*Biochimica et Biophysica Acta*, 438 (1976) 61—70


BBA 67820

# OXIDATION OF SARCOSINE AND *N*-ALKYL DERIVATIVES OF GLYCINE BY D-AMINO-ACID OXIDASE

MAKOTO NAOI and KUNIO YAGI

*Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya (Japan)*

(Received October 24th, 1975)

## Summary

1. Sarcosine was oxidized by D-amino-acid oxidase (D-amino-acid: $O_2$ oxidoreductase (deaminating), EC 1.4.3.3) to yield methylamine and glyoxylic acid. A series of *N*-alkyl glycines were also oxidized by this enzyme.

2. *N*-Acetyl- and *N*-phenylglycine inhibited the oxidase by competing with the substrate, while *N*-methyl-*N*-acetylglycine did not bind to the enzyme. This suggests the requirement of at least one unsubstituted hydrogen atom at the amino group of glycine for binding.

3. The primary step in the reaction was the release of a proton from the substrate, indicating the formation of a substituted imino acid, which was spontaneously hydrolyzed to glyoxylic acid and an amine.

## Introduction

Sarcosine is oxidized by either sarcosine oxidase reported by Handler et al. [1] or glycine oxidase reported by Ratner et al. [2]. It was later found by Neims and Hellerman [3] that glycine oxidase and D-amino-acid oxidase (D-amino-acid: $O_2$ oxidoreductase (deaminating), EC 1.4.3.3) are identical. However, oxidation of sarcosine was not studied in the paper of Neims and Hellerman and glycine oxidase reported by Ratner et al. [2] was a crude preparation and the authors did not study the oxidation of other *N*-alkyl derivatives of glycine. This paper reports that sarcosine and other *N*-alkyl glycines are oxidized by highly purified D-amino-acid oxidase, which has been known to oxidize D-α-hydroxy acids [4], L-proline [5,6] and nitroalkanes [7] besides D-isomers of α-amino acids. The mechanism of the reaction is compared with that of the oxidation of D-α-amino acids.

Abbreviation: $Me_3Si$, trimethylsilyl.

## Materials and Methods

D-Amino-acid oxidase was prepared from pig kidney by the method of Yagi et al. [8,9]. When checked by (sodium dodecyl sulfate)-disc electrophoresis, the enzyme sample was found to contain a single component with a molecular weight of 39 000.

Sarcosine, *N*-phenylglycine, *N*-dimethylglycine, betaine, methylamine and other alkyl amines were purchased from Nakarai Chemicals Co., Kyoto. Glyoxylic acid was purchased from Sigma Co., St. Louis, Mo. and fluorescamine from Roche Diagnostics, N.J. *N*-Acetylglycine and *N*-methyl-*N*-acetylglycine were synthesized as follows: 100 mmol of glycine or sarcosine were dissolved in 10 ml of ethanol and 110 mmol of acetic anhydride were added at 5°C. The reaction was allowed to proceed for 24 h at room temperature. The solvent was removed by evaporation and the product was recrystallized from an ethanol/water mixture.

*N*-Ethylglycine was synthesized by a modified method of Michaelis and Schubert [10]. To 20 ml of an aqueous solution of monochloroacetic acid (100 mmol), 20 ml of a chilled aqueous solution of ethylamine (110 mmol) were added dropwise and the pH of the mixture was adjusted to 11.0 with 8 M NaOH. After 10 min, the pH of the solution was adjusted to 11.0 by further addition of 8 M NaOH. The reaction was continued for 6 h under reflux. After evaporation of the solvent, the *N*-ethylglycine formed was dissolved in hot ethanol and condensed in the presence of HCl. Crystals were obtained on addition of ethyl ether. Other *N*-alkyl derivatives of glycine were synthesized in the same way as above. The purity of the samples was checked by thin-layer chromatography using ethyl acetate/acetic acid/water (3 : 2 : 1, v/v) as a developing system. The data of elemental analyses of a series of *N*-alkyl glycines are summarized in Table I. The chemical structures of these compounds were confirmed by their proton magnetic spectra which were measured in a JEOL JNM-PS-100 NMR spectrometer.

