Franks, F. (1978) in *Characterization of Protein Conformation and Function* (Franks, F., Ed.) p 37, Symposium Press, London.
Fung, C. M., Chang, C. C., & Gupta, R. K. (1979) *Biochemistry 18*, 457.
Gabel, D., Rasse, D., & Scheraga, H. A. (1976) *Int. J. Pept. Protein Res. 8*, 237.
Harada, I., Takamatsu, T., Shimanouchi, T., Miyazawa, T., & Tamiya, N. (1976) *J. Phys. Chem. 80*, 1153.
Hseu, T. H., Liu, Y. C., Wang, C., Chang, H., Hwang, D. M., & Yang, C. C. (1977) *Biochemistry 16*, 2999.
Hseu, T. H., Chang, H., Hwang, D. M., & Yang, C. C. (1978) *Biochim. Biophys. Acta 537*, 284.
Inagaki, F., Miyazawa, T., Hori, H., & Tamiya, N. (1978) *Eur. J. Biochem. 89*, 433.
Jirgensons, B., & Capetillo, S. (1970) *Biochim. Biophys. Acta 274*, 1.
Karlsson, E., & Sundelin, J. (1976) *Toxicon 14*, 295.
Lauterwein, J., Wüthrich, K., Schweitz, H., Vincent, J.-P., & Lazdunski, M. (1977) *Biochem. Biophys. Res. Commun. 76*, 1071.
Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265.
Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searle, F. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991.
Menez, A., Bouet, R., Tamiya, N., & Fromageot, P. (1976) *Biochim. Biophys. Acta 453*, 121.
Nèmethy, G., & Scheraga, H. A. (1977) *Q. Rev. Biophys. 19*, 233.
Parrish, J., & Blout, E. R. (1971) *Biopolymers 10*, 1431.
Parrish, J., & Blout, E. R. (1972) *Biopolymers 11*, 1001.
Ryden, L., Gabel, D., & Eaker, D. (1973) *Int. J. Pept. Protein Res. 5*, 261.
Seto, A., Sato, S., & Tamiya, N. (1970) *Biochim. Biophys. Acta 214*, 483.
Tanaka, S., & Scheraga, H. A. (1977) *Macromolecules 10*, 305.
Timasheff, S. N. (1970) *Acc. Chem. Res. 3*, 62.
Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1.
Tsernoglou, D., & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 971.
Tsetlin, V. I., Arseniev, A. S., Utkin, Y. N., Gurevich, A. S., Senyavina, L. B., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1979) *Eur. J. Biochem. 94*, 337.
Tu, A. T. (1973) *Annu. Rev. Biochem. 42*, 337.
Urry, D. W., Long, M. M., Ohnishi, T., & Jacobs, M. (1974) *Biochem. Biophys. Res. Commun. 61*, 1427.
Visser, L., & Blout, E. R. (1971) *Biochemistry 10*, 743.
Visser, L., & Louw, A. I. (1978) *Biochim. Biophys. Acta 533*, 80.
Woody, R. W. (1974) *Pept. Polypeptides Proteins, Proc. Rehovot Symp., 2nd*, 338.
Woody, R. W. (1978) *Biopolymers 17*, 1451.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, Elsevier/North-Holland, New York.
Yang, C. C. (1965) *J. Biol. Chem. 240*, 1616.
Yang, C. C. (1974) *Toxicon 12*, 1.
Yang, C. C. (1976) *Snake 8*, 57.
Yang, C. C., Chang, C. C., Hamaguchi, K., Ikeda, K., Hayashi, K., & Suzuki, T. (1967) *J. Biochem. (Tokyo) 61*, 272.
Yang, C. C., Chang, C. C., Hayashi, K., Suzuki, T., Ikeda, K., & Hamaguchi, K. (1968) *Biochim. Biophys. Acta 168*, 373.
Yonath, A., Podjarny, A., Honig, B., Sielecki, A., & Traub, W. (1977) *Biochemistry 16*, 1418.

# Purification and Characterization of Avian Dopamine β-Hydroxylase†

Robert A. Long, Roger M. Weppelman,* Joyce E. Taylor, Richard L. Tolman, and George Olson

ABSTRACT: Dopamine β-hydroxylase (EC 1.14.17.1) has been purified from the chromaffin granules of avian adrenals. The enzyme has a molecular mass of approximately 320K daltons and consists of four apparently identical subunits joined in pairs by disulfide bonds. Analysis of the products formed from dopamine tritiated in the β position indicated that 1.72 times as much tritium was retained in norepinephrine as was released as water. Ferrocyanide could serve as a reductant, but ascorbate at equal concentrations afforded higher rates. The enzyme had a pH optimum of 5–6 and was activated by either fumarate or acetate, with fumarate being far more effective. Kinetic experiments varying the concentrations of the substrates ascorbate and dopamine and those of the products dehydroascorbate and norepinephrine suggested that the mechanism was un-uni bi-uni ping pong. By this mechanism, the enzyme released dehydroascorbate after being irreversibly reduced by ascorbate and then sequentially bound oxygen and dopamine and released the product norepinephrine. The enzyme was inhibited by high but probably physiological concentrations of the substrate ascorbate and was activated by low concentrations of the product dehydroascorbate. Ascorbate inhibition was noncompetitive with dopamine, and dehydroascorbate activation was due to an increase in the enzyme's affinity for ascorbate with little or no change in its $V_{max}$. Substrate inhibition by ascorbate and product activation by dehydroascorbate might together ensure that the rate of norepinephrine synthesis in vivo remains relatively unaffected by changes in the ratio of ascorbate to dehydroascorbate within chromaffin granules.

The monoxygenase dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], which catalyzes the conversion of dopamine to norepinephrine, is present in the chromaffin granules of adrenal medullary tissue (Levin et al., 1960). To date, this enzyme has been purified from bovine (Friedman & Kaufman, 1965), ovine (Rush & Geffen, 1972), and rat (Grzanna & Coyle, 1976) adrenal medullas as well as from human pheochromocytoma (Stone et al., 1974) and

† From Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065. *Received February 27, 1981.*

0006-2960/81/0420-7423$01.25/0 

plasma (Frigon & Stone, 1978). No purifications of dopamine β-hydroxylase from nonmammalian tissue have been reported.

The reaction catalyzed by the bovine enzyme, which has been the most studied, was suggested to proceed by a ping-pong mechanism, as described by Cleland (1963), by which the product dehydroascorbate was released before the substrates dopamine and oxygen bound. This mechanism was based on the observation that parallel kinetic patterns resulted when the concentrations of ascorbate and dopamine were varied (Goldstein et al., 1968), which indicated that an irreversible step, presumed to be release of dehydroascorbate, separated the steps in which these two substrates bound.

This mechanism has been questioned by Ljones & Flatmark (1974), who noted that the reduction potential of ascorbate was sufficiently low vis-à-vis the enzyme's active site Cu(I) that reduction of the enzyme was probably irreversible thermodynamically. Thus, reduction rather than release of dehydroascorbate could account for the apparent ping-pong relationship between ascorbate and dopamine. When these authors used $Fe(CN)_6^{4-}$, whose reduction potential is approximately equal to that of the enzyme, instead of ascorbate, kinetic patterns that appeared to converge resulted when dopamine and $Fe(CN)_6^{4-}$ were varied, which suggested that the reaction might be sequential. As a result of these data, these authors proposed a ter-bi sequential mechanism by which dehydroascorbate was released only after $O_2$ and dopamine had bound. End product inhibition experiments that could distinguish between sequential and ping-pong mechanisms have not been reported, and thus the mechanism of dopamine β-hydroxylase remains in dispute.

