# Studies on Crystalline D-Amino Acid Oxidase. III. Substrate Specificity and $\sigma$–$\rho$ Relationship*

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ABSTRACT: The capability of crystallized hog kidney D-amino acid oxidase as catalyst for the oxidation of series of *meta*- and *para*-substituted *C*-phenylglycines and phenylalanines has been investigated. A brief account of the synthesis of certain of these amino acid substrates is included. Various parameters of the enzymatic oxidations have been evaluated in terms of the $\sigma$–$\rho$ relationship of Hammett. Plots of log maximal rate of *C*-phenylglycine oxidation with respect to $\sigma$ are asymmetrically biphasic, with a maximum corresponding to a $\sigma$ value of 0.04. With electron-donating substituents, a $\rho$ value of 5.44 (correlation coefficient, $r = 0.97$) is derived; with electron-attracting substituents, the $\rho$ value is $-0.40$ ($r = 0.69$). Variation of reaction temperature or oxygen partial pressure did not alter significantly the results presented above. In the case of substituted phenylalanines, a biphasic plot was again observed, with a maximum at $\sigma$ 0.23, and with $\rho$ values of 0.73 ($r = 0.98$) and $-0.93$. Michaelis constants for the amino acids were generally independent of substituent. With sheep kidney D-amino acid oxidase, correlation with $\sigma$ of the relative rates of oxidation at equimolar substrate concentrations with respect to the *C*-phenylglycine series again provided an asymmetrically biphasic plot with a maximum at $\sigma$ 0.0 and with $\rho$ values of 3.45 and $-0.65$. The striking capacity of electron-donating substituents to decrease the rate of *C*-phenylglycine oxidation, an effect that necessarily is less pronounced in the phenylalanine series, taken with earlier studies in this laboratory, has permitted development of a working hypothesis with respect to the mechanism of action of this flavoenzyme involving, in part, proton abstraction from the $\alpha$-carbon atom of the substrate amino acid.

Although $\alpha$-amino acids are relatively stable toward oxidation, *e.g.*, by oxygen, there are stereospecific flavoenzymes, widely distributed in nature, that act as efficient catalysts for such reactions. Insight into the detailed mechanism of action of one of these enzymes, mammalian kidney D-amino acid oxidase, has evolved from studies in this laboratory concerned with inhibitory processes (Hellerman *et al.*, 1946; Frisell *et al.*, 1956; Hellerman *et al.*, 1965) and with the "trapping" of an enzyme–substrate intermediate through the reductive action of sodium borohydride (Coffey *et al.*, 1965). Clarification has been afforded also by the correlation of substrate specificity data with respect to the $\sigma$–$\rho$ relationship of Hammett (1940). A more exhaustive treatment of this correlation is the subject of this report.[1]

The flavin–adenine dinucleotide linked D-amino acid oxidase (EnFAD)[2] is catalyst for the oxidative deamination of certain D-$\alpha$-amino acids[3] to the corresponding $\alpha$-keto acids and ammonia (Krebs, 1935); in an aerobic environment, there is concomitant reduction of molecular oxygen to hydrogen peroxide. $\alpha$-Imino acids have been considered to be intermediates in the over-all catalytic event. In the presence of catalase, conventional equations are

$$RCH(NH_2)COO^- + {}^1/_2O_2 \xrightarrow[\text{catalase}]{\text{EnFAD}} RC(=NH)COO^- + H_2O$$

$$RC(=NH)COO^- + H_2O \longrightarrow RCOCOO^- + NH_3$$

In the current investigation, study of the substrate specificity of the oxidase has been extended to include the maximal rates and Michaelis constants for series of *meta*- and *para*-substituted (a) *C*-phenylglycines ($\alpha$-aminophenylacetic acids) and (b) phenylalanines. This

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[1] Part of this study has been presented: Neims and Hellerman, (1961) and Neims (1961).

[2] Abbreviations used: EnFAD, flavin–adenine dinucleotide linked D-amino acid oxidase; DPN, diphosphopyridine nucleotide.

[3] Recent reports have indicated that D-amino acid oxidase exhibits catalytic activity in the oxidation of glycine (Neims and Hellerman, 1962) and also measurable activity for L-proline and L-3,4-dehydroproline (Wellner and Scannone, 1964).

$XC_6H_4CH(NH_2)COO^-$ (a) $XC_6H_4CH_2CH(NH_2)COO^-$ (b)

report includes an outline of the sources or methods of preparation of these amino acid substrates.

The semiempirical $\sigma$–$\rho$ relationship, a linear free-energy formulation that quantitatively relates both thermodynamic and kinetic reaction parameters with the electron-attracting and electron-donating properties of substituents within a homologous series of compounds, has been applied broadly to the investigation of certain areas of reaction mechanism (Jaffe, 1953; Leffler and Grunwald, 1963). Successful applications of the linear free-energy relationships to problems of enzymatic catalysis are rare inasmuch as difficulties can arise owing to the generally rigid steric requirements of enzyme–substrate interaction. Enzymatic reactions in which the $\sigma$–$\rho$ relationship has proved of interest involve primarily hydrolases (Ormerod, 1953; Gawron *et al.*, 1953; Nath and Rydon, 1954; Mounter, 1958; Bergmann *et al.*, 1958; Caplow and Jencks, 1962; Bender and Nakamura, 1962; Sager and Parks, 1964), but oxidative processes are beginning to receive attention (Deitrich *et al.*, 1962).

The broad specificity of D-amino acid oxidase (Krebs, 1935) permits $\sigma$–$\rho$ analysis of the data reported here concerning the oxidation of substituted *C*-phenylglycines and phenylalanines. Plots of log maximal rate with respect to $\sigma$ provide good correlation and are biphasic for each substrate series. Biphasic character persists notwithstanding variations in reaction temperature and oxygen concentration. It is of interest that biphasic Hammett plots involving other enzyme-catalyzed reactions have been reported by Deitrich *et al.* (1962) and Mazrimas *et al.* (1963).[4] In the current study, a significant sensitivity of the maximal rates of enzymatic oxidation of the *C*-phenylglycines with respect to "electron-donating" substituents has been observed.

## Experimental Section

*Materials.* Crystalline D-amino acid oxidase was prepared from fresh hog kidney by the method of Massey *et al.* (1961). Stage 4 of this procedure was repeated until enzyme of constant specific activity was obtained (17.1–23.3 $\mu$moles of D-alanine oxidized/min per mg of protein with conditions as defined below). The enzyme preparation appeared to be homogeneous on the basis of ultracentrifugal results and molecular sieve chromatography.

D-Amino acid oxidase from fresh sheep kidney was purified according to the procedure of Negelein and Brömel (1939); sheep kidney preparations catalyzed the oxidation of 6.4–6.8 $\mu$moles of D-alanine/min per mg of protein with conditions as defined below. Crystalline bovine liver catalase was obtained from Worthington.

