Studies of Enzyme-mediated Reactions. Part I.[1] Syntheses of Deuterium- or Tritium-labelled (3*S*)- and (3*R*)-Phenylalanines: Stereochemical Course of the Elimination catalysed by L-Phenylalanine Ammonia-lyase

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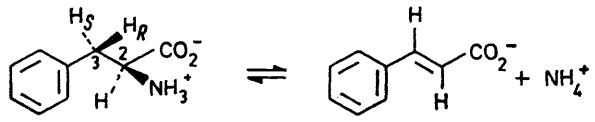
Two rational syntheses of L-phenylalanine [≡ (2*S*)-isomer] have been devised which allow labelling with isotopic hydrogen at C-3 in configurations established by chemical correlation with [2-$^2H_1$]succinic acid. The labelled samples have been used to prove that L-phenylalanine ammonia-lyase eliminates the 3-*pro-S* hydrogen atom of L-phenylalanine together with ammonia to give *trans*-cinnamic acid (*a*) with the isolated enzyme from potatoes and (*b*) during the biosynthesis of colchicine in intact *Colchicum autumnale* plants.

The biochemical formation of the C_6–C_1 unit required for the construction of haemanthamine (30) in *Amaryllidaceae* plants involves loss of the hydrogen atom from C-3 of cinnamic acid.

ENZYMES possess three capacities of outstanding importance: their specificity for substrates and conversion of a substrate by precise chemical reactions into product; their stereospecificity; and the remarkable rate enhancements they are able to achieve. All three aspects have been studied with increasing intensity since Pasteur's time,[2] and recent reviews [3-8] survey the enormous progress which has been made. The present series of papers will mainly describe research on the stereochemistry of the changes which occur when a substrate is converted into product for a selected set of enzymic reactions. Such knowledge can give valuable information about the mechanism of the reaction.[9] Further, precise stereochemical information should interlock in the future with growing knowledge, particularly from *X*-ray analysis, of the shape of active sites of enzymes and of the disposition of amino-acid residues around the sites. The stereochemical data should point to the key residues which take a direct part in the reaction of interest.

[1] Preliminary report, K. R. Hanson, R. H. Wightman, J. Staunton, and A. R. Battersby, *Chem. Comm.*, 1971, 185.
[2] L. Pasteur, *Compt. rend.*, 1858, **46**, 615.
[3] J. W. Cornforth, *Quart. Rev.*, 1969, **23**, 125.
[4] D. Arigoni and E. L. Eliel, *Topics Stereochem.*, 1969, **4**, 127.
[5] R. Bentley, 'Molecular Asymmetry in Biology,' Academic Press, New York, 1969, vol. I; 1970, vol. II.
[6] 'The Enzymes,' ed. P. D. Boyer, Academic Press, New York, 1970, vols. I and II, 3rd edn.
[7] *Progr. Bio-organic Chem.*, 1971, **1**.
[8] R. E. Dickerson and I. Geis, 'The Structure and Action of Proteins,' Harper and Row, New York, 1969.
[9] Reviewed by (*a*) G. Popják in ref. 6, vol. II, p. 116; (*b*) I. A. Rose, *ibid.*, p. 281; (*c*) K. R. Hanson, *Ann. Rev. Plant Physiol.*, 1972, **23**, 335.

L-Phenylalanine ammonia-lyase [10] (EC 4.3.1.5) occurs widely in higher plants and in some fungi; it catalyses the elimination of a proton and ammonia from L-phenylalanine (1) [≡ (2*S*)-phenylalanine] to give *trans*-cinnamate (2). This is then transformed in higher plants into a

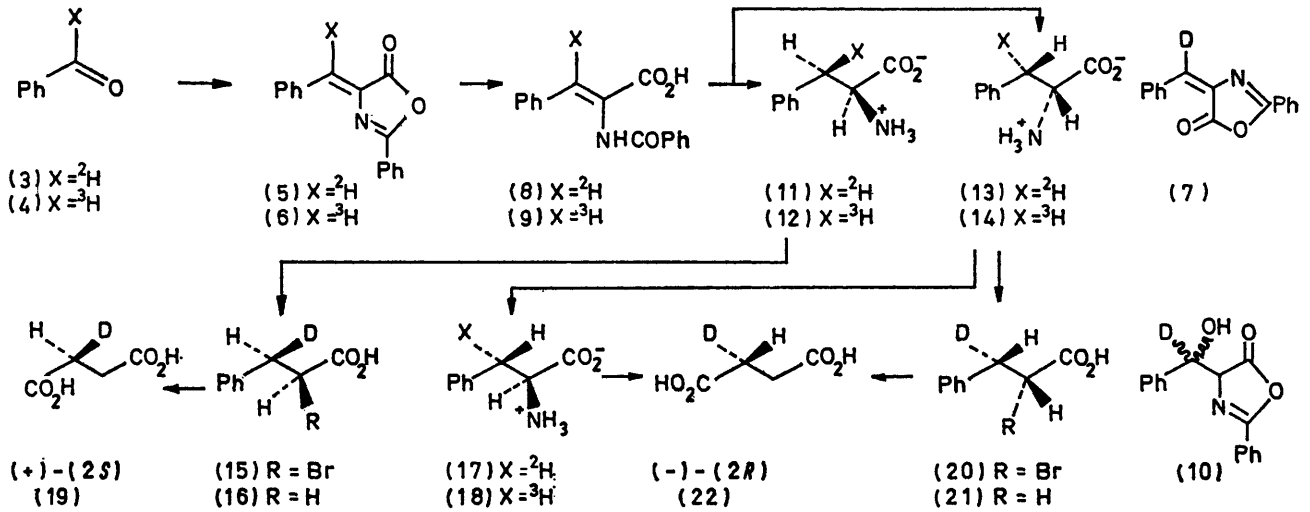


(2*S*) ≡ L (1) (2)

wide range of phenylpropanoid derivatives such as lignin, alkaloids, flavanoids, and anthocyanins.[11] It thus seems that the enzyme acts as a switching point in metabolism whereby phenylalanine from the amino-acid pool used for protein synthesis is diverted to the biosynthesis of the foregoing secondary metabolites. Recently, the enzyme from potato tubers has been extensively purified and its properties and kinetics have been studied.[12] We report a study of the stereochemistry of the elimination process (1) ⟶ (2) in two different systems.

This work took advantage of the fact that C-3 of phenylalanine is a prochiral centre carrying two diastereotopic hydrogen atoms.[13] If the enzymic elimination reaction is stereospecific, loss of either the 3-*pro*-*R* or the 3-*pro*-*S* hydrogen atom [H_R and H_S in structure (1)] will occur. The stereochemical problem can thus be solved by synthesis of phenylalanine carrying at C-3 deuterium or tritium labels of known configuration. Two rational routes to these labelled substances are now described.

One synthesis involved the conversion of [*formyl*-^{2}H]-benzaldehyde (3) with *N*-benzoylglycine into the azlactone (5) [or possibly (7)], which was shown by n.m.r. spectroscopy and chromatography to be homogeneous. The stereochemistry of this product has been disputed [14a-d] but the set of chemical correlations reported here fully support the illustrated *Z*-configuration, with the bulky substituents *trans*. A more recent *X*-ray analysis [14e] on α-benzamidocinnamic acid interlocks with our work. Surprisingly, a minor but significant loss of deuterium occurred during the conversion (3) ⟶ (5), possibly by slight H-for-D exchange at C-3 in the intermediate (10); benzaldehyde containing 0·98 atom equiv. of deuterium gave rise to the azlactone (5) with 0·90 atom equiv. of deuterium. The azlactone was ring-opened with sodium hydroxide (this reaction is known [14a] to occur with retention of configuration) to yield the benzamidocinnamic acid (8). Catalytic hydrogenation over palladium afforded *N*-benzoylphenylalanine, which was hydrolysed. It was expected [15] that the hydrogenation would be cleanly *syn*-stereospecific to generate finally [3-$^2H_1$]phenylalanine as the racemate [2*SR*,3*RS*; (11) + (13)].