Avian dopamine β-hydroxylase was of special interest to us since the alcohol deterrent antabuse, a potent inhibitor of dopamine β-hydroxylase (Goldstein et al., 1964), and several related dithiocarbamates adversely affect the fertility of laying hens (Weppelman et al., 1980). To investigate the possible connection between the dithiocarbamates' inhibition of dopamine β-hydroxylase and their antifertility properties, we have purified avain dopamine β-hydroxylase essentially to homogeneity. The purification, characterization, and kinetic properties of this enzyme are the subject of this paper.

## Materials and Methods

*Materials.* Norepinephrine bitartrate and sterile solutions of bovine liver catalase were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Dehydroascorbic acid was synthesized by Fluka A.G. (Switzerland) and was obtained from Tridom Co. (Hauppauge, NY). When subjected to reversed-phase high-pressure liquid chromatography (HPLC), the dehydroascorbate was found to be contaminated with less than 1% ascorbic acid. Dopamine, labeled with tritium in the β position, and Aquasol-2 were from New England Nuclear (Boston, MA). SexSal laying hens, age 8–16 months, were obtained from Kerr Hatchery (Frenchtown, NJ).

*Assay of Enzyme Activity.* The following tritium-release assay was adapted from similar assays published previously (Kaufman et al., 1968; Kaufman, 1971; Wilcox & Beaven, 1976). Unless stated otherwise, each reaction mixture contained 400 units of catalase (based on the supplier's statement of activity), 0.6 μmol of ascorbic acid, 12.5 μmol of fumarate, 15.7 nmol of pargyline, 5 nmol of dopamine, and 0.1 μCi of dopamine tritiated in the benzylic position (5–10 Ci/mmol) in a total volume of 0.1 mL. The pH of the fumarate, ascorbate, and dehydroascorbate solutions was adjusted to 5.5.

Twenty microliters of enzyme was added to the reaction mixture in 12 × 75 mm borosilicate glass culture tubes. The tubes were then gassed with $O_2$ and were sealed and incubated at 40 °C (the chicken's body temperature) with gentle shaking. After 1 h, 100 μL of water was added, and the entire contents were frozen in dry ice, thawed on their periphery by dipping in warm water, transferred to a Thunberg tube, and placed on dry ice. The tube was evacuated and the side arm of the tube was placed in dry ice while the bottom was incubated at room temperature. After about 1 h, sufficient sublimination occurred to permit removal of 100 μL of water from the head of the tube. This sample was added to 10.0 mL of Aquasol-2 and was counted with about 35% efficiency.

Reaction mixtures containing either no enzyme or boiled enzyme yielded backgrounds of 0.75–1.5% of their total radioactivity, depending on the batch of radioactive dopamine. Background was determined daily and all assays were corrected for it.

For calculation of activity, the assumptions were made that there were no isotope effects and that the label was equally distributed between *R*- and *S*-benzylic positions. This does not take into account the data in Figure 2 that indicate that the primary isotope effect is 1.72-fold greater than the secondary isotope effect, again assuming equal distribution of label between the *R*- and *S*-benzylic positions. Thus, the rate of tritiated water release would reflect 58% of the rate of norepinephrine synthesis in the absence of a secondary isotope effect or less than 58% depending on the magnitude of the secondary effect. For all experiments, units are equal to nanomoles of norepinephrine, calculated as described above, formed per minute. For all experiments investigating the enzyme's kinetics, 0.006–0.012 unit of enzyme was used.

*Analysis and Interpretation of Kinetic Data.* All graphs, unless indicated otherwise, were fitted by eye, and interpretations were based on the publications of Cleland (1963, 1970) and Segal (1975).

*Assay of Epinephrine.* The fractions to be assayed were adjusted to 0.8 N perchloric acid. The resulting precipitate was removed by centrifugation, and the epinephrine content of the supernatant was determined by a modification of the procedure of Von Euler & Hamburg (1949). The iodine oxidation was conducted at pH 6, and the absorbance was determined at 529 nm. Since epinephrine yields more than 5 times as much color as norepinephrine, dopa, or dopamine, interference from these compounds was neglected.

*Assay of Protein.* Protein content was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin as the standard (Bradford, 1976). This dye-binding assay was chosen since it is not subject to interference from catecholamines.

*Purification of Avian Chromaffin Granules.* Fifty to three hundred SexSal laying hens, age 8–16 months, were decapitated and exsanguinated. The adrenals were removed within 5 min after death and placed in 20 mM sodium phosphate buffer, pH 7.2, containing 0.3 M sucrose at 0 °C. Sodium phosphate buffer at this pH was used throughout the purification. The adrenals were then removed from buffer and trimmed of extraneous tissue. Since avian adrenals are diffuse and lack a defined medulla, removal of cortex was not possible. The granules were then purified from intact avian adrenals by the following procedure that was adapted from that used by Foldes et al. (1972) to purify granules from bovine adrenals.

The trimmed adrenals were added to 20 mM phosphate, containing 0.3 M sucrose. Approximately 10 mL of buffer was used for each gram wet weight of adrenals. The suspension was then ground in a Ten Broeck tissue grinder with a glass pestle rotated at 200–400 rpm for approximately 2 min at 0 °C. Debris was removed by centrifugation at 270*g* in a

Sorvall SS 34 rotor for 6 min at 4 °C. The supernatant was decanted and was centrifuged at 27000*g* in a Sorvall SS 34 rotor at 4 °C for 15 min. This supernatant was discarded, and the pellet containing the granules was resuspended in the same buffer and was again centrifuged as described above. This pellet was again resuspended, and the procedure was repeated for a total of three washes. The fourth sequential pellet from the 27000*g* centrifugation served as the chromaffin granule preparation.

*Lysis of Chromaffin Granules and Ammonium Sulfate Fractionation.* The granule preparation was resuspended in 20 mM phosphate, containing 0.1% Triton X-100. After 30 min of stirring at room temperature to permit lysis, debris was removed by centrifugation at 27000*g* for 25 min in a Sorvall SS 34 rotor at 4 °C. The supernatant containing the released enzyme was decanted, and sufficient ammonium sulfate was added to yield 80% saturation. After centrifugation at approximately 270*g* for 3 min, the liquid was removed and the particulate material was resuspended in 20 mM phosphate, which was 50% saturated with ammonium sulfate, the volume of which was one-fourth the volume of the Triton X-100 supernatant. After centrifugation at approximately 270*g* as above, the liquid was removed and the particulates were again resuspended in an equal volume of 20 mM phosphate, 50% saturated with ammonium sulfate. The centrifugation was repeated, the liquid was again removed, and the particulate material was dissolved in 150 mM phosphate.

*Hydroxylapatite Batch Fractionation.* The ammonium sulfate fractionated enzyme in 150 mM phosphate was then added to a slurry of hydroxylapatite (Bio-Gel HT from Bio-Rad Laboratories) equilibrated with this buffer. One milliliter of slurry was used for every 4 mg of protein in the enzyme preparation. The hydroxylapatite was permited to settle, and the supernatant was decanted and saved. An equal volume of 150 mM phosphate buffer was then added, and, after the slurry again settled, this supernatant was decanted and added to the first supernatant. These combined supernatants comprised the hydroxylapatite batch fractionated enzyme.