*Synthetic Work.* The purpose of this section is to outline sufficient data to permit an appreciation of the identity and authenticity of the substrates investigated in this study. A full account is to be published in another place.

The substituted DL-*C*-phenylglycines were prepared by alkaline hydrolysis of the appropriate 5-arylhydantoins except as noted. 5-Arylhydantoins were synthesized essentially as described by Bucherer and Leib (1934) from commercially available substituted benzaldehydes. *p*-Methylmercaptobenzaldehyde was prepared from methyl *p*-methylmercaptobenzoate as described by Baker *et al.* (1952) and converted to the amino acid *via* the hydantoin in the usual manner. Methyl *p*-methylmercaptobenzoate was prepared from *p*-aminobenzoic acid (Campaigne and Meyer, 1962). *m*-Nitro-DL-*C*-phenylglycine was prepared (with Dr. Irving Cooperstein) by direct nitration of DL-*C*-phenylglycine essentially as reported by Plöchl and Loë (1885). *m*-Amino-DL-*C*-phenylglycine was prepared by reduction of the corresponding *m*-nitro compound either with tin and hydrochloric acid (Plöchl and Loë, 1885) or catalytically (Friis and Kjaer, 1963). *p*-Amino-DL-*C*-phenylglycine was obtained from ethyl *p*-nitrophenylacetate by the series of reactions described by Davis *et al.* (1955). *p*-Dimethylamino-DL-*C*-phenylglycine was prepared by catalytic reduction (10% Pd–C) of *p*-dimethylaminobenzoylformic acid oxime in absolute ethanol which contained 2 equiv of anhydrous hydrogen chloride. The oxime was obtained by reaction of *p*-dimethylaminobenzoylformic acid with hydroxylamine in aqueous solution, pH 8.0, at room temperature for 24 hr. *p*-Dimethylaminobenzoylformic acid was prepared from dimethylaniline and oxalyl chloride (Staudinger and Stockman, 1909). *p*-Carboxy-*C*-phenylglycine was prepared in this laboratory by Dr. Frances Zwanzig.

*p*-Trimethylammonium-DL-*C*-phenylglycine chloride hydrochloride ([*p*-(DL-aminocarboxymethyl)phenyl]trimethylammonium chloride hydrochloride) was synthesized in this laboratory by Dr. John Wein, starting with *p*-dimethylaminobenzoylformyl chloride. Details are to be described in a separate publication. The identity of the product was determined by its physical properties, analysis, and conversion to 4-trimethylammoniumbenzaldehyde chloride by oxidation with ninhydrin. The aldehyde and its derivatives possessed the properties recorded in the literature.

Nuclear magnetic resonance and infrared spectra were consistent with assigned structures of substrates requiring special study. Nuclear magnetic resonance spectra, evaluated with the kind collaboration of Dr. Donald P. Hollis, were determined in dilute sodium deuterioxide on a Varian A-60 instrument. The infrared spectra were determined on a Perkin-Elmer 21 instrument by the potassium bromide disk technique. Analytical data for the substituted *C*-phenylglycines are given in Table I.

DL-4-Trimethylammoniumphenylalanine chloride hydrochloride ([*p*-(DL-2-amino-2-carboxyethyl)phenyl]-trimethylammonium chloride hydrochloride) was pre-

[4] A note outlining a study with snake venom L-amino acid oxidase also presents a biphasic Hammett plot (Radda, 1964).

TABLE 1: Analytical Data Relating to the Substrates Synthesized.

| Compound | Dec Pt, °C[a] | Formula | Carbon[b] Calcd, % | Carbon[b] Found, % | Hydrogen[b] Calcd, % | Hydrogen[b] Found, % | Nitrogen[b] Calcd, % | Nitrogen[b] Found, % | α-Amino Nitrogen[c,d] Calcd, % | α-Amino Nitrogen[c,d] Found, % |
|---|---|---|---|---|---|---|---|---|---|---|
| DL-*C*-Phenylglycine | 260[e] | $C_8H_9NO_2$ | 63.6 | 63.4 | 6.0 | 5.9 | 9.3 | 9.3 | 9.3 | 9.2 |
| *p*-Methyl- | 257–258 | $C_9H_{11}NO_2$ | 65.4 | 65.6 | 6.7 | 6.7 | 8.5 | 8.4 | 8.5 | 8.2 |
| *m*-Methyl-[f] | 238–241 | $C_9H_{11}NO_2$ | 65.4 | 65.0 | 6.7 | 6.7 | 8.5 | 8.5 | 8.5 | 8.5 |
| *p*-Chloro- | 260–261 | $C_8H_8ClNO_2$ | 51.8 | 51.5 | 4.4 | 4.4 | 7.6 | 7.7 | 7.6 | 7.5 |
| *m*-Chloro- | 253–254 | $C_8H_8ClNO_2$ | 51.8 | 51.6 | 4.4 | 4.4 | 7.6 | 7.8 | 7.6 | 7.5 |
| *p*-Methoxy- | 264–265 | $C_9H_{11}NO_3$ | 59.7 | 59.4 | 6.1 | 6.2 | 7.7 | 7.9 | 7.7 | 7.7 |
| *m*-Methoxy-[f] | 220–222 | $C_9H_{11}NO_3$ | 59.7 | 59.8 | 6.1 | 6.3 | 7.7 | 8.0 | 7.7 | 7.4 |
| *p*-Fluoro-[f] | 270–271[g] | $C_8H_8FNO_2$ | 56.8 | 56.7 | 4.8 | 5.0 | 8.3 | 8.0 | 8.3 | 8.0 |
| *m*-Fluoro-[f] | 253 | $C_8H_8FNO_2$ | 56.8 | 57.0 | 4.8 | 5.0 | 8.3 | 8.3 | 8.3 | 8.0 |
| *p*-Hydroxy- | 229–230 | $C_8H_9NO_3$ | 57.5 | 57.2 | 5.4 | 5.5 | 8.4 | 8.4 | 8.4 | 8.3 |
| *m*-Hydroxy-[f] | 221–222 | $C_8H_9NO_3$ | 57.5 | 57.3 | 5.4 | 5.5 | 8.4 | 8.5 | 8.4 | 8.5 |
| *p*-Amino- hydrochloride | *h* | $C_8H_{11}ClN_2O_2$ | 47.4 | 47.2 | 5.5 | 5.5 | 13.8 | 13.2 | 6.9 | 7.1 |
| *m*-Amino- | 220–221 | $C_8H_{10}N_2O_2$ | 57.8 | 57.5 | 6.1 | 6.4 | 16.9 | 16.8 | 8.4 | 8.2 |
| *m*-Nitro- | 172–173 | $C_8H_8N_2O_4$ | 49.0 | 48.6 | 4.2 | 4.3 | 14.3 | 14.1 | 7.1 | 7.2 |
| *p*-Dimethylamino-[f] | 231–232 | $C_{10}H_{14}N_2O_2$ | 61.8 | 61.5 | 7.3 | 7.4 | 14.4 | 14.2 | 6.1 | 5.9[i] |
| *p*-Methylmercapto-[f] | 230–231 | $C_9H_{11}NO_2S$ | 54.8 | 55.0 | 5.6 | 5.7 | 7.1 | 7.4 | 7.1 | 7.0 |
| *p*-Carboxy-[f] | 226–228 | $C_9H_9NO_4$ | 55.4 | 55.4 | 4.7 | 4.8 | 7.2 | 6.9 | 7.2 | 6.7 |
| *p*-Trimethylammonium- chloride hydrochloride[f] | 220–222 | $C_{11}H_{18}Cl_2N_2O_2$ | 47.0 | 46.8 | 6.5 | 6.6 | 10.0 | 10.3 | 5.0 | 5.0 |
| 3,4-Dimethoxy- | 240–242 | $C_{10}H_{13}NO_4$ | 56.9 | 56.9 | 6.2 | 6.3 | 6.6 | 6.5 | 6.6 | 6.6 |
| *p*-Dimethylamino-DL-phenylalanine | 275 | $C_{11}H_{16}N_2O_2$ | 63.4 | 63.5 | 7.7 | 7.7 | 13.4 | 13.6 | 6.7 | 6.8 |
| *m*-Amino-DL-phenylalanine hydrochloride[f] | 237–238 | $C_9H_{13}ClN_2O_2$ | 49.9 | 50.3 | 6.0 | 6.4 | 13.0 | 13.2 | 5.5 | 4.9 |
| *p*-Trimethylammonium-DL-phenylalanine chloride hydrochloride[f] | 244 | $C_{12}H_{20}Cl_2N_2O_2$ | 48.8 | 48.6 | 6.8 | 7.0 | 9.5 | 9.5 | 4.8 | 4.6 |

