Stereochemistry of Reactions Catalyzed by Glutamate Decarboxylase[†]

Hidenori Yamada and Marion H. O'Leary*

ABSTRACT: When the decarboxylation of L-glutamic acid by the glutamate decarboxylase from *Escherichia coli* is carried out in D_2O, the product γ-aminobutyric acid contains a single deuterium atom. The stereochemistry of this material was established by conversion to levorotatory methyl 4-phthalimido[4-^{2}H]butyrate. The dextrorotatory isomer of the latter compound was synthesized from S-[2-^{2}H]glycine by a series of reactions not affecting the stereochemistry at the chiral center. Thus, the decarboxylation of glutamic acid occurs with retention of configuration. Decarboxylation of L-α-methylglutamic acid by this enzyme produces levorotatory γ-aminovaleric acid and thus also occurs with retention of configuration.

Studies of the stereochemistry of reactions catalyzed by pyridoxal-P dependent enzymes have begun to provide a consistent picture for a variety of enzymes (Dunathan, 1971). These reactions generally proceed first by formation of a Schiff base between enzyme-bound pyridoxal-P and the amino acid substrate, followed by cleavage of one of the bonds to the α carbon of the amino acid, forming a quinoid intermediate. It has been suggested (Dunathan & Voet, 1974) that all bond-making and breaking steps may occur on the same face of this quinoid intermediate, and cases studied to date seem to bear out this suggestion.

Glutamate decarboxylase (EC 4.1.1.15) requires pyridoxal-P for activity. Decarboxylation of glutamic acid in D_2O results in incorporation of a single atom of deuterium into the product γ-aminobutyric acid (Mandeles et al., 1954; Yamada & O'Leary, 1977) and the enzyme does not catalyze hydrogen exchange between this product and the solvent (Yamada & O'Leary, 1977). The enzyme also catalyzes the decarboxylation of α-methylglutamic acid (Huntley & Metzler, 1967; Sukhareva & Torchinsky, 1966) at a rate which is approximately 600 times slower than the rate of decarboxylation of L-glutamic acid (Yamada & O'Leary, unpublished). In approximately 1% of decarboxylations of α-methylglutamic acid the reaction products are γ-ketovaleric acid and pyridoxamine-P as a result of transamination following decarboxylation (Huntley & Metzler, 1967; Yamada & O'Leary, unpublished). The stereochemistry of the tritiated pyridoxamine-P formed when this decarboxylation is carried out in tritiated water has been determined by Sukhareva et al. (1972). Although the stereochemistry of two other pyridoxal-P dependent enzymatic decarboxylations has been determined (Belleau & Burba, 1960; Leistner & Spenser, 1975), the stereochemistry of the decarboxylation of glutamic acid and of α-methylglutamic acid has not been determined. The latter result is of particular interest because of the existence of the decarboxylation-dependent transamination.

Experimental Section

General. All chemicals were the highest purity available. Glutamate decarboxylase was prepared as described previously (O'Leary, 1969). Serine transhydroxymethylase was provided by Dr. L. V. Schirch. Mass spectra were obtained with an MS-9 mass spectrometer, NMR spectra with a Jeol MH-100, and optical rotations were measured with a Durrum-Jasco Model J-20 spectropolarimeter.

(R)-(−)-4-Amino[4-^{2}H]butyric Acid. To 100 mL of D_2O (99.8 atom % D) was added 3 mg of solid glutamate decarboxylase, followed by 100 mg of pyridoxal-P, 50 mg of dithiothreitol, 1 g of monosodium L-glutamate, and 1 g of L-glutamic acid. Over a 3-day period the mixture was stirred at 20 °C and a total of 10 g of L-glutamic acid was added. The pD was maintained at approximately 4.5 by occasional addition of DCl. The enzyme was removed by precipitation with trichloroacetic acid and centrifugation, after which the solution was lyophilized. The residue was redissolved and chromatographed on Amberlite XE 64 (H^+ form, 2 × 75 cm). Unreacted glutamic acid was eluted with water, and then (R)-(−)-4-amino[4-^{2}H]butyric acid was eluted with a gradient of 1 L of water and 1 L of 0.5 N acetic acid. The product was lyophilized and recrystallized from ethanol-water, mp 203-204 °C (dec) (lit. 202 °C (dec); Tafel & Stern, 1900). A small portion of this material was converted by pyrolysis to 4-butyrolactam (Yamada & O'Leary, 1977) and its mass spectrum showed the presence of 1.00 ± 0.02 deuterium atom.

