Stereochemistry and Kinetic Isotope Effects in the Bovine Plasma Amine Oxidase Catalyzed Oxidation of Dopamine†

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ABSTRACT: The stereochemistry of the bovine plasma amine oxidase catalyzed oxidation of 2-(3,4-dihydroxyphenyl)-ethylamine (dopamine) has been investigated by comparing $^3H/^{14}C$ ratios of 3,4-dibenzyloxyphenethyl alcohols, derived from 3,4-dihydroxyphenylacetaldehydes, to starting dopamines chirally labeled at C-1 and C-2. The oxidation of [2*RS*-^{3}H]-, [2*R*-^{3}H]-, and [2*S*-^{3}H]dopamine leads to products which have retained 53, 59, and 47% of their tritium. Similarly, oxidation of [1*RS*-^{3}H]-, [1*R*-^{3}H]-, and [1*S*-^{3}H]dopamine leads to an 80, 80, and 92% retention of tritium. The configurational purity of tritium at C-2 of dopamine and C-1 of the dopamine precursor 3-methoxy-4-hydroxyphenethylamine has been confirmed employing dopamine-β-hydroxylase (specific for the *pro-R* hydrogen at C-2) and pea seedling amine oxidase (specific for the *pro-S* hydrogen at C-1). In addition, chromatographically resolved isozymes of bovine plasma amine oxidase have been demonstrated to lead to the same stereochemical result as pooled enzyme fractions. We have been able to rule out carbon interchange and tritium transfer in the ethylamine side chain of dopamine as the source of the apparent nonstereospecificity. Estimated primary tritium isotope effects are 1 for [2-^{3}H]dopamines and 5–6 and 26–34 for [1*R*-^{3}H]- and [1*S*-^{3}H]dopamine, respectively. We propose the presence of alternate dopamine binding modes, characterized by absolute but opposing stereochemistries and differential primary tritium isotope effects at C-1.

The Cu^{2+}-containing plasma amine oxidases are a group of enzymes catalyzing the oxidative deamination of primary amines:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

The overall equation represented above is generally considered to be the product of two partial reactions: oxidation of the amine substrate to the corresponding Schiff base, which is subsequently hydrolyzed to aldehyde and ammonia, followed by reoxidation of reduced enzyme by molecular oxygen (Oi et al., 1970; Suva & Abeles, 1978):

$$E_{ox} + RCH_2NH_2 + H_2O \rightarrow [E_{red}\cdot RCH{=}NH] \rightarrow E_{red} + RCHO + NH_3$$
$$E_{red} + O_2 \rightarrow E_{ox} + H_2O_2 \quad (2)$$

The enzyme isolated from bovine plasma has been shown to consist of two identical 85 000 mol wt subunits, covalently linked via an interchain disulfide bond (Achee et al., 1968). Although this enzyme is referred to as spermine oxidase, it has a broad substrate specificity and oxidatively deaminates numerous amines such as benzylamine and dopamine[1] at comparable rates (Yamada & Yasunobu, 1962). In contrast to the membrane-bound mitochondrial monoamine oxidases which contain FAD and may have an important function in the oxidative deamination of biogenic amines, both the chemical nature of the active site group(s) and the physiologic significance of plasma amine oxidases are unclear.

Mechanistic investigations of the bovine plasma amine oxidase reaction have been largely concerned with either potential valency changes of the enzyme-bound Cu^{2+} or the presence of pyridoxal phosphate; the latter had been inferred from the spectral properties of plasma amine oxidases, the sensitivity of these enzymes to carbonyl-reacting reagents, and by mechanistic comparison with other pyridoxal phosphate containing enzymes such as transaminases and decarboxylases. In an early study, Yamada et al. (1963) ruled out changes in the valence of enzyme-bound Cu^{2+} upon formation of reduced enzyme. In addition, extensive investigations to detect pyridoxal phosphate or a pyridoxal-like cofactor have failed to implicate a role for such a functional group. For example, Inamasu et al. (1974) observed that BH_4 reduction of enzyme under conditions of benzylamine turnover results in the attachment of benzaldehyde to the ϵ-amino group of a lysine residue rather than pyridoxal phosphate. Recently, Suva & Abeles (1978) have shown that incubation of enzyme under anaerobic conditions with the substrate [α-^{3}H]ethyl glycinate does not lead to the incorporation of nonexchangeable tritium into the enzyme, making a transamination mechanism extremely unlikely. As an alternative to a carbonyl functional group at the active site, both a sulfenic acid residue (Benitez & Allison, 1974; Allison et al., 1973) and the interchain disulfide bond of plasma amine oxidase (Neumann et al., 1975) have been proposed as the chemically relevant functional group. Suva & Abeles (1978) have now demonstrated that the reduced enzyme contains a single reactive cysteine per subunit which is not available in the oxidized enzyme. They propose a prosthetic group comprised of a cysteine sulfur, and an as yet unidentified group "X", –"X"–S–.

Neumann et al. (1975) reported that bovine plasma amine oxidase catalyzes an elimination of HCl from β-chlorophenethylamine to form phenylacetaldehyde, at a rate comparable to the normal oxidative reaction, and considered

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[1] Abbreviations used: dopamine, 2-(3,4-dihydroxyphenyl)ethylamine; ConA, concanavalin; DMF, dimethylformamide; THF, tetrahydrofuran; Me_4Si, tetramethylsilane.

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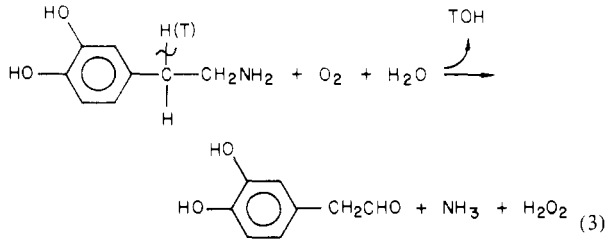
the elimination reaction evidence for proton abstraction in the normal oxidative process. Additional support for an enzyme-mediated proton abstraction derives from the observation that the plasma amine oxidase catalyzed oxidation of dopamine results in a concomitant release of tritium from C-2 of this molecule (Lovenberg & Beaven, 1971), eq 3. Although such



(3)

tritium release could occur directly to solvent from an activated form of the substrate, a more likely mechanism involves base catalysis by an enzyme bound residue since approximately 50% of label was observed to be lost from dopamine, randomly labeled at C-2.

In this paper we report the results of an investigation of the stereochemistry of hydrogen activation in the oxidation of dopamine, chirally labeled with tritium at both C-1 and C-2. Parallel studies indicate that benzylamine oxidation involves the stereospecific loss of the *pro-S* hydrogen at C-1 (Battersby, A. R., Staunton, J., Klinman, J. P., & Summers, M. C., 1979; Suva & Abeles, 1978), analogous to the stereochemistry observed for the pea seedling amine oxidase reaction (Battersby et al., 1976a,b). Surprisingly, we find that dopamine oxidation involves nonstereospecific loss of tritium from both C-1 and C-2. The implications of these results to the mechanism and active site configuration of bovine plasma amine oxidase are considered.

Experimental Section

Materials

Potassium [^{13}C]cyanide (98% atom excess) was obtained from Prochem, B.O.C. Limited, London, palladium on charcoal (10%) from Koch-Light Laboratories, $NaBH_4$ from Fisons Chemicals, and $LiAlH_4$ from BDH Chemicals Limited. *N,N*-Dimethylformamide (Aldrich Chemical Co.) was dried over CaH_2 and distilled under reduced pressure. Boron trifluoride etherate (BDH Chemicals Ltd.) was distilled under reduced pressure from 2% CaH_2, 10% anhydrous ether. Tetrahydrofuran (Aldrich Chemical Co.) and dioxane were stored over CaH_2 and distilled from $LiAlH_4$ immediately before use. Benzyl chloride (Aldrich Chemical Co.) was dried over anhydrous $MgSO_4$, distilled under reduced pressure and stored over CaH_2. Anhydrous methanol was prepared by heating AR methanol (BDH Chemicals Ltd.) at reflux with magnesium turnings, followed by distillation. Ether, benzene, and petroleum ethers were distilled with P_2O_5, and, when necessary, stored over sodium wire. α-Methylmannoside was purchased from Calbiochem. Preparative thin-layer chromatography was carried out on glass plates coated with silica gel obtained from Merck, Darmstadt, West Germany. Bio-Rex 70 and hydroxylapatite resins were from Bio-Rad Laboratories. ConA–Sepharose and Sephadex G-200 were purchased from Pharmacia, and DE-52 cellulose was from Whatman.

Enzymes

Liver alcohol dehydrogenase, yeast aldehyde dehydrogenase, and catalase were from Sigma. A partially purified preparation of solubilized rat liver mitochondrial monoamine oxidase was kindly provided by Professor K. F. Tipton, Cambridge. Dopamine β-hydroxylase was prepared from bovine adrenal glands as described by Rush et al. (1974).

Three different forms of bovine plasma amine oxidase, referred to as A1, A2, and B, were purified by modification of the procedure of Yamada & Yasunobu (1962). Briefly, two major peaks of enzyme activity were separated on DE-52; fraction A eluted with 0.05 M phosphate, pH 7.0, and fraction B with 0.07 M phosphate, pH 7.0. Fraction A was further resolved into two peaks of enzyme activity on hydroxylapatite; fraction A1 eluted with 0.01 M phosphate, pH 6.8, and fraction A2 with 0.06 M phosphate, pH 6.8. Fraction B gave a single peak on hydroxylapatite which was eluted with 0.06 M phosphate, pH 6.8. Finally, the three separate fractions were purified by chromatography on Sephadex G-200. Enzyme activity, expressed in international units, was assayed as described by Neumann et al. (1975). The total yield of purified amine oxidase was 20%, and this consisted of 9.6, 22.5, and 11.3 U of specific activity 0.23, 0.29, and 0.36 U/mg for fractions A1, A2, and B, respectively.