[a] Decomposition points were determined with a Thomas–Hoover capillary melting point apparatus and are uncorrected. Samples were introduced to the oil bath which had been preheated to 20° below the decomposition point and heated at the rate of 2°/min. Data for compounds described previously are cited for informational purposes. [b] Analyses performed by Mr. J. Walter, Dept. of Chemistry, Johns Hopkins University. [c] Analyses were performed in these laboratories by application of the quantitative ninhydrin assay (Moore and Stein, 1954). [d] α-Amino nitrogen was determined also on the following compounds: D-phenylalanine, Calcd 8.5, Found 8.5; D-tyrosine, Calcd 7.7, Found 7.7; *m*-fluoro-DL-phenylalanine, Calcd 7.7, Found 7.9; *p*-fluoro-DL-phenylalanine, Calcd 7.7, Found 7.6; *p*-chloro-DL-phenylalanine, Calcd 7.2, Found 6.9; *p*-nitro-DL-phenylalanine, Calcd 6.7, Found 6.5. [e] Sample sublimed. [f] To the best of our knowledge this compound has not been described earlier. [g] Sample started to sublime at 260°. [h] Decomposition point indefinite. [i] Analysis performed on the hydrochloride salt.

pared by Dr. John Wein in this laboratory, starting with DL-*N*-phthaloyl-4-dimethylaminophenylalanine ethyl ester (Bergel and Stock, 1959). *p*-Dimethylaminophenylalanine was prepared by the conventional erlenmeyer synthesis with *p*-dimethylaminobenzaldehyde as an initial reactant. For the preparation of DL-*m*-aminophenylalanine, *m*-nitrobenzaldehyde was an initial reactant in a similar reaction series, the intermediate α-benzoylamino-*m*-nitrocinnamic acid (Na salt) being reduced catalytically (Adams Pt) to produce the cor-

TABLE II: Maximal Rates for the *C*-Phenylglycine Series as Substrates of Hog Kidney D-Amino Acid Oxidase.[a]

| *C*-Phenylglycine Substituent | $\sigma$[b] | $V_M$ (μmoles of substrate oxidized/min per mg of protein) 38° Air | 38° Oxygen | 25° Air | 25° Oxygen |
|---|---|---|---|---|---|
| *p*-$N(CH_3)_2$ | −0.83 | 0.0003 | — | — | — |
| *p*-$NH_2$ | −0.66 | 0.0016 | — | — | — |
| *p*-OH | −0.37 | 0.015 | 0.020 | — | — |
| *p*-$OCH_3$ | −0.27 | 0.092 | 0.11 | — | — |
| *p*-$CH_3$ | −0.17 | 0.25 | 0.30 | 0.14 | 0.14 |
| *m*-$NH_2$ | −0.16 | 3.2 | 6.0 | 2.2 | 2.3 |
| *m*-$CH_3$ | −0.07 | 3.5 | 6.8 | 1.9 | 2.6 |
| H | 0.00 | 7.8 | 20.0 | 5.4 | 11.3 |
| *p*-F | 0.06 | 6.7 | 13.0 | 3.7 | 6.4 |
| *m*-OH | 0.12 | 5.0 | 6.0 | 1.6 | 2.1 |
| *m*-$OCH_3$ | 0.12 | 1.9 | 2.7 | 0.50 | 0.52 |
| *p*-Cl | 0.23 | 4.1 | 7.5 | 2.6 | 5.5 |
| *m*-F | 0.34 | 7.2 | 14.0 | 5.1 | 9.6 |
| *m*-Cl | 0.37 | 5.9 | 12.2 | 3.5 | 6.2 |
| *m*-$NO_2$ | 0.71 | 3.2 | 4.3 | 1.9 | 3.2 |
| *p*-$COO^-$ | 0.00 | 0.002 | — | — | — |
| *p*-$N^+(CH_3)_3$ | 0.82 | <0.0001 | — | — | — |

[a] Reaction rates were determined by assay of the product phenylglyoxylic acids (see Experimental Section). $V_M$ was estimated graphically and each value represents the average obtained from at least three independent experiments (38°, air) or two independent experiments (other conditions). The initial concentration of each substrate varied from $3 \times 10^{-4}$ to $3 \times 10^{-3}$ M; a greater concentration range was used where assay sensitivity and substrate solubility permitted. Determinations were conducted in 89 mM sodium pyrophosphate buffer, pH 8.5. A racemic mixture of each substrate was added; calculations were based on the concentration of the D isomer. The precision of the spectrophotometric assay was studied by statistical evaluation of $V_M$ obtained from least-square analyses of the data of 20 independent experiments with *C*-phenylglycine as substrate at 38° in air; standard error for $V_M$ is 0.046. [b] From McDaniel and Brown (1958).

responding amino derivative, antecedent to subsequent reduction of the latter compound with sodium amalgam to α-*N*-benzoyl-*m*-aminophenylalanine. The identity and purity of all synthetic analogs of phenylalanine were established by their chemical and physical properties and by analysis (Table I).