The oxidation of sarcosine and other *N*-alkyl glycines was measured polarographically as reported previously [8]. For simultaneous of $O_2$ uptake and pH change, the reaction mixture contained 0.1 mmol of *N*-alkyl glycine dissolved in 10 ml of 1 mM Tris/HCl buffer (pH 9.0) containing 1 mmol KCl and 0.1 μmol of FAD. The reaction was initiated by addition of the enzyme. The oxygen uptake was recorded using an oxygen electrode (Beckman oxygen sensor), and the change of pH was followed in a Hitachi-Horiba F-5 pH meter connected to a recorder [11]. The absorbance spectra were measured using a Beckman DK-2A recording spectrophotometer.

The reaction products were identified by paper and thin-layer chromatography. 1 mmol of sarcosine was reacted with 0.1 μmol of the enzyme in 5 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3). The reaction was continued with shaking for 24 h at 20°C, and aliquots of the mixture were directly applied to paper or thin-layer chromatography. The following solvents were used: *t*-butanol/acetic acid/water (3 : 1 : 1, v/v), *n*-propanol/acetic acid/water (10 : 1 : 9, v/v) and phenol/water (4 : 1, v/v). Toyo Filter Paper No. 51, Toyo Roshi Co., Tokyo and precoated silica gel thin-layer plate (TLC Aluminium Sheets, Silica gel 50 $F_{254}$), E. Merck Co., Darmstadt, were used for chromatography. For

TABLE I

DATA OF THE ELEMENTAL ANALYSES OF SYNTHESIZED *N*-ALKYL GLYCINES

| *N*-Alkylglycines | Calculated (%) | | | Found (%) | | |
|---|---|---|---|---|---|---|
| | C | H | N | C | H | N |
| *N*-Ethylglycine HCl ($C_4H_9O_2N$ HCl) | 34.41 | 7.22 | 10.04 | 33.94 | 7.21 | 10.02 |
| *N*-*n*-Propylglycine HCl ($C_5H_{11}O_2N$ HCl) | 39.09 | 7.87 | 9.11 | 39.25 | 7.62 | 9.01 |
| *N*-*n*-Butylglycine HCl ($C_6H_{13}O_2N$ HCl) | 42.99 | 8.42 | 8.36 | 43.00 | 8.40 | 8.27 |
| *N*-*n*-Amylglycine HCl ($C_7H_{15}O_2N$ HCl) | 46.27 | 8.88 | 7.71 | 46.04 | 9.08 | 7.63 |
| *N*-*n*-Hexylglycine HCl ($C_8H_{17}O_2N$ HCl) | 49.68 | 9.28 | 7.16 | 48.83 | 9.22 | 7.32 |
| *N*-Benzylglycine HCl ($C_9H_{11}O_2N$ HCl) | 53.60 | 6.00 | 6.95 | 53.54 | 6.10 | 6.79 |
| *N*-Phenethylglycine HCl ($C_{10}H_{13}O_2N$ HCl) | 55.69 | 6.54 | 6.49 | 55.28 | 6.53 | 6.47 |

detection of amino groups, a 0.2% ninhydrin in 95% aqueous ethanol was used. For detection of secondary amines, chromatograms were sprayed with a mixture of sodium nitroprusside and carbonate [12]. For the detection of glyoxylic acid, a freshly prepared solution of 1% phenylhydrazine hydrochloride in 0.5 M HCl was sprayed, dried in hot air, and the paper or plate was sprayed with 3% hydrogen peroxide in 0.5 M HCl.