Substrates

Dopamine hydrochloride was purchased from Koch-Light Laboratories. Benzylamine (Sigma) was converted to the hydrochloride salt and crystallized from methanol/ethyl acetate. NADH, NAD^+, and $NADP^+$ were from Boehringer. [2-^{3}H]Dopamine samples were a kind gift of Professor A. R. Battersby, Cambridge. Specific activities were 2.5×10^6, 1.7×10^6, and 2.6×10^6 cpm/μmol for the [2*RS*-^{3}H]-, [2*R*-^{3}H]-, and [2*S*-^{3}H]dopamines, respectively. The syntheses of [1-^{3}H]dopamines, from the corresponding 3-methoxy-4-hydroxyphenethylamine derivatives (also a gift from Professor Battersby), and [1-^{13}C]dopamine are described in detail below.

Preparation of [1RS]-, [1R]-, and [1S-^{3}H]Dopamine

The same synthetic procedure was used to prepare the three tritiated dopamine samples. Typically, a small sample of [1*RS*]-, [1*R*]-, or [1*S*-^{3}H]-2-(3-methoxy-4-hydroxyphenyl)ethylamine hydrochloride (4–5 mg; total cpm $\sim 3 \times 10^7$) was dissolved in 1 mL of freshly distilled 48% HBr and heated at reflux for 5 h, under nitrogen gas. After 5 h, solvent was removed by vacuum transfer. The ^{3}H-labeled dopamine sample was purified on preparative silica gel thin-layer chromatographic plates developed with *n*-BuOH:AcOH:H_2O (12:3:5): R_f dopamine, 0.39. Dopamine was extracted with MeOH:AcOH (3:1), and the solvent was subsequently removed on the rotary evaporator. To the residue was added 25 μmol of unlabeled dopamine (together with a small amount of ^{14}C labeled dopamine) in 2.0 mL of potassium phosphate buffer (50 μmol, pH 7.0), which was passed down a short column of Bio-Rex 70 (H^+ form, 0.8 × 3.0 cm). The column was washed extensively with H_2O, followed by 100 mM HCl. Fractions containing dopamine were pooled and freeze-dried. The purified [^{3}H]dopamine was stored in sealed vials (1–5 μmol per vial) under vacuum and kept at −15 °C. The overall yield was 50–60%. Specific activities were 6.5×10^5, 2.7×10^5, and 2.1×10^5 cpm/μmol for the [1*RS*-^{3}H]-, [1*R*-^{3}H]-, and [1*S*-^{3}H]dopamines, respectively.

*Synthesis of [1-^{13}C]Dopamine [Scheme I (**5**)]*

[cyano-^{13}C]-3-Methoxy-4-benzyloxyphenylacetonitrile [(**2**) *R = Me*]. Solid $K^{13}CN$ (225 mg, 3.41 mmol; 98% atom excess) was added to a stirred solution of 3-methoxy-4-benzyloxybenzyl chloride (800 mg, 3.36 mmol) in anhydrous dimethylformamide (DMF), and left at room temperature for 42 h. The solution was poured onto an ice/water (250 g)

mixture and mixed vigorously for 30 min. The resulting pale yellow solid was extracted into chloroform (2 × 150 mL) which was subsequently washed with H_2O (100 mL) and saturated brine (50 mL) and dried over anhydrous $MgSO_4 \cdot H_2O$. The residue obtained on evaporation was dried in vacuo over P_2O_5 to remove the last traces of DMF and finally crystallized from benzene/petroleum ether (30–40 °C) to give an amorphous white solid (640 mg, 83%), mp 65–67 °C [lit. (Benington & Morin, 1967) 69–70 °C].

[1-^{13}C]-2-(3-Methoxy-4-benzyloxyphenyl)ethylamine Hydrochloride (***3***). The aforementioned [^{13}C]nitrile [(***2***) R = Me, 550 mg] was added to a solution of $NaBH_4$ (1.0 g) in anhydrous tetrahydrofuran (THF) (60 mL). Freshly distilled boron trifluoride ether (8 mL) was rapidly added and the mixture heated at reflux for 5 h, under dry nitrogen gas. Water (20 mL) was added, followed by the careful addition of 3 N HCl to decompose excess borohydride. The solvent was evaporated to a small volume and the residue partitioned between 1 N NaOH and ether. The aqueous layer was extracted with more ether. The combined organic layer was washed with H_2O and saturated brine and dried over anhydrous Na_2SO_4. The amine was precipitated as the hydrochloride salt by the addition of ethereal hydrogen chloride and crystallized from methanol/ethyl acetate (460 mg, 73%), mp 174–175 °C [lit. (Battersby et al., 1964) 176–178 °C].

[1-^{13}C]Dopamine (***5***). This was prepared from ***3*** using the procedure described earlier for the synthesis of stereospecifically labeled [1-^{3}H]dopamines. Since the reaction was carried out on a larger scale, the material was recrystallized several times from methanol/ethyl acetate to give colorless plates, mp 230–238 °C [lit. (*The Merck Index*, 1968) 241 °C]. For NMR assignment (see Figure 1c) a small amount of ^{13}C-material was diluted with unlabeled dopamine to give about 6% enriched sample, which was similarly recrystallized from methanol/ethyl acetate.

*Synthesis of Reference Sample: [1-^{13}C]-2-(3,4-Dibenzyloxyphenyl)ethyl Alcohol [Scheme I (**7**)]*

[cyano-^{13}C]-3,4-Dibenzyloxyphenylacetonitrile [(***2***) *R = PhCH$_2$*]. A similar procedure to the one described above was used, leading to a recrystallized yield of 84%, mp 52–53 °C [lit. (Battersby et al., 1971) 53–54 °C].

[carboxyl-^{13}C]-3,4-Dibenzyloxyphenylacetic Acid (***6***). The corresponding [^{13}C]nitrile (100 mg; 304 μmol) was heated at 140 °C for 22 h in ethylene glycol (1.0 mL) and H_2O (0.31 mL) containing KOH (250 mg). The cooled solution was diluted with H_2O (4.0 mL) and extracted with ether (2 × 5 mL). The aqueous phase was adjusted to pH 2, by the addition of concentrated HCl, and extracted again with ether (3 × 10 mL). The combined organic phase was washed with H_2O and saturated brine and dried over anhydrous Na_2SO_4. The residue obtained on evaporation of the ether was crystallized several times from toluene/*n*-heptane: yield, 64 mg (61%); mp 108–109 °C [lit. (Carlsson et al., 1962) 109 °C].

[1-^{13}C]-2-(3,4-Dibenzyloxyphenyl)ethyl Alcohol (***7***). The above alcohol was synthesized in two steps from **6** as follows: the [^{13}C]carboxylic acid (54 mg) was dissolved in methanol (0.5 mL) and treated with excess diazomethane in ether for 10 min. Solvent was removed in a stream of nitrogen gas and the residue dried in vacuo. The methyl ester was used without further purification. R_f (silica, ether solvent): methyl ester, 0.75; carboxylic acid, 0.13; τ (^{12}C-labeled material) 2.6 (m, 10 H), 3.2 (m, 3 H), 4.9 (s, 2 H), 6.4 (s, 3 H), 6.5 (s, 2 H).

The ^{13}C-labeled methyl ester in anhydrous THF (8 mL) was slowly added to a stirred suspension of $LiAlH_4$ (50 mg) in the same solvent (5.0 mL). The reaction was heated at reflux overnight. After workup from aqueous NaOH (Fieser & Fieser, 1967), the crude ^{13}C-labeled alcohol was crystallized from benzene/*n*-heptane/petroleum ether (40–60 °C): yield, 28 mg (54% from acid); τ (^{12}C-labeled material) 2.7 (m, 10 H), 3.2 (m, 3 H), 4.9 (s, 2 H), 6.25 (t, J_{HH} = 6 Hz, 2 H), 7.3 (t, J_{HH} = 6 Hz, 2 H), 8.6 (s (broad), 1 H). Finally 6.5 mg of ^{13}C-labeled alcohol was mixed with unlabeled alcohol (81 mg) and recrystallized three times. This material was then used to assign the C-1 and C-2 carbon atoms of the ethyl side chain (see Figure 2b): the ^{13}C enrichment at C-1 is ca. 7.3%.

Methods

Amine substrates were assayed enzymatically using dansyl chloride (Creveling & Daly, 1971). Radioactivity was determined in toluene–ethanol cocktails on Intertechnique or Packard Tri-Carb scintillation spectrometers, and standardized using radiolabeled [^{3}H]- and [^{14}C]-*n*-hexadecane (Radiochemical Centre, Amersham). Counting efficiency for ^{3}H was ~20%, and for ^{14}C ~50%. In double-label experiments, the windows were set such that there was no overlap of ^{3}H into the ^{14}C channel, and ^{14}C overlapped by a factor of 2.0 ± 0.2 into the ^{3}H channel.