D-Tyrosine was the valued gift of the late Dr. J. P. Greenstein. DL-*m*-Fluorophenylalanine, DL-*p*-fluorophenylalanine, DL-*p*-nitrophenylalanine, D-phenylalanine, and DL-*p*-chlorophenylalanine were commercial products found to be of suitable purity. Sources of other reagents were: flavin–adenine dinucleotide (Grade III) and D-alanine, Sigma; sodium pyruvate, Schwarz BioResearch; sodium phenylpyruvate, Calbiochem. These and other commercially available reagents were suitably purified and characterized before use.

*Assays of Enzymatic Activity.* The rate of the enzyme-catalyzed process was evaluated by following the rate of oxygen consumption manometrically and *routinely* by the more precise spectrophotometric procedure for accumulation of α-keto acid oxidation product. Final concentrations are given within parentheses. The enzymatic reaction was initiated by addition of 0.3 ml of a mixture of D-amino acid oxidase, to be designated, and FAD (10 μM) in sodium pyrophosphate buffer (89 mM), pH 8.5, to 2.2 ml of a mixture of buffer, substrate, and catalase (0.065 μM), pH 8.5. The temperature, gas phase, and initial substrate concentration are specified in the tables. For determination of the specific activities of the hog and sheep kidney oxidases, the temperature was 38°, the gas phase, air, and the D-alanine concentration, 25 mM. Sufficient enzyme was used with the different amino acids to act as catalyst for the oxidation of 0.003–1.0 μmole of substrate/min; the reaction rate was essentially a linear function of protein concentration over the range of enzyme required. Manometric assays were performed with the standard Warburg apparatus, the enzyme and cofactor being added from the side arm after temperature equilibration.

*Spectrophotometric Assay.* In the spectrophotometric assay, the timed addition of enzyme and cofactor was delayed until other components of the reaction mixture had reached bath temperature in a Dubnoff metabolic

apparatus; the enzymatic reaction was stopped 20 min later by introduction of 0.5 ml of 30% (w/v) trichloroacetic acid. Product α-keto acid anions were estimated as the respective 2,4-dinitrophenylhydrazone derivatives according to a modification of the procedure of Friedemann and Haugen (1943). After removal of precipitated protein by centrifugation, variable aliquots of the reaction mixture were brought to a total volume of 1.0 ml with water; the solution was allowed to react with 0.2 ml of 1 M HCl saturated with 2,4-dinitrophenylhydrazine for at least 15 min at room temperature before addition of 3.0 ml of 2 M NaOH; absorbancy at 525 mμ was evaluated no later than 30 min after introduction of base. Inasmuch as the molar absorptivities of the 2,4-dinitrophenylhydrazones derived from pyruvic, phenylpyruvic, *p*-chlorophenylglyoxylic, *p*-fluorophenylglyoxylic, and *p*-dimethylaminophenylglyoxylic acids exhibited only minor differences at 525 mμ, enzymatic assay data were compared to a sodium pyruvate standard curve. Comparative results of the manometric and spectrophotometric assays agreed consistently within 5% for those substrates for which the less sensitive manometric assay had proved applicable.

*Protein Determination.* Both a modified biuret procedure and the Folin-biuret method (Lowry *et al.*, 1951) were used; each was calibrated to the dry weight of D-amino acid oxidase which had been dialyzed for 48 hr at 0° against 8 l. of glass-distilled water.

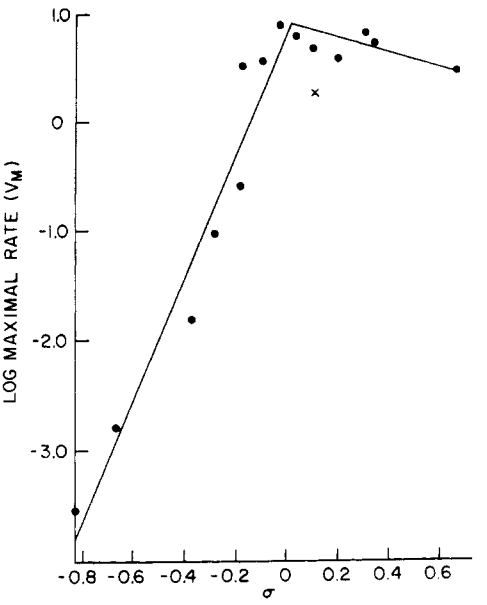


FIGURE 1: Logarithm of the maximal rate ($V_M$) for the substituted *C*-phenylglycines as substrates of hog kidney D-amino acid oxidase, plotted against σ (see footnote *b* of Table II). The respective maximal rates, taken from Table II, refer to micromoles of substrate oxidized per minute per milligram of protein at 38° in air. x refers to *m*-methoxy-*C*-phenylglycine (see text).

## Results

*σ–ρ Relationship and Maximal Rates, $V_M$, for the C-Phenylglycine Series.* The maximal rates of oxidative deamination observed with the *C*-phenylglycine series as substrates of hog kidney D-amino acid oxidase are tabulated in Table II. Logarithms of the maximal rates obtained at 38° in air are plotted against σ in Figure 1.[5] The magnitude of σ is an experimentally derived measure (McDaniel and Brown, 1958) of the electron-attracting or electron-donating properties pertaining to individual substituent groupings. "Hydrogen" (*i.e.*, absence of substituent) has arbitrarily been assigned a σ value of 0.00; by convention, the greater the electron-attracting property of a substituent, the more positive is the designation of σ. Although a racemic mixture of each amino acid was used experimentally, calculations are based on the concentration of the D isomer only. Evidence supporting the assumption that L-amino acids are enzymatically inactive in this system will be presented below. In this regard, it is interesting to note that glycine, itself, is oxidized to glyoxylic acid in the presence of D-amino acid oxidase (Neims and Hellerman, 1962).

Here, with the *C*-phenylglycine series and D-amino acid oxidase, the log maximal rate–σ correlation is biphasic and exhibits a maximum near the σ value of 0.04. It is apparent from the results recorded in Table II and Figure 1 that as the electron-donating character of a substituent grouping increases (σ <0) relative to hydrogen as "substituent," the maximal rate for enzyme-catalyzed oxidation of substrate decreases markedly. For example, the maximal rate observed with *p*-dimethylamino-*C*-phenylglycine is four to five orders of magnitude less than the rate obtained with *C*-phenylglycine. The eight members of the *C*-phenylglycine series that possess substituents with σ constants between −0.83 and 0.00 were grouped as an independent reaction series; the data were evaluated according to the procedure advocated by Jaffe (1953). The marked sensitivity of the rate of the catalytic process to "electron-donating" substituents is reflected in a calculated ρ value of 5.44, with a standard deviation, $s_\rho$, of 0.54. The correlation coefficient, *r*, is 0.97.

Electron-*attracting* groupings also decrease the rate of substituted *C*-phenylglycine oxidation, but their effect is much less pronounced. The log maximal rate (38°, air)–σ correlation for members of the *C*-phenylglycine series with substituent σ constants between 0.00 and 0.71 is characterized by a ρ value of −0.40 ($s_\rho$ = 0.20, *r* = 0.69).