The amines formed by the enzyme were also identified by gas chromatography. To the reaction mixture containing 300 μmol of sarcosine and 30 nmol of FAD in 3.0 ml of 0.2 M sodium borate/sodium hydroxide buffer (pH 11.0) or 0.1 M sodium pyrophosphate buffer (pH 8.3), 2—3 nmol of the enzyme were added. In the case of short-chained *N*-alkyl amines, an aliquot (usually 1 μl) of the reaction mixture was withdrawn at definite time and applied to a gas chromatograph, Shimadzu GC-4BM, directly. The conditions of the chromatography were as follows: a 2 m glass column, packed with 10% THEED on Chromosorb WAW (60—80 mesh), was used, and nitrogen carrier gas was flowed at a rate of 40 ml per min. The column temperature was 60°C, and the temperature of injector and detector (hydrogen flame detector) 160°C. For identification of the reaction products of *N*-benzyl- and *N*-phenethylglycine, 3 ml of the reaction mixture were mixed with 6 ml of 50% *n*-butanol. pH of the mixture was brought to 11.0 with 8 M NaOH. The butanol phase was washed twice with water, evaporated and dried over phosphorous pentoxide in a desiccator overnight. The samples were dissolved in 10 μl of dry methylene chloride and 100 μl of trifluoroacetic anhydride were added. Trifluoroacetylation was completed in 10 min at room temperature. Aliquots were injected into a gas chromatograph equipped with a 2 m glass column packed with 3% silicone SE-30 on Gas chrom-Q (100—120 mesh). The column temperature was 120°C and the temperature of injector and hydrogen flame detector 250°C. The flow rate of carrier gas was 45 ml per min.

The quantitative analysis of amines produced was carried out fluorometrically using fluorescamine, 4-phenylspiro-(furan-2(3H), 1′-phthalan)-3,3′-dione

[13,14]. To the reaction mixture containing 30 μmol of sarcosine in 3.0ml of 0.2 M sodium borate/sodium hydroxide buffer (pH 11.0), 2 nmol of the enzyme were added. At several time intervals, 0.1 ml of the mixture was taken and mixed with 3.0 ml of the borate buffer (pH 11.0). The amine was extracted three times with each 1.5 ml of *n*-hexane and then extracted with 1.0 ml of 1 M HCl from the combined *n*-hexane phase. A 0.5 ml aliquot of the water phase was mixed with 1.5 ml of the borate buffer (pH 11.0), and 0.5 ml of fluorescamine solution (3 mg fluorescamine/10 ml dry acetone) was added. The fluorescence intensity was measured in a corrected recording fluorospectrophotometer, Shimadzu RF 502, at room temperature. The excitation wavelength was 390 nm, and fluorescence intensity was measured at 490 nm.

Glyoxylic acid formed was also identified by isolating its hydrazone. 2 mmol of sarcosine were reacted with 0.2 μmol of the enzyme in 5 ml of 0.1 M sodium pyrophosphate buffer (pH 6.8) *. The reaction mixture was gently shaken under aerobic condition at room temperature for 24 h. To terminate the reaction, half the volume of 6 M HCl was added and the denatured protein removed by centrifugation. The supernatant solution was then mixed with a theoretical amount of 2,4-dinitrophenylhydrazine in 2 M HCl. The crude hydrazone was recrystallized from aqueous ethanol.

The determination of glyoxylic acid was also carried out by gas chromatography using its trimethylsilyl ($Me_3Si$) derivative. An aliquot of the abovementioned reaction mixture was taken, passed through a column (0.5 × 5 cm) of Dowex 50 W (100—200 mesh, $H^+$-form), evaporated and dried over phosphorous pentoxide in a desiccator overnight. The sample was trimethylsilylated in a mixture of dry pyridine/bis($Me_3Si$)acetamide/trimethylchlorosilane (2 : 2 : 1, v/v) at room temperature for 30 min. $Me_3Si$-glyoxylic acid was injected into a gas chromatography, Shomadzu GC-4BM equipped with a hydrogen flame detector. A 2 m glass column packed with 3% SE-30 on Gas Chrom-Q (100—120 mesh) was used. The conditions were as follows: column temperature 100°C, injector and detector temperature 200°C. Nitrogen gas was flowed at the rate of 40 ml per min.

Formaldehyde was chacked by the reaction with dimedone [15].