Proton nuclear magnetic resonance spectra were recorded on either a Perkin-Elmer R-12B (60 MHz) or Varian HA-100 (100 MHz) spectrometer using approximately 5% w/v solutions in deuteriochloroform or methanol-d_4 with tetramethylsilane (Me_4Si) as internal standard. Each signal is described above in terms of chemical shift (in τ values from Me_4Si), intensity, multiplicity, and coupling constant (J_{HH} in Hz) with use of the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet.

Carbon-13 NMR spectra were recorded at 20 MHz on a CFT-20 spectrometer with proton noise decoupling. All spectra were recorded using the following conditions: 4000-Hz sweep width; 1.023-s acquisition time; pulse length of 10 μs. There was no pulse delay. Amine samples were recorded in dilute hydrochloric acid with a deuterium oxide insert as internal lock. The sample concentrations were 0.17–0.5 M in a volume of 1 mL in 12-mm diameter tubes. Internal dioxane (67.4 ppm downfield from Me_4Si) was used to assign chemical shifts of relevant ^{13}C nuclei. The carbon-13 spectra of the alcohol samples were recorded in benzene-d_6 which also served as internal lock. Chemical shifts were assigned using the signals due to benzene: 129.2, 128.0, and 126.8 ppm downfield from Me_4Si.

Chemical Conversion of 2-(3,4-Dihydroxyphenyl)ethyl Alcohol to 2-(3,4-Dibenzyloxyphenyl)ethyl Alcohol. In the tritium and carbon-13 studies, the dihydroxyphenethyl alcohol was converted to the protected *O,O'*-dibenzyl derivative by the following procedure. The residue (100–200 μmol of dihydroxyphenethyl alcohol), obtained on evaporation of the ethyl acetate extract, was dried over P_2O_5 in vacuo, and subsequently dissolved in absolute methanol (5 mL) containing benzyl chloride (0.4 mL). Anhydrous K_2CO_3 (400 mg) was added and the mixture heated at reflux for 14 h, under dry nitrogen gas. Methanol was removed on the rotary evaporator, and the slurry partitioned between H_2O (10 mL) and ether (10 mL). The aqueous layer was extracted with more ether (2 × 10 mL). The combined organic phase was washed with H_2O and saturated brine and dried over anhydrous $MgSO_4 \cdot H_2O$. Excess benzyl chloride was removed using a vacuum pump and the crude *O,O'*-dibenzyl alcohol purified by TLC on a single 20 × 20 × 0.25 cm silica plate developed with $CHCl_3$:MeOH (5:1). The alcohol was then crystallized several times from benzene/*n*-heptane/petroleum ether (40–60 °C). The overall yield after two recrystallizations was about 25%.

Table I: Stereochemistry of [2-^{3}H]Dopamine Oxidation Catalyzed by Bovine Plasma Amine Oxidase[a]

dopamine	$^3H/^{14}C$	3,4-dibenzyloxyphenethyl alcohol $^3H/^{14}C$	% ^3H retention
[2*RS*-^{3}H]-	10.6	5.6	53
[2*R*-^{3}H]-	9.2	5.4	59
[2*S*-^{3}H]-	8.7	4.1	47

[a] Two micromoles of [2-^{3}H]dopamine, mixed with [1-^{14}C]dopamine to give the desired $^3H/^{14}C$ ratios, was incubated with plasma amine oxidase (0.21 U), NADH (4 μmol), liver alcohol dehydrogenase (1 U), 100 μmol of 100 mM P_i, pH 7.2, in a total volume of 1 mL. After 45 min, the reaction was stopped by the addition of 25 μL of concentrated HCl, unlabeled 3,4-dihydroxyphenethyl alcohol (60 μmol) was added, and the aqueous solution was extracted with ethyl acetate (4 × 2.0 mL). The organic phase was washed with water (2 × 3 mL) and saturated brine (2 mL) and dried. 3,4-Dihydroxyphenethyl alcohol was converted to 3,4-dibenzyloxyphenethyl alcohol as described in the Methods section.

Deprotection of 2-(3,4-Dibenzyloxyphenyl)ethyl Alcohol. 2-(3,4-Dihydroxyphenyl)ethyl alcohol is conveniently stored as the *O,O'*-dibenzyl derivative. In those experiments where unlabeled dihydroxyphenethyl alcohol was added to the reaction medium, deprotection of the relevant amount of dibenzyl derivative was carried out *immediately* before dilution of labeled material was required. Quantitative yields of deprotected material were obtained by catalytic hydrogenolysis. Typically, 3,4-dibenzyloxyphenethyl alcohol (22 mg, 60 μmol) was dissolved in 95% ethanol (5 mL) containing 1 drop of 3 N HCl. Palladized charcoal (10%, 12 mg) was added and hydrogenolysis carried out at ambient temperature and 1 atm of hydrogen until uptake was complete. The catalyst was removed by filtration through a small pad of Celite. The filtrate was evaporated to very small volume, diluted with H_2O, and used directly. The same procedure was used to deprotect 3,4-dibenzyloxyphenylacetic acid when 3,4-dihydroxyphenylacetic acid was required.

Isolation and Purification of 2-(3,4-Dihydroxyphenyl)ethyl Alcohol and 3,4-Dihydroxyphenylacetic Acid. As shown in Tables III and V, the $^3H/^{14}C$ ratio of labeled 3,4-dihydroxyphenethyl alcohol and phenylacetic acid resulting from the amine oxidase conversion of dopamine coupled to alcohol and aldehyde dehydrogenase, respectively, was obtained as follows. The residue, after vacuum transfer, was dissolved in methanol (0.2 mL) containing 10 μmol of unlabeled material. The solution was transferred to a single 20 × 20 × 0.1 cm silica plate and developed with $CHCl_3$:MeOH:AcOH (18:2:1). The plate was dried in air for at least 2 h and then extracted with dioxane:acetic acid (2 mL; 10:1). Insoluble material was removed by centrifugation at low speed, and an aliquot of supernatant removed for scintillation counting. R_f (silica, $CHCl_3$:MeOH:AcOH, 18:2:1): dihydroxyphenethyl alcohol, 0.3; dihydroxyphenylacetic acid, 0.2.

Results

Stereochemistry of [2-^{3}H]Dopamine Oxidation. The stereochemistry of tritium loss from C-2 of dopamine upon oxidation with plasma amine oxidase was determined by comparing the $^3H/^{14}C$ ratios of the appropriately labeled dopamine to 3,4-dibenzyloxyphenethyl alcohol. A coupled assay in which product aldehyde is converted directly to alcohol was carried out, in order to eliminate any nonspecific loss of tritium from C-2 of 3,4-dihydroxyphenylacetaldehyde (Scheme II, A). Benzylation of the ring hydroxyl groups gives 3,4-dibenzyloxyphenethyl alcohol, which is air stable and hence easily purified. As summarized in Table I, oxidation of [2*RS*-^{3}H]dopamine leads to a derivative which has retained 53% of its tritium, consistent with the observation of Lovenberg & Beaven (1971) of an approximately 46% release of tritium into water.

Table II: Stereochemistry of [2-^{3}H]Dopamine Hydroxylation Catalyzed by Dopamine-β-Hydroxylase[a]

dopamine	^3H in H_2O	^3H in residue (cpm)	% ^3H volatile
[2*RS*-^{3}H]-	1.44×10^4	1.74×10^4	45
[2*R*-^{3}H]-	2.38×10^4	0.18×10^4	93
[2*S*-^{3}H]-	0.004×10^4	1.78×10^4	0.22

[a] Tritiated dopamines together with carrier dopamine (~0.6 μmol total) were incubated with 15 μmol of ascorbate, 15 μmol of fumarate, catalase (1760 U), dopamine-β-hydroxylase (0.12 U), and 75 μmol of sodium acetate, pH 5.5, in a volume of 1.5 mL. At 30-min intervals, 0.1 mL of the reaction was added to an equal volume of 5% $HClO_4$ and assayed for norepinephrine production according to Von Euler & Floding (1955). Tritiated water was separated from labeled product by bulb-to-bulb distillation in vacuo of frozen, acidified reaction mixtures. The above data correspond to 0.1 mL of the reaction at >90% product formation.

Table III: Stereochemistry of [2-^{3}H]Dopamine Oxidation Catalyzed by Purified Plasma Amine Oxidase Isozymes[a]

		3,4-dibenzyloxyphenethyl alcohol $^3H/^{14}C$			% retention			
dopamine	$^3H/^{14}C$	A1	A2	B	A1	A2	B	
[2*RS*-^{3}H]-	14.6	8.46	8.17	8.34	58	56	57	56 ± 1
[2*R*-^{3}H]-	12.6	7.18	6.34	7.46	57	50	59	55 ± 4
[2*S*-^{3}H]-	12.3	6.07	6.17	6.19	49	50	50	50

[a] Bovine plasma amine oxidase isozymes A1, A2, and B were prepared as described in the Methods section. Two micromoles of [2-^{3}H]dopamine, mixed with [1-^{14}C]dopamine to give the desired $^3H/^{14}C$ ratios, was incubated with plasma amine oxidase (0.051 U), liver alcohol dehydrogenase (0.75 U), NADH (0.70 μmol), dithiothreitol (0.2 μmol), 200 μmol of KP_i buffer, pH 7.2, in a final volume of 2.0 mL. After conversion of 0.16 μmol of dopamine to the corresponding alcohol, the reaction was terminated by addition of 0.2 mL of 2 N $HClO_4$ acid. Following removal of denaturated protein and excess perchlorate, the reaction mixture was passed through a Bio-Rex-70 ion-exchange column (5 × 0.6 cm). Volatile counts were removed by vacuum transfer, and 10 μmol of 3,4-dihydroxyphenethyl alcohol was converted to 3,4-dibenzyloxyphenethyl alcohol as described in the Methods section.

