Studies of Enzyme-mediated Reactions. Part II.[1] Stereochemistry of the Elimination of Ammonia from L-Tyrosine catalysed by the Enzyme from Maize

By **P. G. Strange, J. Staunton, H. R. Wiltshire,** and **A. R. Battersby,*** University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW
K. R. Hanson and **E. A. Havir,** Biochemistry Department, The Connecticut Agricultural Experiment Station, P.O. Box 1106, New Haven, Connecticut 06504, U.S.A.

A stereoselective synthesis has been used to yield two samples of (2*RS*)-tyrosine (≡ DL-tyrosine) in which the (2*S*)-form of one carries a (3*R*)-[3-$^{3}H_{1}$] label and that of the other, a (3*S*)-[3-$^{3}H_{1}$] label. The configurations of these products at C-3 have been rigorously determined by degradation *via* aspartic acid and malic acid to fumaric acid, the final step being enzymic. It has been proved, by use of the labelled samples of tyrosine, that the enzyme from maize eliminates the 3-*pro-S* hydrogen atom of (2*S*)-tyrosine together with ammonia to give *trans-p*-hydroxycinnamic acid.

Experiments with stereospecifically labelled (2*S*)-phenylalanine have shown that the degree of 3-*pro-S* specificity of the maize enzyme is indistinguishable from that of the potato enzyme. Studies of partial conversions with the maize enzyme have confirmed that the same active site acts on both (2*S*)-tyrosine and (2*S*)-phenylalanine.

THE presence in various plants of an enzyme which converts L-tyrosine † (1) into *p*-hydroxycinnamic acid [as (2)] was demonstrated [2] in 1961; the enzyme was originally called tyrase [2] and barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.) were found to be particularly suitable sources. It is found generally in grasses [2] and in some fungi; [3] its occurrence has been reviewed [4] and it has been suggested that the production of *p*-hydroxycinnamic acid is linked with lignification in the plant concerned. More recently, the enzyme has been known as L-tyrosine ammonia-lyase, but several lines of evidence have shown [5] that a single enzyme in maize is responsible for elimination of ammonia from both (2*S*)-phenylalanine and (2*S*)-tyrosine and that the same active site acts on both substrates. In contrast, the enzyme from potatoes possesses almost no tyrosine ammonia-lyase activity (<0·5%). There was thus added interest in determining whether the

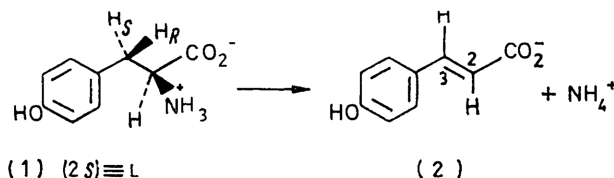
† The *RS*-system will be used throughout the remainder of this paper to designate configuration at both C-2 and C-3 of the labelled amino-acids. Thus, L-tyrosine is equivalent to (2*S*)-tyrosine.

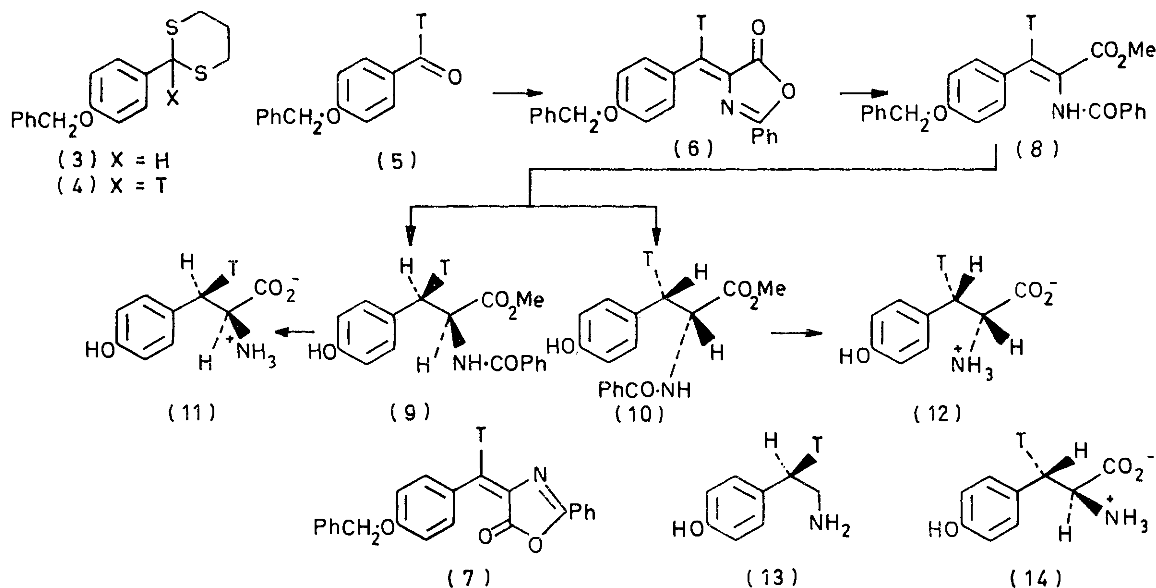
[1] Part I, R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, preceding paper.
[2] A. C. Neish, *Phytochemistry*, 1961, **1**, 1.
[3] R. J. Bandoni, K. Moore, P. V. Subba Rao, and G. N. H. Towers, *Phytochemistry*, 1968, **7**, 205.
[4] M. R. Young, G. N. H. Towers, and A. C. Neish, *Canad. J. Bot.*, 1966, **44**, 341.
[5] H. V. Marsh, jun., E. A. Havir, and K. R. Hanson, *Biochemistry*, 1968, **7**, 1915; E. A. Havir, P. D. Reid, and H. V. Marsh, jun., *Plant Physiol.*, 1971, **48**, 130; 1972, **49**, in the press.

stereochemistry of the elimination process catalysed by the enzyme from maize corresponds to that established in the preceding paper [1] for L-phenylalanine ammonia-lyase from potatoes.[6]

A solution to the stereochemical problem depends on the synthesis of two samples of tyrosine (1) carrying a hydrogen isotope at C-3 of known configuration, *i.e.* *R* and *S* [the 3-*pro-R* and 3-*pro-S* positions are marked on structure (1)]. The azlactone route [1] * was used and later confirm the stereochemistry as in (6). Cleavage of this product with methoxide anion [10] gave the methyl benzamidocinnamate (8), which was hydrogenated to form *N*-benzoyltyrosine [(9) + (10)]. Hydrolysis then afforded (2*RS*)-[3-$^3H_1$]tyrosine.

If the hydrogenation is entirely *syn*-stereospecific, then the configurations at C-2 and C-3 are related and the product should be the racemate (2*SR*,3*RS*)-[3-$^3H_1$]-tyrosine [(11) + (12)]. Clearly, a separation of the





tritium was chosen as the label for all the current work; this demanded a new configurational proof (*cf.* ref. 1) which will be outlined here.