(3) X = ^{2}H (4) X = ^{3}H
(5) X = ^{2}H (6) X = ^{3}H
(8) X = ^{2}H (9) X = ^{3}H
(11) X = ^{2}H (12) X = ^{3}H
(13) X = ^{2}H (14) X = ^{3}H
(7)
(+)-(2*S*) (19)
(15) R = Br (16) R = H
(17) X = ^{2}H (18) X = ^{3}H
(-)-(2*R*) (22)
(20) R = Br (21) R = H
(10)

N.m.r. spectroscopy supported this conclusion. The spectrum of undeuteriated (2*RS*)-phenylalanine showed an ABX system corresponding to the protons at C-2 and C-3; the AB portion (C-3) gave signals of clearly distinct chemical shift (see Figure 1) with J_{AX} 8·5 and J_{BX} 4·5 Hz. If it is assumed that the predominant conformer in

[10] Reviewed by K. R. Hanson and E. A. Havir, (*a*) 'Recent Advances in Phytochemistry, 4,' Appleton-Century-Crofts, New York, 1972, p. 45; (*b*) 'The Enzymes,' ed. P. D. Beyer, Academic Press, New York, 1972, vol. 7, 3rd edn., p. 75.

[11] T. A. Geissman and D. H. G. Crout, 'Organic Chemistry of Secondary Plant Metabolism,' Freeman, Cooper, and Co., San Francisco, 1969.

[12] E. A. Havir and K. R. Hanson, (*a*) *Biochemistry*, 1968, **7**, 1896, 1904; (*b*) *Methods Enzymol.*, 1970, **17***a*, 575; (*c*) *Arch. Biochem. Biophys.*, 1970, **141**, 1.

[13] K. R. Hanson, *J. Amer. Chem. Soc.*, 1966, **88**, 2731; H. Hirchmann and K. R. Hanson, *European J. Biochem.*, 1971, **22**, 301.

[14] (*a*) R. Filler, *Adv. Heterocyclic Chem.*, 1965, **4**, 95; (*b*) K. Brocklehurst, H. S. Price, and K. Williamson, *Chem. Comm.*, 1968, 884; (*c*) A. P. Morgenstern, C. Schutij, and W. Th. Nauta, *Chem. Comm.*, 1969, 321; (*d*) H. E. Carter and W. C. Risser, *J. Biol. Chem.*, 1941, **139**, 255; (*e*) K. Brocklehurst, R. P. Bywater, R. A. Palmer, and R. Patrick, *Chem. Comm.*, 1971, 632.

[15] *E.g.*, T. T. Tchen and H. van Milligan, *J. Amer. Chem. Soc.*, 1960, **82**, 4115.

solution is the staggered form [*e.g.* (11)], with phenyl and carboxy-groups in remote positions,[16] then one predicts $J_{H\text{-}2,H_S} < J_{H\text{-}2,H_R}$ for the 2*S*-isomer [L-isomer]. In support, (2*S*,3*S*)-[2-$^2H_1$]aspartic acid shows[17] $J_{H\text{-}2,H_R}$ 8·3 Hz. The protons at C-2 and C-3 in the spectrum of [(11) + (13)] appeared as a clean AX double doublet (see Figure 1) with *J* 4·5 Hz, in agreement with the suggested (2*SR*,3*RS*)-stereochemistry. Rigorous degradative evidence is adduced later.

The *N*-chloroacetyl derivatives of [(11) + (13)] were resolved with hog kidney acylase-I [18] to yield (2*S*,3*R*)-phenylalanine (11) and (2*R*,3*S*)-*N*-chloroacetyl[3-$^2H_1$]-phenylalanine; the latter was hydrolysed with acid and the product was racemised at C-2 with acetic anhydride

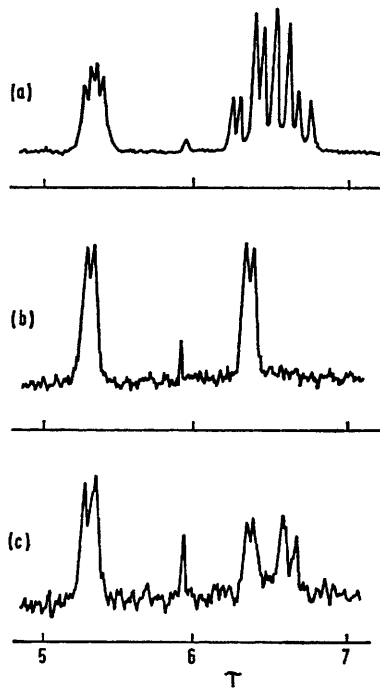


FIGURE 1 N.m.r. spectra (^1H) at 100 MHz; solutions in trifluoroacetic acid; (a) (2*RS*)-phenylalanine, (b) (2*SR*,3*RS*)-[3-$^2H_1$]phenylalanine [(11) + (13)], (c) (2*RS*,3*SS*)-[3-$^2H_1$]-phenylalanine [(13) + (17)]

in acetic acid [19] to give (2*RS*,3*SS*)-[3-$^2H_1$]phenylalanine [(13) + (17)]. The n.m.r. spectrum of this product showed the expected two doublets of equal intensity corresponding to the proton at C-3 (see Figure 1), with *J* 8·5 and 4·5 Hz.

The foregoing *N*-chloroacetylation was repeated in deuterium oxide with unlabelled (2*RS*)-phenylalanine; analysis of the product for deuterium by mass spectrometry showed that some racemisation had occurred at C-2 during this step. The deuterium content found in the control experiment showed that the acylation step in the resolution of the foregoing (2*SR*,3*RS*)-[3-$^2H_1$] phenylalanine produced 7 ± 2% of each enantiomer in the racemate, (2*RS*,3*RS*)-[3-$^2H_1$]phenylalanine. This means that the [3-$^2H_1$]phenylalanines (11) and [(13) + (17)] so prepared contain *ca.* 93% of the compound with the desired C-3 configuration.* These products (11) and [(13) + (17)] were examined mass spectrometrically as their ethyl ester hydrochlorides [20] and 0·90 atom equiv. deuterium was found in each case; no loss had thus occurred during the stages from the azlactone (5).

Monodeuteriosuccinic acid [21] was chosen as the reference standard for rigorous determination of the absolute configurations of (11) and (13). Isomer (11) was converted by treatment with nitrous acid–hydrogen bromide [22] into (2*S*,3*R*)-2-bromo-3-phenyl[3-$^2H_1$]propionic acid (15), which was catalytically reduced to (3*S*)-3-phenyl[3-$^2H_1$]propionic acid (16). Destruction of the aromatic nucleus with ozone followed by a peracetic acid work-up gave [2-$^2H_1$]succinic acid having a deuterium content of 0·62 ± 0·04 atom equiv., markedly less than the level in the starting material. However, o.r.d. measurements,[21] carried out with the help of Professor. J. W. Cornforth, Dr. D. R. Robinson, and Dr. G. Ryback at Sittingbourne, showed that the deuteriated succinic acid contained 81 ± 8% of the (2*S*)-isomer (19). Thus, deuterium loss presumably occurs during the catalytic hydrogenation step, but we have not examined this point further.

A similar degradation of compound (13) by way of intermediates (20) and (21) gave [2-$^2H_1$]succinic acid carrying 0·60 ± 0·4 atom equiv. of deuterium; o.r.d. showed that the deuteriated acid contained 77 ± 8% of the (2*R*)-isomer (22).

The foregoing degradative sequences establish the absolute configurations of the [3-$^2H_1$]phenylalanines (11) and (13), but they do not in themselves give information with fully satisfactory accuracy on configurational purity at C-3. Experiments described later cover this aspect.