Unexpectedly, we observe that the 2*R*-^{3}H and 2*S*-^{3}H samples also retained 59 and 47% of their tritium. This apparent nonstereospecificity of tritium loss from C-2 of dopamine led us to confirm the stereochemical purity of our tritium samples using dopamine-β-hydroxylase, an enzyme demonstrated to activate the *pro-R* hydrogen at C-2 of dopamine (Battersby et al., 1976a). It has previously been shown that the dopamine-β-hydroxylase catalyzed hydroxylation of dopamine is characterized by relatively small tritium isotope effects (2–3) in the presence of saturating levels of the activator fumarate (Klinman et al., 1977). Consequently, tritium release to water, rather than the tritium content of product norepinephrine, was monitored. Our results, summarized in Table II, indicate a high degree of configurational purity in chemically synthesized [2-^{3}H]dopamines.

Bovine plasma amine oxidase has been demonstrated to be a mixture of isozymes characterized by differing affinities for DEAE-cellulose (Yasunobu et al., 1976). We therefore investigated the possibility that the observed nonstereospecificity of hydrogen activation at C-2 of dopamine was a consequence of the presence of isozymes characterized by opposing ster-

Table IV: Stereochemistry of [1-^{3}H]Dopamine Oxidation, Catalyzed by Bovine Plasma Amine Oxidase[a]

dopamine	$^3H/^{14}C$	3,4-dibenzyloxyphenethyl alcohol $^3H/^{14}C$	% ^3H retention
[1*RS*-^{3}H]-	19.2	15.4	80
[1*R*-^{3}H]-	9.7	7.8	80
[1*S*-^{3}H]-	10.7	9.8	92

[a] The conditions were identical with those described in the legend of Table I, with the exception that 4.8 U of liver alcohol dehydrogenase and 1 mM dithiothreitol were used in a volume of 2.4 mL; the incubation time was 60 min.

eochemistries in our amine oxidase preparation. Isozymes A1, A2, and B were prepared as described in the Methods section. As summarized in Table III, the observed percent tritium retention in 3,4-dibenzyloxyphenethyl alcohol is independent of isozyme, and similar to that observed using pooled fractions of enzyme (Table I).

Stereochemistry of [1-^{3}H]Dopamine Oxidation. The stereochemistry of tritium loss from C-1 was determined as described above for [2-^{3}H]dopamine. We observe that oxidation of both racemic and chirally labeled [1-^{3}H]dopamines leads to 3,4-dibenzyloxyphenethyl alcohols characterized by $^3H/^{14}C$ ratios 80–92% that of the starting material, Table IV. These data indicate a nonstereospecific loss of tritium from C-1 of dopamine, analogous to our results for hydrogen abstraction from the C-2 position (Table I).

Although the absolute configuration of the stereospecifically labeled [1-^{3}H]dopamine samples has not been confirmed directly, the absolute configuration and stereochemical purity of the immediate synthetic precursor in each case, [1*R*-^{3}H]- and [1*S*-^{3}H]-2-(3-methoxy-4-hydroxyphenyl)ethylamine, have been demonstrated to be 96–98%, employing pea seedling amine oxidase (Summers, 1974; Battersby et al., 1978). We believe, for a number of reasons, that randomization of label does not occur during deprotection by 48% hydrobromic acid. First, the [2-^{3}H]dopamine samples used in the present study were similarly prepared using hydrobromic acid as the final deprotection step, and as shown in Table II these materials are of high stereochemical purity. Second, there is less than 0.1% of total radioactivity in the distilled HBr at the end of the reaction, indicating negligible exchange of tritium with solvent during deprotection. Finally, as indicated below, the carbon-13 spectra of [1-^{13}C]dopamine prepared in the same manner shows that no rearrangement of the ethylamine side chain occurs during protection.

Absence of Phenyl Migration and Tritium Transfer during Dopamine Oxidation. Phenyl Migration. The possibility that the observed nonstereospecific loss of hydrogen during dopamine oxidation was due to formation of a *symmetric* intermediate leading to phenyl migration was investigated using dopamine labeled with carbon-13 at C-1 (Scheme III). The synthesis of [1-^{13}C]dopamine and reference sample of [1-^{13}C]-2-(3,4-dibenzyloxyphenyl)ethyl alcohol has been described in detail in the Experimental Section. The relevant carbon-13 spectra of both amine and alcohol are shown in Figures 1 and 2.

Figure 1a shows the natural abundance carbon-13 spectrum of 3-methoxy-4-benzyloxyphenethylamine hydrochloride. Although the complete spectrum is recorded, the region of the spectrum of most interest in the present study is situated 30–75 ppm downfield from Me_4Si. This region of the spectrum contains four well-resolved singlets which may be easily assigned as: $-CH_{2\beta}-$, 33.2 ppm; $-CH_{2\alpha}-$, 41.7 ppm; CH_3O-, 56.7 ppm; $-CH_2O-$, 71.7 ppm downfield from Me_4Si. Figure 1b shows the carbon-13 spectrum of chemically synthesized [1-^{13}C]-2-(3-methoxy-4-benzyloxyphenyl)ethylamine hydrochloride (ca. 10% atom excess). The important feature of this spectrum is the singly enriched peak at 41.7 ppm downfield from Me_4Si which may be unequivocally assigned as $-CH_{2\alpha}-$. Finally Figure 1c shows the spectrum of a sample of [1-^{13}C]dopamine, prepared by deprotection of **3** (Scheme I) using 48% HBr. The several notable features of this spectrum include the complete absence of signals at 56.9 and 71.7 ppm downfield from Me_4Si, the single enriched peak attributable to $-CH_\alpha-$, and the smaller upfield peak due to $-CH_{2\beta}-$ at 33 ppm.

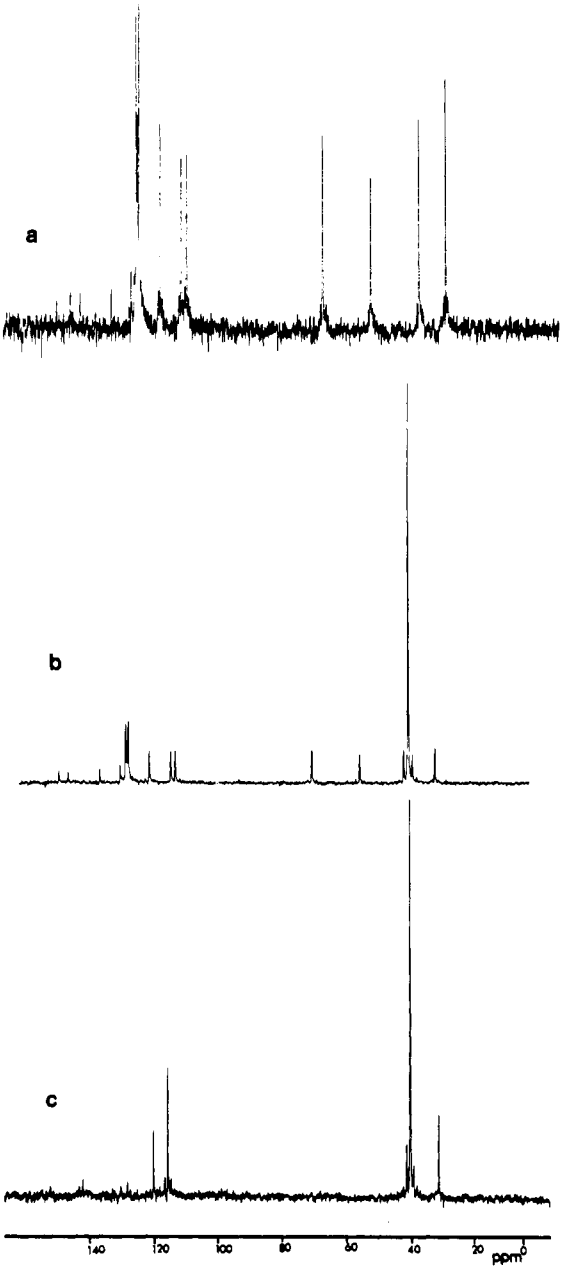
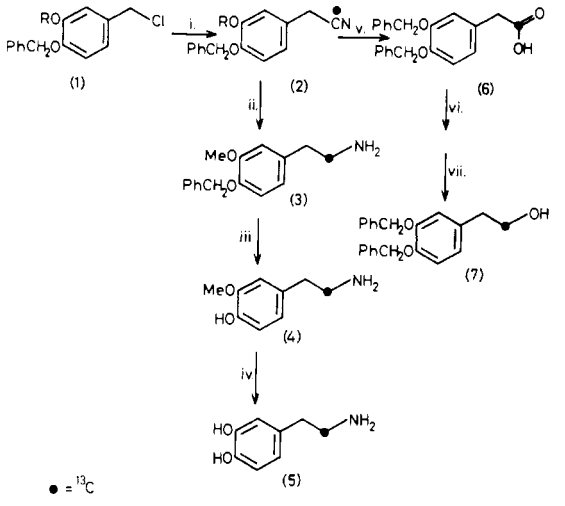


FIGURE 1: Carbon-13 NMR spectra of phenethylamine derivatives. (a) Proton-decoupled Fourier transform carbon-13 NMR natural abundance spectrum of 3-methoxy-4-benzyloxyphenethylamine hydrochloride in H_2O. Sample concentration, 170 mM; 36 592 transients. (b) [1-^{13}C]-2-(3-Methoxy-4-benzyloxyphenyl)ethylamine hydrochloride in H_2O. Sample concentration, 500 mM, approximately 10% enriched, 9300 transients. (c) [1-^{13}C]Dopamine hydrochloride in H_2O. Sample concentration, 270 mM; approximately 6% enriched; 15 000 transients.