The anion derived from the dithian (3) was treated with 1 equiv. of n-butyl lithium at $-78°$ and the mixture was quenched with [^{3}H]trifluoroacetic acid to yield the corresponding [2-^{3}H]dithian (4). Parallel studies in the deuterium series showed that use of an excess of n-butyl-lithium or more forcing conditions resulted in deuteriation at the α-position of the *O*-benzyl group. The recovered [8] 4-benzyloxy[*formyl*-^{3}H]benzaldehyde (5) was converted by the usual method [1,9] into the azlactone (6) [or possibly (7)]. The product was homogeneous chromatographically and spectroscopically and results described

2*S*- and 2*R*-enantiomers will also effect separation of 3*R*- and 3*S*-labelled forms.

The foregoing racemate was mixed with an appropriate amount of (2*RS*)-[2-^{14}C]tyrosine and part was treated with tyrosine carboxylyase (E.C. 4.1.1.25), which is specific for the (2*S*)-isomer.[11] The resultant mixture of (2*R*,3*S*)-[2-^{14}C,3-$^3H_1$]tyrosine (12) and (2*R*)-[1-^{14}C,2-$^3H_1$]-tyramine (13) was separated by ion-exchange.[12] Finally, the purified amino-acid (12) was racemised at C-2 by treatment with hot acetic anhydride in acetic acid followed by acidic hydrolysis to afford (2*RS*,3*SS*)-[2-^{14}C,3-$^3H_1$]tyrosine [(12) + (14)]. The correct conditions for the racemisation step were determined by trial experiments on unlabelled tyrosine with acetic anhydride

* This route has been developed independently by Kirby and Michael.[7]

[6] See also R. Ife and E. Haslam, *J. Chem. Soc.* (*C*), 1971, 2818.

[7] G. W. Kirby and J. Michael, *Chem. Comm.*, 1971, 187.

[8] D. Seebach, *Synthesis*, 1969, **1**, 17.

[9] H. E. Carter, *Org. Reactions*, 1946, **3**, 198.

[10] R. E. Buckles, R. Filler, and L. Hilfman, *J. Org. Chem.*, 1952, **17**, 233.

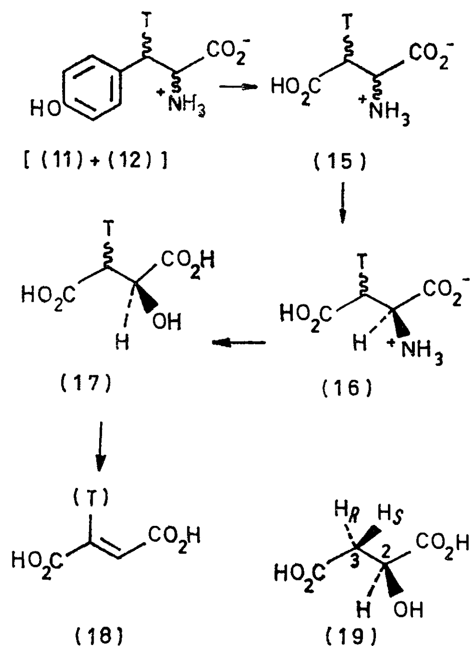
[11] H. M. R. Epps, *Biochem. J.*, 1944, **38**, 242.

[12] H. B. F. Dixon, *J. Chromatog.*, 1966, **24**, 199, and personal communication.

and deuterioacetic acid (analysis by n.m.r. spectroscopy).

The (2*R*,3*S*)-tyrosine (12) and the racemic sample [(12) + (14)] were examined by isotope dilution analysis [13] with use of a vast excess of radioinactive (2*S*)-tyrosine. These products (12) and [(12) + (14)] were thereby shown to contain <0·05 and 49·6% of the (2*S*)-isomer, respectively.

Proof that the configurations drawn for the labelled tyrosines are correct representations was obtained in the following way. The racemate (2*SR*,3*RS*)-[3-$^3H_1$]-tyrosine [(11) + (12)] was degraded by treatment with ozone followed by performic acid to yield (2*RS*)-[3-$^3H_1$]-aspartic acid (15), which was diluted with a large excess of radioinactive (2*S*)-aspartic acid [as (16)] and then converted into its copper salt [14] for purification. The recovered aspartic acid was crystallised to constant specific activity so bringing about a resolution to give optically and radiochemically pure (2*S*)-[3-$^3H_1$]aspartic acid (16). This was mixed with a suitable quantity of generally ^{14}C-labelled (2*S*)-aspartic acid and converted



with nitrous acid [13] and hydrochloric acid into (2*S*)-[3-$^3H_1$]malic acid (17). For Experiment 4 (Table 1) the tyrosine used at the outset was ^{3}H,^{14}C-labelled and so it was unnecessary to add any ^{14}C-labelled aspartic acid. A by-product in the deamination step was 2-chlorosuccinic acid, which was separated chromatographically. Release of tritium into the aqueous medium during the conversion (16) ⟶ (17) was shown to be <0·3% in Experiment 3 (Table 1). Further, when malic acid was kept in D_2O–DCl under the conditions of treatment with nitrous acid, there was no detectable exchange (n.m.r.) over a period twenty times the length of the deamination step.

Malate hydro-lyase (E.C. 4.2.1.2; traditionally called fumarase and often fumarate hydratase) is known to convert (2*S*)-malic acid [as (17)] into fumaric acid by stereospecific removal of the 3-*pro-R* hydrogen atom [15] [shown as H_R on (19)]. This enzyme can thus be used to establish the configuration of our ^{3}H-labelled (2*S*)-malic acid from the foregoing degradative work. Incubation of the (2*S*)-[3-$^3H_1$]malic acid (17) with the enzyme gave fumaric acid (18), which was rigorously purified; the Experimental section describes the various crystalline derivatives used in purification and counting of the set of compounds (16)—(18). The results in Table 1 establish that the major component (>86 ± 4%; $N = 3$) in our synthetic [3-$^3H_1$]tyrosine samples [*e.g.* (11)] is as represented in the stereoformulae. The minor loss of configurational purity at C-3 may be the result of slight lack of stereospecificity in the hydrogenation step or, perhaps more likely, loss of strict stereochemical control as the azlactone (6) is opened to form the methyl benzamidocinnamate (8). Any addition–elimination of methoxide anion to the αβ-unsaturated ester could result in some loss of stereochemical integrity. However, the important point is that the (3*R*)-, and (3*S*)-[3-$^3H_1$]-tyrosine samples are *complementary* by their preparation and are of ample configurational purity at C-3 for rigorous study of the enzyme from maize.

