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MECHANISM OF ENZYME ACTION

VI. KINETIC ISOTOPE EFFECT ON D-AMINO ACID OXIDASE REACTION

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SUMMARY

1. The rate of formation of the purple intermediate of D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] in the anaerobic reaction of this enzyme with its substrate was reduced by substitution of the α-hydrogen of the substrate for deuterium. This indicates that removal of the α-hydrogen of the substrate occurs prior to or in concert with the formation of the purple intermediate. The extent of the deuterium kinetic isotope effect differed for different substrates.

2. The α-deuteration of the substrate affected neither the rate of conversion of the purple intermediate to the fully reduced enzyme nor the rate of oxidation of the purple intermediate with O_2.

3. The kinetic isotope effect on the catalytic oxidation of the substrate was less than that on the formation of the purple intermediate. This indicates that the purple intermediate formation partially determines the overall rate of the enzymic reaction, but the effect on the overall rate is thought to result merely from the effect on the process of the purple intermediate formation. The kinetic isotope effect on the catalytic oxidation was increased by decreasing the enzyme concentration. This may be interpreted to mean that the process of the purple intermediate formation is more rate controlling in the monomeric enzyme than in the dimeric enzyme.

INTRODUCTION

The crystalline purple complex obtained by mixing D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] with its substrate D-alanine under anaerobic conditions[1] was identified with the purple intermediate appearing in a rapid reaction of this enzyme[2,3]. Upon mixing the oxidized enzyme (E_{ox}) with neutral D-amino acid (S) under anaerobic conditions, the purple intermediate ($E' \cdot S'$) is formed rapidly and converted slowly to the fully reduced enzyme–product complex ($E_{red} \cdot P$).

$$E_{ox} + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}\cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'\cdot S' \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_{red}\cdot P \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E_{red} + P \quad (1)$$

Under aerobic conditions, the purple intermediate reacts with O_2 to form the oxidized enzyme-product complex ($E_{ox}\cdot P$) and H_2O_2 (refs 4 and 5).

$$E'\cdot S' + O_2 \overset{k_{+5}}{\rightarrow} E_{ox}\cdot P + H_2O_2 \quad (2)$$

$$E_{ox}\cdot P \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} E_{ox} + P \quad (3)$$

Although the purple intermediate is known to be composed of equimolar amounts of the enzyme and substrate[1] and assigned to a strong charge-transfer complex, an "inner complex" (ref. 6), between the donor substrate and the acceptor flavin of the enzyme[7], further information is required to elucidate this unique intermediate of the flavoenzyme. This necessarily involves a study of the chemical reactions, involving the substrate moiety, which occur up to the formation of the purple intermediate. Since the cleavage of the α-CH bond of the substrate during the enzymic reaction has been proposed[8,9] and is supported by our previous results from H–^{2}H exchange study of this enzymic reaction[10], the α-CH bond cleavage has been examined in relation to the purple intermediate formation.

In our previous paper[11], the kinetic isotope effect on the formation of the purple intermediate using α-deuterated substrates was reported. Later, Walsh *et al.*[12] reported the kinetic isotope effect on the overall reaction, but did not consider our data in their interpretation of the mechanism of this enzyme. The present paper deals with the kinetic isotope effect on each process of the reaction in relation to that on the overall reaction.

The α-CH bond cleavage is assumed to be a proper enzymic process. This is on the basis of our result[13] that the imino acid, formed as the primary oxidation product of the substrate D-amino acid, is hydrolyzed nonenzymically. Thus the present study is expected to delineate a main part of the mechanism of action of this enzyme.

MATERIALS AND METHODS

D-Amino acid oxidase holoenzyme was prepared by the method of Yagi *et al.*[14]. The concentration of the enzyme was expressed in terms of the enzyme-bound FAD. DL-Alanine, DL-valine and DL-leucine were purchased from Tanabe Amino Acid Research Foundation, Osaka, and DL-α-aminobutyric acid and DL-norvaline from Nakarai Chemicals, Kyoto. DL-[α-^{2}H]Alanine, DL-[α-^{2}H]aminobutyric acid, DL-[α-^{2}H]norvaline, DL-[α-^{2}H]valine and DL-[α-^{2}H]leucine* were prepared by the reactions of the respective racemic amino acids with salicylaldehyde and $CuSO_4$ in ^2H_2O