It was known [12a] that the rate at which D-phenylalanine [≡ (2*R*)-phenylalanine] is affected by phenylalanine ammonia-lyase is negligible in comparison with the rate of attack on the L-isomer [≡ (2*S*)-isomer]. Thus, it was readily possible to treat (2*SR*,3*RS*)-[3-$^2H_1$]-phenylalanine [(11) + (13)] with the enzyme from potato tubers until all the (2*S*)-form had been converted into cinnamate. The pure cinnamic acid isolated from the incubation was analysed by mass spectrometry and parallel spectra were measured on non-deuteriated cinnamic acid and on [3-$^2H_1$]cinnamic acid of >98% deuterium content prepared from [*formyl*-^2H]benzaldehyde and malonic acid. The results showed that the

* This calculation is based upon the original racemate [(11) + (13)] being configurationally 100% pure; the figure of 93% is thus a maximum value. Evidence for high configurational purity of [(11) + (13)] in the [3-$^2H_1$]-series will be given later.

[16] J. R. Cavanaugh, *J. Amer. Chem. Soc.*, 1968, **90**, 4533.
[17] J. L. Bada and S. L. Miller, *J. Amer. Chem. Soc.*, 1970, **92**, 2774.
[18] J. P. Greenstein, *Adv. Protein Chem.*, 1954, **9**, 172.
[19] J. P. Greenstein and M. Winitz, ' Chemistry of the Amino Acids,' Wiley, New York, 1961, p. 2174.
[20] K. Beimann, J. Seibe, and F. Gapp, *J. Amer. Chem. Soc.*, 1961, **83**, 3795.
[21] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback, and G. J. Schroepfer, jun., *Proc. Roy. Soc. B*, 1966, **163**, 436.
[22] *E.g.*, J. F. Lane and H. W. Heine, *J. Amer. Chem. Soc.*, 1951, **73**, 1348.

enzymically formed cinnamic acid contained 0·88 ± 0·04 atom equiv. of deuterium. Since the labelled substrate contained 10% of non-deuteriated material, this result is entirely in keeping with a stereospecific enzymic elimination of the 3-*pro*-*S* hydrogen atom from (2*S*)-phenylalanine (1). The n.m.r. spectrum of the deuteriated cinnamic acid showed a broadened singlet (0·9H) at τ 3·58 and very small doublets at τ 2·20 and 3·56 (*J* 16·5 Hz) corresponding to the non-deuteriated material.

A similar treatment of (2*RS*,3*SS*)-[3-$^2H_1$]phenylalanine [(13) + (17)] with the enzyme until complete conversion of the (2*S*)-isomer had occurred yielded cinnamic acid containing 0·08 ± 0·02 atom equiv. of deuterium. The n.m.r. spectrum of this product was virtually indistinguishable from that of non-deuteriated cinnamic acid. These results interlock with those of the foregoing paragraph.

To allow measurements of greater accuracy and to permit the work in higher plants which will be described later, the phenylalanine synthesis was also carried out from [*formyl*-^{3}H]benzaldehyde (4) as starting material. In one series of experiments,[10a] the azlactone (6) was ring-opened with ethoxide and four separate hydrogenations were carried out to yield finally four samples of racemic [3-$^3H_1$]phenylalanine [(2*SR*,3*RS*); (12) + (14)]. When these four samples of the racemate [(12) + (14)] were treated separately with the isolated potato enzyme until spectroscopic measurements showed that the (2*S*,3*R*)-isomer had been fully converted into cinnamic acid, only 0·5% of the tritium present in the original (2*S*)-phenylalanine was released into the medium in each case. More precisely, the four results showed that the fraction of tritium released was 0·48 ± 0·07% ($N = 4$); thus there is a 19 out of 20 chance that the fraction retained was >99·3%. The algebraic relationship between purity of substrate and enzyme stereospecificity will be discussed elsewhere. Here we note that if the synthesis were completely stereospecific *i.e.* if the substrate were 100% (3*R*)-labelled, the enzyme is 99·3% specific in removing the 3-*pro*-*S* hydrogen atom. Alternatively, if the enzyme is 100% specific for the 3-*pro*-*S* hydrogen atom, then the substrate was >99·3% (3*R*)-labelled. For all intermediate situations, the empirical 99·3% is less than the lower limit for either stereospecificity or configurational purity; to a first approximation, if the configurational purity of the (2*S*,3*R*)-isomer is (100 − *x*)%, then the stereospecificity of the enzyme is >(99·3 + *x*)%. In combination, all the foregoing results prove that a highly stereospecific loss of the 3-*pro*-*S* hydrogen atom occurs in the elimination step.

Resolution of the racemic [3-$^3H_1$]phenylalanine (prepared in this case by using hydroxide ion to open the azlactone) was carried out as in the ^{2}H-series to yield the (2*S*)-sample (12) for direct enzymic study; the (2*R*)-product (14) was racemised with acetic anhydride to yield (2*RS*,3*SS*)-[3-$^3H_1$]phenylalanine [(14) + (18)]. A second resolution step by the same procedure then afforded (2*S*,3*S*)-[3-$^2H_1$]phenylalanine (18). Table 1 shows the results obtained by treating these products with phenylalanine ammonia-lyase. The change in ^{3}H : ^{14}C ratio during the conversion of phenylalanine into cinnamic acid confirms that it is the 3-*pro*-*S* hydrogen atom which is removed.

The ^{3}H : ^{14}C values for the phenylalanine recovered from reactions run to partial completion are of interest in showing the operation of a statistically significant isotope effect discriminating against tritium. The figures in Table 1 show that the discrimination factor is *ca.* 1·4 when one takes into account the configurational purity at C-3 in the [3-$^3H_1$]phenylalanine.

TABLE 1

Enzymic conversion of [3-$^3H_1$]phenylalanine into cinnamic acid

Initial phenylalanine			Cinnamic acid ^{3}H : ^{14}C ratio	Recovered phenylalanine ^{3}H : ^{14}C ratio
Configuration	^{3}H : ^{14}C ratio	% Conversion	(% retention)	(% retention)
2*S*,3*R* (12)	6·1 ± 0·2	100	5·3 ± 0·2 (87 ± 4)	
2*S*,3*R* (12)	6·1 ± 0·2	45	5·0 ± 0·2 (82 ± 4)	6·2 ± 0·2 (101 ± 4)
2*S*,3*S* (18)	5·9 ± 0·2	100	0·70 ± 0·05 (12 ± 1)	
2*S*,3*S* (18)	5·9 ± 0·2	45	0·65 ± 0·05 (11 ± 1)	7·0 ± 0·2 (119 ± 4)

In order to interpret such tritium isotope effects one needs to know the limiting value of the discrimination at zero conversion of substrate into products.[23] This was estimated (Figure 2) by comparing tritium release from (2*S*,3*S*)-[3-$^3H_1$]phenylalanine (18) with cinnamate formation from zero to complete conversion. The ratio of the specific activity of the (3*S*)-labelled substrate, estimated by complete conversion, to that of the substrate actually transformed at the limit of zero conversion is the isotope effect on the apparent first-order velocity constant k_f ($= V_{max.}/K_m$) for the enzyme.[23] Since $k_f = [E_0]k_{bi}$, where k_{bi} is the apparent bimolecular rate constant, it follows that $k_i^H/k_f^T = k_{bi}^H/k_{bi}^T = ca.$ 1·3. A similar value is obtained if the specific activity at 40% rather than zero conversion is used in the calculation; thus there is excellent agreement with the results of Table 1. The following paper[24] reports a similar isotope effect when (2*S*,3*S*)-[3-$^3H_1$]tyrosine is a substrate for the enzyme from maize; both results are briefly discussed there.

A second synthesis of phenylalanine stereospecifically deuteriated at C-3 made use of liver alcohol dehydrogenase (LAD) to introduce the asymmetry. [*formyl*-^{2}H]-Benzaldehyde (3) was reduced with LAD in the presence

[23] H. Simon and D. Palm, *Angew. Chem. Internat. Edn.*, 1966, **5**, 920.