Table 2 collects the results obtained by treating the two doubly labelled tyrosine samples separately with the maize enzyme. The presence of the (2*R*)-isomer (≡ D-tyrosine) can be ignored since it is not a significant substrate for the enzyme; indeed, it acts as an inhibitor.[5] The high retention of tritium in Experiments 5 and 6 and the complementary small retention in Experiments 7 and 8 firmly establish that the enzymic elimination removes the 3-*pro-S* hydrogen atom from tyrosine (1). The stereochemistry of the process thus matches that found for phenylalanine ammonia-lyase from potatoes and in *Colchicum autumnale* plants.[1]

When partial conversions of labelled (2*S*)-tyrosine were carried out (Table 2, Experiments 6 and 8), the $^3H:^{14}C$ values found for the recovered amino-acid showed a primary isotopic discrimination against tritium, similar to that observed when phenylalanine was treated with the potato enzyme [1] (*ca.* 1·3). These results provide [1] an approximate value for the isotope effect [16] on the apparent bimolecular rate constant for the enzymic reaction (k_{bi}^H/k_{bi}^T). The observed ratio may be less than the true isotope effect if it is the resultant of a discrimination against tritium and an enzyme-mediated exchange of tritium from the (3*S*)-labelled

[13] W. R. Waterfield, *J. Chem. Soc.*, 1964, 541.
[14] S. Englard, *J. Biol. Chem.*, 1958, **233**, 1003; A. I. Krasna, *ibid.*, p. 1010.
[15] Reviewed by R. Bentley, 'Molecular Asymmetry in Biology,' Academic Press, New York, 1970, vol. II, p. 127. The enzyme is at least 99·99% 3-*pro-R*-specific; H. F. Fischer, C. Frieden, J. S. McKinley McKey, and R. A. Alberty, *J. Amer. Chem. Soc.*, 1955, **77**, 4436; see also G. E. Lienhard and I. A. Rose, *Biochemistry*, 1964, **3**, 190.
[16] H. Simon and D. Palm, *Angew. Chem. Internat. Edn.*, 1966, **5**, 920.

substrate that takes place without release of the olefinic acid. However, the results can be accommodated by a kinetic model involving no exchange and with the observed small discriminations attributed to isotope effects in one or more of the equilibria prior to the rate-limiting step in k_{bi}.[17b] Further work will be necessary to define precisely the origin(s) of the isotopic discrimination.

The results in Tables 1 and 2 are consistent with there being complete stereospecificity for the 3-*pro-S* hydrogen atom of (2*S*)-tyrosine in the action of the enzyme from maize. Further, by a statistical treatment of the values found in the following work on (2*S*)-phenylalanine, a lower limit can be set in this case on the degree of stereospecificity which has been established experimentally. Samples of the same batch of (2*SR*,3*RS*)-[3-$^3H_1$]phenylalanine[1,*] were treated with the potato enzyme and with that from maize; Table 3 collects the results from ten experiments. Since the fraction of tritium released was found to be independent of the substrate–enzyme ratio, there can be no slow release of tritium from the (2*R*)-component present, whereas conversion of the (2*S*)-component is complete. The statistics[18,†] show that there is roughly a two-in-three chance that the observed difference in tritium released, $r_m - r_p$, would have been obtained if the true value is zero. Alternatively, if the degrees of stereospecificity of the maize and potato enzymes do differ, then there is a nineteen-in-twenty chance that the difference is <1·4%.

It is known[17] that cinnamate is released from the potato enzyme prior to ammonia and that the 'amino-enzyme' can either hydrolyse or react with cinnamate to regenerate (2*S*)-phenylalanine. From previous work on the maize enzyme[5] one would expect that the 'amino-enzyme' formed from (2*S*)-tyrosine should react with cinnamate to yield (2*S*)-phenylalanine and similarly, that the 'amino-enzyme' formed from (2*S*)-phenylalanine should convert *p*-hydroxycinnamate into (2*S*)-tyrosine. Table 4 shows that both expectations have been realised

TABLE 1

Determination of absolute configuration at C-3 of labelled tyrosines

Expt. no.	Tyrosine configuration	(2*S*)-Aspartic acid ^3H : ^{14}C ratio	(2*S*)-Malic acid ^3H : ^{14}C ratio	Fumaric acid ^3H : ^{14}C ratio	Retention (%)
1	[(11) + (12)]	4·9 ± 0·2	4·8 ± 0·2	3·8 ± 0·2	79
2	[(11) + (12)]	5·2 ± 0·2	4·8 ± 0·2	4·4 ± 0·2	91
3	[(11) + (12)]	4·1 ± 0·2	3·8 ± 0·2	3·3 ± 0·15	87
					Av. 86 ± 4
4	[(14) + (12)]	7·9 ± 0·2	8·2 ± 0·2	1·2 ± 0·05	15 ± 3

TABLE 2

Enzymic conversion of [3-$^3H_1$]tyrosine into *p*-hydroxycinnamic acid

Expt. no.	Initial tyrosine: Configuration [a]	Initial tyrosine: ^3H : ^{14}C ratio	% Conversion of (2*S*)-isomer	*p*-Hydroxycinnamic acid ^3H : ^{14}C ratio (% retention)	Recovered (2*S*)-tyrosine ^3H : ^{14}C ratio
5	[(11) + (12)]	10·1 ± 0·2	100	9·1 ± 0·2 (90 ± 2)	
6	[(11) + (12)]	10·1 ± 0·2	*ca.* 40	8·5 ± 0·2 (84 ± 2)	11·5 ± 0·2
				Av. 87	
7	[(14) + (12)]	7·9 ± 0·2	100	0·70 ± 0·07 (9 ± 1)	
8	[(14) + (12)]	7·9 ± 0·2	*ca.* 40	0·70 ± 0·07 (9 ± 1)	10·0 ± 0·2
				Av. 9	

[a] Only (11) and (14) are substrates; the enzyme does not act on (12).

TABLE 3

Comparison of the stereospecificities of the maize and potato enzymes with respect to the hydrogen atoms at C-3 of (2*S*)-[3-$^3H_1$]phenylalanine

	Maize enzyme	Potato enzyme
Number of experiments	5	5
Concentration (μmol l^{-1}) of (2*S*)-[3-$^3H_1$] phenylalanine [present in (2*RS*)-mixture] [a]	33—120	24—160
Total enzyme employed (in 3 ml; pH 8·7; 20 h; 30°) [a]	0·07—0·14 units	0·4—0·6 units
Fraction ^3H released from (2*S*)-substrate [b]	r_m = 9·81 ± 0·32%	r_p = 9·53 ± 0·35%
Difference in ^3H released (an estimate of the stereospecificity difference, if any) [b]	$r_m - r_p$ = 0·28 ± 0·48%	
t-Test (8 DF)	*t* = 0·59 equiv. to *P* = 0·6	
95% Confidence limits in $r_m - r_p$ (8 DF)	1·39 to −0·82%	

[a] A range of experimental conditions was employed. [b] Means and standard errors of means.

* It must be remembered that the method of preparation does not generate 100% configurational purity at C-3. Thus some (2*SR*,3*SR*)-[3-$^3H_1$]phenylalanine is also present.[1]

† The algebraic treatment of the relation between configurational purity of labelled substrate and stereospecificity of the enzyme will be given elsewhere by K. R. H.