When enzyme was purified from the adrenals of fewer than 75 hens, hydroxylapatite batch fractionation was omitted. In this case, the ammonium sulfate fractionated enzyme was resuspended in 20 mM buffer, rather than 150 mM buffer, and was then dialyzed and applied to an hydroxylapatite column as described below.

*Hydroxylapatite Column Chromatography.* The hydroxylapatite batch purified enzyme was dialyzed against 100 volumes of 20 mM phosphate at 4 °C and was then applied to a column containing hydroxylapatite equilibrated with 20 mM phosphate. The internal diameter of the column was 2.5 cm and a 1.0-mL bed volume was used for each 4 mg of protein in the batch-purified enzyme. The flow rate was approximately 1.0 mL/min, and 2.0-mL fractions were collected. The column was washed with 20 mM buffer until the eluate contained less than 0.04 mg of protein/mL and was then eluted with a linear gradient of 20–150 mM phosphate, the total volume of which was approximately 4 times the bed volume of the column. Peak fractions, which together contained about 80% of the total activity recovered, were then pooled.

*Diethylaminoethyl (DEAE) Chromatography.* The enzyme purified by hydroxylapatite column chromatography was dialyzed against 20 mM phosphate and was then diluted with 9 volumes of water, and the resulting pH was adjusted to 7.2. The dilute enzyme was then applied to a DEAE column (DEAE-Bio-Gel A from Bio-Rad Laboratories) equilibrated with 2 mM buffer. Internal diameter of the column was 1.5 cm, and 1 mL of bed volume was used for each 4 mg of protein. After the enzyme had been applied, the column was washed with 2 column volumes of 2 mM buffer and then eluted with a linear gradient of from 2 to 20 mM buffer, the volume of which was approximately 4 times the bed volume of the column. The flow rate was 1 mL/min, and 1-mL fractions were collected. Peak fractions containing about 60% of the activity recovered were pooled and served as the final enzyme preparation, which was used for most of the experiments described in the present report. These preparations contained 0.16–0.6 unit/mL and had specific activities of 3–12 units/mg of protein.

*Molecular Mass Determination.* An aliquot of hydroxylapatite fractionated enzyme containing 5 μg of protein was applied to a column of 20-cm length and 1.5-cm diameter containing Bio-Gel A 0.5 M resin, 100–200 mesh (Bio-Rad Laboratories), equilibrated with 20 mM phosphate buffer containing 0.1 M KCl. The flow rate was 0.22 mL/min, and fractions of 0.45 mL were collected and assayed for enzyme activity.

For calibration of the column, 1 mg of ferritin, 5 mg of catalase, 5 mg of bovine serum albumin, and 5 mg of hen albumin, obtained as components of Combithek Calibration Proteins II (Boehringer Mannheim, Indianapolis, IN), and 1 mg of dextran blue 2000 (Pharmacia Fine Chemicals, Piscataway, NJ) were chromatographed separately. Elution peaks were located by the standard protein assay except for dextran blue which was located by its absorbance at 625 nm. Elution volume was determined from the fraction on the leading edge of the peak at which half the peak height was attained. The total volume of the column was 31 mL, and the elution volume of dextran blue was 9.7 mL. The molecular mass of avian dopamine β-hydroxylase was determined from its elution volume as described by Reiland (1971).

*Electrophoresis.* Gel electrophoresis of native protein was performed at either pH 7 or pH 9 by the procedure of Williams & Reisfeld (1964). The stacking gels contained 4.5% acrylamide and the running gels 5.0%. Two to four micrograms of protein was applied to each gel. Electrophoresis was conducted at 4 °C and at an amperage of 5 mA/sample. When done at pH 9, electrophoresis was terminated when the bromphenol blue reached the bottom of the gel. When done at pH 7, electrophoresis was continued until about 0.5 h after the dye left the bottom of the gel. The samples were then stained with Coomassie brilliant blue by the procedure of Chrambach et al. (1967).

Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was performed by the procedure of Weber & Osborn (1969). When samples were prepared without mercaptoethanol, this compound was omitted when the samples were boiled. Approximately 2 μg of protein was used for each gel. Molecular mass standards of 53K, 106K, 159K, and 212K daltons were obtained from BDH Biochemicals (Poole, England).

*Analysis of Reaction Products by HPLC.* For verification that release of tritiated water from dopamine reflected norepinephrine synthesis in the standard assay, samples from an assay mixture were subjected to preparative HPLC, and the distribution of radioactivity between the substrate, dopamine, and the product, norepinephrine, was determined. The assay mixture was as described above except that its total volume was 7.2 mL and it contained 2.4 mg of ammonium sulfate fractionated protein, $9.5 \times 10^6$ dpm of radioactive dopamine, and 40 μM $CuSO_4$, which afforded optimum activity for the preparation of enzyme used.

Table I: Purification of Dopamine β-Hydroxylase from Avian Chromaffin Granules[a]

| purification step | vol (mL) | total units of enzyme | $CuSO_4$ for max act. (μM) | total protein (mg) | units of enzyme/mg of protein | x-fold purification | yield (%) |
|---|---|---|---|---|---|---|---|
| Triton X-100 supernatant | 120 | 45.8 | 80 | 600 | 0.076 | 1.0 | 100 |
| ammonium sulfate | 50 | 35.2 | 80 | 100 | 0.352 | 4.6 | 77 |
| hydroxylapatite batch | 75 | 32.3 | 40 | 38 | 0.850 | 11 | 71 |
| hydroxylapatite chromatography | 16 | 15.1 | 20 | 11 | 1.37 | 18 | 33 |
| DEAE-Bio-Gel A chromatography | 5 | 0.8 | 0 | 0.11 | 7.3 | 96 | 1.7 |

[a] This purification was from the granules of 300 hens.

After 0, 10, 15, 30, 45, or 60 min of incubation at 40 °C, 1.2-mL samples were removed and added to 1.2 mL of 0.8 N perchloric acid. To each 0.5-mL aliquot of this mixture was added 0.5 mg of both norepinephrine and dopamine to serve as internal standards. The mixture was incubated at 0 °C, and the resulting precipitate was removed by centrifugation at 120*g* for 10 min. The precipitates contained less than 15% of the radioactivity and were discarded. To the supernatant was then added sufficient 4 N KOH to adjust the pH to 6.0, and the mixture was incubated at 0 °C to permit the potassium perchlorate to precipitate. After centrifugation at 120*g* for 10 min, the supernatants, containing more than 90% of the radioactivity, were decanted. Aliquots of 150 μL were removed from the supernatants and were subjected to sublimation to remove water, and the radioactivity in the sublimate was determined. The residue was resuspended in 150 μL of water and filtered through a Millipore 0.45-μm filter, and 20-μL samples were subjected to preparative reversed-phase HPLC by the procedure of Mell & Gustafson (1977). A Waters Associates (Milford, MA) Model 440 chromatograph and a Waters reversed-phase μBondapak $C_{18}$ column were used. Absorbance was determined at 280 nm; 12-s fractions (containing 0.2 mL) were collected, and their radioactivity was determined. For all samples, 77–90% of the radioactivity subjected to chromatography was recovered during elution.