The two members of this series with substituents that bear a formal charge, *p*-carboxy-*C*-phenylglycine and

[5] Inasmuch as the Michaelis constants derived for the various substrates involved in the current investigation differ by less than one order of magnitude (Table IV), plots of log maximal rate or of log relative rate (at equimolar substrate concentration) with respect to σ are similar. It follows from usual enzyme kinetics that maximal rates are proportional to constants applicable to the rate-limiting step(s) of the catalytic process subsequent to the initial interaction of enzyme and substrate.

TABLE III: Maximal Rates for the Phenylalanine Series as Substrates of Hog Kidney D-Amino Acid Oxidase.[a]

| Phenylalanine Substituent | $\sigma$ | $V_M$ ($\mu$moles substrate oxidized/min per mg protein) |
|---|---|---|
| $p$-$N(CH_3)_2$ | −0.83 | 4.3 |
| $p$-OH | −0.37 | 12.8 |
| $m$-$NH_2$ | −0.16 | 10.4 |
| H | 0.00 | 18.4 |
| $p$-F | 0.06 | 20.2 |
| $p$-Cl | 0.23 | 22.7 |
| $m$-F | 0.34 | 12.5 |
| $p$-$NO_2$ | 0.78 | 6.0 |
| $p$-$N^+(CH_3)_3$ | 0.82 | 2.6 |

[a] See footnotes *a* and *b* of Table II. The experiments were conducted at 38° in air. Each value represents the average from at least two independent experiments.

the $p$-trimethylammonium-substituted derivative, $(CH_3)_3N^+C_6H_4CH(NH_2)COO^-$, exhibited minimal substrate activity. These results are consistent with earlier observations of the small degree of activity of such substrates as D-glutamic acid, D-aspartic acid, and D-lysine (Krebs, 1935). Data pertaining to the aforementioned amino acids are omitted from Figure 1, inasmuch as in either case there could be involved direct electrostatic interaction between the substituent group and enzyme.

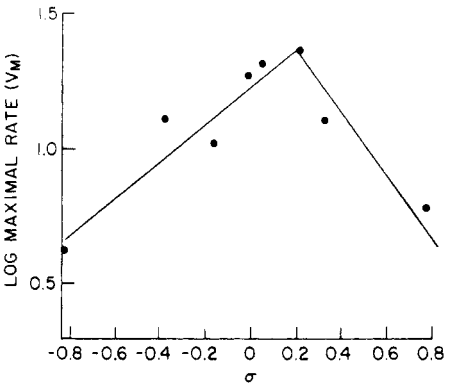


FIGURE 2: Logarithm of the maximal rate ($V_M$) for the substituted phenylalanines as substrates of hog kidney D-amino acid oxidase, plotted against $\sigma$ (see footnote *b* of Table II). The respective maximal rates, taken from Table III, refer to micromoles of substrate oxidized per minute per milligram of protein at 38° in air.

*m*-Methoxy-*C*-phenylglycine, depicted as x in Figure 1, presents an interesting variation. The rate of oxidation of this amino acid is unusually sensitive to reaction temperature (Table II), and its Michaelis constant is anomalously high (Table IV). It may be noted that the methoxy grouping projects farther, perpendicularly, from the plane of the aromatic ring, than any of the other *meta* substituents investigated. Steric interference by this grouping in the *meta* position with respect to the interaction of enzyme and substrate seems probable [compare hydroxyproline (Wellner and Scannone, 1964)]. The opposing electronic effects of methoxy substituents in the *meta* and *para* positions prompted an investigation of *m,p*-dimethoxy-*C*-phenylglycine as a substrate of D-amino acid oxidase, but, as with the *m*-methoxy derivative, a low rate of oxidation, an elevated sensitivity to temperature variation, and a high Michaelis constant were observed.[6]

*Temperature and Oxygen Concentration as Variables in the Oxidation of Substituted C-Phenylglycines.* Inasmuch as changes in the rate-limiting step and (or) mechanism of a reaction can evoke irregularities (*i.e.*, biphasic character) in a Hammett correlation (Leffler and Grunwald, 1963), the plot of log maximal rate for enzymatic oxidation of the substituted *C*-phenylglycines with respect to $\sigma$ was studied as a function of certain experimental variables. As seen in Table II, the biphasic relationship persisted notwithstanding changes in reaction temperature and oxygen concentration. The variations in experimental conditions were not found to affect significantly either the positive or the negative $\rho$ value. The rate stimulation evoked by increasing the partial pressure of oxygen seems to be a "function" of the reaction rate observed in air; the more rapid the enzymatic oxidation proceeds in air, the greater is the rate acceleration consequent with the replacement of air by oxygen. Observations with a few substrates indicate that the results presented in Figure 1 do not vary materially within a narrow range of pH.

*$\sigma$–$\rho$ Relationship and Maximal Rates, $V_M$, for the Phenylalanine Series.* The maximal rates for the phenylalanine series as substrates of hog kidney D-amino acid oxidase at 38° in air are tabulated in Table III. The substituted phenylalanines are generally efficient substrates for the oxidase. It is noteworthy that the maximal rates observed with phenylalanine and *C*-phenylglycine at 38° in air are 17.8 and 7.8 $\mu$moles of substrate oxidized/min per mg of protein, respectively. In Figure 2, logarithms of the maximal rates tabulated in Table III are plotted with respect to $\rho$. Again a biphasic plot is obtained, but the maximum occurs at a $\sigma$ value of 0.23, corresponding to *p*-chlorophenylalanine.

[6] The maximal rate of oxidation of *p*-methylmercapto-DL-*C*-phenylglycine was of lesser magnitude than predicted by $\sigma$–$\rho$ correlation (McDaniel and Brown, 1958). Studies in our laboratory indicate also that *p*-methylmercaptobenzoate ion is a poor substrate-competitive inhibitor of the oxidase. It is noteworthy that anomalies with respect to $\sigma$–$\rho$ correlations with sulfur-containing substituents have been observed in nonenzymatic reactions (Leffler and Grunwald, 1963).

TABLE IV: Michaelis Constants for the *C*-Phenylglycine and Phenylalanine Series as Substrates of Hog Kidney D-Amino Acid Oxidase.[a]

| Substituent | $K_m \times 10^3$ | |
|---|---|---|
| | *C*-Phenylglycine | Phenylalanine |
| *p*-$N(CH_3)_2$ | 10 | 8 |
| *p*-$NH_2$ | 8 | — |
| *p*-OH | 6 | 2 |
| *p*-$OCH_3$ | 8 | — |
| *p*-$CH_3$ | 4 | — |
| *m*-$NH_2$ | 8 | 4 |
| *m*-$CH_3$ | 3 | — |
| H | 2 | 3 |
| *p*-F | 3 | 2 |
| *m*-OH | 9 | — |
| *m*-$OCH_3$ | 18 | — |
| *p*-Cl | 3 | 3 |
| *m*-F | 2 | 2 |
| *m*-Cl | 2 | — |
| *m*-$NO_2$ | 6 | — |
| *p*-$NO_2$ | — | 2 |
| *p*-$N^+(CH_3)_3$ | — | 7 |

[a] See footnotes *a* and *b* of Table II. These experiments were conducted at 38° in air. $K_m$ was estimated graphically and each value represents the average from at least three independent experiments.