(R)-(−)-4-Phthalimido[4-^{2}H]butyric Acid. To 4.7 mL of water containing 283 mg of the deuterated 4-aminobutyric acid prepared above and 310 mg of sodium carbonate was added 730 mg of N-carboethoxyphthalimide and the mixture was stirred at 20 °C for 30 min. The solution was filtered and then acidified, and the precipitated (R)-(−)-4-phthalimido[4-^{2}H]butyric acid was filtered, washed with cold water, and dried, mp 114-115 °C (lit. 115-117 °C; Gabriel & Colman, 1908).

Methyl (R)-(−)-4-Phthalimido[4-^{2}H]butyrate. Esterification of the above compound with diazomethane in ether gave a quantitative yield of methyl (R)-(−)-4-phthalimido[4-^{2}H]butyrate, which was recrystallized from ether, mp 88-89 °C (lit. 89-90 °C; Gabriel & Colman, 1908).

(S)-(−)-[2-^{2}H]Glycine. Hydrogen exchange of the *pro-S* hydrogen of glycine (345 mg) was conducted in 23 mL of 0.01 M phosphate buffer in D_2O, pD 7.8, containing 1.5 mg of pyridoxal-P, 3 mg of tetrahydrofolic acid, and 3.3 mg of serine transhydroxymethylase under nitrogen for 5 days at 20 °C. The enzyme was then removed by precipitation with trichloroacetic acid and centrifugation and the solution was applied to a column of Dowex 50W (100-200 mesh, H^+ form, 1 × 35

[†] From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received August 10, 1977.* This research was supported by Grant NS-07657 from the National Institutes of Health.

0006-2960/78/0417-0669$01.00/0

cm). The column was eluted with water and then with 0.1 N NH_4OH. The recovered glycine was lyophilized and chromatographed on Amberlite XE-64 (H^+ form, 1 × 35 cm) with water. Lyophilization gave pure (*S*)-(−)-[2-^{2}H]glycine. A small amount of this material was acetylated and its mass spectrum showed that it was 8% undeuterated, 86% monodeuterated, and 6% dideuterated.

(S)-(+)-N-Phthaloyl[2-^{2}H]glycine. To a solution of 335 mg of the deuterated glycine prepared above and 478 mg of Na_2CO_3 in 8 mL of water was added 1 g of *N*-carboethoxyphthalimide and the mixture was stirred at 20 °C for 10 min. The solution was filtered, then acidified, and the precipitated (*S*)-(+)-*N*-phthaloyl[2-^{2}H]glycine was filtered, washed with cold water, and recrystallized from acetone, mp 191–193 °C (lit. 191–192 °C; Armarego et al., 1976).

Methyl (S)-(+)-3-Phthalimido[3-^{2}H]propionate. The acid prepared in the previous step (560 mg) was refluxed in 30 mL of $SOCl_2$ for 1 h and then remaining $SOCl_2$ was removed by distillation at reduced pressure. The residue was dissolved in ether and the solution was cooled and excess diazomethane was added. After 12 h the solvent was evaporated and the residue was dissolved in anhydrous methanol. The solution was warmed to 40 °C and 400 mg of Ag_2O was added over a 40-min period, after which the mixture was refluxed for 24 h. After cooling, the mixture was treated with Norit and filtered. The filtrate was concentrated and chromatographed on silica gel with 1% ethyl acetate–99% benzene. The product methyl (*S*)-(+)-3-phthalimido[3-^{2}H]propionate was recrystallized from benzene–hexane, mp 65.5–66 °C.

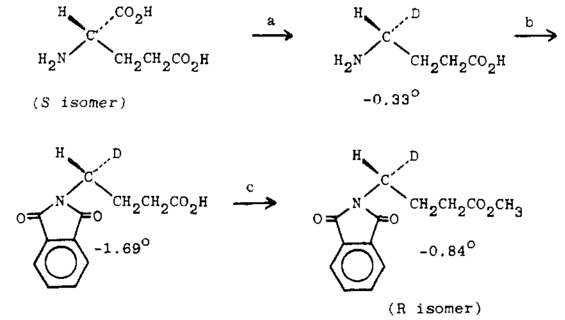
Methyl (S)-(+)-4-Phthalimido[4-^{2}H]butyrate. The ester prepared above (386 mg) was hydrolyzed by refluxing in 50 mL of acetic acid containing 1 mL of 48% HBr for 1 week. The solvent was removed by distillation and the residue was chromatographed on silica gel with acetone. The product was reacted with thionyl chloride, then diazomethane, and then methanol, as described above. The product methyl (*S*)-(+)-4-phthalimido[4-^{2}H]butyrate was chromatographed on silica gel with a gradient of benzene and 3% ethyl acetate–97% benzene and then recrystallized from ether, mp 88 °C.