[24] P. G. Strange, J. Staunton, H. R. Wiltshire, A. R. Battersby, K. R. Hanson, and E. A. Havir, following paper.

of an excess of ethanol and a catalytic quantity of NAD to give (+)-(*S*)-[*methylene*-$^2H_1$]benzyl alcohol [25] (23). The corresponding *p*-tolylsulphonyl ester [26] (24) reacted

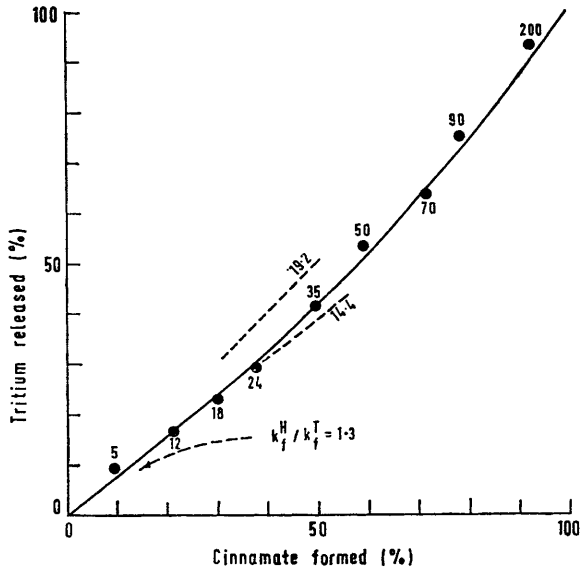
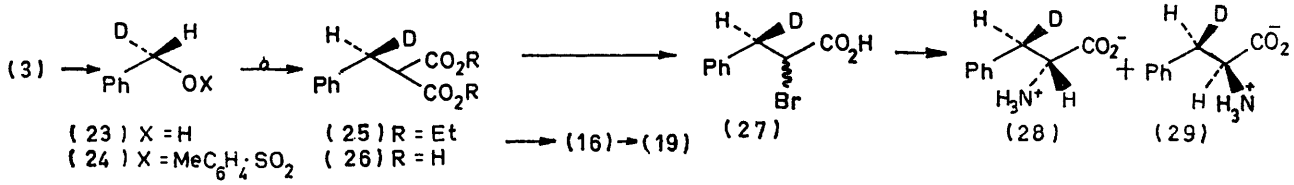


FIGURE 2 Discrimination by phenylalanine ammonia-lyase against tritium release from (2*S*,3*S*)-[3-$^3H_1$]phenylalanine; the numbers against the experimental points indicate the elapsed time (min) at the time of sampling; numbers on the dotted lines are activities in counts min^{-1} $nmol^{-1}$

with sodiomalonic ester to yield ethyl (*S*)-2-ethoxycarbonyl-3-phenyl[3-$^2H_1$]propionate (25), which was hydrolysed to the acid (26), brominated, and decarboxylated to form the bromo-acid (27). Treatment with ammonia then gave the diastereoisomeric mixture of equal amounts



of (2*S*,3*R*)-[3-$^2H_1$]phenylalanine and the (2*R*,3*R*)-isomer [(28) + (29)]. Mass spectrometric and n.m.r. analysis showed 0·96 ± 0·04 atom equiv. of deuterium to be present.

Decarboxylation of the malonic acid (26) gave compound (16) which was degraded as earlier to (+)-(2*S*)-[2-$^2H_1$]succinic acid (19) containing 0·92 ± 0·04 atom equiv. of deuterium and shown by o.r.d. to be virtually configurationally pure (95 ± 5%). Treatment of the diastereoisomeric mixture [(28) + (29)] with the ammonia-lyase enzyme until complete conversion of the (2*S*)-form (29) had occurred gave cinnamic acid containing 0·98 ± 0·04 atom equiv. of deuterium. Further, the n.m.r. spectrum of this sample was identical with that of authentic [3-$^2H_1$]cinnamic acid.

This result is of interest in two ways: (*a*) it provides a rigorous chemical link between two networks [4,10b] of configurationally related chiral substances, and (*b*) it clearly establishes, with the results already described, that the 3-*pro*-*S* hydrogen atom is removed from phenylalanine in the enzymic reaction. Further, when (*RS*)-[2-$^3H_1$]phenylalanine [27] [as (1)] was incubated with the enzyme, less than 1% of the tritium in the (2*S*)-isomer (which was completely converted into cinnamate) was released into the medium. Clearly the elimination process does not involve loss or exchange of the C-2 hydrogen atom in (2*S*)-phenylalanine.

The rigorously defined stereochemistry for the enzymic elimination process is consistent with a mechanism in which the 3-*pro*-*S* hydrogen atom of phenylalanine (1) and the product of the reaction between the substrate's $-\overset{+}{N}H_3$ and the enzyme's prosthetic group [12c] are eliminated in an antiperiplanar manner to generate *trans*-cinnamate (see further discussion in ref. 10*b*).

Results leading to the same conclusions have been obtained by Ife and Haslam; [28a] also, the azlactone route to labelled amino-acids has been developed independently by Kirby and Michael.[28b,*]

The stereochemical course of the phenylalanine ammonia-lyase reaction corresponds to that in which (2*S*)-histidine (≡ L-isomer) is converted by L-histidine ammonia-lyase into urocanic acid.[29] Also, the conversion of aspartic acid into fumaric acid by L-aspartate ammonia-lyase [30] has the same stereochemical specificity.

As an extension of our studies of phenylalanine ammonia-lyase, we wished to study the stereochemistry of its action in two different systems; *Amaryllidaceae* and *Colchicum* plants were selected and for this work, ^3H,^{14}C-labelled materials are required. Earlier work [31] had shown ring A and the starred carbon atom of the

* We thank both these groups for exchange of information.

[25] *Cf.* A. Streitwieser, J. R. Wolfe, and W. D. Schaeffer, *Tetrahedron*, 1959, **6**, 338; V. E. Althouse, D. M. Feigl, W. A. Sanderson, and H. S. Mosher, *J. Amer. Chem. Soc.*, 1966, **88**, 3595; A. Horeau and H. Nouaille, *Tetrahedron Letters*, 1966, 3953.

[26] A. Streitweiser, J. R. Wolfe, and W. D. Shaeffer, *Tetrahedron*, 1959, **6**, 338.

[27] R. B. Johns and D. J. Whelan, *Austral. J. Chem.*, 1966, **19**, 2143.

[28] (*a*) R. Ife and E. Haslam, *J. Chem. Soc.* (*C*), 1971, 2818; (*b*) G. W. Kirby and J. Michael, *Chem. Comm.*, 1971, 187.

[29] I. L. Givot, T. A. Smith, and R. H. Abeles, *J. Biol. Chem.*, 1969, **244**, 6341; J. Rétey, H. Feirz, and W. P. Zeylemaker, *F.E.B.S. Letters*, 1970, **6**, 203.

[30] S. Englard, *J. Biol. Chem.*, 1958, **233**, 1003; A. I. Krasna, *ibid.*, p. 1010 (note that their conclusions must be reversed since they were based upon the supposed stereospecificity of fumarate hydrase which was then incorrectly assigned).

[31] A. R. Battersby, R. Binks, S. W. Breuer, H. M. Fales, W. C. Wildman, and R. J. Highet, *J. Chem. Soc.*, 1964, 1595; D. A. Archer, S. W. Breuer, R. Binks, A. R. Battersby, and W. C. Wildman, *Proc. Chem. Soc.*, 1963, 168.

Amaryllidaceae alkaloids, *e.g.* haemanthamine (30), are derived from phenylalanine by way of cinnamic acid with subsequent cleavage of two carbon atoms from the latter to generate a C_6–C_1 precursor. The presence of phenylalanine ammonia-lyase in *Amaryllidaceae* plants has been demonstrated.[32]

Accordingly, (2*S*,3*S*)-[3-$^3H_1$,3-^{14}C]phenylalanine [as (18)] in equimolar admixture with its diastereoisomer (2*R*,3*S*)-[3-$^3H_1$,3-^{14}C]phenylalanine (14) was prepared by racemisation as before of compound (14) and addition of the appropriate quantity of (*RS*)-[3-^{14}C]phenylalanine. The doubly labelled material was introduced as usual[31] into *Narcissus pseudonarcissus* plants (King Alfred daffodils) and the haemanthamine (30) was isolated after Fuganti (Milan);* he is studying the mechanistic implications further. This result, though of considerable interest, frustrated the original plan; the same problem did not arise, however, in *Colchicum* plants.