In Figure 2, dpm per sample refers to the dpm present in the 0.5-mL aliquots from which protein and potassium perchlorate were removed. Each of these aliquots in turn corresponded to 0.25 mL of the original reaction mixture. For all samples, the radioactivity recovered in water, dopamine, and norepinephrine accounted for 77–80% of the radioactivity added as dopamine ($3.3 \times 10^5$ dpm/0.25 mL of assay mixture = 100%).

As a control, enzyme that had been boiled for 2 min was incubated for 60 min and treated identically as the other samples. The results of the control are shown in Figure 1.

## Results

*Isolation of Chromaffin Granules.* Because of the difficulties associated with assaying dopamine hydroxylase in crude preparations (Kaufman, 1971), epinephrine was used as an indication of granule purification. A typical homogenate of avian adrenals contained 4 mg of epinephrine and 61 mg of protein per g wet wt of tissue. Several cycles of centrifugation yielded a granule preparation that contained 63% of the epinephrine present in the homogenate but only 29% of the protein. Thus, the granules were purified about 2-fold.

*Purification of Enzyme from Chromaffin Granules.* Table I describes the results of a typical purification from chromaffin granules prepared from 300 chickens. After the granules were lysed with Triton X-100 and the membranes were removed by centrifugation, virtually all protein, epinephrine, and enzyme activity were recovered in the supernatant. At this stage of purification $CuSO_4$ was needed to reverse inhibition by sulfhydryl-containing contaminants in the extract (Nagatsu et al., 1967). The concentration needed for maximum activity was 80 μM, which indicates that the level of endogenous inhibitors was relatively high.

Ammonium sulfate fractionation resulted in the elimination of epinephrine and nearly a 5-fold increase in specific activity. However, the level of endogenous inhibitors was not decreased since the concentration of $CuSO_4$ needed to eliminate inhibition remained at 80 μM.

Hydroxylapatite batch fractionation and column chromatography together resulted in an additional 4-fold increase in specific activity and a decrease in the $CuSO_4$ concentration required for maximum activity to 20 μM.

When subjected to DEAE chromatography, activity eluted in a peak at 5–10 mM phosphate. Though the enzyme was not well resolved from trailing proteins, the fractions pooled for the final preparation all had about the same specific activity. The enzyme did not require $CuSO_4$ (Table I) and was apparently free of inhibitors. The poor recovery of activity from DEAE chromatography was probably due to the enzyme's tendency, when present in dilute solutions, to adhere to glass and chromatographic resins. Use of Bio-Gel A 0.5 M resin in place of DEAE also resulted in considerable loss of activity even though both this resin and DEAE itself afforded excellent recoveries when used to purify ammonium sulfate fractionated enzyme. This tendency defeated attempts at further purification.

*Molecular Mass and Subunit Composition.* When ammonium sulfate purified enzyme was chromatographed on Bio-Gel A 0.5 M resin, activity eluted at a position that suggested that the enzyme's molecular mass was about 316000 daltons.

Native gel electrophoresis at either pH 7 or pH 9 resulted in one slowly migrating protein band. When $NaDodSO_4$ gel electrophoresis was done with mercaptoethanol, a major band whose mobility suggested a molecular mass at 80000 daltons resulted. $NaDodSO_4$ gel electrophoresis done without mercaptoethanol yielded a band whose molecular mass was 160000 daltons. This suggests that native enzyme, whose molecular mass is approximately 320000 daltons, consists of four subunits joined in pairs by disulfide bonds. The bovine enzyme is of similar molecular mass and subunit composition (Craine et al., 1973; Wallace et al., 1973). It should be noted that $NaDodSO_4$ gel electrophoresis also yielded several minor rapidly migrating protein species. Whether these are degradation products, as has been claimed for human plasma hydroxylase (Frigon & Stone, 1978), or contaminants is not known.

*Additional Properties.* Activity was stable for at least 6 weeks when frozen and stored at −20 °C. The enzyme bound quantitatively to Con A–Sepharose, suggesting that it might be a glycoprotein like the bovine enzyme (Wallace & Lo-

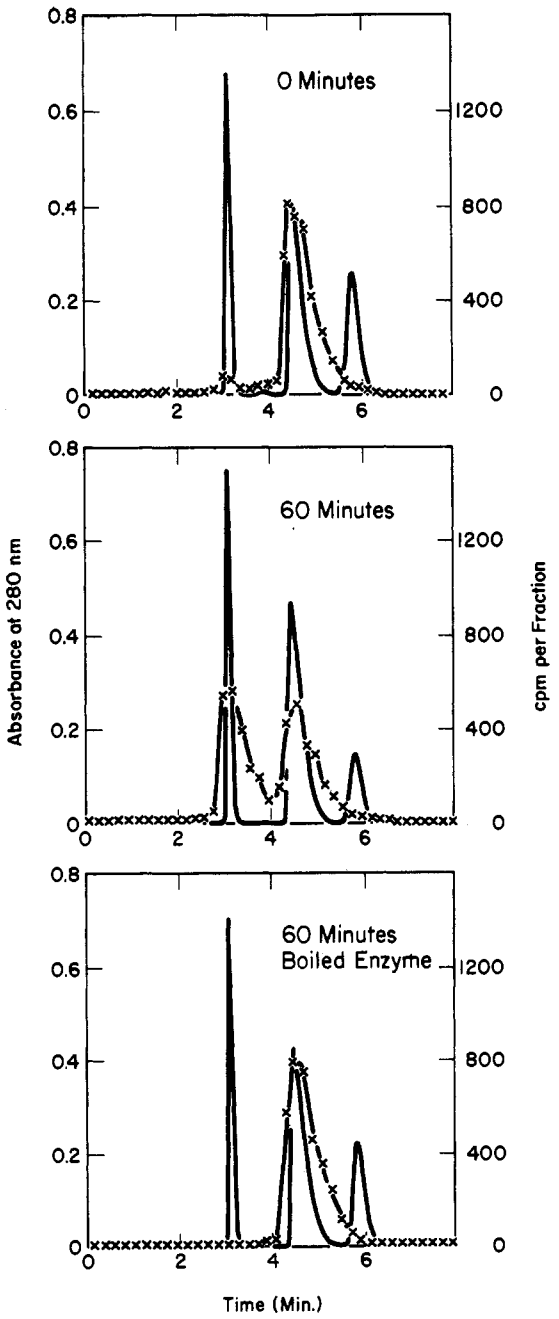



FIGURE 1: HPLC analysis of products formed in reaction mixtures from dopamine tritiated in the benzylic position. Either 0 (top) or 60 min (middle) after addition of enzyme, a reaction mixture was sampled and subjected to HPLC to determine the distribution of radioactivity. The bottom panel shows the results from an identical reaction mixture incubated for 60 min with boiled enzyme. The abscissa is the elution time in minutes from injection. The continuous line is the absorbance at 280 nm, and the absorbance peaks are norepinephrine, dopamine, and fumarate, in order of elution. The discontinuous line describes the radioactivity present in the fractions collected.

venberg, 1974; Miras-Portugal et al., 1975). The bound enzyme was about 50% active but could not be eluted with saturated methyl α-D-mannopyranoside or with 0.4 M NaCl, either singly or together. Similar observations have been made for the rat hydroxylase (Grzanna & Coyle, 1976).

*Analysis of Reaction Products.* For verification that release of tritiated water from dopamine reflected norepinephrine synthesis, samples were taken from an assay mixture at different times, and the radioactivity in water was determined by sublimation while the radioactivity in norepinephrine and dopamine was determined by preparative HPLC.