TABLE V: Relative Rates ($v$) of Oxidation of the *C*-Phenylglycine Series as Substrates of Sheep Kidney D-Amino Acid Oxidase.[a]

| *C*-Phenylglycine Substituent | $\sigma$[b] | $v$ (μmoles of substrate oxidized/min per mg of protein) | |
|---|---|---|---|
| | | Manometric Assay | Spectrophotometric Assay |
| *p*-$N(CH_3)_2$ | −0.83 | <0.1 | 0.002 |
| *p*-OH | −0.37 | <0.1 | 0.035 |
| *p*-$OCH_3$ | −0.27 | <0.1 | 0.039 |
| *m*-$NH_2$ | −0.16 | 0.43 | 0.45 |
| H | 0.00 | 1.4 | 1.5 |
| *p*-Cl | 0.23 | 0.78 | 0.82 |
| *m*-$NO_2$ | 0.71 | 0.45 | 0.49 |
| *p*-$COO^-$ | 0.00 | <0.1 | <0.001 |
| *p*-$N^+(CH_3)_3$ | 0.82 | <0.1 | <0.001 |

[a] Relative rates were assayed manometrically and spectrophotometrically (see Experimental Section) at 37° in air. The initial concentration of all racemic amino acids was $5 \times 10^{-2}$ M where solubility permitted; an equivalent suspension was otherwise used. [b] See footnote *b* of Table II.

With the phenylalanine substrates, the sensitivity of the catalytic process with respect to electron-donating substituents necessarily is decreased substantially, owing to interposition of an insulating methylene group (Jaffe, 1953). The positive $\rho$ values of 5.44 and 0.73 ($s_\rho = 0.04$, $r = 0.98$) obtained under identical reaction conditions for the *C*-phenylglycine series and phenylalanine series, respectively, may be compared with $\rho$ values of 2.4 and 0.8 reported for the base-catalyzed hydrolysis of ethyl benzoates and ethyl phenylacetates, respectively (Jaffe, 1953).

Without inclusion of data for the *p*-trimethylammonium-substituted analog of phenylalanine, the somewhat unfavorable effect of electron-attracting substituents in a few members of the phenylalanine series is reflected by the $\rho$ value, −0.93. Although the *p*-trimethylammonium-substituted derivative of *C*-phenylglycine is inactive as a substrate, the maximal rate of oxidation of the phenylalanine analog is displaced only slightly from the predicted rate of enzymatic oxidation (Table III).

*σ–ρ Relationship and the Michaelis Constants, $K_m$, in the C-Phenylglycine and Phenylalanine Series.* The Michaelis constants for the *C*-phenylglycine and phenylalanine series as substrates of hog kidney D-amino acid oxidase at 38° in air are tabulated in Table IV. It can be seen that the Michaelis constants for the two substrate series do not differ significantly. But little relationship is observed between $K_m$ and $\sigma$; the over-all variation of $K_m$ for all substrates is less than one order of magnitude.

*σ–ρ Relationship and the Relative Rates of Oxidation of Substituted C-Phenylglycines in the Presence of Sheep Kidney D-Amino Acid Oxidase.* Although maximal rates were not evaluated, the relative rates at equimolar concentrations of sample members of the *C*-phenylglycine series have been studied as substrates of sheep kidney D-amino acid oxidase at 37° in air (Table V). The marked sensitivity of reaction rate with respect to electron-donating substituents, and, in addition, the biphasic character of the Hammett plot obtained with the hog kidney oxidase again are apparent with this enzyme. With electron-donating substituents, a $\rho$ value of 3.45 is obtained ($s_\rho = 0.57$, $r = 0.95$) while, with electron-attracting substituents, the $\rho$ value is −0.65. The poor substrate efficiency for *p*-carboxy-*C*-phenylglycine and the *p*-trimethylammonium derivative again is observed with the sheep enzyme.

*Determination of $V_M$ and $K_m$.* Certain factors that pertain to the experimental derivation of maximal rates, relative rates, and Michaelis constants require elaboration. Although a racemic mixture of each amino acid was used experimentally, rate calculations were based on the concentration of the substrate D isomer only. The assumption of substrate inactivity with respect to the L isomers of the various phenylglycines and

TABLE VI: $pK_{a,NH_3^+}$ for Substituted *C*-Phenylglycines and Phenylalanines.[a]

| Substituent | $pK_{a,NH_3^+}$ | |
|---|---|---|
| | *C*-Phenylglycine | Phenylalanine |
| *p*-$N(CH_3)_2$ | 9.22 | 9.20 |
| *p*-$OCH_3$ | 9.06 | 9.16 |
| H | 8.97 | 9.07 |

[a] Temperature, 30°; Γ/2, 0.005. Measurements were taken with a sensitive McGinnis-type glass electrode assembly by Miss Pauline Ramsdell who assisted also with earlier synthetic and analytical work.

phenylalanines in the presence of hog kidney D-amino acid oxidase is substantiated by the following observations. Inasmuch as the oxidation of substrate proceeds essentially irreversibly in an aerobic environment, the enzymatic oxidation of racemic mixtures of tyrosine, phenylalanine, and sample *C*-phenylglycines (DL-α-aminophenylacetic acid and *p*-methyl, *p*-chloro, and *p*-fluoro analogs) in each instance was allowed to proceed to depletion of substrate. In the presence of D-amino acid oxidase and of catalase, only 0.45–0.50 of each of the original racemic mixtures, presumably the D isomer, could be recovered as the corresponding α-keto acid. In addition, L-tyrosine and L-phenylalanine did not exhibit substrate activity when assayed under the experimental conditions described in Table II.

The possible complications of substrate inhibition, L-amino acid inhibition, and product inhibition were also evaluated. L-Tyrosine, L-phenylalanine, racemic mixtures of sample *C*-phenylglycines including *p*-hydroxy, *p*-methyl, *p*-chloro, *m*-fluoro, and *m*-nitro analogs, phenylglyoxylate ion, and *p*-dimethylaminophenylglyoxylate ion were examined individually for possible inhibitory action in the enzymatic oxidation of D-alanine. The latter substrate is oxidized at least fourfold more rapidly than any of the *C*-phenylglycines. No significant inhibition was observed when the initial concentrations of D-alanine and potential inhibitor were 2.5 and 3.0 mM, respectively. In addition, when D-alanine was added to reaction vessels 30 min after D-amino acid oxidase had been admixed with sample *C*-phenylglycines under catalytically functional conditions, no inhibition of the enzymatic oxidation of the former substrate was observed. Any weak inhibitory action of the substituted phenylpyruvates did not interfere with determination of $V_M$ and $K_m$ for the substituted phenylalanines. Essentially linear double-reciprocal plots were observed with every substrate.