## Results

On addition of sarcosine, under aerobic conditions, the absorbance spectrum of the enzyme changed, as shown in Fig. 1, within 1 s and remained unchanged until the oxygen dissolved in the medium was consumed (about 2 min). The absorbance peak at 455 nm shifted to 460 nm with considerable hypochromism and an increase in the absorbance in the longer-wavelength region. The splitting of the absorbance band at the shorter-wavelength region disappeared and the maximum showed a blue shift to 370 nm with hypochromism. The spectrum changed gradually into that of the reduced form after the oxygen in the solution was consumed. Under anaerobic conditions, the spectrum of the enzyme was converted into that of the reduced form immediately.

The oxidation of sarcosine by the oxidase was dependent on the amount of

* This pH was preferred, because glyoxylic acid was unstable at higher pH.

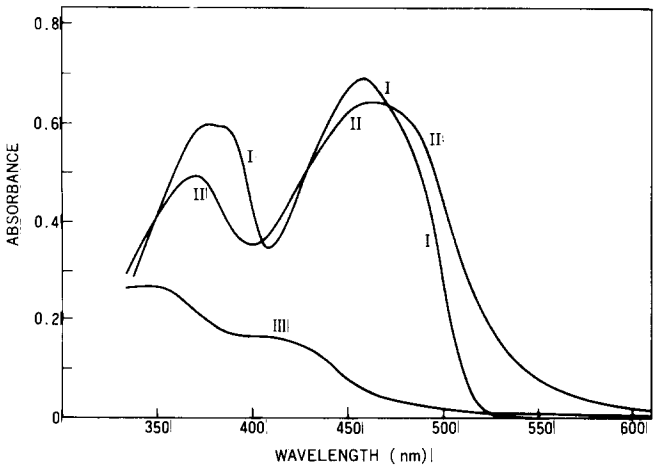



Fig. 1. Spectral change of the enzyme upon addition of sarcosine. Curve I, the enzyme ($6.9 \cdot 10^{-5}$ M in final concentration) dissolved in 3.0 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3). Curve II, within 1 min after the addition of sarcosine (100 μmol) to I. Curve III, 3 min after II.

enzyme and the Michaelis constant ($K_m$) and the maximum velocity ($V$, expressed in terms of molar activity) were obtained by the double-reciprocal plot of Lineweaver and Burk. The apparent $K_m$ and $V$ values for sarcosine were obtained as 16 and 3 mM at pH 8.3 and 52 and 72 mM at pH 11.0, respectively (Table IV).

The pH versus activity curve is shown in Fig. 2. The optimum pH is about 11.0. The oxidation rates fall off very sharply to either side of the optimum. At pH 8.3, where the catalytic activity of this enzyme for many amino acids has been measured, the enzymatic activity for sarcosine was found to be far smaller than the maximum.

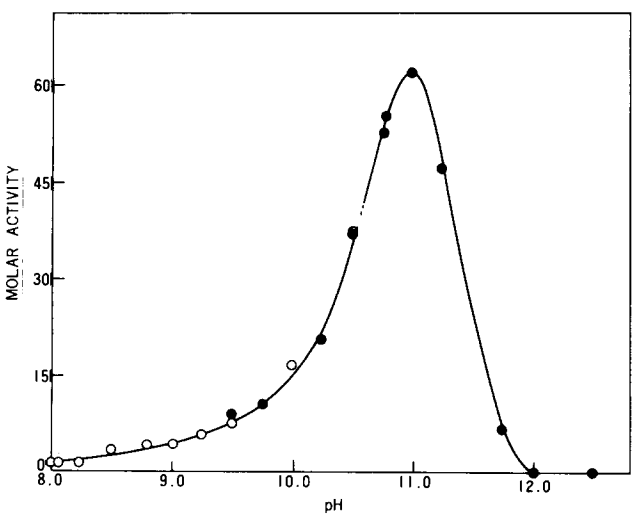



Fig. 2. Optimum pH for oxidation of sarcosine. Sarcosine (1 M in final concentration) was dissolved in 10.0 ml of 0.1 M sodium pyrophosphate buffer (○) or 0.2 M sodium borate buffer (●), and oxygen consumption was measured with an oxygen electrode. The enzyme ($2.4 \cdot 10^{-6}$ M in final concentration) and FAD ($1 \cdot 10^{-5}$ M) were added to the substrate solution.