* Evans *et al.*[15] reported that D-amino acid oxidase catalyzed the H–^{3}H exchange in the α-position of L-amino acids. We examined this phenomenon by incubating L-leucine with the enzyme in ^2H_2O for several hours, but could not detect by NMR any exchange of the α-hydrogen of the amino acid for deuterium. Therefore, the above-mentioned H–^{3}H exchange is considered to be due to the contamination with other enzyme(s) in their D-amino acid oxidase preparations as discussed by them. Accordingly, L-isomers in racemic amino acids used in the present study were thought to act merely as competitive inhibitors[16, 17], but not as substances in which deuterium exchange occurs.

by adopting the method of Tamiya and Oshima[18]. For the preparation of these α-deuterated amino acids, base-catalyzed hydrolytic ring-opening of the oxazolones corresponding to the respective *N*-acetylamino acids in ^2H_2O (ref. 19) was also utilized. Their purities were checked by paper chromatography, infrared spectroscopy and NMR; it was confirmed that the isotopic substitution of the α-hydrogen of the amino acids was almost complete, and substitution at the β-position did not occur.

The absorbance change at 550 nm caused by anaerobic or aerobic mixing of the enzyme with the substrate was measured at 20 °C using a Yanaco SPS-1 stopped-flow spectrophotometer connected to a storage oscilloscope and a pen recorder. Solutions of the enzyme and of the substrate, containing 0.017 M PP_i–HCl buffer, pH 8.3, were made anaerobic by bubbling with Ar which had been washed with an alkaline pyrogallol solution, and by consuming the trace of O_2 with the glucose oxidase ($1 \cdot 10^{-7}$ M)–glucose ($5 \cdot 10^{-3}$ M) system, as described previously[3].

The O_2 consumption due to the enzymic oxidation of the substrate under aerobic conditions was measured polarographically with a Kyusui Kagaku Bioxygraph as reported previously[14].

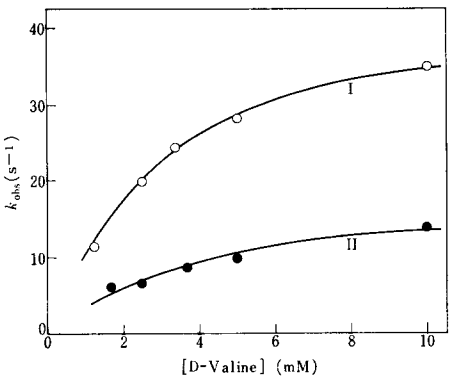
RESULTS

Kinetic isotope effect on the formation of the purple intermediate

The rapid formation of the purple intermediate followed by its slow conversion to the fully reduced enzyme in the anaerobic reaction of D-amino acid oxidase with neutral D-amino acids can be traced by the absorbance change at 550 nm (ref. 5). Accordingly, the kinetic isotope effect on the formation of the purple intermediate was studied by comparing the rapid increase in absorbance at 550 nm due to the reaction for α-H-substrate with that for α-^{2}H-labelled substrate. The stopped-flow traces followed essentially first-order kinetics. The pseudo-first-order rate constant (k_{obs}) of the reaction was plotted against the concentration of the substrate. Fig. 1 shows the result obtained with valine*. With this substrate, the rate of formation of the purple intermediate levelled off in the range of the substrate concentration investigated, so the rate is considered to be controlled by the process $E_{ox} \cdot S \rightarrow E' \cdot S'$, *viz.* by k_{+2} in Eqn 1. The result is similar to that obtained with leucine[11]. Although in these cases the reaction is inhibited by L-amino acid present, it is clear from Fig. 1 that substitution of the α-hydrogen of the substrate for deuterium caused the rate of formation of the purple intermediate to decrease to approximately one-third of the original at the substrate concentration range where the rate levelled off.

On the other hand, substitution of the α-hydrogen of norvaline for deuterium resulted in a slight decrease in the observed rate of formation of the purple intermediate, as shown in Fig. 2. In the case of α-aminobutyric acid, reduction of the rate of the purple intermediate formation by the α-deuteration was also slight. This feature is similar to that obtained with alanine[11]. Even though the kinetic isotope effect on the formation of the purple intermediate in these cases is minute, the fact that the rate of the reaction with norvaline showed saturation with respect to the concentration of the substrate indicates that the kinetic isotope effect is also due to

* A similar result was reported preliminarily at the Symposium on Structure and Function of Oxidation– Reduction Enzymes held in Stockholm, 1970 (ref. 20).