It is established that ring A and carbon atoms 5, 6, and 7 of colchicine (32) arise from phenylalanine[33,34] and that cinnamic acid is an intermediate.[33] Since demecolcine (33) is a later precursor of colchicine,[35] the same holds true also for this base. The next intermediate to be recognised[35] on the biosynthetic pathway beyond cinnamic acid is the 1-phenethylisoquinoline (34), which undergoes stepwise oxygenation eventually to form autumnaline[35] (35). It is not yet known whether the reduction of the double bond in cinnamic acid

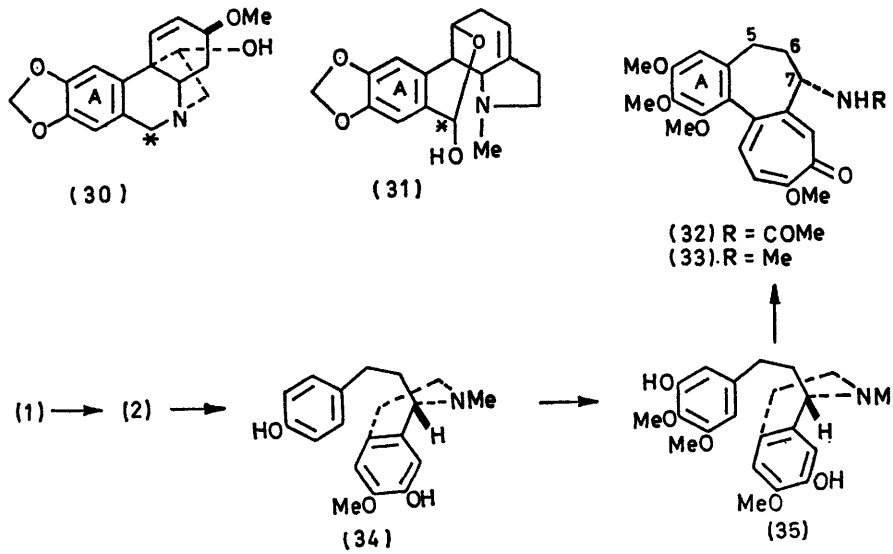


TABLE 2

Tracer experiments on *Colchicum autumnale*

Precursor phenylalanine			Colchicine	Demecolcine	
Configuration of ^3H-labelled sample	^3H : ^{14}C ratio	Incorpn. (%)	^3H : ^{14}C ratio (% retention)	Incorpn. (%)	^3H : ^{14}C ratio (% retention)
(2*S*,3*R*) (12)	4·9 ± 0·1	0·8%	4·4 ± 0·2 (90 ± 4)	0·011%	3·9 ± 0·3 (80 ± 9)
(2*R*,3*S*) + (2*S*,3*S*) equimolar [(14) + (18)]	3·5 ± 0·1	1·14%	0·43 ± 0·06 (12 ± 2)	0·008%	0·35 ± 0·1 (10 ± 3)

several days. A second experiment, using (2*S*,3*R*)-[3-$^3H_1$,3-^{14}C]phenylalanine in equal mixture with (2*R*,3*R*)-[3-$^3H_1$,3-^{14}C]phenylalanine was run in parallel. Satisfactory incorporations with respect to carbon-14 were obtained in each case into haemanthamine (30): 0·10% from the 3*S*-experiment and 0·18% from the 3*R*-experiment. Oduline (31) was also isolated from the plants in both cases and gave (respectively) incorporations of 0·055 and 0·11%. Surprisingly, there was no significant retention of tritium in either case. This finding of loss of tritium from C-3 of phenylalanine and cinnamic acid during incorporation into *Amaryllidaceae* alkaloids has been observed independently by Dr. C. precedes or follows the construction of the isoquinoline ring.

(2*S*,3*R*)-[3-$^3H_1$]Phenylalanine (12) was mixed with (2*S*)-[1-^{14}C]phenylalanine to provide one precursor. The second was prepared by mixing (2*SR*,3*SS*)-[3-$^3H_1$]-phenylalanine [(18) + (14)] (see before) with (2*RS*)-[1-^{14}C]phenylalanine. The results derived by feeding these samples to *C. autumnale* plants are collected in Table 2. The findings for colchicine clearly prove that it is the 3-*pro*-*S* hydrogen atom of phenylalanine (1) which is eliminated in forming cinnamic acid (2); this is

* We thank Dr. Fuganti for this information.

[32] R. J. Suhadolnik, A. G. Fischer, and J. Zulalian, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 208; *Proc. Chem. Soc.*, 1963, 132.

[33] A. R. Battersby and J. J. Reynolds, *Proc. Chem. Soc.*, 1960, 364; A. R. Battersby, R. Binks, J. J. Reynolds, and D. A. Yeowell, *J. Chem. Soc.*, 1964, 4257.

[34] E. Leete and P. E. Nemeth, *J. Amer. Chem. Soc.*, 1960, **82**, 6055; 1961, **183**, 2192.

[35] A. R. Battersby, *Pure Appl. Chem.*, 1967, **14**, 117.

then built into colchicine without further loss of tritium. The values for demecolcine (33) are less accurate because of the lower specific activities for counting, but the values show the same stereochemical control.

Thus, our studies establish that the stereochemistry of the enzymatic elimination reaction (1) ⟶ (2) is the same for the isolated enzyme from potatoes and for the enzyme of intact *Colchicum* plants. It seems probable that this will hold generally. Further evidence in support of this view is presented in the following paper.[24]

EXPERIMENTAL

General directions are given in Part II.[24] Deuterium analyses were carried out on A.E.I. MS9, MS12, and MS902 mass spectrometers by direct insertion of the sample. Spectra of labelled and unlabelled materials were run consecutively and in duplicate; the deuterium content was calculated as in ref. 36.

[formyl-^{2}H]*Benzaldehyde* (3).—This was prepared by the dithian route; [37] the deuteriated dithian showed no n.m.r. signal at τ 4·93 and contained 98% deuteriated species (M^+, 197). The recovered aldehyde,[37] b.p. 87—90° at 35 mmHg, showed no n.m.r. signal at τ 0·05.

4-([α-^{2}H]*Benzylidene*)-2-*phenyl*-Δ^2-*oxazolin*-5-*one* (5).—The foregoing aldehyde (3·0 g, 0·028 mol), *N*-benzoylglycine (4·8 g, 0·028 mol), anhydrous sodium acetate (2·3 g), and acetic anhydride (8 g) were heated together at 80° for 2 h and then cooled. Ethanol (10 ml) was added, the mixture was cooled to 0°, and the precipitate was collected, washed with ethanol at 0°, then with hot water, and finally recrystallised from benzene to give the *deuteriated oxazolinone* (4·86 g, 73%), m.p. 165—166° (lit.,[38] 167—168° for unlabelled material); τ 2·7 (v. weak s, CH=C, non-deuteriated material); *m/e* 250 (M^+, 100%), 249 (14), 105 (90), 77 (98), and 51 (48); 0·90 ± 0·04 atom equiv. deuterium.

2-*Benzamido*[3-^{2}H]*cinnamic Acid* (8).—A suspension of the deuteriated oxazolinone (2·24 g, 9 mmol) in water (200 ml) was treated with sodium hydroxide (0·44 g, 11 mmol) in water (5 ml). The mixture was heated under reflux until all the solid had dissolved and the cooled solution was acidified with concentrated hydrochloric acid to yield 2-*benzamido*[3-^{2}H]*cinnamic acid* (2·19 g, 90%), m.p. (ill-defined) *ca.* 220° (lit.,[39] 210—228° for unlabelled material); $\nu_{max.}$ (Nujol) 3300, 1695, 1650, and 1520 cm^{-1}.