[17] E. A. Havir and K. R. Hanson, (*a*) *Biochemistry*, 1968, **7**, 1904; (*b*) 'The Enzymes,' ed. P. D. Boyer, Academic Press, New York, 1972, vol. 7, 3rd edn.

[18] C. I. Bliss, 'Statistics in Biology,' McGraw-Hill, New York, 1967, vol. 1, p. 213.

(compare test and control runs) and these results confirm that one active site on the maize enzyme is effective for both (2*S*)-phenylalanine and (2*S*)-tyrosine.

The reversibility of the ammonia-lyase enzymes from potatoes and maize and also the foregoing demonstrations of interconversion of substrates have implications for biosynthetic work. The possibility that such partial reactions may occur *in vivo* must be borne in mind when analysing the results of studies in which labelled substrates for the ammonia-lyase * are administered as precursors to plants or fungi.

TABLE 4

Partial reactions catalysed by the enzyme from maize

Expt. no.[a]	Reaction mixture (0·3 ml; pH 6·8)	Total radioactivity (nCi) in amino-acid after 90 min	% Conversion into amino-acid
		In phenylalanine	
9t	(2*S*)-Tyrosine (1·67 mmol l^{-1}) + enzyme + [*U*-^{14}*C*] cinnamate (50 nCi)	2·6	5·2
9c	NH_4^+ (1 mmol l^{-1}) + enzyme + [*U*-^{14}*C*] cinnamate (50 nCi)	0·17	0·35
		In tyrosine	
10t	(2*S*)-Phenylalanine (6·67 mmol l^{-1}) + enzyme + *p*-hydroxy-[*U*-^{14}C] cinnamate (130 nCi)	7·6	5·75
10c	NH_4^+ (2 mmol l^{-1}) + enzyme + *p*-hydroxy[*U*-^{14}C] cinnamate (130 nCi)	1·2	0·90

[a] t = Test, c = control.

In summary, the enzyme from maize acts in a highly stereospecific way on (2*S*)-tyrosine (1) with removal of the *pro-S* hydrogen atom from C-3 to generate *trans-p*-hydroxycinnamic acid (2); this is consistent with an antiperiplanar elimination process. The same active site is involved in the enzymic reaction with both (2*S*)-tyrosine and (2*S*)-phenylalanine and there is no appreciable difference in the degree of stereospecificity of the maize and potato enzymes towards (2*S*)-phenylalanine.

EXPERIMENTAL

General Directions.—Solutions were dried over anhydrous sodium sulphate and evaporated below 40°. M.p.s were determined on a Kofler hot-stage apparatus. Strictly anhydrous aprotic solvents were dried finally by distillation from lithium aluminium hydride. Unless otherwise indicated, t.l.c. was carried out on plates coated with Merck GF_{254} and alumina for chromatography was grade III neutral. Radioactive samples were counted in Cambridge on a Packard Tricarb liquid scintillation spectrometer (model 3320) (internal standardisation with ^{3}H- or ^{14}C-hexadecane) and in New Haven on a 720 Nuclear-Chicago liquid scintillation counter. Unless otherwise stated, u.v. spectra were recorded on a Unicam SP 800 instrument for solutions in 95% ethanol, i.r. spectra for solutions in chloroform on Unicam SP 200 and SP 1000 or Perkin-Elmer 257 spectrometers, and n.m.r. spectra for solutions in deuteriochloroform on Varian HA100 and XL100 and on Perkin-Elmer R12B spectrometers (tetramethylsilane standardisation). Mass spectra were determined by direct insertion on A.E.I. MS9, MS12, and MS902 machines. Assays by u.v. spectroscopy of enzymic activity were carried out on a Gilford 2400 recording spectrophotometer coupled to an accurate temperature control system.

* That is, (2*S*)-phenylalanine, (2*S*)-tyrosine, cinnamate, or *p*-hydroxycinnamate.

2-(4-*Benzyloxyphenyl*)-1,3-*dithian* (3).—A solution of 4-benzyloxybenzaldehyde (2·12 g) and propane-1,3-dithiol (1 ml) in chloroform (50 ml) was saturated with dry hydrogen chloride, stirred for 30 min, then washed successively with water (2 × 50 ml), 10% potassium hydroxide (4 × 50 ml), and water (2 × 50 ml), and dried. After treatment with charcoal and filtration, the solution was evaporated and the residue crystallised from chloroform–light petroleum (b.p. 40—60°) to give the *dithian* (2·26 g), m.p. 144—145° (Found: $\lambda_{max.}$ 231 nm; $\nu_{max.}$ 3000, 1610, 1590, and 1520 cm^{-1}; τ 2·57 (2H, m, aryl H), 2·62 (5H, s, aryl H), 3·10 (2H, d, *J* 10 Hz, aryl H), 4·90 (s, 1H, methine), 4·98 (s, 2H, O·CH_2Ar), 7·10 (4H, m, dithian CH_2·S), and 7·95 (2H, m, dithian CH_2); *m/e* 302 (M^+, 25%), 228 (10), 211 (5), and 91 (100).

2-(4-*Benzyloxyphenyl*)-1,3-[2-^{3}H]*dithian* (4).—The foregoing dithian (1·0 g, 3·3 mmol), rigorously dried, in anhydrous tetrahydrofuran (80 ml) was stirred at −74° under >1 atm pressure of dry nitrogen. A solution of n-butyl-lithium (15% in hexane; 1·69 ml, 3 mmol) was then injected through a serum cap, and the mixture was stirred for 1·5 h at −74° before quenching by injection of [^{3}H]trifluoroacetic acid (0·15 ml, 2·34 mmol) [prepared [1] from trifluoroacetic anhydride (0·15 ml, 1·1 mmol) with [^{3}H]water (21 mg, 1·17 mmol; 30 mCi)]. After 2 min water (*ca.* 20 ml) was added and the mixture was warmed to 20° and adjusted to pH 4·5 with 0·05M-hydrochloric acid. The organic solvent was evaporated off; extraction of the aqueous suspension with chloroform (extract dried over potassium carbonate) gave the [^{3}H]dithian (1·02 g; 21·5 mCi), m.p. 141—143°, of suitable purity for the next stage.

In trial experiments under these conditions but involving quenching of the anion with [^{2}H]water it was shown by n.m.r. that only the expected proton (τ 4·90) had been replaced.