Scheme I: Chemical Synthesis of [1-^{13}C]Dopamine and [1-^{13}C]-2-(3,4-dibenzyloxyphenyl)ethyl Alcohol (R = CH_3- or $PhCH_2$-)



i. K-^{13}CN/DMF; ii. B_2H_6/THF; iii. H_2/Pd/C; iv. 48% HBr; v. KOH/ethylene glycol; vi. CH_2N_2; vii. $LiAlH_4$/THF.

Figures 2a–c show the carbon-13 spectra of three different samples of 3,4-dibenzyloxyphenethyl alcohol—the ultimate product of the amine oxidase catalyzed oxidation of dopamine. The natural abundance carbon-13 spectrum of the alcohol in the region 20–100 ppm downfield from Me_4Si is shown in Figure 2a. This region of the spectrum contains three prominent peaks: $-CH_{2\beta}-$, 39.3 ppm; $-CH_{2\alpha}-$, 63.8 ppm; and $-CH_2O-$, 71.7 ppm downfield from Me_4Si. One would have expected two peaks at about 71 ppm, one peak for each $-CH_2O-$ of the benzyl protecting groups. Indeed, scale expansion of this region shows that the single peak centered on 71.67 and 71.78 ppm. Figure 2b shows the spectrum of [1-^{13}C]-2-(3,4-dibenzyloxyphenyl)ethyl alcohol (ca. 7.3% atom excess), which confirms the assignments from the natural abundance spectrum of the same alcohol. In addition, comparison of peak heights indicates an enrichment at $-CH_{2\alpha}-$ of ~7% which is in accord with the percent enrichment expected from dilution of the 98% enriched material (see Experimental Section). Finally, Figure 2c shows the spectrum of a sample of 3,4-dibenzyloxyphenethyl alcohol derived from enzyme-catalyzed oxidation of [1-^{13}C]dopamine. Clearly, the only carbon-13 enriched signal corresponds to the α carbon. As outlined in the Methods section, the carbon-13 enrichment expected in the product alcohol based on substrate turnover and subsequent dilution with unlabeled material corresponds to a C-13 enrichment of 5.25%. Comparison of peak heights indicates an enrichment at C-1 of 5.5%. Thus, all the label can be assigned to the α carbon of product alcohol, and phenyl migration does not account for the unusually high tritium retention values observed with this enzyme.

Tritium Transfer. As an alternative to carbon interchange, tritium transfer between C-1 and C-2 in the course of substrate oxidation was considered as a source of the apparent nonstereospecificity. [2*R*-^{3}H]- and [2*S*-^{3}H]dopamines were converted to 3,4-dihydroxyphenylacetic acid by coupling the amine oxidase reaction to yeast aldehyde dehydrogenase (Scheme IIB). The products of the reaction mixture, following a ≥95% conversion of amine to acid, were separated by DE52-cellulose chromatography. The elution profile resulting from [2*S*-^{3}H]dopamine oxidation is illustrated in Figure 3. Approximately 56% of total counts, corresponding to tritiated water

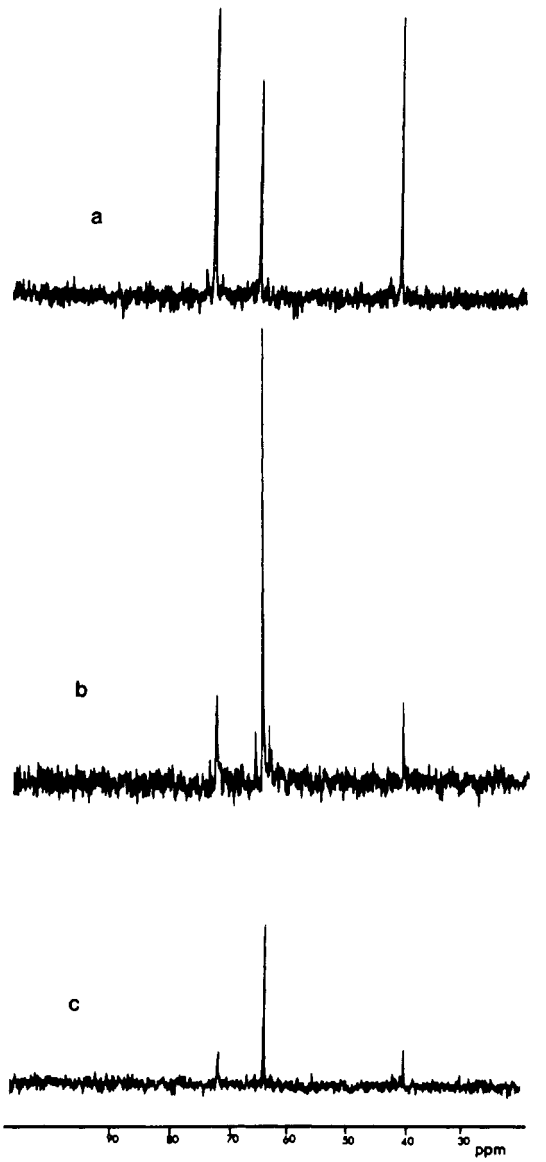
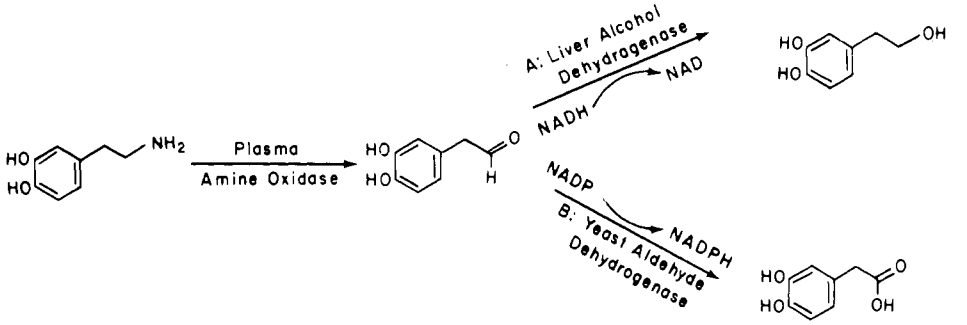


FIGURE 2: Carbon-13 NMR spectra of phenethyl alcohol derivatives. (a) Proton-decoupled Fourier transform carbon-13 NMR natural abundance spectrum of 3,4-dibenzyloxyphenethyl alcohol in benzene-d_6. Sample concentration, 300 mM; 6600 transients. (b) [1-^{13}C]-2-(3,4-Dibenzyloxyphen)ethyl alcohol in benzene-d_6. Sample concentration, 190 mM; approximately 7% enriched; 6600 transients. (c) [1-^{13}C]-2-(3,4-Dibenzyloxyphenyl)ethyl alcohol derived from incubation of [1-^{13}C]dopamine with plasma amine oxidase. Sample concentration 62 mM; 67000 transients. The reaction mixture contained [1-^{13}C]dopamine (9.47 mg, 50 μmol; 98% atom excess), KP_i (1 mmol, pH 7.2), NADH (10 μmol), DTT (4 μmol), plasma amine oxidase (0.2 U, fraction A1), and liver alcohol dehydrogenase (15 U) in a total volume of 10 mL. The reaction course was monitored by the removal of an aliquot of the reaction mixture, diluting it in water and determining OD at 340 nm. After 60 min, a further 10 μmol of NADH was added. At 200 min, the ΔOD at 340 nm corresponded to the formation of 11.3 μmol of 3,4-dihydroxyphenethyl alcohol. Unlabeled 3,4-dihydroxyphenethyl alcohol (200 μmol) in H_2O (2.0 mL) was added to the reaction mixture which was rapidly adjusted to pH 3 by the addition of concentrated HCl, and subsequently extracted with ethyl acetate (3 × 10 mL). The mixture of labeled and unlabeled alcohols was converted to the *O,O'*-dibenzyl derivative as described in the Methods section. The dilution corresponds to a final ^{13}C enrichment of approximately 5.25% atom excess, or, if migration occurs, 2.62% atom excess at both C-1 and C-2.

together with a small amount of unreacted dopamine, appeared in the void volume. The recovery of counts in the major radioactive peak, fractions 74–90, was 9%. In the absence of tritium transfer and assuming 100% recovery of 3,4-di-

Scheme II[a]



[a] Plasma amine oxidase catalyzed oxidation of dopamine leads to 3,4-dihydroxyphenylacetaldehyde, which was reduced to 3,4-dihydroxyphenethyl alcohol (A) or oxidized to 3,4-dihydroxyphenylacetic acid (B).

