A STUDY ON 
D-AMINO-ACID OXIDASE

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LIST OF ENZYMES USED THROUGHOUT THIS THESIS

In this thesis the non-systematic, trivial names of the enzymes are used. This list includes the trivial and the systematic names of the enzymes; also is included the enzyme number as established by the Commision for Enzymes of the International Union of Biochemistry.

<table>
<thead>
<tr>
<th>EC number</th>
<th>Systematic name</th>
<th>Trivial name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.3.4</td>
<td>D-Glucose: ( \text{O}_2 )-oxidoreductase</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>1.4.3.2</td>
<td>L-Amino acid: ( \text{O}_2 )-oxidoreductase (deaminating)</td>
<td>L-Amino-acid oxidase (deaminating)</td>
</tr>
<tr>
<td>1.4.3.3</td>
<td>D-Amino acid: ( \text{O}_2 )-oxidoreductase (deaminating)</td>
<td>D-Amino-acid oxidase</td>
</tr>
<tr>
<td>1.11.1.6</td>
<td>Hydrogen-peroxide: hydrogen-peroxide oxidoreductase</td>
<td>Catalase</td>
</tr>
<tr>
<td>4.1.2.10</td>
<td>Mandelonitrile benzaldehyde lyase</td>
<td>D-Oxynitrilase (Hydroxynitrile lyase) Shethna flavoprotein</td>
</tr>
</tbody>
</table>

LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EDTP</td>
<td>Ethylenediaminetetrapropionate</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide, oxidised or quinone form</td>
</tr>
<tr>
<td>FADH*</td>
<td>Flavin adenine dinucleotide, half-reduced or semiquinone form</td>
</tr>
<tr>
<td>FADH(_2)</td>
<td>Flavin adenine dinucleotide, fully reduced or hydroquinone form</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide, oxidised or quinone form</td>
</tr>
<tr>
<td>FMNH*</td>
<td>Flavin mononucleotide, half-reduced or semiquinone form</td>
</tr>
<tr>
<td>FMNH(_2)</td>
<td>Flavin mononucleotide, fully reduced or hydroquinone form</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>(K_D)</td>
<td>Dissociation constant of the enzyme-substrate complex</td>
</tr>
<tr>
<td>(K_i)</td>
<td>Dissociation constant of the enzyme-inhibitor complex</td>
</tr>
<tr>
<td>(K_m)</td>
<td>Michaelis constant (substrate concentration required for half maximal velocity of an enzyme reaction)</td>
</tr>
<tr>
<td>ORD</td>
<td>Optical rotatory dispersion</td>
</tr>
<tr>
<td>Tris</td>
<td>Tri(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>(V_{max})</td>
<td>Maximum velocity at infinite substrate concentration but not infinite acceptor concentration</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

This study is concerned with the non-metal flavoproteins, especially with the enzyme D-amino-acid oxidase. With the exception of mouse liver (SHACK, 1943) this enzyme is found in the kidney and liver of almost all mammals studied (KREBS, 1935, 1959). Although this enzyme is widely spread in nature the physiological role of D-amino-acid oxidase is still unknown. The enzyme was discovered by KREBS (1935) and it was shown by WARBURG and CHRISTIAN (1938a, b) that the prosthetic group was flavin adenine dinucleotide (FAD).

It was first thought that D-amino-acid oxidase was an iron-containing flavoprotein and that this iron could be reduced by the addition of substrate (KUBO et al., 1958, 1960). Later it was found by MASSEY et al. (1961) that the first preparations contained an iron-protein as contamination, which could be separated from D-amino-acid oxidase on a calcium phosphate cellulose column. YAGI and OZAWA (1963) reported that the preparations of MASSEY et al. (1961) were not pure, but contained a complex between the enzyme and the competitive inhibitor benzoate (benzoate was added to stabilize the enzyme during the heat-treatment of the isolation). By treatment with an excess of substrate the enzyme could be freed of benzoate.

The spectrum of D-amino-acid oxidase resembles that of FAD with maxima at 377 and 453 nm. The binding of benzoate induces a red shift in the spectrum. The maximum at 453 shifts to 462 nm, and a pronounced shoulder appears at 485 nm. VEGER et al. (1966) distinguished two types of enzyme-inhibitor complexes with benzoate derivatives by the changes in the absorption spectrum of the flavoprotein, which occurred either at 480 to 500 nm or from 500 to 700 nm. MASSEY and GANTHER (1965) found that the carboxylic group is essential for these interactions. They suggested that the changes in flavin absorption in the 480 to 500 nm region are due to the disruption of a hydrogen bond between the flavin and the protein, with the result that the flavin comes in a more hydrophobic environment. These effects are also partly related with the effects of solvents on the absorption bands of 3-methyl lumiflavin (HARBURY and FOLEY, 1958; HARBURY et al., 1959).

The behaviour of D-amino-acid oxidase in the ultracentrifuge is very complex. The enzyme dialysed against FAD shows a single peak below a concentration of 1%, but when the enzyme is dialysed in the absence of FAD (during which some flavin is lost) a heavier component appears (MASSEY et al., 1961). This suggests that the removal of FAD favours polymerization. A minimum molecular weight of 45, 700 per mole FAD was found. In the presence of FAD the sedimentation constant increases with increasing protein concentration (CHARLWOOD et al., 1961). Below a concentration of 1%, an almost symmetrical peak is found, while at 2% a heavier component appears. In a 0.54% solution dialysed against a buffer free of FAD, three components are observed. The average molecular weight in the presence of FAD varies between 132,000 and 205,000, in the protein concentration range 0.19–1.16%.