4-*Benzyloxy*[formyl-^{3}H]*benzaldehyde* (5) (cf. *ref.* 7).—A solution of the [^{3}H]dithiane (4) (1·0 g) in acetonitrile (185 ml) and water (20 ml) was treated with a solution of *N*-chlorosuccinimide (1·68 g) and 2,6-lutidine (1·48 ml) in acetonitrile (22 ml). After 5 s, silver nitrate (2·42 g) in water (7·25 ml) was added, followed 5 min later by formic acid (2·2 ml) and after a further 1 min by water (220 ml) and sodium hydrogen carbonate (7·26 g). The suspension was swirled until evolution of carbon dioxide ceased, then it was filtered; the solid was washed with dichloromethane (100 ml) and the latter washings were retained. The aqueous filtrate was freed from organic solvents by evaporation, then extracted with dichloromethane (200 ml), and the extracts and washings were combined, dried, and evap-

orated. The residue, in benzene, was chromatographed on alumina (40 g); elution was continued with benzene to yield the [^{3}H]benzaldehyde (0·59 g), m.p. 72—73° [from ether–light petroleum (b.p. 60—80°)]. The material was identified by comparison with a sample prepared in a parallel radioinactive run which gave the following data; $\lambda_{max.}$ 224 and 287 nm; $\nu_{max.}$ 2810, 2720, 1680, and 1600 cm^{-1}; τ 0·16 (1H, s, CHO), 2·24 (2H, d, *J* 9 Hz, aryl H) 2·64 (5H, s, aryl H), 3·00 (2H, d, *J* 9 Hz, aryl H), and 4·95 (2H, s, C*H*$_2$Ar); *m*/*e* 212 (11%), 92 (9), 91 (100), and 65 (16).

4-(4-Benzyloxy [α-^{3}H]*benzylidene)-2-phenyl-*Δ^2*-oxazolin-5-one* (6).—(This and the following two preparations with Dr. J. L. McHugh). The foregoing [^{3}H]aldehyde (0·59 g, 2·8 mmol) was dissolved at 80° in acetic anhydride (0·78 ml); anhydrous sodium acetate (0·23 g) was then added and when dissolved was followed by *N*-benzoylglycine (0·55 g, 2·9 mmol) and acetic anhydride (0·5 ml). After 10 min, the mixture solidified to a yellow mass, which was heated at 80° for a further 2 h, then cooled to 0° before the gradual addition of ethanol (7 ml; pre-cooled to 0°) all at 0°. The solid was collected and washed with ethanol (10 ml) at 0° and with hot water (10 ml); recrystallisation from benzene afforded the [^{3}H]*oxazolinone* (0·69 g, 70%), m.p. 158—160°. Preparative t.l.c. of the mother liquors gave unchanged starting aldehyde (0·11 g).

An identical radioinactive run gave material for characterisation (Found: C, 77·6; H, 5·0; N, 4·1. $C_{23}H_{17}NO_3$ requires C, 77·7; H, 4·8; N, 3·9%), $\lambda_{max.}$ 395 nm; $\nu_{max.}$ 1788, 1768, 1655, and 1600 cm^{-1}; τ 1·85 (4H, d, *J* 9 Hz, overlapping aryl H), 2·3—2·7 (3H, m, overlapping aryl H), 2·6 (5H, s, aryl H), 2·82 (1H, s, methine), 2·94 (2H, d, *J* 10 Hz, aryl H), and 4·90 (2H, s, C*H*$_2$Ar); *m*/*e* 355 (M^+, 22%), 105 (51), 91 (100), and 78 (28).

Methyl α-Benzamido-4-benzyloxy[β-^{3}H]*cinnamate* (8).—The foregoing oxazolinone (0·68 g, 1·9 mmol), suspended in anhydrous methanol (25 ml), was treated at *ca.* 50° with sodium methoxide (0·21 g, 3·8 mmol). The mixture was kept at 20° for 1 h, then acidified with hydrochloric acid, and the precipitated *methyl cinnamate* (8) was recrystallised from glacial acetic acid or methanol; yield 0·69 g (93%), m.p. 153—154°.

A parallel radioinactive run gave identical material (Found: C, 74·6; H, 5·5; N, 3·4. $C_{24}H_{21}NO_4$ requires C, 74·4; H, 5·4; N, 3·6%); $\lambda_{max.}$ 233 and 312 nm; $\nu_{max.}$ 3400, 1710, 1680, and 1605 cm^{-1}; τ 2·15 (3H, m, aryl H), 2·68 (5H, m, aryl H), 3·15 (2H, d, *J* 9 Hz, aryl H), 5·03 (2H, s, C*H*$_2$Ar), and 6·26 (3H, s, OMe).

(2RS)-N-*Benzoyl-O-benzyl*[3-$^3H_1$]*tyrosine Methyl Ester*.—The methyl cinnamate (8) (82 mg) in methanol (5 ml) was shaken with hydrogen and Adams platinum oxide (47 mg) at 760 mmHg and 19° until uptake was complete. The solution was filtered and evaporated and the residue crystallised from aqueous acetone to give the [3-$^3H_1$]*tyrosine ester* (77 mg), m.p. 110°.

Radioinactive material was similarly prepared (Found: C, 74·4; H, 6·3; N, 3·4. $C_{24}H_{23}NO_4$ requires C, 74·0; H, 6·0; N, 3·6%), $\lambda_{max.}$ 235 and 280 nm.

(2SR,3RS)-N-*Benzoyl*[3-$^3H_1$]*tyrosine Methyl Ester* [(9) + (10)].—The foregoing preparation was unreliable in that occasionally the *O*-benzyl group was removed to some extent. The main preparation was therefore continued as follows. A solution of the methyl cinnamate (8) (0·69 g) in methanol (200 ml) and concentrated hydrochloric acid (3 drops) was shaken with hydrogen and palladised charcoal (10%; 70 mg) until uptake was complete and the band at 312 nm in the u.v. spectrum had disappeared. The solution was filtered and evaporated; crystallisation from methanol (carried out only on radioinactive material) gave the (2*RS*)-ester [(9) + (10)], m.p. 167—168° (92%). The product was identified by comparison with authentic material prepared from (2*RS*)-tyrosine, and by the following data (Found: C, 68·5; H, 5·6; N, 4·7. $C_{17}H_{17}NO_4$ requires C, 68·2; H, 5·7; N, 4·7%), $\lambda_{max.}$ 227 and 275 nm (shifted to 243 and 296 nm on addition of sodium hydroxide); $\nu_{max.}$ (Nujol), 3380, 3260, 1730, 1640, and 1615 cm^{-1}; τ [$(CD_3)_2SO$] 1·25 (1H, d, *J* 9 Hz, NH), 2·1—2·44 (5H, aryl H), 2·75 (2H, d, *J* 8 Hz, aryl H), 3·25 (2H, d, *J* 8 Hz, aryl H), 5·3 (1H, m, C*H*·CO$_2$Me), 6·3 (3H, s, OMe), and 6·9 (2H, d, *J* 9 Hz, C*H*$_2$Ar); *m*/*e* 299 (M^+, 3%), 178 (88), 147 (20), 122 (47), 107 (57), 105 (100), and 77 (69).