4-Aminovaleric Acid. DL-α-Methylglutamic acid hemihydrate (2.04 g) in 150 mL of water containing 100 mg of pyridoxal-P and 10 mg of dithiothreitol at pH 4.5 was decarboxylated with 25 mg of glutamate decarboxylase at 37 °C for 5 days. The enzyme was then removed by precipitation with trichloroacetic acid and centrifugation and the solution was lyophilized. The NMR spectrum of the redissolved residue indicated that decarboxylation had proceeded to the extent of approximately 48% (assuming that only one enantiomer of the substrate reacts). The solution was chromatographed on Amberlite XE 64 (H^+ form, 2.5 × 30 cm). Elution with water produced unreacted α-methylglutamic acid. Elution with a gradient from water to 0.05 N HCl gave 4-aminovaleric acid. α-Methylglutamic acid was further purified by chromatography on Dowex 50W (H^+ form) eluted with a gradient from water to 0.4 N HCl and was then recrystallized repeatedly from water–pyridine (pH 3), mp 160–161 °C (dec). 4-Aminovaleric acid was chromatographed on Amberlite XE-64 (H^+ form) eluted with a gradient from water to 0.1 N acetic acid and then repeatedly recrystallized from water–ethanol–ether, mp 212–213 °C (lit. 217–218 °C; Lucente et al., 1965).

Results

L-Glutamic acid was decarboxylated by glutamate decarboxylase in D_2O. The purified product contained a single deuterium atom. This product was converted to methyl 4-phthalimido[4-^{2}H]butyrate as shown in Scheme I. This material had a specific rotation at 340 nm of −6.28°C. Specific rotations of all compounds are shown in Scheme I.

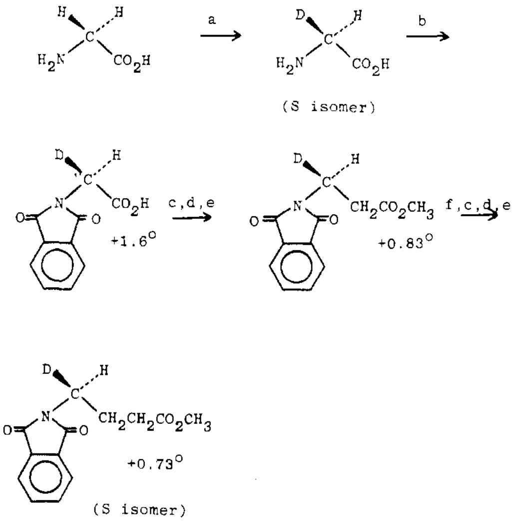
SCHEME I: Conversion of L-Glutamic Acid to Methyl 4-Phthalimido[4-^{2}H] butyrate.[a]



[a] Specific rotations (589 nm) are shown below each structure. Reagents: (a) glutamate decarboxylase in D_2O; (b) *N*-carboethoxyphthalimide; (c) diazomethane.

(*S*)-[2-^{2}H]Glycine was prepared by hydrogen exchange between glycine and D_2O in the presence of serine transhydroxymethylase (Schirch & Jenkins, 1964; Jordan & Akhtar, 1970). Following purification, a small sample of the product was acetylated and the mass spectrum showed that it was 86% monodeuterated. The optical rotation at 238 nm was −33.9° and at 227 nm was −71.1°, in agreement with previous values (Armarego et al., 1976; Arigoni & Eliel, 1969; Battersby et al., 1976). The deuterated glycine was converted into methyl-4-phthalimido[4-^{2}H]butyrate as shown in Scheme II.

SCHEME II: Conversion of Glycine to Methyl 4-Phthalimido[4-^{2}H]-butyrate.[a]



[a] Specific rotations (589 nm) are shown below each structure. Reagents: (a) serine transhydroxymethylase in D_2O; (b) *N*-carboethoxyphthalimide; (c) $SOCl_2$; (d) diazomethane; (e) Ag_2O; (f) HBr/CH_3CO_2H.