The reaction products were identified by paper and thin-layer chromatography as summarized in Tables II and III. In the case of sarcosine, one of the products was found to be methylamine. In gas chromatography, the retention time of the product obtained by the reaction at either pH 8.3 or 11.0 was identical with that of the standard methylamine. The retention time of the main component of $Me_3Si$ derivatives of the reaction products corresponded to that of $Me_3Si$-glyoxylic acid. The 2,4-dinitrophenylhydrazone of the reaction product was isolated and the melting point was 188—189°C, which is in agreement with the reported value for the hydrazone of glyoxylic acid.

Glycine, which was reported as a product of oxidative demethylation of sarcosine by sarcosine oxidase [1], was not found by paper chromatography (Table II). Another reaction product, formaldehyde, also was not found by the reaction with dimedone.

The above results suggests the occurrence of the following reaction:

$$CH_3NHCH_2COOH + O_2 + H_2O \rightarrow CH_3NH_2 + CHOCOOH + H_2O_2 \quad (1)$$

To verify the above reaction, oxygen uptake and reaction products were measured. In the reaction mixture containing 300 μmol of sarcosine, 30 nmol of FAD and 2 nmol of the enzyme in 3 ml of borate buffer (pH 11.0), 27.2 nmol of oxygen were consumed per ml per min, while 27.0 nmol of methylamine and 27.4 nmol of glyoxylic acid were formed per ml per min. In the presence of catalase, the oxygen consumption in the mixture was reduced to half of the above; in the case of the oxidation of *N*-*n*-butylglycine at pH 8.3, oxygen uptake was reduced from 20.0 to 10.5 mol (per mol of the enzyme per min). This indicates that $H_2O_2$ is formed accompanied by the oxidation of *N*-alkyl glycines.

*N*-Ethyl and other *N*-alkyl derivatives of glycine were also found to be oxidized by the enzyme and $K_m$ and $V$ for the oxidation of these compounds at pH 8.3 and 11.0 are summarized in Table IV.

On the other hand, amino-substituted compounds of glycine shown in Table V were found not to be oxidized by the enzyme when examined by oxygen consumption. However, the absorbance spectrum of the enzyme was perturbed by *N*-acetyl- or *N*-phenylglycine (see Fig. 3). These two compounds were found to inhibit the enzyme in competition with the substrate (see Fig. 4). Data are

TABLE II

IDENTIFICATION OF A PRODUCT IN THE REACTION OF SARCOSINE WITH D-AMINO-ACID OXIDASE BY USING PAPER CHROMATOGRAPHY

The chromatography was carried out at 20°C for 24—28 h, and the spots were visualized by ninhydrin reaction. Solvent systems: I, *t*-butanol/acetic acid/water (3 : 1 : 1, v/v); II, *n*-propanol/acetic acid/water (10 : 1 : 9, v/v); III, phenol/water (4 : 1, v/v).

| Compound | Solvent systems | | |
|---|---|---|---|
| | I | II | III |
| Glycine | 0.47 | 0.49 | 0.39 |
| Sarcosine | 0.57 | 0.55 | 0.68 |
| Methylamine | 0.70 | 0.62 | 0.54 |
| Reaction product | 0.70 | 0.62 | 0.55 |

TABLE III

IDENTIFICATION OF A PRODUCT IN THE REACTION OF SARCOSINE WITH D-AMINO-ACID OXIDASE BY USING THIN-LAYER CHROMATOGRAPHY

The spots were detected using a phenylhydrazine/hydrogen peroxide spray [12]. Solvent systems: I, *t*-butanol/acetic acid/water (3 : 1 : 1, v/v); II, *n*-propanol/acetic acid/water (10 : 1 : 9, v/v).