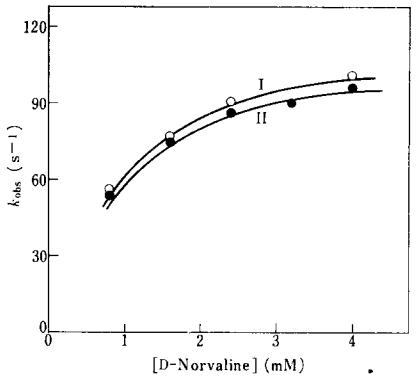


Fig. 1. Kinetic isotope effect on the formation of the purple intermediate of D-amino acid oxidase with D-valine. At various concentrations of valine, the formation of the purple intermediate was measured. Equal volumes of the anaerobic solutions of the enzyme ($2.88 \cdot 10^{-5}$ M) and of DL-valine of various concentrations were mixed and the absorbance changes at 550 nm due to the reaction at pH 8.3 and 20 °C were followed by a stopped-flow spectrophotometer (light path, 10 mm). The pseudo-first-order rate constants (k_{obs}) obtained from the reaction traces were plotted against the concentrations of D-valine. Curve I, with DL-[α-H]valine; Curve II, with DL-[α-^{2}H]valine.

Fig. 2. Kinetic isotope effect on the formation of the purple intermediate of D-amino acid oxidase with D-norvaline. The conditions were the same as those of Fig. 1. The pseudo-first-order rate constants (k_{obs}) obtained from the reaction traces were plotted against the concentrations of D-norvaline. Curve I, with DL-[α-H]norvaline; Curve II, with DL-[α-^{2}H]norvaline.

a decrease in k_{+2}. Accordingly, it is obvious from the above findings that the cleavage of the α-CH bond occurs prior to or in concert with the formation of the purple intermediate.

Kinetic isotope effect on the conversion of the purple intermediate to the fully reduced enzyme

The conversion of the purple intermediate to the fully reduced enzyme under anaerobic conditions followed first-order kinetics and was almost independent of the concentration of the substrate in accord with the previous reports[5,21]. The reaction traces of this process were obtained by the measurement of the absorbance change at 550 nm with [α-H]- and [α-^{2}H]leucine. The calculated rate constants were the same within the experimental error; $7.3 \cdot 10^{-3}$ s^{-1} and $7.6 \cdot 10^{-3}$ s^{-1} with [α-H]- and [α-^{2}H]-leucine, respectively. Similar results were obtained with other substrates, alanine, valine, α-aminobutyric acid and norvaline. Thus, substitution of the α-hydrogen for deuterium did not affect the rate of conversion of the purple intermediate to the fully reduced enzyme.

Kinetic isotope effect on the reaction of the purple intermediate with O_2

When D-amino acid oxidase is mixed with neutral D-amino acids such as D-alanine in the presence of O_2, the stopped-flow trace of the absorbance at 550 nm becomes like a "spike" as reported by Massey and Gibson[5]. The height and shape of this "spike" are determined by the velocities of the purple intermediate formation and of the reaction of this intermediate with O_2. Thus the effect of substitution of the α-hydrogen of the substrate for deuterium on the reaction of the purple inter-

mediate with O_2 can be examined by observing the "spike" under the conditions where the rates of formation of the purple intermediate with α-H- and α-^{2}H-labelled amino acid are the same.

The traces of the "spike" at 550 nm observed with [α-H]- and [α-^{2}H]norvaline are shown in Fig. 3. The extent of formation of the purple intermediate was the same in these two traces, and the values of the half-time of disappearance of the purple intermediate calculated from the traces with deuterated and non-deuterated norvaline were the same (approx. 30 ms). With DL-alanine and DL-α-aminobutyric acid, the results were the same as above, indicating that the rate of oxidation of the purple intermediate with O_2 is not affected significantly by the α-deuteration.