None of the reactions used for this conversion should affect the absolute configuration of the asymmetric carbon atom, so the absolute configuration of the product should correspond to that of the glycine starting material. The product had a specific rotation at 340 nm of +6.23°. Other specific rotations are shown in Scheme II.

As shown in Scheme II, the product derived from glycine should be of the *S* configuration. The product derived from glutamic acid (Scheme I) has an equal and opposite rotation to that derived from glycine; thus the product derived from glutamic acid has the *R* configuration and the decarboxylation of glutamic acid occurs with retention of configuration.

Racemic α-methylglutamic acid was decarboxylated by glutamate decarboxylase and the product 4-aminovaleric acid was isolated and purified, after which it had $\alpha_D = -11.5°$. The *R* isomer of this compound had $\alpha_D = +12°$ (Lucente et al., 1965). Recovered α-methylglutamic acid had a negative optical rotation and is thus principally the *R* isomer (Kagan et al., 1965; Izumi et al., 1965). Thus, the *S* isomer is decarboxylated to form an *S* product and this decarboxylation also occurs with retention of configuration.

Discussion

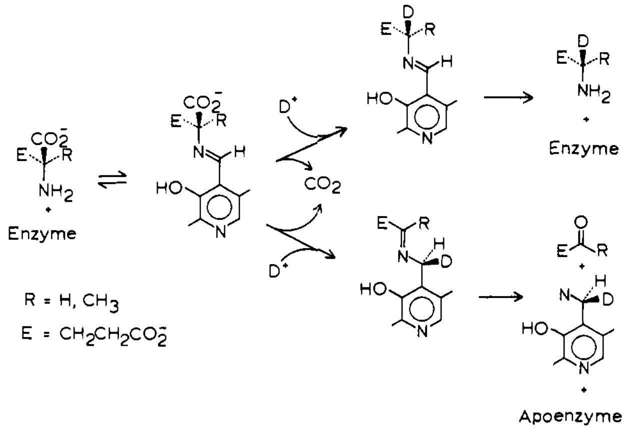
The decarboxylation of L-glutamic acid by bacterial glutamate decarboxylase in D_2O produces (*R*)-4-amino[4-^{2}H]-butyric acid; thus, reaction occurs with retention of configuration. The magnitude of the optical rotation of the product indicates that the reaction is virtually stereospecific, but it is impossible to eliminate the possibility that there might be a small nonstereospecific component.

Two other pyridoxal-P dependent decarboxylases, tyrosine decarboxylase (Belleau & Burba, 1960) and lysine decarboxylase (Leistner & Spenser, 1975) have also been shown to catalyze decarboxylation with retention of configuration. Thus, the results with glutamate decarboxylase continue the general pattern of consistency suggested by Dunathan & Voet (1974), according to which all pyridoxal-P dependent enzymes are derived from a common ancestor and all reactions are expected to show a consistent stereochemistry.

Although a number of amino acid decarboxylases slowly decarboxylate α-methyl amino acids, in no case has the stereochemistry of that process been determined until now. The optical rotation of the α-methylglutamic acid remaining after decarboxylation of DL-α-methylglutamic acid by glutamate decarboxylase indicates that only the L isomer of this compound is decarboxylated, and the optical rotation of the γ-aminovaleric acid produced by decarboxylation indicates that the reaction occurs with retention of configuration. The decarboxylation of α-methylglutamic acid is accompanied by a small amount of decarboxylation-dependent transamination, in which the protonation of the quinoid intermediate produced by decarboxylation occurs on the 4′ carbon of the coenzyme, rather than on the α-carbon of the amino acid. Sukhareva et al. (1972) have established that the labeled pyridoxamine-P produced by conducting the decarboxylation of α-methylglutamic acid in tritiated water has the *S* configuration, which is the same as that produced by conducting a variety of enzymatic transaminations in tritiated water (Dunathan, 1971).

Two principal steric constraints appear to operate in reactions of enzymes which require pyridoxal-P (Dunathan, 1971). First, the pyridine ring of the coenzyme, the 5′ carbon, the imine nitrogen, and the α carbon of the substrate must be coplanar in order for reaction to occur. Second, the bond between the α carbon of the substrate and the atom or group being removed (the carboxyl group for decarboxylation, the α hydrogen for transamination) must be as far out of this plane as possible in order to align the appropriate orbitals for reaction.