(2SR,3RS)-[3-$^3H_1$]*Tyrosine* [(11) + (12)].—A suspension of the foregoing methyl ester (0·53 g) in concentrated hydrochloric acid (125 ml) was heated under reflux for 20 h, then cooled, extracted with ether, and evaporated to dryness. The residue was dissolved in water (50 ml) and the pH adjusted to 5·7 with ammonium hydroxide to yield the labelled tyrosine [(11) + (12)] (0·3 g). The purity of this product and its identity were established (*a*) by paper chromatography against authentic (2*RS*)-tyrosine in butanol–acetic acid–water (3 : 1 : 1 v/v) and (*b*) by i.r. (Nujol) and u.v. comparison with (2*RS*)-tyrosine.

(2R,3S)-[2-^{14}C,3-$^3H_1$]*Tyrosine* (12) *and* (2R)-[1-^{14}C,2-$^3H_1$]-*Tyramine* (13).—The [3-$^3H_1$]tyrosine (14·05 mg, *ca.* 0·5 mCi) from the previous experiment was dissolved with (2*RS*)-[2-^{14}C]tyrosine (*ca.* 0·05 mCi) (Radiochemical Centre, Amersham) in N-hydrochloric acid (0·5 ml). N-Sodium hydroxide (0·05 ml) was added (to pH 5·7) and the crystals were collected (15·9 mg); this provided ^{3}H,^{14}C-labelled [(11) + (12)]. A sample (*ca.* 0·5 mg) was diluted with radioinactive (2*RS*)-tyrosine (100 mg); this material was recrystallised as before to constant specific activity and ^{3}H : ^{14}C ratio.

A stirred solution of a similar preparation of ^{3}H,^{14}C-labelled [(11) + (12)] (20 mg) in 0·2M-acetate buffer (pH 5·5; 25 ml) was incubated at 30° for 3 h with tyrosine decarboxylase (10 mg; 5 units; *Str. faecalis*; Cambrian Chemicals Ltd.) and pyridoxal phosphate (*ca.* 2 mg). More enzyme (5 units) was then added and incubation was continued for a further 3 h. After the solution had been boiled, the protein was removed by centrifugation; the solution was then evaporated and the residue in 0·2M-pyridinium acetate buffer (pH 5·0; 20 ml) was applied to an ion-exchange column prepared as follows.

Zeocarb 225 SRC 16 resin was treated first with an excess of 2N-sodium hydroxide, then in a column with 3N-hydrochloric acid until free of Na$^+$, and after thorough washing with water (eluant pH 3—4) was transferred to a beaker and pyridine was added (to final pH 7). A column of this resin (1 × 20 cm) was washed with 0·2M-pyridinium acetate buffer (pH 5·0) until the pH values of eluant and eluate were the same.

The (2*R*)-tyrosine was eluted from this column with the same buffer (150 ml needed) and was followed by u.v. spectroscopy; evaporation of the appropriate fractions, dissolution of the residue in water (2 ml), and adjustment to pH 5·7 gave (2*R*,3*S*)-[2-^{14}C,3-$^3H_1$]tyrosine (12) (9·5 mg). Part of this product (0·446 mg) was diluted with (2*S*)-tyrosine (100 mg) and recrystallised as before six times; the specific activity of the material then showed that the original sample contained $<$0·05% of the (2*S*)-isomer.

The (2*R*)-tyramine was eluted with 4N-ammonium hydroxide (200 ml), from which it was recovered by evaporation; the residue was dissolved in methanol (0·5 ml) and collected as (2*R*)-[1-^{14}C,2-^{3}H$_1$]tyramine hydrochloride by addition of ethereal hydrogen chloride.

(2RS,3SS)-[2-^{14}C,3-^{3}H$_1$]*Tyrosine* [(12) + (14)].—The foregoing tyrosine (12), diluted with radioinactive material (total 15 mg), was suspended in glacial acetic acid (1 ml), heated under reflux, and treated with acetic anhydride (0·32 ml). After 15 min, the solution was cooled to 20° and kept for 2 h, then evaporated to dryness. The residue was heated under reflux for 4·5 h with 4N-hydrochloric acid. The mixture was evaporated and the residue in water was adjusted to pH 5·7. The (2*RS*)-tyrosine [(12) + (14)] (13 mg) was precipitated; dilution analysis as before showed that complete racemisation had occurred [within experimental error 49·6% of (2*S*)-tyrosine present].

Degradation of (2SR,3RS)-[3-^{3}H$_1$]*Tyrosine* [(11) + (12)] *to* (2S,3S)-[3-^{3}H$_1$]*Aspartic Acid* [as (16)].—A solution of the [3-^{3}H$_1$]tyrosine [(11) + (12)] (3·7 mg) and radioinactive (2*RS*)-tyrosine (16·3 mg) in aqueous 5% formic acid (50 ml) was treated with a stream of ozonised oxygen [19] (*ca.* 5%) at 0° for 10 h. Nitrogen was then passed to remove ozone and after the addition of formic acid (1 ml) and hydrogen peroxide (100 vol.; 3 ml) the mixture was heated under reflux for 75 min. Palladised charcoal (10%; *ca.* 20 mg) was added to the cooled solution, which, after effervescence had ceased, was filtered and evaporated. The residue in water (20 ml) was adjusted to pH 3 with hydrochloric acid, and radioinactive (2*S*)-aspartic acid (0·25 g) was added. This solution was heated at 100° with an excess of copper(II) carbonate until precipitation of the copper aspartate was complete. The solid was collected, suspended in water (100 ml), and treated several times with an excess of hydrogen sulphide (with intermittent heating) until complete conversion into copper sulphide had been achieved. The solution was filtered and evaporated and the residue was recrystallised from aqueous ethanol to constant specific activity. Randomly ^{14}C-labelled (2*S*)-aspartic acid was added to give a ^{3}H : ^{14}C ratio of *ca.* 5; the product was converted as usual into its diethyl ester, which was crystallised to constant ^{3}H : ^{14}C ratio as the hydrochloride (from ethyl acetate–hexane), m.p. 95° (decomp.) and as the N-(*α-naphthylureido)-derivative*, m.p. 113—115° (from benzene–hexane). The latter was prepared by adding one drop of ethanolic phenolphthalein to diethyl (2*S*)-aspartate hydrochloride (30 mg) followed by saturated aqueous sodium hydrogen carbonate until the solution was just pink. The solution was evaporated to dryness and diethyl aspartate (18 mg) was distilled (short-path) from the residue at 100° and 0·5 mmHg. This in dry hexane (1 ml) was treated with a slight excess of α-naphthyl isocyanate; the precipitate was collected and recrystallised from benzene–hexane; yield 27 mg (Found: C, 63·7; H, 6·2; N, 8·0. $C_{19}H_{22}N_2O_5$ requires C, 63·7; H, 6·2; N, 7·8%), $\lambda_{max.}$ 227 and 292 nm; $\nu_{max.}$ 3400, 1730, and 1670 cm^{-1}; *m/e* 358 (M^+, 17), 312 (24), 229 (16), 228 (26), 211 (14), 169 (64), 144 (15), and 143 (100).