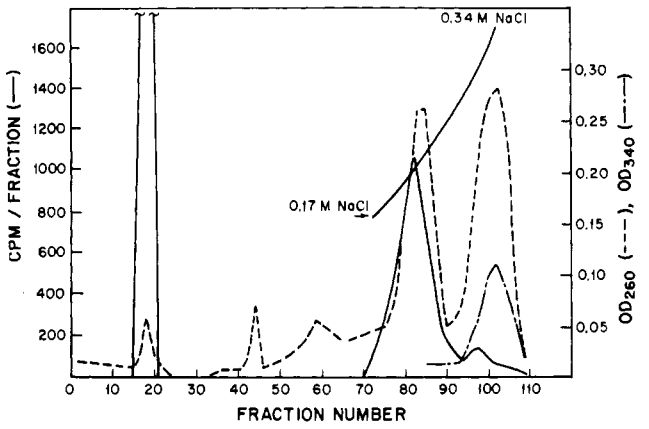


FIGURE 3: DE-52 chromatography of reaction products from incubation of dopamine with bovine plasma amine oxidase in the presence of $NADP^+$ and yeast aldehyde dehydrogenase. The reaction mixture contained [2*S*-^{3}H]dopamine (0.4 μmol, 3.4×10^5 cpm/μmol), $NADP^+$ (0.6 μmol), KP_i buffer (80 μmol, pH 8.1), yeast aldehyde dehydrogenase (20 units, 20 U/mg), catalase (2340 units, 39000 U/mg), and bovine plasma amine oxidase (0.005 unit, 0.14 U/mg) in a total volume of 1 mL. The reaction proceeded to ~95% conversion, based on the change in optical density at 340 nm. An 0.9-mL sample of the reaction mixture was added to a DE-52 column (0.8 × 78) equilibrated at pH 7.2 in 500 mM Tris-HCl. The flow rate was 15 mL/h and 2.2-mL fractions were collected. A nonlinear gradient from 0–0.4 M NaCl was initiated at fraction 16. Of the 1.22×10^5 cpm added to the column, 0.69×10^5 or 56% of the total counts appeared in the breakthrough. The recovery of counts in the major radioactivity peak, 74–90, was 0.11×10^5 or 9% of the total counts. The yield of NADPH based on OD_{340} was 0.3 μmol; a total of 0.14×10^4 cpm occurs in fractions 94–108.

hydroxyphenylacetic acid, 44% of total counts were expected in this peak indicating a ≥20% recovery of this product. In contrast to our relatively poor recovery of 3,4-dihydroxyphenylacetic acid, NADPH was obtained in an 83% yield based on absorbance at 340 nm. A small number of counts (~1% of the total added to the column) were observed to cochromatograph with NADPH.

Our ability to detect tritium at C-1 of aldehyde in NADPH is limited by the (unknown) magnitude of a primary hydrogen isotope effect in the yeast aldehyde dehydrogenase reaction. This consideration, together with our consistently low yields of 3,4-dihydroxyphenylacetic acid from ion-exchange chromatography, led us to compare $^3H/^{14}C$ ratios of product prepared according to Scheme IIB with 3,4-dibenzyloxyphenethyl alcohols (Scheme IIA, followed by derivatization of ring hydroxyls). A reduced ratio of $^3H/^{14}C$ in the oxidized vs. reduced product is a direct measure of tritium at C-1 of intermediate 3,4-dihydroxyphenylacetaldehyde. As summarized in Table V, conversion of 3,4-dihydroxyphenylacetaldehyde to its oxidized product leads to $^3H/^{14}C$ ratios which are slightly reduced relative to the corresponding phenethyl alcohol derivatives: 92, 99, and 92% for products of [2*RS*-^{3}H]-, [2*R*-^{3}H]-, and [2*S*-^{3}H]dopamines.

Table V: Comparison of $^3H/^{14}C$ Ratios in Dopamine to 3,4-Dibenzyloxyphenethyl Alcohol (A) and 3,4-Dihydroxyphenylacetic Acid (B) Obtained by Reaction with Bovine Plasma Amine Oxidase and to 3,4-Dihydroxyphenethyl Alcohol (C) by Reaction with Mitochondrial Monoamine Oxidase

		plasma amine oxidase[a]		monoamine oxidase[b]
dopamine		A	B	C
[2*RS*-^{3}H]-	14.60	8.46	7.80	14.20
[2*R*-^{3}H]-	12.62	7.18	7.09	12.90
[2*S*-^{3}H]-	12.29	6.07	5.61	11.40

[a] The reaction conditions for 3,4-dibenzyloxyphenethyl alcohol production are those in Table III. In the case of 3,4-dihydroxyphenylacetic acid, yeast aldehyde dehydrogenase and NADP (1 μmol) replaced liver alcohol dehydrogenase and NADH. The reaction mixture was deproteinized, subjected to Bio-Rex-70 ion exchange and vacuum transferred to remove volatile counts. Ten micromoles of unlabeled 3,4-dihydroxyphenylacetic acid was added to the resulting residue and purified as described in the Methods section. [b] The reaction conditions were those in Table III, with the exception that 0.0023 U of monoamine oxidase replaced plasma amine oxidase. The reaction was terminated after ΔOD = 0.2 at 340 nm, corresponding to a turnover of 0.064 μmol of [^{3}H]dopamine.

The combined results of Figure 3 and Table V indicate ≤8% of total tritium at the C-1 position of product. Consequently, our failure to observe a stereospecific hydrogen loss from C-2 cannot be due to tritium transfer. The limited amount of tritium at C-1 of 3,4-dibenzyloxyphenethyl alcohols is attributed to "contaminating" tritium in starting dopamines; since oxidation of [2*RS*-^{3}H]-, [2*R*-^{3}H]-, and [2*S*-^{3}H]dopamines with mitochondrial monoamine oxidase, an enzyme which catalyzes the same reaction as plasma amine oxidase with the exception of hydrogen activation at C-2 (Lovenberg & Beaven, 1971), leads to a 3, 0, and 7% loss of tritium to the medium (Table V).

Kinetic Isotope Effect Measurements. Primary tritium isotope effects have been investigated by measuring tritium release to water as a function of product formation. In the case of [2*RS*-^{3}H]-, [2*R*-^{3}H]-, and [2*S*-^{3}H]dopamine oxidation, approximately constant ratios of volatile counts to product formation of 0.47 ± 0.06, 0.42 ± 0.06, and 0.54 ± 0.01 were observed, Table VI. Although the insensitivity of these ratios to the extent of product formation would normally indicate an absence of isotopic discrimination, this need not be the case for a reaction involving alternate pathways leading to a nonstereospecific hydrogen loss. However, two features of

Table VI: Relationship between Tritium Release and Product Formation for [2-^{3}H]Dopamine Oxidation[a]

dopamine	product formation, P (%)	^{3}H (cpm) volatile	^{3}H (cpm) residue	volatile/residue × 100, V (%)	V/P
[2*RS*-^{3}H]-	0	142	13 055	1.1	
	10	742	10 540	6.5 (5.5)	0.55
	19	1227	10 848	10.2 (9.1)	0.48
	54	3124	9 300	25.1 (24.0)	0.44
	100	5439	7 451	42.2 (41.1)	0.41
					0.47 ± 0.06
[2*R*-^{3}H]-	0	99	10 117	0.97	
	9.5	490	8 010	5.8 (4.8)	0.51
	19	733	8 162	8.2 (7.2)	0.38
	48	1656	7 364	18.4 (17.4)	0.36
					0.42 ± 0.06
[2*S*-^{3}H]-	0	137	9 141	1.5	
	10	570	7 567	7.0 (5.5)	0.55
	20	991	7 485	11.7 (10.2)	0.51
	49	2424	6 241	28.0 (26.5)	0.54
	100	5042	3 860	56.6 (55.1)	0.55
					0.54 ± 0.01

[a] Incubations contained [2-^{3}H]dopamine, together with carrier dopamine (0.2 μmol total), 80 μmol of KP_i, pH 7.1, 0.48 μmol of NADH, 0.091 U of plasma amine oxidase, liver alcohol dehydrogenase (0.3 U), and catalase (3900 U) in a volume of 1.0 ml. Product formation was assayed by following the disappearance of NADH absorbance at 340 nm. At the appropriate times, 0.1 mL of the reaction mixture was removed and added to 0.1 mL of 5% $HClO_4$. The percentage of volatile counts was determined following removal of water in vacuo. [b] The numbers in parentheses have been corrected for the percent volatile counts at zero time.

tritium loss from the C-2 position of dopamine indicate isotope effects of one for hydrogen abstraction from this position: (1) the sum of percent volatile counts from the *pro-R* and *pro-S* positions is 42 (± 6) + 54 (± 1) = 96 (± 7)%; and (2) the sum of tritium retained (Table I) and tritium lost (Table VI) is 59 + 42 = 101% and 47 + 54 = 101% for the *pro-R* and *pro-S* isomers, respectively.

The properties of tritium release from the C-1 position of dopamines are in marked contrast to the C-2 position. The amount of tritium lost from C-1 at low conversion is exceedingly small, precluding accurate measurements. Even at 100% conversion, relatively few counts were observed in water: 5.4 ± 0.8, 8.5 ± 1.9, and 1.6 ± 0.7% for [1*RS*-^{3}H]-, [1*R*-^{3}H]-, and [1*S*-^{3}H]dopamine oxidation, respectively (Table VII). Several aspects of the data in Table VII are consistent with nonstereospecific hydrogen activation involving large primary hydrogen isotope effects at C-1; these include the less than 50% loss of tritium from [1*RS*-^{3}H]dopamine at 100% conversion and the fact that tritium loss from [1*R*-^{3}H]- and [1*S*-^{3}H]dopamines does not sum to 100%. Whereas the data indicate a larger isotope effect for [1*S*-^{3}H]- than [1*R*-^{3}H]-dopamine oxidation, the absolute magnitude of these effects cannot be estimated without certain assumptions concerning mechanism.