SCHEME III: Stereochemistry of the Decarboxylation of Glutamic Acid.



A mechanism which accommodates the stereochemical results for glutamate decarboxylase is shown in Scheme III. One important ambiguity remains in this scheme. We have assumed, but cannot rigorously prove, that the proton transfers in the normal decarboxylation and in the decarboxylation-dependent transamination of α-methylglutamic acid occur on the same face of the planar, conjugated system. By analogy to transaminases, it is likely that this is the case. In addition, in this stereochemical scheme the same catalytic group might be responsible for both the normal and abnormal protonations, and the decarboxylation-dependent transamination can be viewed simply as resulting from the inability of that catalytic group to control the position of protonation with absolute specificity. If correct, this stereochemical picture continues the pattern of consistency among various pyridoxal-P dependent enzymes (Dunathan & Voet, 1974), according to which all bond making and breaking processes occur on the same face of the coenzyme.

In this paper we have derived an internally consistent scheme for the stereochemistry of reactions catalyzed by glutamate decarboxylase. An analogous scheme has previously been derived for reactions catalyzed by aspartate aminotransferase (Dunathan, 1971). An interesting relationship exists between the stereochemical assignments in the two enzymes. It appears that all bond-making and bond-breaking steps (carbon–hydrogen bond making and breaking and carbon–carbon bond breaking) occur on the same side (in the absolute sense) of the plane formed by the pyridine ring, the aldehyde carbon, the amino acid nitrogen, and the amino acid α carbon. In order for this to be true, the carboxyethyl side chain (which confers specificity in the reaction) must occupy two different positions, 120° apart, in the two enzymes. If Dunathan's argument about evolution of pyridoxal enzymes from a common ancestor is correct, it is interesting that it is the stereochemistry around the coenzyme which is conserved, rather than the position of the substrate's specificity-conferring side chain.

Acknowledgments

We are grateful to Dr. L. V. Schirch for supplying serine transhydroxymethylase and to Dr. G. Lucente for advice concerning the absolute configuration of γ-aminovaleric acid.

References

Arigoni, D., & Eliel, E. L. (1969) *Top. Stereochem. 4,* 127.

Armarego, W. L. F., Milloy, B. A., & Pendergast, W. (1976) *J. Chem. Soc., Perkin Trans. 1,* 2229.

Battersby, A. R., Staunton, J., & Summers, M. C. (1976) *J. Chem. Soc., Perkin Trans. 1,* 1052.

Belleau, B., & Burba, J. (1960) *J. Am. Chem. Soc. 82,* 5751.

Dunathan, H. C. (1971) *Adv. Enzymol. 35,* 79.

Dunathan, H. C., & Voet, J. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 3888.

Gabriel, S., & Colman, J. (1908) *Chem. Ber. 41,* 513.

Huntley, T. E., & Metzler, D. E. (1967) Abstracts, p 201c, 154th National Meeting of the American Chemical Society, Chicago.

Izumi, Y., Tatsumi, S., Imaida, M., Fukuda, Y., & Akabori,

S. (1965) *Bull. Chem. Soc. Jpn. 38,* 1338.

Jordan, P. M., & Akhtar, M. (1970) *Biochem. J. 116,* 277.

Kagan, H. M., Manning, L. R., & Meister, A. (1965) *Biochemistry 4,* 1063.

Leistner, E., & Spenser, I. D. (1975) *J. Chem. Soc., Chem. Commun.,* 378.

Lucente, G., Cagnetta, G., Fiorentini, G., & Romeo, A. (1965) *Gazz. Chim. Ital. 95,* 1335.

Mandeles, S., Hanke, M. E., & Koppelman, R. (1954) *J. Biol. Chem. 209,* 327.

O'Leary, M. H. (1969) *Biochemistry 8,* 1117.

Schirch, L. V., & Jenkins, W. T. (1964) *J. Biol. Chem. 239,* 3801.

Sukhareva, B. S., & Torchinsky, Y. M. (1966) *Biochem. Biophys. Res. Commun. 25,* 585.

Sukhareva, B. S., Dunathan, H. C., & Braunstein, A. E. (1972) *FEBS Lett. 15,* 241.