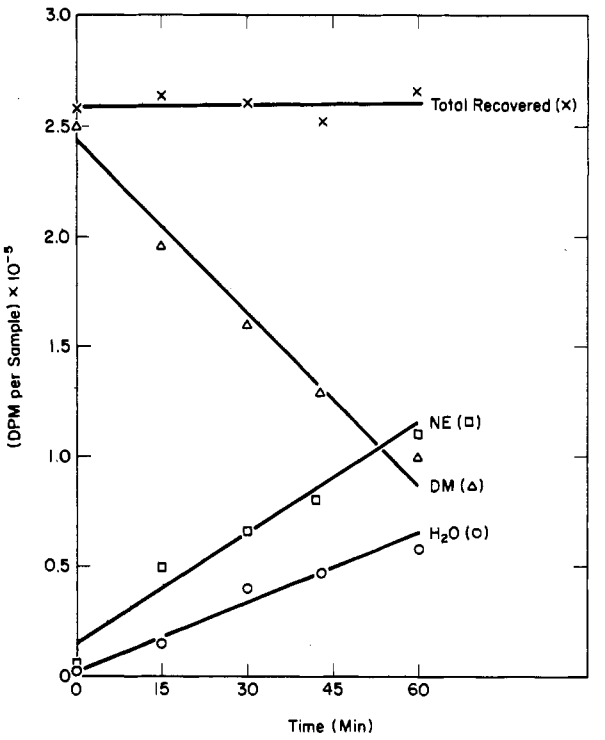



FIGURE 2: Distribution of radioactivity among the substrate dopamine and the products norepinephrine and water in a reaction mixture at different times. Radioactivity in dopamine and norepinephrine was from the chromatograph shown in Figure 1 and from similar chromatographs done on the 15-, 30-, and 45-min samples. Radioactivity in water was determined from sublimation.

In the chromatographs shown in Figure 1, the absorbance peaks are norepinephrine, dopamine, and fumarate, in order of elution. In all cases the peaks of radioactivity are broader than the corresponding absorption peaks because diffusion occurred between the photocell and the fraction collector, a distance of about 50 cm. At 0 min, more than 97% of the radioactivity recovered from HPLC was as dopamine while at 60 min 47% was as dopamine and the remainder was as norepinephrine. For a reaction mixture incubated for 60 min with boiled enzyme, all radioactivity was recovered as dopamine.

Figure 2 shows that distribution of radioactivity among dopamine, norepinephrine, and water at various times during the 60 min incubation. The results indicate a time-dependent increase in the radioactivity associated with norepinephrine and water and a decrease in that associated with dopamine. The total dpm recovered in norepinephrine, water, and dopamine was constant, which suggests that no radioactive products other than norepinephrine and water were formed from dopamine.

Regression analysis of the data in Figure 2 yielded the following relationship between radioactivity recovered in norepinephrine ($y$) and that recovered in water ($x$).

$$y = 0.022 + 1.72x$$

$$r^2 = 0.96$$

The intercept of this regression was not significantly different from zero ($p > 0.05$), and there was an apparent tritium isotope effect of 1.72 in favor of retaining radioactivity in norepinephrine. This effect would correspond to the ratio of secondary to primary isotope effects if tritium were equally distributed between the *R*- and *S*-benzylic positions. A similar isotope effect has also been noted for the bovine enzyme (Bachan et al., 1974; Taylor, 1974).

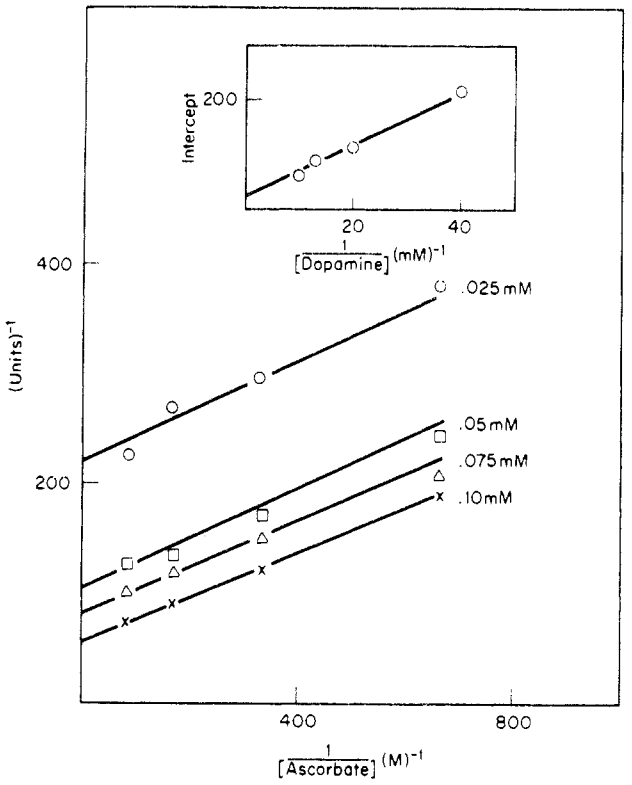



FIGURE 3: Double-reciprocal plots of velocity against ascorbate concentration at different dopamine concentrations. Dopamine concentrations are given to the right of the plots. The inset is a secondary plot of intercepts against reciprocal dopamine concentrations.

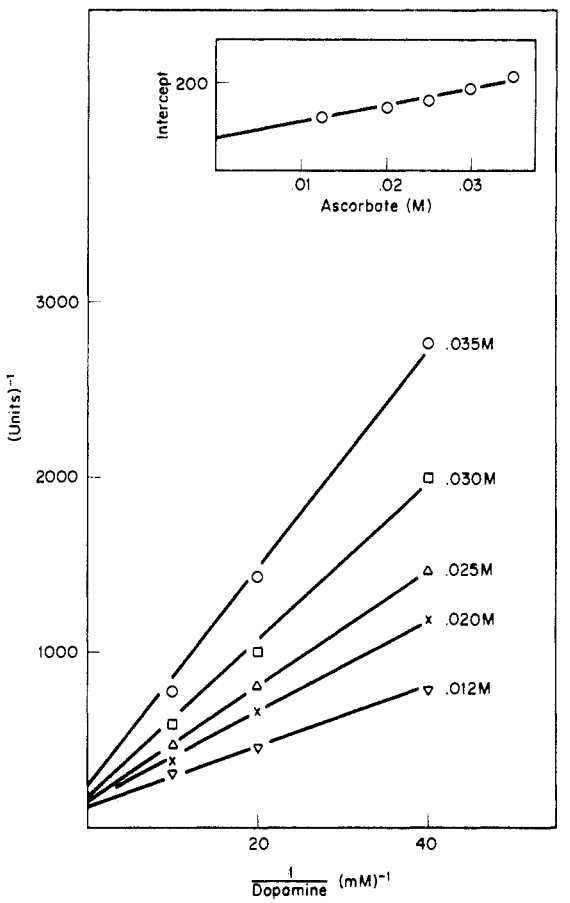



FIGURE 4: Double-reciprocal plots of velocity against dopamine concentration at different ascorbate concentrations. Ascorbate concentrations are given to the right of the plots. The inset is a secondary plot of intercepts against ascorbate concentrations.