(2SR,3RS)-N-*Benzoyl*[3-^{2}H$_1$]*phenylalanine*.—A solution of the foregoing acid (2·1 g) in glacial acetic acid (150 ml) was hydrogenated at 1 atm and 20 °C over 10% palladised charcoal (0·2 g) (uptake 1·03 mol. equiv.). The solution was filtered and evaporated to yield the racemic *N*-benzoylphenylalanine, m.p. 187° (from ethanol) (lit.,[40] 187—188° for unlabelled material), $\nu_{max.}$ 3315, 1723, 1625, 1580, and 1540 cm^{-1}; *m/e* 270 (M^+, 6%), 149 (34), 148 (23), 105 (100), 92 (12), and 77 (30); 0·89 ± 0·04 atom equiv. deuterium.

(2SR,3RS)-[3-^{2}H$_1$]*Phenylalanine* [(11) + (13)] *and the Ethyl Ester Hydrochloride*.—The foregoing *N*-benzoyl derivative (5·0 g) suspended in 6N-hydrochloric acid (150 ml) was heated under reflux for 7 h. The cooled clear solution was extracted exhaustively with ether, the aqueous solution was evaporated to dryness, the residue was resuspended in water, and the solvent was again evaporated off. A solution of the final residue in the minimum volume of hot water was adjusted to pH 5 with ammonium hydroxide and the precipitate [(11) + (13)] which separated at 0° was collected (2·76 g, 90%); $\nu_{max.}$ (Nujol) 1630, 1600, 1510, 1415, 1355, 1325, 1280, and 830 cm^{-1}; τ (CF_3·CO_2H) 2·55 (5H, m, aryl H), 5·26 [1H, d, *J* 4·5 Hz, C(2)H], and 6·37 [1H, d, *J* 4·5 Hz, C(3)H].

Dry hydrogen chloride was passed for 2 min into a suspension of the product [(11) + (13)] (10 mg) in anhydrous ethanol (3 ml); the solution was heated under reflux for 2 h and then was evaporated. The residue crystallised from ethanol–ether to give the ethyl ester hydrochloride, m.p. 126° (lit.,[41] 127° for non-deuteriated material); *m/e* 194 (M^+, 2%), 121 (M − CO_2Et, 100), 120 (non-deuteriated M − CO_2Et, 10), 102 (M − PhCHD, 85), 92 ($PhCHD^+$, 20), and 74 (38).

(2SR,3RS)-N-*Chloroacetyl*[3-^{2}H$_1$]*phenylalanine*.—A stirred solution of the foregoing amino-acid (2·42 g, 14·5 mmol) in N-sodium hydroxide (20 ml) was treated alternately and dropwise at 0° with a solution of chloroacetyl chloride (2·82 g, 25 mmol) in dry ether (20 ml) and N-sodium hydroxide (40 ml). After 2 h the solution was acidified and extracted thrice with ethyl acetate, and the extracted material was crystallised from water to yield the *N*-chloroacetyl derivative (2·57 g, 73%), m.p. 128—129° (lit.,[42] 130°, non-deuteriated); $\nu_{max.}$ (Nujol) 3395, 1745, 1725, and 1660 cm^{-1}.

(2S,3R)-[3-^{2}H$_1$]*Phenylalanine* (11) *and* (2R,3S)-[3-^{2}H$_1$]-*Phenylalanine* (13).—A suspension of the foregoing racemate (2·56 g) in water (80 ml) was adjusted to pH 7·3 by addition of ammonium hydroxide followed by acetic acid. To the resultant solution was added hog kidney acylase I (B.D.H.; 40 mg), and after the mixture had been kept at 38° for 9 h the pH was re-adjusted to 7·3 and more acylase I (20 mg) was added. After a further 40 h, the pH was adjusted to 5 and the solution was boiled briefly with charcoal, filtered, acidified to pH 1·7 with 2N-nitric acid, and extracted thrice with ethyl acetate. The organic solution afforded (2*R*,3*S*)-*N*-chloroacetyl[3-^{2}H$_1$]phenylalanine (1·11 g), m.p. 124—125° (from water) (lit.,[43] m.p. 125°, non-deuteriated); $\nu_{max.}$ (Nujol) 3320, 1710, 1645, and 1550 cm^{-1}.

Concentration of the aqueous phase to 30 ml, adjustment with ammonium hydroxide to pH 5, and further concentration to crystallisation point, gave (2*S*,3*R*)-[3-^{2}H$_1$]phenylalanine (11) (0·45 g); $\nu_{max.}$ (Nujol) 1560, 1495, 1405, 1310, 1275, 940, 820, 750, and 740 cm^{-1}.

A mixture of the (2*R*,3*S*)-*N*-chloroacetyl[3-^{2}H$_1$]phenylalanine (1·1 g) and 3N-hydrochloric acid (25 ml) was heated under reflux for 4 h; the solution was then evaporated, water was added, and the mixture was evaporated again. The residue, in the minimum volume of hot water, was adjusted to pH 5 to yield (2*R*,3*S*)-[3-^{2}H$_1$]phenylalanine (13) (0·65 g); its i.r. spectrum was identical with that of its enantiomer (11).

(2RS,3SS)-[3-^{2}H$_1$]*Phenylalanine* [(13) + (17)].—Acetic anhydride (0·4 ml) was added dropwise at 100° to a stirred solution of (2*R*,3*S*)-[3-^{2}H$_1$]phenylalanine (146 mg) in glacial acetic acid (2 ml). After 1 h at 100°, the solution was evaporated and the residue was heated under reflux with 3N-hydrochloric acid (5 ml) for 4 h. This solution was then

[36] K. Biemann, 'Mass Spectrometry,' McGraw-Hill, New York, 1962, p. 223.
[37] D. Seebach, B. W. Erickson, and G. Singh, *J. Org. Chem.*, 1966, **31**, 4303.
[38] R. M. Herbst and D. Shemin, *Org. Synth.*, 1943, Coll. Vol. II, p. 490.
[39] E. Erlenmeyer, *Annaln*, 1893, **275**, 8.
[40] Ref. 19, p. 1271.
[41] Ref. 19, p. 932.
[42] Ref. 19, p. 746.
[43] Ref. 19, p. 2173.

evaporated and the residue was treated as in the foregoing preparation to yield the racemate [(13) + (17)] (116 mg, 74%); τ (CF_3·CO_2H) 2·55 (10H, m, aryl H), 5·3br [2H, d, C(2)H], 6·37 [1H, d, *J* 4·5 Hz, C(3)H], and 6·64 [1H, d, *J* 7·5 Hz, C(3)H]. The mass spectrum of the corresponding ethyl ester hydrochloride was identical with that of the isomer [(11) + (13)] and showed 0·9 ± 0·04 atom equiv. deuterium.

Degradation of (2R,3S)-[3-$^2H_1$]*Phenylalanine* (13) *and of the* (2S,3R)-*Isomer* (11).—A solution of the (2*R*,3*S*)-amino-acid (0·4 g) in 35% hydrobromic acid (8 ml) was treated at −10° dropwise during 30 min with sodium nitrite (0·5 g, 2 equiv.) in water (5 ml). After the mixture had been kept <0° for a further 1 h, it was allowed to warm to 20° and extracted with ether. The extracts were shaken with aqueous sulphurous acid, washed with water, dried, and evaporated to give (2*R*,2*S*)-2-bromo-3-phenylpropionic acid (565 mg). A solution of this in 3 : 2 (v/v) dioxan–water (6 ml) was shaken with hydrogen and 10% palladised charcoal (150 mg) (uptake 1·04 mol. equiv.) The solution was filtered, most of the solvent was evaporated off, and water and ether were added. Three extractions with ether gave (*R*)-3-phenyl[3-$^2H_1$]propionic acid (0·3 g, 80%), m.p. 45° [from light petroleum (b.p. 40—60°)] (lit.,[44] m.p. 48°, non-deuteriated material).