Tafel, J., & Stern, M. (1900) *Chem. Ber. 33,* 2230.

Yamada, H., & O'Leary, M. H. (1977) *J. Am. Chem. Soc. 99,* 1660.

Studies of the Control of Luminescence in *Beneckea harveyi:* Properties of the NADH and NADPH:FMN Oxidoreductases[†]

Edward Jablonski and Marlene DeLuca*

ABSTRACT: Highly purified NADH and NADPH:FMN oxidoreductases from *Beneckea harveyi* have been characterized with regard to kinetic parameters, association with luciferase, activity with artificial electron acceptors, and the effects of inhibitors. The NADH:FMN oxidoreductase exhibits single displacement kinetics while the NADPH:FMN oxidoreductase exhibits double displacement or ping-pong kinetics. This is consistent with the formation of a reduced enzyme as an intermediate in the reaction catalyzed by the NADPH:FMN oxidoreductase. Coupling of either of the oxidoreductases to the luciferase reaction decreases the apparent K_ms for NADH, NADPH, and FMN, supporting the suggestion of a complex between the oxidoreductases and luciferase. The soluble oxidoreductases are more efficient in producing light with luciferase than is a NADH dehydrogenase preparation obtained from the membranes of these bacteria. The soluble enzymes use either FMN or FAD as substrates for the oxidation of reduced pyridine nucleotides while the membrane NADH dehydrogenase is much more active with artificial electron acceptors such as ferricyanide and methylene blue. FMN and FAD are very poor acceptors. The evidence indicates that neither of the soluble oxidoreductases is derived from the membranes. Both enzymes are constitutive and do not depend on the synthesis of luciferase.

The production of light by extracts of luminescent bacteria is due to the interaction of reduced flavin mononucleotide, $FMNH_2$,[1] a long chain aldehyde, molecular oxygen, and a specific enzyme, luciferase (Strehler & Cormier, 1953; McElroy et al., 1953; Becvar & Hastings, 1975; Hastings & Wilson, 1976). Reaction 1 describes the overall process for light emission.

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\nu \quad (1)$$

The generation of reduced FMN is dependent on the presence of specific pyridine nucleotide oxidoreductases which can use either NADH or NADPH as electron donors (reaction 2).

$$NAD(P)H + H^+ + FMN \rightarrow NAD(P)^+ + FMNH_2 \quad (2)$$

In *Photobacterium fischeri* there appears to be only one oxidoreductase which uses either NADH or NADPH (Duane & Hastings, 1975). *Beneckea harveyi* has been shown to have two distinct oxidoreductases, one specific for NADH and another specific for NADPH (Gerlo & Charlier, 1975). These enzymes use soluble FMN as a substrate unlike many other flavin utilizing enzymes which contain the flavin as a tightly bound cofactor. Similar oxidoreductases have been found in *Photobacterium phosphoreum, Escherichia coli, Azotobacter vinelandii,* and *Clostridium perfringens* (Puget & Michelson, 1972).

We report here further characterization of the NADH and NADPH:FMN oxidoreductases from *B. harveyi* with regard to kinetics and their association with luciferase. The effect of artificial electron acceptors and various inhibitors on the activity of these two enzymes was also investigated. The properties of the soluble oxidoreductases were compared with the membrane-bound NADH dehydrogenase.[2]

Materials and Methods

Chemicals. NADH, NADPH, AMP, and dithiothreitol were obtained from Calbiochem. FAD, FMN, decanal, rotenone, dicoumarol, 2-heptyl-4-hydroxyquinoline *N*-oxide, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, Triton X-100, and DNase I were purchased from Sigma Chemical Co. Dichlorophenolindophenol, menadione, and methylene blue were from the Aldrich Chemical Co. 5,5'-Dithiobis(2-nitrobenzoic

---

[†] From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093. *Received September 19, 1977.* This research was supported by a grant from the National Science Foundation, BMS 72-02405. E.J. is supported by a U.S. Public Health Service Predoctoral Training Grant.

[1] Abbreviations used: FAD, flavin adenine dinucleotide; $FMNH_2$ and FMN, reduced and oxidized riboflavin 5-phosphate; DCIP, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoate; DNP, dinitrophenol.

[2] We will refer to the soluble enzymes which use FMN as a substrate as oxidoreductases and the membrane bound enzyme as NADH dehydrogenase.

0006-2960/78/0417-0672$01.00/0