*Acid Dissociation Constants, $K_{a,NH_3^+}$, for Substituted C-Phenylglycines and Phenylalanines.* For comparative purposes, $K_{a,NH_3^+}$ for a few substituted *C*-phenylglycines and phenylalanines was evaluated with the use of a sensitive McGinnis-type glass electrode assembly. The respective p*K* values are reported in Table VI. Approximate ρ values of 0.30 and 0.14 were derived from correlation of the log of the acid dissociation constants with respect to σ for the *C*-phenylglycine and phenylalanine series, respectively. It seems unlikely that the degree of α-ammonium ionization has any significant bearing upon the Hammett correlations presented above.

## Discussion

When the logarithm of the maximal rate derived for each substituted *C*-phenylglycine as substrate for crystallized hog kidney D-amino acid oxidase is plotted against σ, a biphasic graph is obtained. Qualitatively similar results are obtained when the various phenylalanines are used as substrates. A Hammett plot with biphasic character can be the result of a change in either the rate-limiting step or the mechanism of a reaction (Leffler and Grunwald, 1963). In the current investigation, the two possibilities, rate-limiting step or mechanism alteration, seem indistinguishable on the basis of available data; further discussion will be limited to considerations based on the assumption that different steps in the over-all catalytic process are rate limiting for substrates included within the two limbs of Hammett correlations.

Certain aspects of the results reported here support the assumptions necessary for application of the σ–ρ relationship. All linear segments of the two Hammett plots display satisfactory correlation between log rate and σ; such conformity of data emphasizes the importance of intramolecular substituent electronic effects and would not be anticipated on the basis of steric differences among the substituents. A difference between the peak positions in the two Hammett plots also is consistent with the importance of substituent electronic effects.

The marked sensitivity of the rate of the catalytic process to electron-donating substituents ($0.83 \leqq \sigma \leqq 0.00$), specifically in the *C*-phenylglycine series (ρ value, 5.44), is noteworthy. Reactions that exhibit comparably high sensitivity in aqueous media to substituent variation have been observed only rarely for nonenzymatic systems, and hitherto not at all for interactions involving catalysis by enzymes. It must be noted, however, that as the dielectric constant of solvent decreases, the ρ value applicable to certain organic reactions usually increases (Jaffe, 1953). Inasmuch as the state of solvation of the substrate molecule, while interacting with the enzyme, is unknown, the contribution of such solvent effects to the ρ value cannot be evaluated. Notwithstanding these considerations, it is reasonable to emphasize a ρ factor of such magnitude and to postulate, therefore, the likelihood of development of a significant partial negative charge associated with the substrate's α-carbon atom during the course of enzymatic oxidation.

*Hypothesis.* This conclusion and the results of a concurrent study in this laboratory concerned with the isolation of a stabilized derivative of a D-amino acid oxidase–substrate compound through the reductive

action of sodium borohydride (Coffey *et al.*, 1965) have permitted development of a working hypothesis applicable to the catalytic process. The proposal is presented schematically, with D-alanine as substrate, in Figure 3. It is suggested that initially (Figure 3, part A), in the presence of the flavoenzyme, nucleophilic attack by the substrate's $\alpha$-amino nitrogen atom upon an isoalloxazine position (with potential carbonyl character,[7] *e.g.*, position 2) results in the formation of a labile intermediate related formally to a carbinolamine or azomethine. Appropriate orientation of substrate is ensured by a protein cationic site (d) and strongly nucleophilic groupings at (c); it is of interest that such "three-point" specification could account for the stereospecificity of the enzyme–substrate interaction.

Concomitant with activation of the intermediate, presumably through a process of proton abstraction involving the interaction of particular protein nucleophiles and the substrate's $\alpha$-hydrogen, an internal oxidation–reduction involving probably sequential one-electron transfers (*cf.* Massey and Gibson, 1964) would ensue (Figure 3, part B). The participation of molecular oxygen would ensure fluid restoration of the electron acceptor moiety (EnFAD). The ultimate products of this enzymatic oxidation, namely, $\alpha$-keto acid and ammonia, would be obtained upon "hydrolysis" of the oxidized intermediate.[8]

The conspicuous effect of electron-donating substituents in the *C*-phenylglycine series is consistent with the assumption of a process of substrate activation involving an attractive force upon the substrate's $\alpha$-hydrogen referable to protein nucleophiles, *e.g.*, particular sulfhydryl anions (Hellerman *et al.*, 1965). If, in the case of *C*-phenylglycines suitably substituted with electron-donating groupings, the step involving proton abstraction became rate limiting, a large positive $\rho$ would be anticipated; the development of a significant partial negative charge about the substrate's $\alpha$-carbon atom would be embodied in the transition state for such a reaction step. The $\rho$ values of 5.44 for the hog enzyme (maximal rates) and 3.45 for the sheep enzyme (relative rates) observed here in the oxidation of substituted *C*-phenylglycines may be compared with a $\rho$ value of 3.4 derived for the base-promoted carbonyl-elimination reaction in which various benzyl nitrates are converted to aldehydes and nitrate ion (Baker and Heggs, 1955). For this nonenzymatic oxidation–reduction reaction, Buncel and Bourns (1960) have indeed reported evidence for activation by proton abstraction.

On the other hand, it seems probable that, in contrast to those *C*-phenylglycines with electron-donating substituents, the more active members of the series (those with electron-attracting substituents) resemble more closely the more usual, efficient substrates (D-alanine, D-methionine, and D-proline) of D-amino acid oxidase if the rate-limiting steps of the over-all enzymatic oxidations are considered. On the basis of inhibitory studies (Frisell *et al.*, 1956) and kinetic studies (Massey and Gibson, 1964), it has been suggested that the rate-limiting step with such effective substrates might involve dissociation of an enzyme–product intermediate. But the details of such a dissociation are as yet unknown and in any case the slight detrimental effect of electron-attracting substituents is difficult to interpret.