| Compound | Solvent systems | |
|---|---|---|
| | I | II |
| Glyoxylic acid | 0.27 | 0.54 |
| Reaction product | 0.26 | 0.54 |

TABLE IV

$K_m$ AND $V$ FOR OXIDATION OF *N*-ALKYL DERIVATIVES OF GLYCINE

| Compound | at pH 8.3 * | | at pH 11.0 ** | |
|---|---|---|---|---|
| | $K_m$ (mM) | $V$ *** | $K_m$ (mM) | $V$ *** |
| Sarcosine | 16 | 3 | 52 | 72 |
| *N*-Ethylglycine | 20 | 27 | 45 | 286 |
| *N*-*n*-Propylglycine | 22 | 33 | 50 | 182 |
| *N*-*n*-Butylglycine | 25 | 28 | 26 | 77 |
| *N*-*n*-Amylglycine | 26 | 59 | 25 | 110 |
| *N*-*n*-Hexylgycine | 87 | 7 | 22 | 13 |
| *N*-Benzylglycine | 4 | 9 | 25 | 40 |
| *N*-Phenthylglycine | 6 | 63 | 40 | 125 |
| Glycine | 330 | 50 | 1540 | 222 |

* 0.1 M sodium pyrophosphate buffer was used, and all the reactions were performed at 20°C. Enzyme concentration was $2 \cdot 10^{-5}$ M for sarcosine and 1—5. Initial concentration of oxygen in the mixture was 0.284 μmol/ml. The values of $K_m$ and $V$ are apparent.

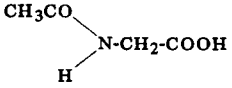
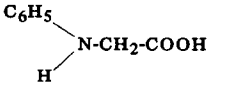
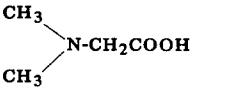
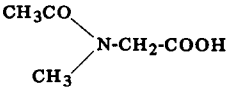
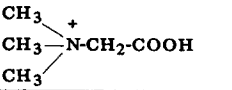
** 0.2 M sodium borate buffer was used. Enzyme concentration was $1 \cdot 10^{-6}$ M.

*** $V$ is expressed in terms of molar activity.

TABLE V

BINDING OF AMINO-SUBSTITUTED DERIVATIVES OF GLYCINE TO D-AMINO-ACID OXIDASE

Binding was judged by the perturbation of the absorbance spectrum of the enzyme. $K_i$ (inhibition constant) was calculated by measuring the inhibition of oxygen uptake using D-alanine as substrate.

| | Binding | $K_i$ |
|---|---|---|
|  | + | 1.11 mM |
|  | + | 0.10 mM |
|  | — | |
|  | — | |
|  | — | |

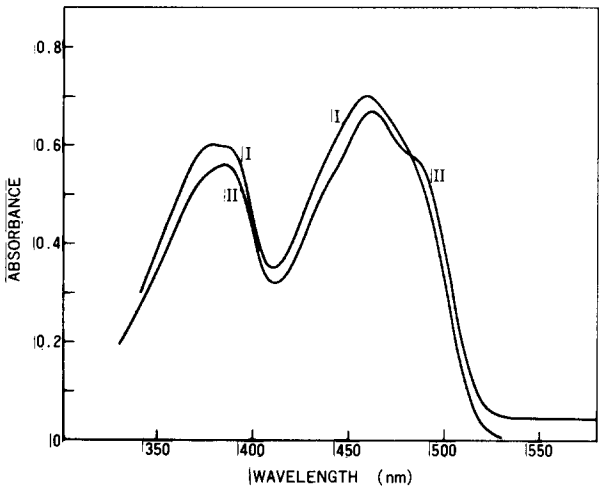



Fig. 3. Change in spectrum of the enzyme upon addition of *N*-phenylglycine. Curve I, the enzyme ($6.2 \cdot 10^{-5}$ M in final concentration) dissolved in 3.0 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3). Curve II, after the addition of *N*-phenylglycine (6 μmol) to I.

summarized in Table V, showing that the compounds which bind to the enzyme have one hydrogen at the α-amino group of glycine.