*Activation by Fumarate, Acetate, or NaCl.* When phosphate buffer was used instead of fumarate buffer, no activity was detected. Fumarate was found to activate the enzyme with concentrations of 0.125–0.25 M resulting in maximum activity and with higher concentrations being somewhat inhibitory. Acetate buffer at these concentrations resulted in only 40% of the activity seen with fumarate while 0.12 M phosphate buffer, containing NaCl, at these same concentrations resulted in only 10%. The bovine enzyme is also activated by fumarate, acetate, and NaCl, listed in descending effectiveness (Goldstein et al., 1968; Craine et al., 1973).

*Specificity of the Enzyme for Reductant.* The ability of potassium ferrocyanide, NADPH, and ferrous sulfate, each at 5 mM, to serve as reductant was tested. Ferrocyanide yielded 10% of the activity yielded by ascorbate, and NADPH and ferrous ion each yielded less than 3%. For bovine enzyme, ferrocyanide and ferrous ions both served as reductants though ferrocyanide is less effective than ascorbate (Ljones et al., 1976) and ferrous ions are the least effective of the three (Friedman & Kaufman, 1965).

*pH Optimum.* Maximum activity was seen between pH 5 and pH 6. There were fairly sharp decreases in activity between pH 4.5 and pH 5.0 and between pH 6.0 and pH 6.5.

*Effect of Variable Ascorbate and Dopamine.* When dopamine and ascorbic acid were both varied, the double-reciprocal plots of velocity vs. ascorbate concentration at different dopamine concentrations were parallel (Figure 3). Ping-pong kinetics were also observed when the data in Figure 3 were plotted with dopamine as the variable substrate. This suggests that an irreversible step separates the form of the enzyme binding dopamine from that binding ascorbate. Secondary plots of these data yielded apparent $K_m$ values for ascorbate and dopamine of 2 mM and 0.2 mM, respectively.

When the ascorbate concentration was raised to more than 12 mM, substrate inhibition, which was noncompetitive with dopamine, resulted (Figure 4). The replot of these data yielded an apparent $K_i$ of 20 mM, which is about 10-fold greater than ascorbate's $K_m$. When increasing amounts of enzyme were assayed in the presence of a constant, inhibitory concentration of ascorbate (20 mM), the resulting plot of activity vs. enzyme added extrapolated to the origin. This indicates that ascorbate is a reversible, noncompetitive inhibitor (Segal, 1975) and rules out the possibility that the observed inhibition was due to the irreversible inactivation of the enzyme by various radicals derived from ascorbate. Thus, the data in Figure 4 suggest that ascorbate forms a dead-end complex with a form of the enzyme that is upstream and reversibly connected to the form that binds dopamine.

*Inhibition by Norepinephrine.* Figure 5 indicates that the product norepinephrine is a competitive inhibitor with respect to ascorbate ($K_i$ = 5 mM), which suggests that these compounds bind to the same form of the enzyme and thus that ascorbate is the first substrate to bind and norepinephrine is the last product to be released.

When reaction mixtures contained one of four concentrations of dopamine between 20 and 200 $\mu$M and either 0, 10, 20, or 30 mM norepinephrine, double-reciprocal plots of velocity against dopamine indicated that norepinephrine was a noncompetitive inhibitor. This suggests that no irreversible steps, such as release of the product dehydroascorbate, separate the forms of the enzyme that bind these compounds.

*Effect of Dehydroascorbate.* When reaction mixtures contained one of four concentrations of dopamine between 20 and 200 $\mu$M and either 0, 30, 40, or 50 mM dehydroascorbate, double-reciprocal plots of velocity vs. dopamine indicated that dehydroascorbate was a noncompetitive inhibitor with a $K_i$ of

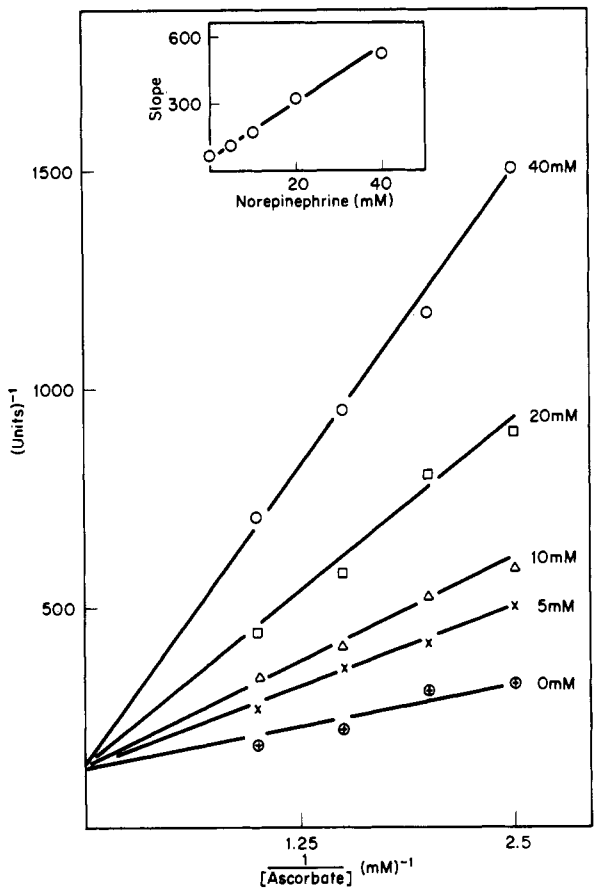



FIGURE 5: Double-reciprocal plots of velocity against ascorbate concentration at different norepinephrine concentrations. The concentrations of norepinephrine are given to the right of the plots. The inset is a secondary plot of intercepts against norepinephrine concentrations.

25 mM. This suggests that these compounds bind to forms of the enzyme that are different but reversibly connected as would occur if oxygen binding preceded dopamine binding and if dehydroascorbate bound to the form of the enzyme that bound oxygen.

When ascorbate and dehydroascorbate were varied, two different effects resulted, depending on the concentration of dehydroascorbate (Figure 6). At concentrations greater than 10 mM, uncompetitive inhibition resulted, which indicates that an irreversible step separates the forms of the enzyme binding these compounds. This would be the case if the reaction proceeded by a ping-pong mechanism and if reduction of the enzyme by ascorbate was thermodynamically irreversible. Surprisingly, dehydroascorbate at concentrations less than 10 mM activated the enzyme, primarily by decreasing its $K_m$ for ascorbate. There was no marked effect on $V_{max}$.

Figure 7 presents two velocity vs. ascorbate plots. In one case no dehydroascorbate was added while in the other dehydroascorbate was added so that the total concentration of ascorbate and dehydroascorbate always equaled 5 mM. Without dehydroascorbate, the velocity varied about 14-fold when ascorbate was increased from 0.05 to 5 mM. With dehydroascorbate, this same change in ascorbate concentration resulted in a velocity change that was only slightly greater than 2-fold.