The (2*RS*,3*SS*)-[2-^{14}C,3-^{3}H$_1$]tyrosine [(12) + (14)] was degraded in the same way to yield (2*S*,3*R*)-[2-^{14}C,3-^{3}H$_1$]-aspartic acid; it was not necessary to add ^{14}C-labelled aspartic acid in this case.

(2S,3S)-[random-^{14}C,3-^{3}H$_1$]*Malic Acid* [as (17)].—A stirred solution of the foregoing (2*S*,3*S*)-[random-^{14}C,3-^{3}H$_1$]-aspartic acid (0·1 g) in N-hydrochloric acid (6 ml) was treated during 1 h with aqueous sodium nitrite (30% w/v; 2 ml). After 16 h at 20° the solution was evaporated, the residue was extracted with hot ethyl acetate (25 and 12 ml), and the extracts were evaporated. A solution of the resultant crude malic acid in 0·5N-sulphuric acid (1 ml) was adsorbed on silicic acid [20] (2 g; Mallinkrodt; freed from 'fines' by suspension in water and decantation several times followed by drying of the heavier particles).

The same coarse silicic acid (16 g) was mixed with 0·5N-sulphuric acid (11 ml) and was slurried into a column in chloroform; the malic acid–silicic acid mixture was then packed on the top of this column as a slurry in chloroform. Elution with butanol–chloroform (3 : 17 v/v; 100 ml) gave 2-chlorosuccinic acid (32 mg), identified as described later. The column was then washed with more butanol–chloroform (7 : 13 v/v; 100 ml and then 1 : 1 v/v; 200 ml). The latter eluant gave malic acid, which was extracted from the eluate into an equal volume of water. The water was evaporated off to give a homogeneous product (67 mg; t.l.c. on cellulose in 3 : 1 : 1 butanol–acetic acid–water), identified by comparison with authentic (2*S*)-malic acid.

One tenth of the radioactive malic acid was diluted with radioinactive (2*S*)-malic acid (40 mg) to allow crystallisation from ethyl acetate and hexane to constant ^{3}H : ^{14}C ratio. The final product was converted into its diphenacyl ester (see later) to confirm the ^{3}H : ^{14}C values.

The 2-chlorosuccinic acid showed τ (CD_3·OD) 5·38 (1H, t, *J* 7 Hz, CH) and 7·05 (2H, dd, 6·99 and 7·05 *J* 7 Hz, CH_2); the mass spectrum was that of the corresponding anhydride (Found: *m/e* 133·9771. $C_4H_3ClO_3$ requires 133·9760): *m/e* 136 (M^+, 17%), 134 (42), 126 (3), 124 (7), 99 (12), 108 (25), 106 (42), 73 (100), 90 (10), and 89 (11), *m** 85·7; 83·8, 62·5, 61·5, and 49·4.

Similar deamination of (2*S*,3*R*)-[2-^{14}C,3-^{3}H$_1$]aspartic acid gave (2*S*,3*R*)-[2-^{14}C,3-^{3}H$_1$]malic acid.

(2S)-*Malic Acid Diphenacyl Ester*.—0·1N-Lithium hydroxide was added to (2*S*)-malic acid (11 mg) to give a solution of pH 8 which was evaporated. The residue was thoroughly dried and dry dimethylformamide (1 ml) and phenacyl bromide (20 mg) were added; the mixture was heated at 90° for 6 h before evaporation. That part of the residue soluble in chloroform was fractionated on thick silica plates in ether, and the component of R_F *ca.* 0·3 was eluted with methanol–ethyl acetate (1 : 9 v/v). Recrystallisation from aqueous ethanol gave (2S-*malic acid diphenacyl ester* (15 mg), m.p. 110·5—111° (Found: C, 65·0; H, 4·8. $C_{20}H_{18}O_7$ requires C, 64·9; H, 4·9%).

Enzymic Conversion of Labelled Malic Acids into Fumaric Acids.—A solution of the ^{3}H,^{14}C-labelled malic acid (60 mg) in water (5 ml) was adjusted to pH 7·4 with N-sodium hydroxide, and 0·01M-phosphate buffer (5 ml; pH 7·4) was added. Malate hydro-lyase (30 μl; 2 mg ml^{-1}; Boehringer) was added, and after the mixture had been kept at 39° for 2 h it was acidified with concentrated hydrochloric acid. Radioinactive fumaric acid (20 mg) was added and the solution was evaporated to dryness. Extraction of the residue with hot ethanol (2 × 25 ml) gave, after evaporation, a product which in 0·5N-sulphuric acid (1 ml) was adsorbed on silicic acid (2 g). Fractionation was continued as in the previous experiment and the fumaric acid was eluted with butanol–chloroform (3 : 17 v/v; 100 ml). Shaking the eluate with an equal volume of water recovered the fumaric

[19] *Cf.* H. Corrodi and E. Hardegger, *Helv. Chim. Acta*, 1955, **38**, 2038.

[20] *Cf.* W. A. Bulen, J. E. Varner, and R. C. Burrel, *Analyt. Chem.*, 1952, **24**, 187.

acid from the organic phase, and the water was evaporated to give pure fumaric acid (19 mg), identified by comparison with authentic material (t.l.c. on cellulose in 3 : 1 : 1 butanol–acetic acid–water). It was recrystallised from water to constant specific activity and ^3H : ^{14}C ratio.

Enzymic Conversions with Phenylalanine Ammonia-lyase (from Maize and Potatoes).—The maize and potato enzymes used in these experiments had been purified as previously described.[5,21] Units for both are expressed in terms of (2*S*)-phenylalanine as the substrate.[21] Where side fractions of low specific activity were employed, they were of good stability (see ref. 1). The enzyme was dialysed against 0·04M-borate (Na^+) buffer, pH 8·7, prior to use.

(a) *Enzymic stereospecificity by* ^3H : ^{14}C *labelling* (Table 2). For Experiments 5 and 7, the labelled (2*RS*)-tyrosine (*ca.* 0·5 mg, 2·76 μmol) was treated in 0·04M-borate (Na^+) buffer, pH 8·7, at 23° for 48 h with the enzyme from maize (*ca.* 1·6 units; spec. act. *ca.* 20 munits per mg; enzyme purification including the agarose column stage); reaction vol. 20 ml; substrate concn. 60 μmol l^{-1} of (2*S*)-isomer. The removal of (2*S*)-tyrosine (complete in 20 h) and the accumulation of *p*-hydroxycinnamate was assayed (25 μl samples) by measurement of radioactivity following t.l.c. on silica gel [benzene–acetic acid–water (2 : 2 : 1 v/v), upper phase; ChromAR sheet 500, Mallinckrodt]. After 48 h, the enzyme in the reaction mixture (for assay: 25 μl) was as active as at zero time. The mixture was dialysed at 0° against 0·04M-borate (Na^+) buffer, pH 8·7 (700 ml), for 20 h and the dialysate was evaporated. The tyrosine and *p*-hydroxycinnamate in the residue were examined as described later.