The release of a proton at the initial step of the oxidation was demonstrated by simultaneous measurements of pH change and oxygen consumption. By addition of the enzyme, the pH of the mixture decreased immediately and subsequently increased towards the initial level, as shown in Fig. 5. The time course of the decrease corresponded to that of the oxygen uptake.

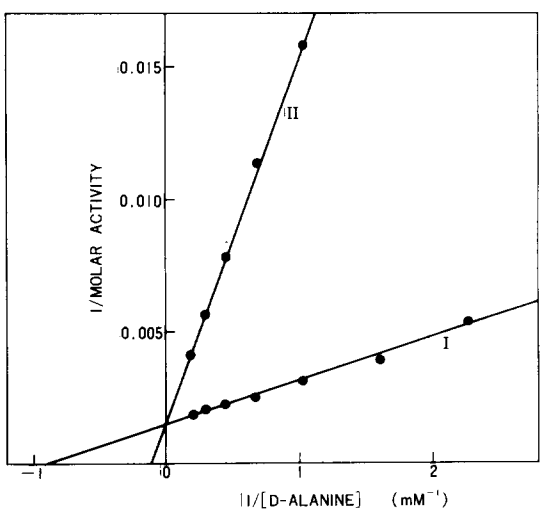



Fig. 4. Inhibition of the enzymatic oxidation of D-alanine by *N*-phenylglycine. The enzyme ($2.5 \cdot 10^{-7}$ M in final concentration) was reacted with graduated concentrations of D-alanine at pH 8.3 and at 20°C. Curve I, in the absence of *N*-phenylglycine. Curve II, in the presence of *N*-phenylglycine ($6.6 \cdot 10^{-4}$ M in final concentration).

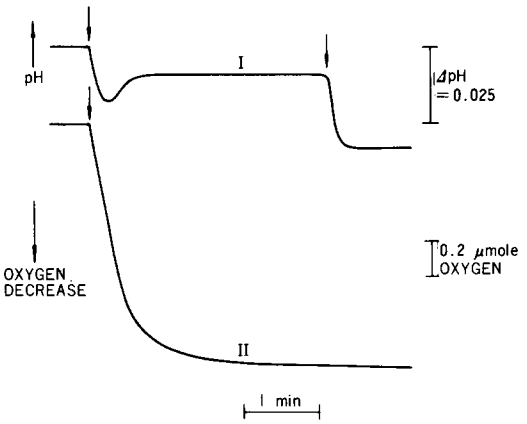



Fig. 5. Simultaneous recording of pH change and oxygen consumption during the reaction of D-amino-acid oxidase with *N*-phenethylglycine. The reaction mixture, which contained 10 mM *N*-phenethylglycine, 10 μM FAD, 100 mM KCl and 1 mM Tris · HCl (pH 9.0) in a total volume of 10.0 ml, was adjusted to pH 9.0 by addition of 0.2 M NaOH. The reaction was initiated by the addition of 0.18 μmol of the enzyme (left-hand arrow). Curve I, pH change. Curve II, oxygen consumption. The change of pH was standardized by the addition of 1 μmol of HCl (right-hand arrow).

## Discussion

In the present study, it was clearly demonstrated that sarcosine was oxidized by highly purified D-amino-acid oxidase to yield methylamine and glyoxylic acid. Quantitative analyses established Eqn. 1. This reaction accords with that of the "glycine oxidase system" reported by Ratner et al. [2]. Although Neims and Hellerman [3] claimed that glycine oxidase is identical with D-amino-acid oxidase, they did not give any direct evidence for the oxidation of sarcosine by this enzyme. Accordingly, the present data afford definite evidence to their proposition. Since neither glycine nor formaldehyde was formed in the present reaction system, it is evident that the demethylation of sarcosine reported by Handler et al. [1] is not the case.