Oxonised oxygen (*ca.* 5%) was passed at 20° for 16 h through a solution of all the foregoing acid in chloroform (50 ml); chloroform was added periodically to maintain the volume. Excess of ozone was then removed in a stream of nitrogen, the solution was evaporated, and the residue was heated under reflux for 4 h with aqueous hydrogen peroxide (100 vol; 1 ml), formic acid (1 ml), and water (3 ml); more hydrogen peroxide (*ca.* 0·5 ml) was added periodically. The residue left by evaporation of the solvents was washed with chloroform to yield a solid which was sublimed at 120° and 0·3 mmHg (yield 180 mg). This was crystallised five times from ethyl acetate to give (*R*)-[$^2H_1$]succinic acid (35 mg), m.p. and mixed m.p. with authentic material 184—185°; the deuteriated acid showed negligible u.v. absorption above 250 nm.

The (2*S*,3*R*)-amino-acid (11) (420 mg) similarly gave (*S*)-[3-$^2H_1$]succinic acid (54 mg), m.p. and mixed m.p. 184—185°.

The mass spectrometric and o.r.d. measurements on the two samples of succinic acid were carried out exactly as described by Cornforth *et al.*[21]

(2RS,3RR)-[3-$^2H_1$]*Phenylalanine* [(28) + (29)].—To a 0·01M-phosphate buffer (800 ml; pH 7·4) were added ethanol (30 ml), albumin (65 mg), NAD (60 mg), and a suspension of liver alcohol dehydrogenase (2 ml; 10 mg per ml *ex* Boehringer). This mixture was stirred at 20° under nitrogen and a solution of [*formyl*-^{2}H]benzaldehyde (2·5 ml) in dioxan (20 ml) was added dropwise during 15 h. After a further 10 h the solution was near-saturated with sodium acetate and extracted with ether. This gave a liquid which was chromatographed on silica gel with dichloromethane and chloroform as eluants to yield unchanged benzaldehyde (392 mg) and (+)-(S)-[α-$^2H_1$]benzyl alcohol (23) (2·16 g, 83%); $\nu_{max.}$ 2140 cm^{-1}; τ 2·8 (5H, s, aryl H) and 5·6 (1H, d, *J* 1 Hz).

This product was converted[26] into its *p*-tolylsulphonyl derivative (60% yield), m.p. 55° (lit.,[26] 58°). Part of this (1·1 g) in tetrahydrofuran (15 ml) was added dropwise to a clear solution prepared by dropwise addition of a solution of diethyl malonate (1·6 g, 10 mmol) in anhydrous tetrahydrofuran (10 ml) to a stirred suspension of sodium hydride (50% in oil; 0·26 g, 5 mmol) in anhydrous tetrahydrofuran (5 ml). After the mixture had been stirred at 20° for 1 day, it was poured into ice and 2N-sulphuric acid. Extraction with ether gave an oil which was fractionated on silica gel with benzene and chloroform as eluants to yield ethyl (*S*)-2-ethoxycarbonyl-3-phenyl[3-$^2H_2$]propionate (25) (850 mg, 75%); τ 2·8 (5H, s, aryl H), 5·9 (4H, q, *J* 7 Hz, CH_2·CH_3), 6·4 (1H, d, *J* 8 Hz, methine), 6·8 (1H, d, *J* 8 Hz, methylene), and 8·9 (6H, t, *J* 7 Hz, CH_2·CH_3).

This ester (711 mg) was heated under reflux for 3 h with potassium hydroxide (2 g) in ethanol (10 ml) and water[45] (5 ml). Extraction of the cooled solution thrice with ether was followed by acidification and re-extraction; the latter extracts yielded the malonic acid (26) (0·5 g), homogeneous by t.l.c. This product in dry ether (15 ml) was treated dropwise with a solution of bromine (430 mg) in dry ether and then heated under reflux for 10 min. After the solution had been shaken with water, it was evaporated and the residual oil (524 mg) was identified as 2-bromobenzylmalonic acid by comparison with an authentic sample.[45] Decarboxylation at 130—140° for 1·5 h gave an oil which was partitioned between water and ether; evaporation of the ether layer left a liquid (346 mg). This was dissolved in ammonium hydroxide (*d* 0·880; 5 ml), and after 6 days at 20° the solution was evaporated and the residue dissolved in the minimum volume of aqueous hydrochloric acid. The resulting solution was adjusted to pH 5 with ammonia. (2*RS*,3*RR*)-[3-$^2H_1$]phenylalanine [(28) + (29)] then separated at 0° (93 mg); i.r. spectrum identical with that of [(13) + (17)] already prepared. Mass spectrometric analysis of the corresponding ethyl ester hydrochloride, prepared as earlier, showed 0·97 ± 0·04 atom equiv. deuterium.

Correlation of the Foregoing Series with (+)-(2S)-[2-$^2H_1$]-*Succinic Acid.*—The malonic acid (26) (0·5 g), prepared as just described, was heated at 130° under nitrogen for 3 h, and the product was crystallised twice from light petroleum (b.p. 40—60°) to give (*S*)-3-phenyl[3-$^2H_1$]propionic acid (16) (0·3 g, 70%), m.p. 46° (lit.,[44] 48°). This was degraded by ozonolysis as in earlier cases to yield (+)-(2*S*)-[2-$^2H_1$]-succinic acid (10 mg), m.p. and mixed m.p. 186°, which was examined as before by mass spectrometry and o.r.d.

2-*Phenyl*-[2-^{3}H]-1,3-*dithian.*—Tritiated water (30 mg, 1·67 mmol; 30 mCi) was transferred by vacuum line into trifluoroacetic anhydride (380 mg, 1·8 mmol); the tube was sealed and kept under vacuum for 16 h at 20° to yield [^{3}H]trifluoroacetic acid.

A solution of n-butyl-lithium (15% w/w in hexane; 3·2 ml, 5 mmol) was added by syringe through a serum cap to a solution of 2-phenyl-1,3-dithian[37] (980 mg, 5 mmol) in anhydrous tetrahydrofuran (10 ml) at −50° under dry nitrogen. After the mixture had been stirred at −50° for 5 h, the foregoing [^{3}H]trifluoroacetic acid was added, followed after 5 min by an excess of water. The solution was warmed to 20° and evaporated to dryness, and the residue in 1 : 1 (v/v) pentane–dichloromethane was washed with aqueous sodium hydrogen carbonate, water, and brine, and then dried and evaporated. Crystallisation of the residue from methanol gave 2-phenyl[2-^{3}H]-1,3-dithian (862 mg, 88%; 25 mCi), m.p. 71° (lit.,[37] 70—71°).

(2S,3R)-[3-$^3H_1$]*Phenylalanine* (12) *and* (2S,3S)-[3-$^3H_1$]-

[44] A. W. Ingersoll and C. S. Marvell, *Org. Synth.*, 1941, Coll. Vol. I, p. 311.

[45] *Cf.* A. W. Ingersoll and C. S. Marvell, *Org. Synth.*, 1955, Coll. Vol. III, p. 706.

Phenylalanine (18).—These were prepared from the foregoing [^{3}H]dithian by exactly the same sequences as used in the ^{2}H-series. In each step of the synthesis, the chemical and radiochemical yields matched, within experimental error.

(2SR,3RS)-[3-$^3H_1$]*Phenylalanine* [(12) + (14)].—The following method [10a] gave material of high configurational purity. Treatment of the [^{3}H]oxazolinone (6) in ethanol with sodium ethoxide [14d] gave the ethyl ester of (9), which was repeatedly recrystallised from aqueous ethanol and then shown to be free from (6) and (9) by t.l.c. This product (8 μmol) in ethanol (3 ml) was shaken at 30° and 760 mmHg with hydrogen and palladium oxide (9 mg) (uptake complete in 70 min). The reduction product was heated under reflux with 6N-hydrochloric acid for 4 h, the resultant solution was extracted with ether, and the aqueous phase was evaporated to give the amino-acids [(12) + (14)] as their hydrochlorides. T.l.c., ninhydrin assay, and the enzymic assay (described later) confirmed chemical and radio-chemical purity. Four essentially identical preparations were carried out in this way. Hydrogenations carried out on a larger scale gave products of lower configurational purity.

