintillation spectrometer. The results of one representative experiment are shown in Table I. The data obtained with the two 9-tritio acids indicate that the hydrogen in the D-position of stearate was removed during the formation of oleate while that in the L-position was not. The recovered methyl stearate remaining after the incubation of the 9D-isomer was considerably enriched with respect to tritium; $H^3:C^{14} = 1.6$ as compared to 1.0 in the original substrate. This finding indicates a primary kinetic isotope effect and suggests that the removal of the 9D-hydrogen is a rate-limiting step in the over-all reaction. Abstraction of hydrogen from C-10 on conversion to oleate was also stereospecific, since it occurred only with one of the two isomers.[12] In this case the methyl stearate recovered after incubation was not enriched with respect to tritium, and we therefore infer from the absence of a significant isotope effect that the removal of hydrogen at C-10 is not a rate-limiting step in the over-all desaturation reaction.

TABLE I

ENZYMATIC DESATURATION OF H^3, C^{14}-STEARIC ACIDS LABELED STEREOSPECIFICALLY WITH TRITIUM

H^3C^{14} Ratios in Stearate and Oleate Recovered after Incubation[a]

Substrate	Methyl stearate	Methyl oleate
9D-H^3-stearic acid-1-C^{14}	1.58	0.35
9L-H^3-stearic acid-1-C^{14}	1.02	.89
10-H^3-stearic acid-1-C^{14} (prepared from (+) 10-hydroxyoctadecanoate)	0.99	.10
10-H^3-stearic acid-1-C^{14} (prepared from (−)-10-hydroxyoctadecanoate)	.85	.87

[a] For ease of comparison, the ratios presented in this table have been calculated in reference to an assigned value of unity in the substrate, *i.e.*, measured $H^3:C^{14}$ ratio in compounds after incubation divided by the measured $H^3:C^{14}$ ratio of the substrate.

Thus, the enzymic desaturation at the 9,10 position of stearic acid is characterized by a rate-limiting, stereospecific removal of the 9D-hydrogen followed by a nonrate-limiting, stereospecific loss of one of the hydrogens at C-10. This is the first report on the stereochemistry of hydrogen elimination on introduction of an isolated double bond into an acyclic compound. It represents a notable example of the stereospecificity of enzyme-catalyzed reactions at *meso* carbon atoms.[13,14]

(5) L. L. Wallen, R. G. Benedict, and R. W. Jackson, *Arch. Biochem. Biophys.*, **99**, 249 (1962). We are indebted to Dr. Wallen for a gift of a culture of this organism.

(6) Optical activity of the methyl hydroxystearates was measured with a Rudolf spectropolarimeter at 23 ± 1° in methanol solution. At 546 mμ the compounds had the following specific rotations (± stand. dev.). I, methyl 9-hydroxyoctadecanoate from Δ^{12}-9-hydroxydecanoic acid, −0.18 ± 0.04°. II, methyl 9D-hydroxyoctadecanoate (synthetic), −0.17 ± 0.5°. III, methyl 9-hydroxyoctadecanoate from the tosylate of I, +0.17 ± 0.04°. IV, methyl 10-hydroxyoctadecanoate, produced by *pseudomonad* culture from oleic acid, −0.16 ± 0.07°. V, methyl 10-hydroxyoctadecanoate from the tosylate of IV, +0.15 ± 0.05°. The magnitude of the rotation of each sample increased with decreasing wave length.

(7) From the results of others (E. R. Alexander, *J. Am. Chem. Soc.*, **72**, 3796 (1950), and G. K. Helmkamp and B. F. Rickborn, *J. Org. Chem.*, **22**, 479 (1957)), we infer that direct replacement of the tosyl function by tritium occurred with inversion of configuration. Thus, the tosylate of methyl 9D-hydroxyoctadecanoate is assumed to yield 9L-H^3-octadecanoic acid. Both in the inversion of the hydroxyl functions and in the hydrogenolysis some racemization is to be expected. The resultant stearic acids will therefore be partially racemized with respect to tritium and the enzymatic conversion to oleic acid (Table I), even if stereospecific, will not proceed with complete removal or retention of labeled hydrogen.

(8) Kindly provided to us by Professor A. M. Pappenheimer.

(9) A. J. Fulco, R. Levy, and K. Bloch, unpublished.

(10) H. Goldfine and K. Bloch, *J. Biol. Chem.*, **236**, 2596 (1961).

(11) The identity of the methyl oleate is based on v.p.c. data, catalytic reduction to methyl stearate, degradation by permanganate–periodate,[9] and demonstration of *cis*-configuration by thin layer chromatography (L. Morris, *Chem. Ind.* (London), 1238 (1962)).

(12) We are not able to specify the absolute configuration of the hydrogen which is removed from carbon atom 10 since the necessary information for assigning configurations to the 10-hydroxyoctadecanoic acids used as starting materials is unavailable.