For Experiments 6 and 8, the labelled tyrosine (*ca.* 0·4 mg, 2·2 μmol) was treated anaerobically in 0·04M-borate (Na^+) buffer, pH 8·7, at 23° for 1 h with a similar preparation of the enzyme (*ca.* 130 munits; spec. act. *ca.* 40 munits per mg); reaction vol. 6 ml; substrate conc. 180 μmol l^{-1} of (2*S*)-isomer. The mixture was then cooled to 0°, assayed for the removal of radioactive tyrosine and treated further as before.

The dry buffer residue from each of the foregoing experiments was partitioned between water (80 ml), N-hydrochloric acid (20 ml), and ethyl acetate (100 ml) to which radioinactive *p*-hydroxycinnamic acid (50—100 mg) and (2*S*)-tyrosine (50—100 mg) were added. The organic layer was separated and the aqueous phase was extracted thrice with ethyl acetate. Evaporation of the combined organic extracts gave crude *p*-hydroxycinnamic acid, which was recrystallised once from water and then from ethyl acetate–hexane to constant specific activity and ^3H : ^{14}C ratio; m.p. 209—210°. A portion (31 mg) was converted into its methyl ester (with diazomethane); yield 25 mg, m.p. 136—137° (from aqueous methanol).

The aqueous layer from the foregoing extraction with ethyl acetate was evaporated, and the residue in water (20 ml) was adjusted to pH 5·7 with sodium hydroxide. Recrystallisation of the precipitated (2*S*)-tyrosine was carried out as described earlier to constant specific activity and ^3H : ^{14}C ratio.

(b) *Sublimation assay: comparisons of enzymic stereospecificity* (*Table* 3). Ten samples of the same preparation [1] of (2*SR*,3*RS*)-[3-$^3H_1$]phenylalanine [as (11) + (12)] (spec. act. 12·4 nCi μmol^{-1}) in 0·04M-borate (Na^+) buffer, pH 8·7, were treated as indicated in Table 3 with enzyme from maize (spec. act. *ca.* 10 munits per mg; purification including the agarose column stage) or from potatoes (spec. act. *ca.* 400 munits per mg; purified to same stage); reaction vol. 2·5 ml. After 20 h, samples (3 × 100 μl) were taken for counting. A further portion (1·9 ml) was made alkaline with 4N-potassium hydroxide (0·1 ml), sampled (3 × 100 μl), then divided into two portions and the tritiated water was sublimed by use of a Y-assembly.[22] Both sublimates were sampled (2 × 100 μl). The results for the four sublimed samples agreed closely with each other and also with further results obtained by a second sublimation from alkali carried out on the pooled first sublimates. No quenching correction was necessary for the alkaline samples. The complete set of values allows *r* for Table 3 to be calculated.

(c) *Partial reactions* (*Table* 4). The enzyme from maize (spec. act. 87 munits per mg; purification including the agarose column stage) was used; separations by t.l.c. were performed as under (a). (2*S*)-[*U*-^{14}C]Phenylalanine (480 nCi $mmol^{-1}$) and (2*S*)-[*U*-^{14}C]tyrosine (396 nCi $mmol^{-1}$) (Radiochemical Centre, Amersham, and New England Nuclear, respectively) were converted into labelled cinnamate and *p*-hydroxycinnamate as follows and the products were used the same day. (2*S*)-[*U*-^{14}C]Phenylalanine (1 μCi; 2·08 nmol) was treated in 17mM-borate (Na^+) buffer, pH 8·7, at 23° for 1 h with the ammonia-lyase (60 munits); reaction vol. 0·3 ml; substrate concn. 6·9 μmol l^{-1}. Samples (5 μl) were taken at 15 min intervals and fractionated with carrier cinnamate by t.l.c.; the cinnamic acid region was assayed by its radioactivity. The reaction reached equilibrium in 30 min. The [*U*-^{14}C]cinnamate in the main reaction mixture was similarly separated but without dilution and was eluted with M-phosphate (K^+) buffer, pH 6·8. *p*-Hydroxy[*U*-^{14}C]cinnamate was similarly prepared by treating (2*S*)-[*U*-^{14}C]tyrosine (5 μCi; 12·8 nmol) in 40mM-phosphate (K^+) buffer, pH 7·6, at 23° for 1 h with the ammonia-lyase (85 munits); reaction vol. 0·3 ml; substrate concn. 42·6 μmol l^{-1}.

For Experiment 9t, the (2*S*)-tyrosine (0·5 μmol; concn. 1·67 mmol l^{-1}) in 40mM-phosphate (K^+) buffer, pH 6·8, was treated at 23° for 5 min with the ammonia-lyase (43 munits). In Experiment 9c, the (2*S*)-tyrosine was replaced by ammonium sulphate (0·15 mmol; ammonium ion concn. 1 mmol l^{-1}). After 5 min, [*U*-^{14}C]cinnamate (50 nCi; 0·1 nmol) was added to each reaction mixture. The incorporation of radioactivity into phenylalanine was checked during 90 min by taking samples (10 μl) at 15 min intervals for t.l.c. as before. The region containing phenylalanine was assayed for radioactivity. For Experiment 10t, (2*S*)-phenylalanine (2 μmol) in the same buffer was treated with the same amount of enzyme (substrate concn. 6·67 mmol l^{-1}), and for Experiment 10c, (2*S*)-phenylalanine was replaced by ammonium sulphate (0·3 mmol) (ammonium ion concn. 2 mmol l^{-1}). After 5 min, *p*-hydroxy[*U*-^{14}C]cinnamate (130 nCi; 0·27 nmol) was added to each mixture. The incorporation of radioactivity into tyrosine was monitored as for phenylalanine. In Experiments 9t and 10t, the incorporations into amino-acid amounted to *ca.* 55, 90, and 100% of the final value at 30, 60, and 90 min. The diminishing rate of incorporation is as expected because the product of the forward reaction competes with labelled cinnamate or *p*-hydroxycinnamate for the amino-enzyme intermediate. After 90 min, the main bulk of the reaction

[21] E. A. Havir and K. R. Hanson, *Methods Enzymol.*, 1970, **17*a***, 575.

[22] K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1.

mixtures 9t and 10t was heated at 100° for 2 min, and centrifuged. That radioactivity was incorporated into the stated amino-acids was checked by further t.l.c. by paper chromatography with two solvent systems,[17a] and by high-voltage electrophoresis (Savant flat-plate apparatus) at pH 1·9, 15°, and 60 V cm^{-1}.[22]

The foregoing experiments were carried out at pH 6·8 because this had proved satisfactory for partial reactions with the enzyme from potatoes.[17a]

We thank the S.R.C. for Research Studentships (to P. G. S. and H. R. W.), Dr. H. B. F. Dixon for his advice, and Drs. J. E. Kelsey and J. L. McHugh for experimental information in advance of publication. We also thank the following for financial support: New Haven, The National Science Foundation; Cambridge, The Nuffield Foundation and the S.R.C.

[2/884 *Received*, 21*st April*, 1972]