Earlier chemical studies dealing with oxidation of $\alpha$-amino acids have some relevance bearing on the foregoing considerations. With ninhydrin (triketohydrindene hydrate) (Van Slyke *et al.*, 1941) as electron acceptor, oxidation seems to involve, initially, nucleophilic attack by the amino acid's $\alpha$-amino grouping upon an activated carbonyl group, with resultant development of an azomethine-type linkage. Furthermore, with hypochlorite (Langheld, 1909) or chloramine-T (Dakin, 1917) as oxidizing reagent in slightly alkaline solution, presumably the *N*-chloramino acid is formed initially. In general, after an initial step involving combination with a suitable electron acceptor entity, internal oxidation–reduction of the complex follows with transfer of electrons through nitrogen to the initially affixed acceptor. Although analogy with Figure 3 is

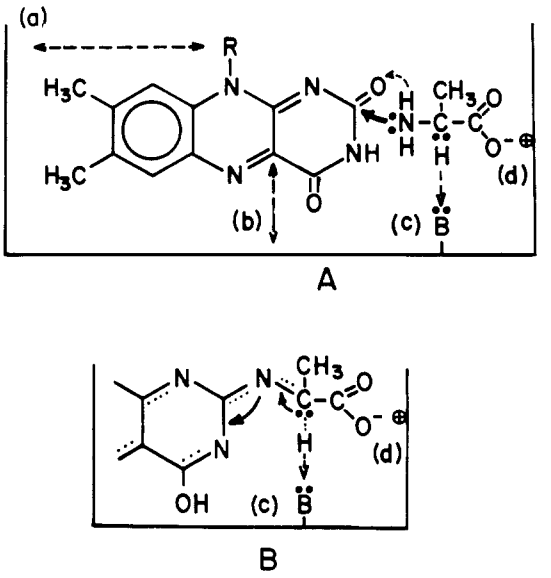


FIGURE 3: Working hypothesis for the interaction between D-alanine and D-amino acid oxidase. One or more linkages between the flavin component and the enzyme–protein are depicted as dashed lines between flavin and (a) and (b). D-Alanine is used as a model substrate. See text for further details. (Figure appeared as Scheme 1 in paper II, Coffey *et al.* (1965); reproduced by permission.)

[7] Compare the discussion of the isoalloxazine moiety by Beinert (1960).

[8] The detailed mechanism of this hydrolysis has received attention in this laboratory and will be treated in a forthcoming paper (paper V of series). It appears that the removal of products involves in part the participation of the $\epsilon$-amino group of a particular lysine residue of the enzyme–protein in an aminolytic step (*cf.* Coffey *et al.*, 1965).

interesting, the products of nonenzymatic oxidation include aldehyde, carbon dioxide, and ammonia, rather than α-keto acid and ammonia, the products of oxidase catalysis. As suggested in Figure 3, enzymatically induced abstraction of the substrate's α-hydrogen nucleus, in combination with electrostatic interaction between the substrate's carboxyl group and a protein cation, contributes to an oxidative pathway permitting formation of keto acid rather than aldehyde.

*Additional Considerations.* Finally, it may be noted that organic reactions (see for example, Santerre *et al.*, 1958; Noyce *et al.*, 1958) involving broadly azomethine-type intermediates exhibit biphasic Hammett correlations, but with positive $\rho$ values of lesser magnitude than we have observed in enzymatic studies. Our working hypothesis for amino acid oxidase does include *formally* analogous steps. Sequences initiating carbinolamine formation and subsequent dehydration have been implicated differentially as responsible for the biphasic character of the Hammett plots concerned in the nonenzymatic studies (compare Jencks, 1964); certain difficulties with the generality of this interpretation have been discussed by Crowell *et al.* (1964).

Additional interest derives from a consideration of the argument developed in a paper by Deitrich *et al.* (1962) dealing with the substrate specificity of diphosphopyridine nucleotide (DPN)-linked aldehyde dehydrogenase in relation to a biphasic Hammett plot observed in their investigation. Here the results suggested a process involving nucleophilic attack by the enzyme upon a substrate's aldehydic carbon atom followed by or accompanied with hydride abstraction at the hand of DPN. A mechanism somewhat analogous to this might be visualized in the case of D-amino acid oxidase. Experiments, now in progress, designed to compare the effects of isotopic substitution of the substrate's α-hydrogen atom among *C*-phenylglycines representative of each linear segment of the biphasic Hammett plot, should permit some additional clarification concerning the details of mechanism.

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# Hydrodynamic and Optical Rotatory Dispersion Studies on Wheat Germ Soluble Ribonucleic Acid*

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ABSTRACT: Wheat germ soluble ribonucleic acid (s-RNA) prepared by the method of D. G. Glitz and C. A. Dekker [(1963) *Biochemistry 2*, 1185] has been subjected to a detailed physicochemical investigation using such techniques as sedimentation velocity, Archibald ultracentrifugation, viscometry, diffusion, and optical rotatory dispersion. The intrinsic sedimentation constant, determined by both schlieren and ultraviolet optics, is 3.98 S, and the $D^0_{20,w}$ value, evaluated from schlieren data by three methods of analysis, averages $6.48 \times 10^{-7}$ cm²/sec. An independent estimate of $D^0_{20,w}$ of 6.42 Fick units was obtained through the use of the Möller method [Möller, W. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 501], which utilizes ultraviolet optics. The molecular weight of the system was estimated from the schlieren sedimentation diffusion data and the use of the Svedberg equation as 31,800 ± 1000. This value compares with Archibald approach to equilibrium estimates of molecular weight ($M$) of 28,200 ± 800, and end-group estimates on the same preparation of ~28,000 based on 80 nucleotides in the chain. The homogeneity of the preparation is evidenced by the symmetries of both the sedimentation and diffusion schlieren patterns, the excellent correspondence of $D^0_{20,w}$ as determined by several methods of analysis, and the good agreement between the number- and weight-average molecular weights. The weight intrinsic viscosity of wheat germ s-RNA was found to be 0.071 dl/g, a value independent of shear rate. Application of the Scheraga–Mandelkern equation to the hydrodynamic data resulted in a $\beta$-shape factor of $2.24 \times 10^6$ corresponding to an axial ratio for the molecule of 5/1. Wheat germ s-RNA has been shown by optical rotatory dispersion (ORD) to exist in a highly ordered secondary structure, as is evidenced by the appearance of large positive Cotton effects in the region of the 260-m$\mu$ absorption band of the constituent bases. The rotation in the visible region accurately follows a one-term Drude equation and is characterized by a dispersion constant, $\lambda_c$, of 250 m$\mu$ in the partly helical form. The positive Cotton decreases in amplitude while the dispersion constant increases upon subjecting the s-RNA to the action of denaturants, and these conformational effects are reversible. Ethylene glycol causes a complete collapse of the secondary structure of the molecule, and since its principal action is to weaken hydrophobic bonds, hydrophobic forces through base–base interactions have been identified as playing a principal role in maintaining the highly asymmetric native structure of the wheat germ s-RNA molecule.

The unique role of soluble ribonucleic acid (s-RNA) in the protein synthetic process is now a well-documented report. Amino acid specific s-RNA molecules react with each of the 20 amino acids and their respective activating enzymes (synthetases) to form aminoacyl s-RNA esters, and these in turn react with the RNA template [messenger RNA (m-RNA)] to promote peptide bond synthesis. While the principle of complementarity of base pairing is a plausible mechanism for the interaction of s-RNA with template RNA, the precise structural features of the s-RNA molecules which define the nature of the specificity of their interaction with their respective synthetases are yet to be determined. In this regard it is probable that a particular sequence of nucleotides in the s-RNA molecule (primary structure) and/or the configuration which that sequence dictates (secondary and tertiary structures) play a key role in the nature of this interaction. For this reason, knowledge of the chemical and physical characteristics of the s-RNA molecule is important in order to define the mechanism of its biological function.

A considerable amount of structural information is

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