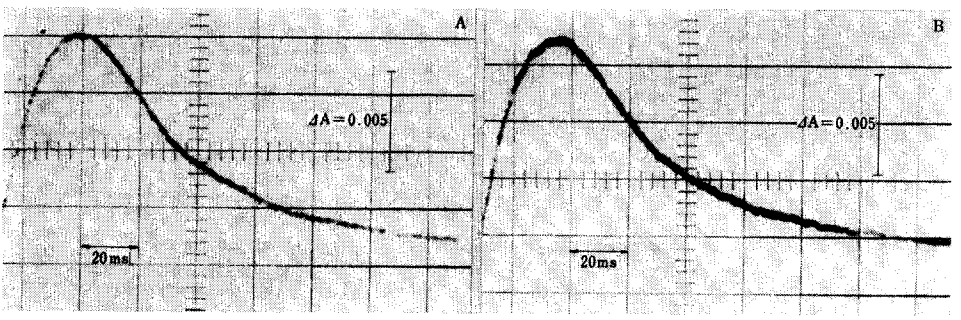


Fig. 3. Photographs of oscilloscope traces of the reaction of the purple intermediate of D-amino acid oxidase with O_2. The absorbance changes at 550 nm due to the reaction of D-amino acid oxidase with [α-H]- and [α-^{2}H]norvaline in the air-saturated buffer (pH 8.3) at 20 °C were followed by a stopped-flow spectrophotometer (light path, 2 mm). The final concentrations of the enzyme and O_2 were $3.80 \cdot 10^{-5}$ and $2.84 \cdot 10^{-4}$ M, respectively. (A) with DL-[α-H]norvaline, $2.0 \cdot 10^{-3}$ M (in final, in terms of D-isomer). (B) with DL-[α-^{2}H]norvaline, $2.5 \cdot 10^{-3}$ M (in final, in terms of D-isomer).

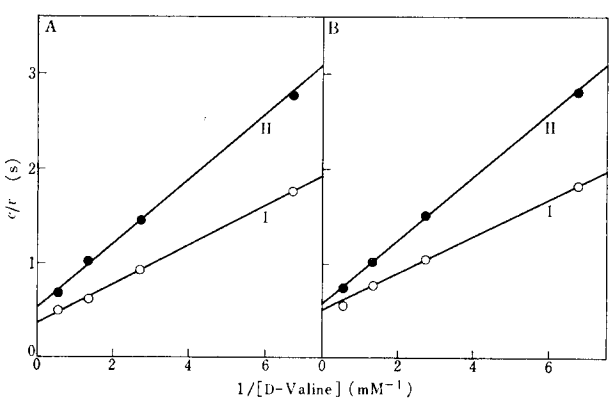


Fig. 4. Kinetic isotope effect on the catalytic oxidation of D-valine by D-amino acid oxidase. O_2 consumption was measured at pH 8.3 and 20 °C with reaction mixtures containing the enzyme [$5.38 \cdot 10^{-7}$ M (A) and $5.38 \cdot 10^{-6}$ M (B)], FAD ($2 \cdot 10^{-5}$ M) and valine (concentrations indicated in the figure). Curve I, with DL-[α-H]valine; Curve II, with DL-[α-^{2}H]valine.

Kinetic isotope effect on the overall reaction catalyzed by D-*amino acid oxidase*

The kinetic isotope effect of the α-deuteration of the substrate on the turnover of this enzyme was examined by measuring O_2 consumption. In Fig. 4A, the data

obtained with [α-H]- and [α-^{2}H]valine are plotted in a double-reciprocal form. By comparing these two plots, it is apparent that a considerable deuterium kinetic isotope effect is observed for the overall reaction; the ratio of the value of V for [α-H]valine to that for [α-^{2}H]valine is 1.5. However, in the cases of alanine, α-aminobutyric acid and norvaline, the effect was found to be marginal.

When the isotope effect on the overall reaction was compared with that on the purple intermediate formation, significant difference was noticed between the two; the former was smaller than the latter. This means that the process of the purple intermediate formation only partially determines the overall rate.

It was also found that the kinetic isotope effect on the overall rate is dependent on the concentration of the enzyme, as shown in Figs 4A and 4B. Upon increasing the enzyme concentration from $5.38 \cdot 10^{-7}$ to $5.38 \cdot 10^{-6}$ M, the ratio of the value of V for [α-H]valine to that for [α-^{2}H]valine was decreased from 1.5 to 1.1.