Discussion

Previous studies of the amine oxidase catalyzed oxidation of benzylamine indicate that both the pea seedling (Battersby et al., 1976b) and bovine plasma (Battersby, A. R., Staunton, J., Klinman, J. P., & Summers, M. C., 1979; Suva & Abeles, 1978) enzymes catalyze the abstraction of the *pro-S* hydrogen at C-1 of substrate. A major finding of the present study is that hydrogen loss from dopamine, stereospecifically labeled with tritium at both C-1 and C-2, occurs nonstereospecifically

Table VII: Tritium Release at 100% Conversion for [1-^{3}H]Dopamine Oxidation[a]

dopamine		^{3}H (cpm) volatile	^{3}H (cpm) residue	volatile counts (%)
[1*RS*-^{3}H]-	(1)	700	10 611	6.2
	(2)	6870	144 000	4.6
				5.4 ± 0.8
[1*R*-^{3}H]-	(1)	338	2 924	10.4
	(2)	6000	87 000	6.5
				8.5 ± 1.9
[1*S*-^{3}H]-	(1)	22	2 293	0.96
	(2)	2400	102 000	2.3
				1.6 ± 0.7

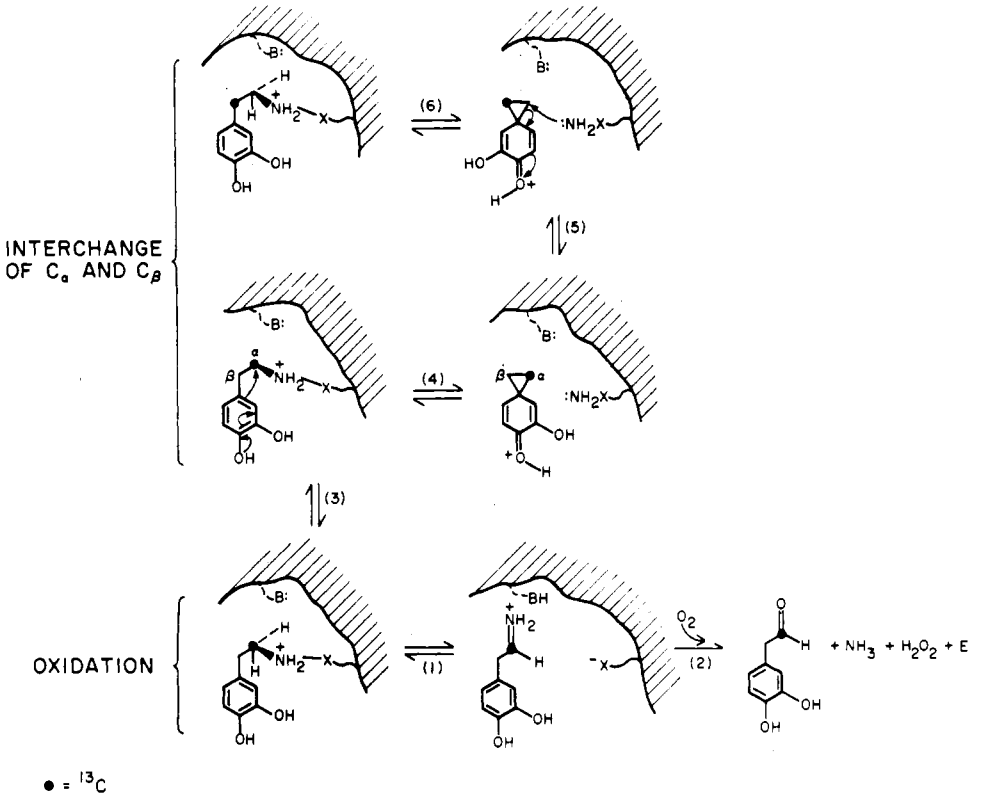
[a] The conditions were similar to those described in Table VI. The distinction between 1 and 2 was the approximately 13–46-fold increase in the specific activity of [1-^{3}H]dopamine in the latter.

(Tables I and IV). The configurational purity of our C-2 tritiated dopamine samples has been confirmed using dopamine-β-hydroxylase which is *pro-R* specific (Table II). Similarly, the immediate synthetic precursor of the C-1 tritiated dopamine samples, 3-methoxy-4-hydroxyphenethylamine, has been correctly assigned using pea seedling amine oxidase which is *pro-S* specific (Summers, 1974; Battersby et al., 1978). In addition, we have shown that oxidation of dopamine with chromatographically resolved isozymes of plasma amine oxidase occurs with the same stereochemical outcome as pooled enzyme fractions (Table I and III). Thus, we are able to rule out racemization of tritium in the course of dopamine synthesis and the presence of plasma amine oxidase isozymes characterized by differing stereospecificities as possible reasons for the apparent nonstereospecificity of enzyme action.

Our current lack of understanding concerning the chemical mechanism and cofactor requirements of bovine plasma amine oxidase led us to consider either phenyl migration, hydrogen migration, or perhaps a combination of these two competing processes to explain the stereochemical data. A reasonable mechanism for phenyl migration resulting from the generation of a symmetric intermediate from phenethylamine derivatives is shown in Scheme III. According to this scheme, amine binding leads to formation of a covalent bond between substrate nitrogen and an active site group, designated "X" (Suva & Abeles, 1978). The resultant intermediate may then undergo either oxidation via hydrogen abstraction at C-1 or phenyl migration leading to interchange of C-α and C-β. Although such a mechanism would reconcile the disparate stereochemical results for benzylamine vs. dopamine oxidation, it is not supported by the analysis of C-13 product derived from [1-^{13}C]dopamine (Figures 1 and 2). Similarly, the possibility of intramolecular tritium transfer between C-1 and C-2 in the ethylamine side chain of dopamine in the course of oxidative deamination has been ruled out by analysis of tritium in NADPH derived from oxidation of dopamine to 3,4-dihydroxyphenylacetic acid (Scheme IIB and Figure 3) and by comparison of ^{3}H/^{14}C ratios of products obtained by enzyme-mediated oxidation vs. reduction of 3,4-dihydroxyphenylacetaldehyde (Scheme IIA,B and Table V).

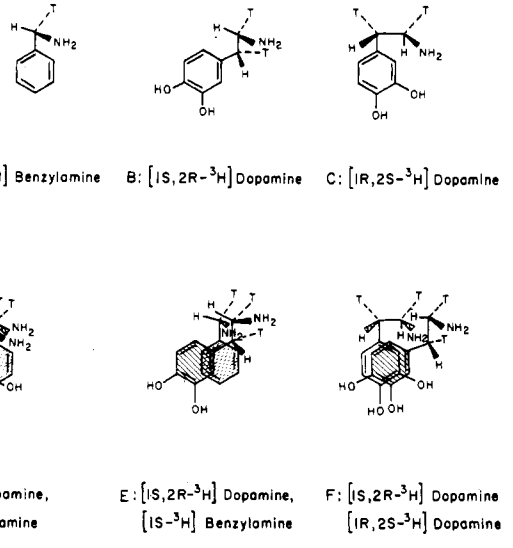
The available data suggest that the most plausible reason for the apparent nonstereospecific oxidation of dopamine is the existence of multiple, catalytically active sites for this substrate. Employing benzylamine binding as a frame of reference, we propose the following provisional picture of the

Scheme III[a]



[a] Amine oxidation has been proposed to involve formation of a covalent bond between substrate nitrogen and an active site group, designated "X" (Suva & Abeles, 1978). According to this scheme, the intermediate formed in the course of dopamine oxidation may undergo *either* proton abstraction at C-1 leading to oxidation or phenyl migration leading to interchange of C-α and C-β.

Scheme IV: Proposed Substrate Binding Modes for Benzylamine and Dopamine at Active Site of Bovine Plasma Amine Oxidase