(13) F. H. Westheimer, H. F. Fisher, E. E. Conn, and B. Vennesland, *J. Am. Chem. Soc.*, **73**, 2402 (1951).

(14) R. H. Levy, P. Talalay, and B. Vennesland in "Progress in Stereochemistry," Vol. 3, P. B. D. De La Mare and W. Klyne, Ed., 1962, p. 299.

(15) Recipient of Research Career Development Award from the National Institutes of Health. On leave of absence from the Department of Biochemistry, University of Minnesota.

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RECEIVED SEPTEMBER 7, 1963

Isolation and Identification from Common Vetch of γ-L-Glutamyl-β-cyano-L-alanine, a Bound Form of the Neurotoxin β-Cyano-L-alanine[1-3]

Sir:

Vicia sativa (common vetch), like a number of other cultivated vetches, is extensively grown and used as a forage plant and soil-improving crop.[4] Incidents of poisoning by common vetch have been noted and reviewed.[5] The recent isolation of the neurotoxic amino acid β-cyano-L-alanine from the seed of common vetch has been of added interest in connection with the implication of this seed as a contaminant in foods which have been associated with causing lathyrism in man.[6,7] However, the concentration of β-cyanoalanine (0.15%) in a contaminant

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(2) Presented at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., 1963, at the Symposium on Deleterious Compounds of Natural Origin in Foods and Feeds.

(3) We thank Miss Hilda Malodeczky, Mrs. Harriet R. Levie, and Mr. Adam Zsolnay for capable technical assistance.

(4) P. R. Henson and H. A. Schoth in "Vetch Culture and Uses," Farmers' Bulletin No. 1740, U. S. Department of Agriculture, Government Printing Office, Washington, D. C., 1961; L. H. Bailey, Ed., "The Standard Cyclopedia of Horticulture," Vol. III, the Macmillan Company, New York, N. Y., 1929–1930, p. 3465.

(5) D. G. Steyn, *Onderstepoort J. Vet. Sci. Animal Ind.*, **1**, 219 (1933).

(6) C. Ressler, *J. Biol. Chem.*, **237**, 733 (1962), and references cited therein.

(7) *Nutr. Rev.*, **21**, 28 (1963).

seemed somewhat low, based on the toxicity of β-cyanoalanine to the rat,[6] to warrant serious consideration of this amino acid as an active factor in lathyrism. We now wish to report that common vetch contains, as well, significant quantities of β-cyano-L-alanine in a bound form which can also be neurotoxic. Moreover, observations with a second species indicate that both the seed of common vetch and its active principles can be far more toxic than experience with the rat had suggested.

Preliminary analytical procedures allowed characterization of the bound β-cyanoalanine as the acidic dipeptide glutamyl-β-cyanoalanine (R_f 0.59, pyridine–water 65:35). The β-cyanoalanine peptide was then isolated from common vetch by chromatography of material in 30% ethanol seed extracts directly on Dowex 1-X4 with pyridinium acetate buffer, pH 4.1, as well as by preparative electrophoresis on blocks of Solka-floc at pH 5.7 and pH 3.5. The purified peptide was then crystallized from water–ethanol, yield 59%, m.p. 185.5–186° dec. (*Anal.* Calcd. for $C_9H_{13}N_3O_5$: C, 44.4; H, 5.39; N, 17.3. Found: C, 44.4; H, 5.52; N, 17.0). Its *dicyclohexylammonium (DCHA) salt* melts at 184–185° dec.; $[\alpha]^{25}D$ +15.1° (*c* 0.6, 2.5% $KHCO_3$). *Anal.* Calcd. for $C_{21}H_{36}N_4O_5 \cdot 0.5\ H_2O$: C, 58.2; H, 8.60; N, 12.9; H_2O, 2.08. Found: C, 58.1; H, 8.46; N, 13.1; H_2O, 2.61. Hydrolysis in 6 *N* HCl for 10 hr. at 110° followed by analysis on the Beckman automatic amino acid analyzer[8] gave a quantitative yield of aspartic acid, glutamic acid, and ammonia, all in a molar ratio to each other of 1:1. On treatment[6] of the DCHA salt with sodium in ammonia containing methanol followed by desalting, hydrolysis, and analysis, less than 1% of aspartic acid was found. Glutamic acid and 2,4-diaminobutyric acid, which were present in molar ratio of 1:1, were obtained in 89% yield.