DISCUSSION

The present study clearly demonstrates that, in the reaction of D-amino acid oxidase with the substrate, the effect of α-deuteration of the substrate is only observed in the process of the purple intermediate formation, but neither in the process of conversion of the purple intermediate to the fully reduced enzyme nor in the process of oxidation of the purple intermediate with O_2. This indicates that the cleavage of the α-CH bond of the substrate occurs prior to or in concert with the formation of the purple intermediate. It is especially noted that the reduction of the rate of formation of the purple intermediate by the α-deuteration is significant with leucine and valine, but only slight with alanine, α-aminobutyric acid and norvaline. The difference in the extent of kinetic isotope effect among these substrates can be ascribed to the difference in the effect on k_{+2}, since the kinetic isotope effect on the formation of the purple intermediate is due to the change in k_{+2} as mentioned in Results. Accordingly, it is likely that the transition state of the cleavage of the α-CH bond is different depending on the kind of alkyl group of the substrate.

The present data also demonstrate the kinetic isotope effect on the overall reaction with valine, as was already observed by Walsh *et al.*[12] with serine and β-chloroalanine. However, in the cases of alanine, α-aminobutyric acid and norvaline, effect is only scarcely observed. This feature can be explained by taking into account the fact that the overall reaction consists of a number of reaction processes (see Eqns 1–3) and the process of the purple intermediate formation is only partially rate controlling.

The extent of the kinetic isotope effect on the overall reaction was dependent on the enzyme concentration; the effect being increased upon dilution of the enzyme solution. This can be explained by considering that the process of the purple intermediate formation is more rate controlling in the monomer than in the dimer. This view is supported by the reported data that the rate of formation of the purple intermediate is larger in the dimer than in the monomer[3], while the rate of the overall reaction is larger in the monomer than in the dimer[22,23], suggesting that the rate of dissociation of $E_{ox} \cdot P$ is larger in the monomer than in the dimer.

The present conclusion that the cleavage of the α-CH bond occurs prior to or in concert with the formation of the purple intermediate provides an essential clue to

elucidate the interaction between the flavin and the substrate moiety in the intermediate. The intermediate has been considered as a sort of strong charge-transfer complex, an "inner complex", which is formed between the donor substrate and the acceptor flavin part of the enzyme[7]. This is based on the fact that it shows a broad absorption band in the longer wavelengths and it gradually changes into the anion and cation radicals. It should be emphasized that such an interaction is attained after the α-hydrogen of the substrate is removed. In connection with this view, "outer" charge-transfer complexes formed between the donor inhibitors and the acceptor flavin of the enzyme should be recalled[17,24]. Examples of such inhibitors are *o*-aminobenzoate and Δ^1-piperidine 2-carboxylate. These compounds possess a carboxyl group and a nitrogen atom having a lone pair in the position spacially similar to those of the substrate amino acid. In complex formation of these inhibitors with the enzyme, the nitrogen lone pair is considered to interact with the flavin. In this case, however, reduction of the flavin does not occur. It seems, therefore, that the cleavage of the α-CH bond adjacent to the nitrogen atom is essential for the substrate to form the "inner complex".

The cleavage of the α-CH bond may occur *via* proton, hydrogen atom or hydride ion transfer. Among these, base-catalyzed proton transfer is the most probable. This mechanism was first suggested by Neims *et al.*[25] who found a linear relationship between the Hammett's σ values of *m*- and *p*-substituents of phenylglycine and the V of the catalytic oxidation of these substrates. Our preliminary results indicated that a similar linear free energy relationship also held (Yagi, K., Nishikimi, M., Takai, A. and Ohishi, N., unpublished) for the reduction of the enzyme with phenylglycine derivatives under anaerobic conditions. Recently, Walsh *et al.*[12] presented evidence for this mechanism by demonstrating the elimination of Cl of β-chloroalanine catalyzed by D-amino acid oxidase. In addition to the above-mentioned base-catalyzed proton abstraction, participation of the amino group of the substrate is thought to be essential to the enzymic reaction. Like the formation of "outer" charge-transfer complex of D-amino acid oxidase with *o*-aminobenzoate or Δ^1-piperidine 2-carboxylate, the substrate's amino group seems to donate electron(s) to the flavin moiety of the enzyme. It is reasonable to suppose that the α-proton abstraction occurs in concert with this interaction, since the latter interaction will reduce the electron density on the α-carbon atom, facilitating the release of the α-proton. In this case the resulting carbanion is thought to be more nucleophilic than the original substrate, thus the "inner complex", *viz.* the purple complex, will be easily formed.