In the case of the oxidation of sarcosine, liberation and fixation of a proton were observed, as in the case of D-alanine [11]. This indicates the occurrence of the following steps of reaction.

$$\begin{array}{l} \mathrm{CH_2\text{-}COO^-} \\ \quad | \\ \mathrm{RNH_2^+} \end{array} + \text{oxidized enzyme} \rightarrow \begin{array}{l} \mathrm{CH\text{-}COO^-} \\ \quad \| \\ \mathrm{RN} \end{array} + \mathrm{H^+} + \text{reduced enzyme}$$

$$\begin{array}{l} \mathrm{CH\text{-}COO^-} \\ \quad \| \\ \mathrm{RN} \end{array} + \mathrm{H_2O} + \mathrm{H^+} \rightarrow \mathrm{RNH_3^+} + \begin{array}{l} \mathrm{CH\text{-}COO^-} \\ \quad \| \\ \mathrm{O} \end{array}$$

$$\text{Reduced enzyme} + \mathrm{O_2} \rightarrow \text{oxidized enzyme} + \mathrm{H_2O_2}$$

From the results obtained by using *N*-substituted derivatives of glycine, it can be concluded that for the binding of these substances to the enzyme at least one unsubstituted hydrogen atom at the amino group is required. It should be noted in this case that the substitution of one hydrogen atom at the amino group by an acetyl or phenyl group resulted in the inability to undergo the enzymatic oxidation, in contrast to the substitution by a methyl group, viz.

formation of sarcosine. It is considered that the substitution by an acetyl or phenyl group influences the mobilization of electron(s) from the amino group. Accordingly, this supports the idea that electron(s) flow from the substrate to the flavin coenzyme via the amino group of the former.

It may also be mentioned that *N*-acetyl or *N*-phenyl derivatives seem to bind with the enzyme, via both their carboxyl and substituted amino groups and their structure is more similar to a substrate, glycine than benzoic acid, which was used as a substrate substitute [16]. Therefore, these substances are considered to be preferable substrate substitutes of this enzyme.

## References


1 Handler, P., Bernheim, M.L.C. and Klein, J.R. (1941) J. Biol. Chem. 138, 211—218
2 Ratner, S., Nocito, V. and Green, D.E. (1944) J. Biol. Chem. 152, 119—133
3 Neims, A. and Hellerman, L. (1962) J. Biol. Chem. 237, PC976—PC978
4 Yagi, K., Ozawa, T. and Naoi, M. (1969) Biochim. Biophys. Acta 185, 31—38
5 Wellner, D. and Scannone, H. (1964) Biochemistry 3, 1746—1749
6 Yagi, K. and Nishikimi, M. (1968) J. Biochem. Tokyo 64, 371—376
7 Porter, D.J.T., Voet, J.G. and Bright, H.J. (1972) J. Biol. Chem. 247, 1951—1953
8 Yagi, K., Naoi, M., Harada, M., Ōkamura, K., Hidaka, H., Ozawa, T. and Kotaki, A. (1967) J. Biochem. Tokyo 61, 580—597
9 Yagi, K., Sugiura, N., Ohama, H. and Kotaki, A. (1970) J. Biochem. Tokyo 68, 573—575
10 Michaelis, L. and Schubert, M.P. (1936) J. Biol. Chem. 115, 221—222
11 Yagi, K., Nishikimi, M., Ohishi, N. and Takai, A. (1970) Biochim. Biophys. Acta 212, 243—247
12 Sweeley, C.C. and Horning, E.C. (1957) J. Am. Chem. Soc. 79, 2620—2625
13 Weigele, M., DeBernardo, S.L., Tengi, J.P. and Leimgruber, W. (1972) J. Am. Chem. Soc. 94, 5927—5928
14 Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871—872
15 Mackenzie, C.G. (1950) J. Biol. Chem. 186, 351—368
16 Yagi, K. and Ozawa, T. (1961) Biochim. Biophys. Acta 56, 413—419