## Discussion

Dopamine β-hydroxylase purified from the adrenals of laying hens generally resembled the various mammalian dopamine β-hydroxylases. Thus, the avian adrenal enzyme, like

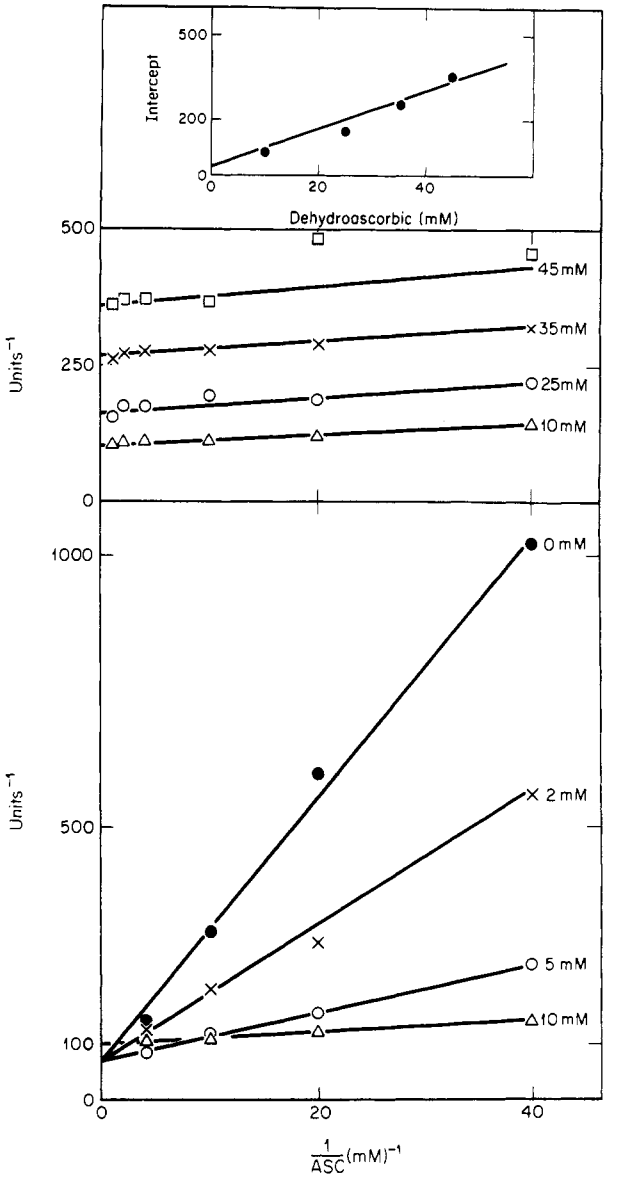
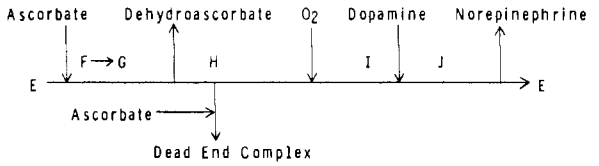



FIGURE 6: Two double-reciprocal plots of velocity against ascorbate concentrations at different dehydroascorbate concentrations. The concentrations of dehydroascorbate are given to the right of the plots. The bottom plots are for 0, 2, 5, and 10 mM dehydroascorbate, and the upper plots repeat the data for 10 mM dehydroascorbate and include data for 25, 35, and 45 mM dehydroascrobate. The inset at the top is a secondary plot of intercepts against dehydroascorbate concentrations for 10, 25, 35, and 45 mM dehydroascorbate.

the bovine adrenal enzyme (Craine et al., 1973; Wallace et al., 1973) and the major form of the enzyme isolated from human serum (Frigon & Stone, 1978), is a tetramer in which two apparently identical monomers are joined by disulfide bonds. The resulting dimers are in turn joined noncovalently to form the native enzyme. Like the rat (Grzanna & Coyle, 1976), bovine (Wallace & Lovenberg, 1974; Miras-Portugal et al., 1975), and human enzymes (Frigon & Stone, 1978), avian hydroxylase binds to Con A–Sepharose (albeit irreversibly) and is probably a glycoprotein. Both avian and bovine enzymes are activated by acetate and more effectively by fumarate (Goldstein et al., 1968; Craine et al., 1973), and both enzymes can utilize ferrocyanide as a reductant though less effectively than ascorbate (Ljones et al., 1976).

Kinetic studies investigating the rate effects of dopamine, ascorbate, norepinephrine, and dehydroascorbate support the following ping-pong model, which is essentially identical with that originally proposed by Goldstein et al. (1968).




This model has been challenged by Ljones & Flatmark (1974), who proposed instead a ter-bi sequential model in which the order of substrate addition was ascorbate, oxygen, and dopamine and in which norepinephrine and dehydroascorbate were released only after all three substrates had bound. By this model reduction of the enzyme by ascorbate rather than release of dehydroascorbate was the irreversible step that accounted for the apparent ping-pong relationship between ascorbate and dopamine. This sequential model predicts that the last product to leave the enzyme should be competitive with ascorbate and uncompetitive with dopamine. Our data indicate that neither norepinephrine nor dehydroascorbate fulfills this prediction.

The observation that ascorbate is a noncompetitive substrate inhibitor with dopamine (Figure 4) is also difficult to reconcile with the sequential model which would require a ternary dead-end complex of reduced enzyme, ascorbate, and dehydroascorbate. This would imply that the reduced enzyme can simultaneously bind both ascorbate and dehydroascorbate. By the ping-pong model, the dead-end complex could be a simple binary complex with ascorbate occupying the site vacated by dehydroascorbate.

Although our data argue against the ter-bi sequential model of Ljones & Flatmark (1974), the data support their proposal, based on redox titration of the enzyme's active site copper, that reduction of the enzyme by ascorbate is virtually irrevesible. Figure 6 demonstrates that dehydroascorbate at concentrations greater than 10 mM is an uncompetitive inhibitor with ascorbate, which indicates that ascorbate binding and dehydroascorbate release are separated by an irreversible step, shown as F → G in the model above and presumed to be reduction of the enzyme by ascorbate.

Though our data generally support the earlier results of Goldstein et al. (1968), there is a point of disagreement. These authors proposed that the sequence I → J in the model was rapid equilibrium ordered since the oxygen concentration did not affect the rate at infinite dopamine. This proposal would predict that inhibitors such as dehydroascorbate or ascorbate would appear competitive with dopamine, whereas both were noncompetitive.

The noncompetitiveness of dehydroascorbate and ascorbate with dopamine also argues against Klinsman's model as described by Walsh (1978). By this model, which was based on an analysis of isotope effects, oxygen and dopamine bind randomly in the absence of fumarate; fumarate serves to direct the reaction flux through the preferred sequence in which dopamine binds first, thereby increasing the rate. This would predict that dehydroascorbate and ascorbate would be competitive with dopamine rather than noncompetitive as observed.

Recently, a semidehydroascorbate radical has been detected in reaction mixtures containing micromolar quantities of dopamine hydroxylase (Ljones & Skotland, 1979; Skotland & Ljones, 1980). These authors have speculated that the predominant catalytic cycle might involve two single-electron transfers from ascorbate to the enzyme, each of which would generate a semidehydroascorbate radical. The radicals would then spontaneously dismutate to produce ascorbate and the ultimate product, dehydroascorbate. Unfortunately the kinetic data available cannot differentiate between the one- and two-electron transfer models. In particular, it should be noted that the single-electron transfer model would also yield linear