The kinetic isotope effect on the reaction of L-amino acid oxidase has also been found by several groups of workers[26–28]. L-Amino acid oxidase also forms a transient long-wavelength absorbing intermediate[29,30]. Accordingly, the presently proposed mechanism for D-amino acid oxidase could also be applicable to L-amino acid oxidase.

REFERENCES

1 Yagi, K. and Ozawa, T. (1964) *Biochim. Biophys. Acta* 81, 29–38
2 Yagi, K., Nishikimi, M., Ohishi, N. and Hiromi, K. (1969) *J. Biochem. Tokyo* 65, 663–666
3 Yagi, K., Nishikimi, M. and Ohishi, N. (1972) *J. Biochem. Tokyo* 72, 1369–1377
4 Nakamura, T., Nakamura, S. and Ogura, Y. (1963) *J. Biochem. Tokyo* 54, 512–519
5 Massey, V. and Gibson, Q. H. (1964) *Fed. Proc.* 23, 18–29
6 Mulliken, R. S. (1952) *J. Phys. Chem.* 56, 801–822

7 Yagi, K., Okamura, K., Naoi, M., Sugiura, N. and Kotaki, A. (1967) *Biochim. Biophys. Acta* 146, 77–90
8 Knoop, F. (1910) *Z. Physiol. Chem.* 67, 489–502
9 Euler, H. v., Adler, E., Günther, G. and Das, N. B. (1938) *Hoppe-Seyler's Z. Physiol. Chem.* 254, 61–103
10 Yagi, K., Ohishi, N. and Nishikimi, M. (1970) *Biochim. Biophys. Acta* 206 181–183
11 Yagi, K., Nishikimi, M., Ohishi, N. and Takai, A. (1970) *FEBS Lett.* 6, 22–24
12 Walsh, C. T., Schonbrunn, A. and Abeles, R. H. (1971) *J. Biol. Chem.* 246, 6855–6866
13 Yagi, K., Nishikimi, M., Ohishi, N. and Takai, A. (1970) *Biochim. Biophys. Acta* 212, 243–247
14 Yagi, K., Naoi M., Harada, M., Okamura, K., Hidaka, H., Ozawa, T. and Kotaki, A. (1967) *J. Biochem. Tokyo* 61, 580–597
15 Evans, E. A., Green, R. H., Spanner, J. A. and Waterfield, W. R. (1963) *Nature* 198, 1301–1302
16 Dixon, M. and Kleppe, K. (1965) *Biochim. Biophys. Acta* 96, 368–382
17 Yagi, K., Naoi, M., Nishikimi, M. and Kotaki, A. (1970) *J. Biochem. Tokyo* 68, 293–301
18 Tamiya, N. and Oshima, T. (1962) *J. Biochem. Tokyo* 51, 78–88
19 Matsuo, H., Fujimoto, Y. and Tatsuno, T. (1965) *Tetrahedron Lett.* 3465–3471
20 Yagi, K. (1972) in *Structure and Function of Oxidation– Reduction Enzymes* (Åkeson, Å. and Ehrenberg, A., eds), pp. 401–417, Pergamon Press, Oxford
21 Yagi, K. (1971) in *Advances in Enzymology* (Nord, F. F., ed.), Vol. 34, pp. 41–78, John Wiley and Sons, New York
22 Shiga, K. and Shiga, T. (1972) *Biochim. Biophys. Acta* 263, 294–303
23 Yagi, K., Sugiura, N., Ōhama, H. and Ohishi, N. (1973) *J. Biochem. Tokyo* 73, 909–914
24 Massey, V. and Ganther, H. (1965) *Biochemistry* 4, 1161–1173
25 Neims, A. H., DeLuca, D. C. and Hellerman, L. (1966) *Biochemistry* 5, 203–213
26 Triggle, D. J. and Moran, J. F. (1966) *Nature* 211, 307–308
27 Porter, D. J. T. and Bright, H. J. (1969) *Biochem. Biophys. Res. Commun.* 36, 209–214
28 Page, D. S. and VanEtten, R. L. (1969) *Biochim. Biophys. Acta* 191, 190–192
29 Beinert, H. (1957) *J. Biol. Chem.* 225, 465–478
30 Massey, V. and Curti, B. (1967) *J. Biol. Chem.* 242, 1259–1264