Enzymic Conversions with Phenylalanine Ammonia-lyase from Potatoes.—The enzyme used in the following experiments was purified as previously described.[12b] For procedures (a) and (b), side fractions from the enzyme purification having relatively low specific activity were used; these preparations were of much higher stability than crude fractions of the same specific activity which had not been through the purification sequence. The enzyme was dialysed against 0·04M-borate (Na^+) buffer, pH 8·7, prior to use.

(a) *With deuteriated substrates; representative procedure.* The deuteriated sample of (2*RS*)-phenylalanine (33·19 mg, 0·2 mmol) was treated at 23° for 28 h in 0·04M-borate (Na^+) buffer (pH 8·7; 55 ml) with phenylalanine ammonia-lyase (4·9 units; spec. act. *ca.* 7 munits per mg; purification up to the calcium phosphate gel step). Formation of cinnamate was followed by observing absorbance at 290 nm (50 μl sample diluted to 3 ml with buffer). Conversion of the (2*S*)-phenylalanine into cinnamate was complete (±1%) after 20 h. Assay of the enzyme in the mixture after 28 h showed complete retention of activity. The mixture was dialysed at 0° against 0·04M-borate (Na^+) buffer, pH 8·7 (1 l) until, as observed by u.v. absorption, equilibrium was reached (*ca.* 20 h). The dialysate was evaporated and the solution of enzyme was used again for the next experiment. A solution of the residue from the dialysate in water (50 ml) was adjusted to pH 1 with hydrochloric acid and extracted with ether (3 × 50 ml). The ethereal extracts afforded cinnamic acid, which was recrystallised several times from aqueous ethanol (yield usually 7 mg); m.p. and mixed m.p. 132—133°.

(b) *With tritiated substrates* (Table 1). The appropriate ^{3}H,^{14}C-labelled sample of (2*S*)-phenylalanine (typically 7·35 mg, 44·5 μmol) was treated with phenylalanine ammonia-lyase (0·4 units; spec. act. *ca.* 4 munits per mg) as in (a) (typical reaction vol. 40 ml). Analysis by u.v. spectroscopy showed complete conversion in 20 h and *ca.* 40% conversion in 2 h. Dialysis and evaporation of the buffer were carried out as in (a) after the appropriate time. The solid residue was dissolved in water (50 ml) and radioinactive cinnamic acid (30 mg) was added; (2*S*)-phenylalanine (30 mg) was also added in runs carried to *ca.* 40% conversion. Isolation of the cinnamic acid and purification to constant specific activity and ^{3}H : ^{14}C ratio was carried out as under (a). For the partial conversions in which the residue contained (2*S*)-phenylalanine, the aqueous phase (after the extraction with ether) was evaporated to dryness and a suspension of the residue in anhydrous ethanol (20 ml) was treated for 2 min with a stream of dry hydrogen chloride. The mixture was heated under reflux for 2 h, then cooled and poured into water (100 ml), and the solution was basified with sodium carbonate. Extraction with ether gave a solution of the required ester, which was converted into its hydrochloride by passing hydrogen chloride into the ether. Evaporation of the resultant solution to dryness and multiple recrystallisation of the residue from ethanol–ether gave (2*S*)-phenylalanine ethyl ester hydrochloride at constant specific activity and ^{3}H : ^{14}C ratio (25 mg); m.p. 150°.

(c) *Sublimation assay: substrate purity and degree of enzyme stereospecificity.* The four samples of (2*RS*)-[3-$^3H_1$]phenylalanine [(12) + (14)] prepared by the ester route were treated separately at 30° for 6 h in 0·04M-borate (Na^+) buffer (pH 8·7; 3 ml) with phenylalanine ammonia-lyase (0·1 units; spec. act. *ca.* 0·2 units per mg; enzyme purification including agarose column stage). Absorbance at 290 nm showed reaction to be complete in 1 h. The percentage of tritium released (r) in each case from the (2*S*)-substrate was determined by sublimation in a Y-assembly [12c] as described in Part II.[24] Assay for radioactivity on the water sample so obtained showed $r = 0{\cdot}485 \pm 0{\cdot}074\%$ ($N = 4$), hence 95% confidence limits in $1 - r = 99{\cdot}52 \pm 0{\cdot}25\%$, *i.e.* $1 - r > 99{\cdot}3\%$.

(d) *Estimation of primary kinetic isotope effect by release of tritium* (*Figure* 2). (2*RS*,3*SR*)-[3-$^3H_1$]Phenylalanine [(12) + (14)] as used in (c) was treated with L-amino-acid oxidase to yield the (2*R*)-component, which was racemised by the acetic acid–acetic anhydride method [19] to give (2*RS*,3*SS*)-[3-$^3H_1$]phenylalanine [(14) + (18)]. A sample of this product was dissolved in 0·04M-borate (Na^+) buffer (pH 8·7; 4·4 ml; substrate concn. 0·235 mmol l^{-1}; 4500 counts min^{-1} ml^{-1} of releasable tritium). Zero time samples (3 × 200 μl) were withdrawn for sublimation and a solution of phenylalanine ammonia-lyase (20 μl; 80 munits; spec. act. *ca.* 0·2 units per mg; enzyme purification including the agarose column stage) was added to it at 30° in a spectrophotometer cuvette. The amount of tritium released at a given time was assayed by adding a sample (200 μl) of the reaction mixture to a solution (300 μl) of N-potassium hydroxide in an O-ring tube of a sublimation Y-assembly.[12c] The tritiated water was separated by sublimation and samples (3 × 100 μl) of the neutral sublimate were taken for scintillation counting; the same micropipette was used throughout. The amount of cinnamate formed was determined by monitoring the change in u.v. absorbance at 300 nm (1 μmol ml^{-1} ≡ 3·40 absorbance units) and calculating by interpolation the absorbance at the time of withdrawing the sample. A correction was made for the small change in absorbance produced on adding the enzyme by extrapolating to zero time. The tritium released at complete reaction was determined for 5 samples (100 μl) after 20 h; absorbance change 0·854.

Tracer Experiments on Plants.—(a) *With* Narcissus pseudonarcissus. The appropriate (2*RS*)-[3-$^3H_1$]phenylalanine sample (*ca.* 40 mg; 0·2 mCi) was mixed with (2*RS*)-[3-^{14}C]phenylalanine (0·05 mCi) (New England Nuclear Corp.) by dissolving in water (3 ml) and evaporation

to dryness. A small sample of the $^{3}H,^{14}C$-labelled material so obtained was diluted with radioinactive (2*RS*)-phenylalanine; the product was converted into the ethyl ester hydrochloride and then crystallised as before to constant label ratio and activity. The remainder of the sample at high specific activity was dissolved in water (3 ml) and administered by injection into the stems of King Alfred daffodils (eleven plants) as the flowers were beginning to wither. After 2 weeks, the plants were worked up for alkaloids by an improved procedure to be described in Part III.[46] The products were haemanthamine (230 mg), m.p. 202—203° (from acetone) (lit.,[47] 203—203·5°), and oduline (120 mg), m.p. 170° (from acetone) (lit.,[47] 168°), identified by comparison with authentic samples.

(b) *With* Colchicum autumnale. The doubly labelled samples were prepared and the accurate ^{3}H : ^{14}C ratio was determined as under (a) save that (2*S*)- and (2*RS*)-[1-^{14}C]-phenylalanine (Radiochemical Centre, Amersham) were used as appropriate (see Table 2). The material at high specific activity (*ca.* 0·2 mCi ^{3}H and 0·05 mCi ^{14}C in 40 mg) was dissolved in water (3 ml) and injected into the capsules of four plants for each experiment. The plants were worked up for alkaloids after 2 weeks by the standard method,[48] to give colchicine (150 mg), m.p. 148—149° (from ethyl acetate) (lit.,[49] 148—150°). The fractions containing demecolcine were diluted with radioinactive demecolcine (20 mg) and the alkaloid was then recrystallised from ethyl acetate–light petroleum (b.p. 60—80°) to constant specific activity and ratio; m.p. 183—184° (lit.,[49] 184—185°).

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