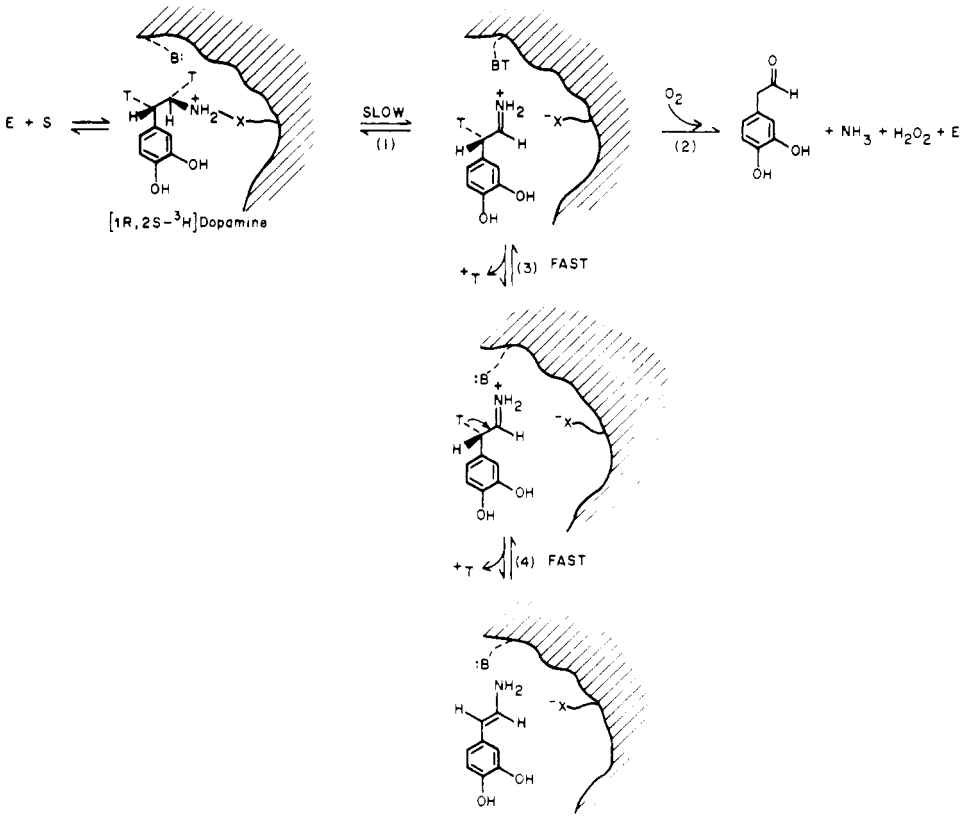


conformation of dopamine at the active site of plasma amine oxidase (Scheme IV). The construction of Scheme IV was predicated on the *single constraint of a fixed geometry for the hydrogen and nitrogen bonds at C-1 undergoing cleavage for all substrates.* By manipulation of molecular models, the two dopamine conformations (B and C) may be readily interconverted by a 60° rotation about an axis perpendicular to the plane of the benzene ring, followed by rotation about the benzylic and ethylamine C–C bonds. An important feature of the scheme is the availability of two binding interactions for dopamine which are characterized by *opposite but absolute stereochemistry.* In the event that a single base catalyzes hydrogen loss from both C-1 and C-2, all bonds cleaved during catalysis can be placed beneath the face of a plane defined by the phenyl ring of bound substrate.

The latter feature implies a defined relationship between the hydrogens abstracted from C-1 and C-2: the loss of the *pro-S* hydrogen at C-2 is linked to *pro-R* abstraction at C-1 (Scheme IVC), and the loss of the *pro-R* hydrogen at C-2 is linked to *pro-S* abstraction at C-1 (Scheme IVB). Although further experiments are necessary to establish this interrelationship, Scheme IV provides a basis for the interpretation of measured kinetic isotope effects. As discussed in detail in the Results section, the isotope effect is one for hydrogen abstraction from C-2 of dopamine; consequently the observed ratios of volatile counts to product formation for [2*R*-^{3}H]- and [2*S*-^{3}H]dopamine oxidation provide a measure of the relative rates of hydrogen abstraction from C-1 of dopamine binding via mode B, 0.42, and mode C, 0.54. Normalizing the tritium release data at 100% formation for [1-^{3}H]dopamine oxidation, we estimate isotope effects of $0.42/0.016 \simeq 26$ for [1*S*-^{3}H]dopamine and $0.54/0.085 \simeq 6.4$ for [1*R*-^{3}H]dopamine.

It should be pointed out that we are currently unable to rule out a two-base mechanism which, in contrast to a single-base mechanism, could lead to either syn or anti abstraction from C-1 and C-2. If hydrogens are removed in a syn fashion, the arguments presented above for a single base mechanism pertain. In the event of an anti loss of hydrogen (leading to [1*S*,2*S*-^{3}H]dopamine in binding mode B and [1*R*,2*R*-^{3}H]-dopamine in binding mode C), the magnitude of isotope effects are estimated to be $0.54/0.016 \simeq 34$ for [1*S*-^{3}H]dopamine and $0.42/0.085 \simeq 4.9$ for [1*R*-^{3}H]dopamine. Experiments are in progress to establish the stereochemical relationship between hydrogen loss from C-1 and C-2 of dopamine, and

Scheme V: Proposed Mechanism for Enzyme-Catalyzed Hydrogen Exchange at C-2 of Dopamine[a]



[a] This mechanism is discussed in detail in the Discussion section.

the precise magnitude of primary tritium isotope effects.

The presence of two binding modes for dopamine is compatible with the enzyme's ability to oxidize a wide range of substrates of differing structural features. Bovine plasma amine oxidase is characterized by an unusually low turnover number (e.g., 0.3–0.4 U/mg with benzylamine), which may reflect an inappropriately defined substrate specificity. In an effort to gain insight into active site topography, we have superimposed [1*S*,2*R*-^{3}H]dopamine onto [1*S*-^{3}H]benzylamine, [1*R*,2*S*-^{3}H]dopamine onto [1*S*-^{3}H]benzylamine, and [1*S*,-2*R*-^{3}H]dopamine onto [1*R*,2*S*-^{3}H]dopamine, Scheme IV, D, E, and F, respectively. Each of the resulting structures will readily accommodate already characterized substrates (e.g., homosulfanilamide, kynuramine, amylamine, heptylamine, and decamethylenediamine; Kapeller-Adler, 1970). In addition, these structures suggest new substrates, characterized by potentially rapid turnover rates and predictable stereochemical pathways for hydrogen abstraction (*pro-R* vs. *pro-S* vs. random). We are currently synthesizing a series of chirally tritiated aromatic amines; a determination of the stereochemistry of the oxidative deamination of these substrates will provide an important test of our proposed model of the bovine plasma amine oxidase active site.

In conclusion, a possible mechanism for the exchange process at C-2 of dopamine is outlined (Scheme V). For ease of discussion we have presented a single base mechanism for the oxidation of [1*S*,2*R*-^{3}H]dopamine. We consider it unlikely that the exchange reaction at C-2 is on the normal enzyme reaction path, since benzylamine, which does not possess a β-carbon atom, is also oxidized. Oxidation of bound substrate by an as yet unknown mechanism gives enzyme-bound imine and protonated enzyme (step 1). To date, all of the proposed mechanisms for the plasma amine oxidase reaction have included an imine-like structure as an obligatory intermediate in the enzyme-catalyzed reaction; recently, Suva & Abeles (1978) have provided direct evidence for such an intermediate by successfully trapping the imine derived from *p*-hydroxybenzylamine using NaB^3H_4. According to Scheme V, the imine intermediate partitions between imine–enamine tautomerization (step 4) and hydrolysis to aldehyde and ammonia (step 2). Our failure to observe tritium isotope effects on the exchange process at C-2 supports a rapid exchange rate relative to imine hydrolysis; the fact that Suva & Abeles (1978) were able to reduce enzyme-bound imine suggests that it is sufficiently long-lived to make imine–enamine tautomerization possible. Thermodynamic considerations favor the enamine structure over the imine structure because of the extra conjugation of the exocyclic double bond with the phenyl ring; the enamine should possess characteristic spectral properties. It would be of interest to know if other primary amines, such as simple alkylamines, undergo a similar exchange reaction at the β-carbon atom.

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Subunit Interaction in Tryptophan Synthase of *Escherichia coli*: Calorimetric Studies on Association of α and β_2 Subunits†

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ABSTRACT: Association of the apo-β_2 and the holo-$(\beta$-PLP$)_2$ subunits of tryptophan synthase from *Escherichia coli* (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) with α subunits of the same enzyme has been studied by microcalorimetry. The results obtained from thermometric titrations clearly demonstrate that only the native complex $\alpha_2\beta_2$ is formed, independent of an excess of α protein. The reaction of the holo-$(\beta$-PLP$)_2$ with α subunits at 25 °C is accompanied by a negative enthalpy change, which is almost twice as large as that for complex formation with the apo-β_2 protein, thus indicating that the interaction enthalpy becomes more favorable in the presence of the coenzyme pyridoxal 5′-phosphate (PLP). Both reaction enthalpies show very large negative temperature coefficients, -3600 ± 100 cal K^{-1} (mol of $\beta_2)^{-1}$ being the value for the formation of the apoenzyme and -2300 ± 100 cal K^{-1} (mol of $\beta_2)^{-1}$ pertaining to formation of the holoenzyme. The studies on the association of α and β_2 subunits in the two buffers revealed that at 25 °C approximately 0.75 proton are absorbed in the presence and absence of the coenzyme, whereas at 35 °C one proton is taken up from the solution when PLP is present, but two if the apo-β_2 complex reacts. These results are a clear indication of energetic linkage between intersubunit interaction, hydrogen ion equilibria, and the binding of the coenzyme.

The interaction of subunits in multimeric enzymes constitutes an informative example of protein–protein interaction. The energetics operative in maintenance of the quaternary structure and the influence exerted by small ligands on the thermodynamic interaction parameters can be particularly well illustrated, if reaction enthalpies and their temperature coefficients are available in addition to the equilibrium constants. Although generalization is not too safe due to the scarcity of relevant data, reaction enthalpies appear to exhibit often larger absolute changes in magnitude than Gibbs free energies, thus rendering them appropriate quantities for the characterization of the energetic aspects of the reaction. The particular reaction chosen is the reconstitution of tryptophan synthase (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) from the β_2 subunits and α subunits in the presence and absence of the coenzyme pyridoxal 5′-phosphate (PLP[1]). Evidence has been presented on the basis of kinetic and binding studies (Faeder & Hammes, 1971; Weischet & Kirschner, 1976; Kirschner et al., 1975a,b; Heyn & Weischet, 1975; Kirschner & Wiscocil, 1972; Bartholmes et al., 1976) performed with the isolated subunits β_2 and α, as well as with the native tetrameric enzyme

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[1] Abbreviations used: DTE, dithioerythritol; PLP, pyridoxal 5′-phosphate.

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