The isolated peptide was established to be N-(γ-L-glutamyl)-β-cyano-L-alanine through synthesis of the latter, accomplished by hydrogenolysis in the presence of a palladium catalyst of the intermediate carbobenzoxy-γ-L-glutamyl-β-cyano-L-alanine α-benzyl ester which was prepared in 69% yield by coupling carbobenzoxy-L-glutamic acid α-benzyl ester and β-cyano-L-alanine by the mixed anhydride procedure with isobutyl chlorocarbonate. Synthetic γ-L-glutamyl-β-cyano-L-alanine, which possessed the expected elementary composition, agreed with the isolated peptide in melting point, and admixture of them, as well as of their DCHA salts, caused no depression in the respective melting points. Optical rotations and infrared spectra of the DCHA salts were the same for both substances. The two materials showed identical behavior on chromatography on paper in two systems as well as on Dowex 1-X4 (acetate) and Amberlite CG-120 (H^+) ion-exchange resin columns, and had the same electrophoretic mobility on paper at pH 5.7 or 8.6. Moreover, the natural and synthetic peptides were readily distinguishable chromatographically and electrophoretically from the product of hydrogenolysis of carbobenzoxy-α-L-glutamyl-β-cyano-L-alanine γ-benzyl ester prepared in a similar way from carbobenzoxy-L-glutamic acid γ-benzyl ester. The natural and synthetic γ-L-glutamyl-β-cyano-L-alanines showed similar toxicities when injected subcutaneously into young rats.

Isolated γ-L-glutamyl-β-cyano-L-alanine is similar in potency on a molar basis to β-cyano-L-alanine when administered to male weanling Sherman rats subcutaneously, or as a single dose by stomach tube. In White Leghorn chicks the dipeptide, when administered subcutaneously or *per os*, is approximately half as toxic as β-cyano-L-alanine. The concentration of γ-glutamyl-β-cyanoalanine is 0.58% in the seed of common vetch and rises to 1.67 to 2.6% (dry wt.) in the young seedling, suggesting the latter stage of development is potentially more toxic than the seed. A mixture of γ-L-glutamyl-β-cyano-L-alanine and β-cyano-L-alanine, incorporated into a basal ration at half the concentration (0.29 and 0.075%) at which these occur in the seed, has an effect in young chicks similar to a 50% common vetch seed ration, the two diets producing within 6 and 6.5 days (average), respectively, terminal convulsive states with a characteristic opisthotonus.[9]

There has been some question of the neurotoxicity of common vetch seed,[7] and in view of seeming conflicting reports in the literature[10-12] we wish to note that toxicity varies strikingly in different species. In general confirmation of recently reported studies with the chick,[13] diets incorporating 20 to 50% of common vetch seed have been found highly neurotoxic to this species, whereas, in our earlier experiments with the rat, similar concentrations of vetch had appeared nontoxic. As in other experiments,[11] even diets very high in vetch (85 and 100%), although tending to retard growth, produced no obvious neurotoxicity in the rat. Considerable species difference holds also for β-cyano-L-alanine, which, in the diets employed, has been found neurotoxic to the chick near the 0.075% level (average survival 10.5 days) in contrast to the rat which tolerates more than 9 times this concentration.[6] On the basis of the concentrations of γ-L-glutamyl-β-cyano-L-alanine and β-cyano-L-alanine in the seed of common vetch and the now established levels of toxicity of these two principals in the two species, one should indeed expect diets which contain 20 to 100% of common vetch seed to be highly neurotoxic to the chick while they could be nontoxic to the rat. The advisability of using diverse species when testing suspect foods or chemicals for neurotoxicity is evident.

These studies constitute the first report of the natural occurrence of β-cyanoalanine in peptide or bound form and also suggest that γ-glutamyl-β-cyanoalanine and β-cyanoalanine are probably the chief neurotoxic principals of common vetch seed.

(8) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(9) The basal ration was commercial Wayne High Fiber Pullet Developer obtained from Allied Mills, Inc., Chicago, Illinois. It contained minimum 14% protein, 2.5% fat, and 9% fiber. The test diets used for the vetch and the isolated principles may not be comparable nutritionally. The isolations were followed chiefly by chemical means due to the lack of a sensitive bioassay at the start of this work.

(10) H. Stott, *Indian J. Med. Res.*, **18**, 51 (1930–1931).

(11) R. McCarrison, *ibid.*, **15**, 797 (1927–1928).

(12) L. A. P. Anderson, A. Howard, and J. L. Simonsen, *ibid.*, **12**, 613 (1924–1925).

(13) J. A. Harper and G. H. Arscott, *Poultry Sci.*, **41**, 1968 (1962).

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Isolation and Reactions of a Stable Enol Phosphonium Salt

Sir:

Enol phosphonium salts I have been postulated[1-3] as intermediates in the Perkow[4] and halogen migration[1]

(1) A. J. Speziale and R. C. Freeman, *J. Am. Chem. Soc.*, **82**, 1903 (1960); A. J. Speziale and L. R. Smith, *ibid.*, **84**, 1868 (1962).

(2) M. S. Kharasch and I. S. Bengelsdorf, *J. Org. Chem.*, **20**, 1356 (1955).

(3) I. J. Borowitz and L. I. Grossman, *Tetrahedron Letters*, 471 (1962).

(4) W. Perkow, *Ber.*, **87**, 755 (1954); W. Perkow, E. W. Krockow, and K. Knoevenagel, *ibid.*, **88**, 662 (1955).