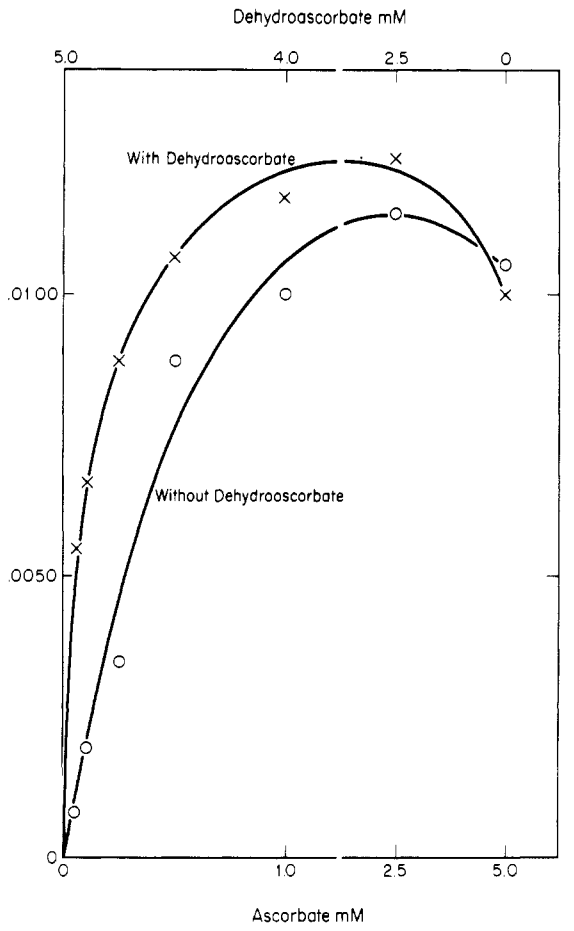



FIGURE 7: Two plots of velocity against ascorbate concentration. For the plot indicated as "with dehydroascorbate", dehydroascorbate was added so that the sum of ascorbate and dehydroascorbate concentrations always equaled 5 mM. For the other plot, which includes the same ascorbate concentrations, dehydroascorbate was omitted.

kinetic plots involving ascorbate since the two ascorbate-binding steps, which it requires, would be separated by the release of the semidehydroascorbate radical.

The discovery that avian hydroxylase is activated by the product dehydroascorbate (Figure 6) at concentrations between 1 and 10 mM was unexpected. The activation was due to a decrease in the enzyme $K_m$ for ascorbate with no significant change in its $V_{max}$. Since it is difficult to explain this activation by an interaction between dehydroascorbate and any of the catalytic sites implied by the model above, this observation suggests that avian dopamine $\beta$-hydroxylase might have regulatory as well as catalytic sites.

It is tempting to speculate about the physiological role that dehydroascorbate activation might play. At least in the rat, very little dehydroascorbate or dioxogulonate, the subsequent metabolite, is released from the adrenal (Lahiri & Lloyd, 1962), which suggests that most dehydroascorbate is reduced to ascorbate within the adrenal and recycled. The recent discovery that bovine chromaffin granules can accumulate dehydroascorbate but not ascorbic acid suggests that the granules are enzymatically capable of reducing dehydroascorbate (Tirrell & Westhead, 1979). If most of the dehydroascorbate within granules is recycled, then any decrease in the concentration of ascorbate would imply an increase in the concentration of dehydroascorbate. This, in turn, would increase the enzyme's affinity for the ascorbate remaining so that the rate of norepinephrine synthesis would be relatively unaffected by the diminished concentration of ascorbate.

In addition to being activated by the product dehydroascorbate, avian dopamine β-hydroxylase was inhibited by the substrate ascorbate with an apparent $K_i$ of 20 mM. For comparison, the enzyme's $K_m$ for ascorbate is 2 mM. Since the concentration of ascorbate within chromaffin granules has been reported to be about 13 mM (Terland & Flatmark, 1975), inhibition might be significant in vivo. Thus, at physiological concentrations, ascorbate may function dually as inhibitor and substrate, which would minimize the effects on rate of changes in ascorbate concentration. Substrate inhibition might then function at the higher ascorbate concentrations to the same end served at the lower concentrations by dehydroascorbate activation. Both activation and inhibition would minimize the role of ascorbate in determining the rate of norepinephrine synthesis.

## References


Bachan, L., Storm, C. B., Wheeler, J. W., & Kaufman, S. (1974) *J. Am. Chem. Soc. 96*, 6799.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Chrambach, A., Reisfeld, R. A., Wyckoff, M., & Zaccari, J. (1967) *Anal. Biochem. 20*, 150.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2*, 1.

Craine, J. E., Daniels, G. H., & Kaufman, S. (1973) *J. Biol. Chem. 248*, 7838.

Foldes, A., Jeffrey, P. L., Preston, B. N., & Austin, L. (1972) *Biochem. J. 126*, 1209.

Friedman, S., & Kaufman, S. (1965) *J. Biol. Chem. 240*, 4763.

Frigon, R. P., & Stone, R. A. (1978) *J. Biol. Chem. 253*, 6780.

Goldstein, M., Anagnoste, B., Lauber, E., & McKereghan, M. R. (1964) *Life Sci. 3*, 763.

Goldstein, M., Tong, H. J., & Garvey, T. Q. (1968) *Biochemistry 7*, 2724.

Grzanna, R., & Coyle, J. T. (1976) *J. Neurochem. 27*, 1091.

Kaufman, S. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 17*, 754.

Kaufman, S., Bridgens, W. F., & Baron, J. (1968) *Oxidation of Organic Compounds*, Vol. 3, p 172.

Lahiri, S., & Lloyd, B. B. (1962) *Biochem. J. 84*, 474.

Levin, E. Y., Levenberg, B., & Kaufman, S. (1960) *J. Biol. Chem. 235*, 2080.

Ljones, T., & Flatmark, T. (1974) *FEBS Lett. 49*, 49.

Ljones, T., & Skotland, T. (1979) *FEBS Lett. 108*, 25.

Ljones, T., Skotland, T., & Flatmark, T. (1976) *Eur. J. Biochem. 61*, 525.

Mell, L. D., & Gustafson, A. B. (1977) *Clin. Chem. (Winston-Salem, N.C.) 23*, 473.

Miras-Portugal, M., Aunis, D., & Mandel, P. (1975) *Clin. Chim. Acta 64*, 293.

Nagatsu, T., Kuzuya, H., & Hidaka, H. (1967) *Biochim. Biophys. Acta 139*, 319.

Reiland, J. L. (1971) *Methods Enzymol. 22*, 287.

Rush, R. A., & Geffen, L. B. (1972) *Circ. Res. 31*, 444.

Segal, I. H. (1975) in *Enzyme Kinetics*, Wiley-Interscience, New York.

Skotland, T., & Ljones, T. (1980) *Biochim. Biophys. Acta 630*, 30.

Stone, R. A., Kirshner, N., Reynolds, J., & Vanaman, T. C. (1974) *Mol. Pharmacol. 10*, 1009.

Taylor, K. B. (1974) *J. Biol. Chem. 249*, 454.

Terland, O., & Flatmark, T. (1975) *FEBS Lett. 59*, 52.

Tirrell, J. G., & Westhead, E. W. (1979) *Neuroscience 4*, 181.

Von Euler, U. S., & Hamberg, U. (1949) *Science (Washington, D.C.) 110*, 561.

Wallace, E. F., & Lovenberg, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3217.

Wallace, E. F., Krantz, M. J., & Lovenberg, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2253.

Walsh, C. (1978) *Annu. Rev. Biochem. 47*, 881.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Weppelman, R. M., Long, R. A., Van Iderstine, A., Taylor, J. E., Tolman, R. L., Peterson, L., & Olson, G. (1980) *Biol. Reprod. 23*, 40.

Wilcox, G., & Beaven, M. A. (1976) *Anal. Biochem. 75*, 484.

Williams, D. E., & Reisfeld, R. A. (1964) *Ann. N.Y. Acad. Sci. 121*, 373.