In sharp contrast to the statements above are the reports of YAGI and co-
workers (Yagi et al., 1961, 1963; Yagi and Ozawa, 1962b, c). They stated that the enzyme under various conditions (apoenzyme, holoenzyme and enzyme-benzoate complex) exhibits a molecular weight of 115,000. These workers found the same molecular weight by sedimentation, diffusion, light-scattering and by FAD analyses. They claimed, in contrast to Charlwood et al. (1961) that the molecular weight was independent of the protein concentration. Some clarification was obtained from a report of Yagi and Ozawa (1964). They overestimated the protein concentration by a factor 2, so that the claimed minimum molecular weight per mole FAD of 115,000 changed to 57,500, which is in fair agreement with the value mentioned above. Recent molecular weight studies (Massey et al., 1965; Antonini et al., 1966) have emphasized the complexity of D-amino-acid oxidase. The molecular weight is dependent on the presence of ions, type of buffer and benzoate, but it is almost certain that the enzyme at infinite dilution has a molecular weight of 100,000–110,000 containing two moles of FAD.

Until recently (Wellner and Scannone, 1964) it was assumed that D-amino-acid oxidase exhibits an absolute specificity for D-amino acids. Evidence has been given (Ratner, 1955) for the existence of a glycine oxidase. However, the report of Neims and Hellerman (1962) suggests that this enzyme is identical with D-amino-acid oxidase. The best substrates for D-amino-acid oxidase are the D-isomers of alanine, methionine, allohydroproline, proline and tyrosine. Less rapidly oxidised are the D-isomers of α amino butyric acid, isoleucine, leucine, phenylalalanine, serine, valine and tryptophan. The D-isomers of arginine, lysine, histidine, glutamic acid, aspartic acid and α aminoadic acid are oxidised at very low rates, as is L-proline (Wellner and Scannone, 1964; Yagi and Nishikimi, 1968); D-glutamine and D-asparagine are not oxidised at all.

The enzyme has a high degree of specificity for oxygen as hydrogen acceptor. A slow reaction occurs with the dyes methylene blue and 2,6 dichlorophenol indophenol, while no reaction occurs with other tested hydrogen acceptors (ferricyanide, cytochrome c, quinones, phenazine metasulphate and NAD+)(Dixon and Kleppe, 1965).

By adding the substrate to D-amino-acid oxidase, it can be seen that the absorbance partially decreases in the 400–500 nm region and that a broad band appears in the region of 530–600 nm (Kubo et al., 1959; Massey et al., 1961). This reaction is very rapid and leads to the formation of a catalytically active intermediate which was first considered to be a flavin semiquinone FADH* (Massey et al., 1961). Further reduction of the enzyme to the FADH2 level takes several minutes, so it is very unlikely that the total reduced enzyme plays kinetically a significant role. By ESR spectroscopy no free radical signal associated with this intermediate could be detected (Kubo et al., 1959; Massey et al., 1961; Nakamura et al., 1963; Yamano, 1964; Massey and Gibson, 1964). It was found by Massey and Gibson (1964) that the spectrum and the rate of reoxidation of the intermediate are dependent on the nature of the different amino acids used as substrates. It was concluded therefore that the intermediate
was a complex with coupled spins between the FADH* and the amino acid radical.

Using a series of FAD analogues modified in the isoalloxazine ring and the ribityl chain, Chassy and McCormick (1965) tested the coenzyme specificity of D-amino-acid oxidase. It was found that the hydroxyl groups of the ribityl chain are not essential for activity. The five carbon chain is essential for activity, although not for binding. If the 3-methylated isoalloxazine was used, neither activity nor binding could be detected. This suggests that the 3(N)-position is involved in the binding on the apoenzyme. Furthermore the results of McCormick et al. (1964) showed that D-amino-acid oxidase exhibits a considerable degree of coenzyme specificity with regard to the AMP portion of FAD.

Besides the classic method for preparing apoenzyme by acid (NH₄)₂SO₄ treatment (Negelein and Brömel, 1939), it can also be prepared by dialysing the enzyme against KBr and EDTA (Massey and Curti, 1966) and by column chromatography (Miyake et al., 1965). These methods are much milder than the classic method. The recombination of the apoenzyme with FAD is a two-step process (Massey and Curti, 1966): a rapid binding of the flavin (this can be followed by fluorescence quenching) followed by a first-order reaction associated with the enzymatic activity. In this second reaction it is assumed that a conformational change is involved.

The starting point of this thesis was the contradiction between the kinetic reaction mechanisms proposed by Dixon and Kleppe (1965) and Massey and Gibson (1964). This contradiction is discussed in the introduction of Chapter 3.

Also studied (Chapter 4) are the conformational changes of D-amino-acid oxidase, as reported by Massey et al. (1966), and the influences of temperature, inhibitor and substrates on the equilibrium between the conformations.

Neims et al. (1966) reported the relationship between the logarithm of the maximum velocities and the σ values of m- and p-substituted substrates. In connection with this report, the relationship between the logarithms of the inhibition constants and the σ values of m- and p-substituted derivatives of benzoic acid was studied.

In Chapter 5 the results of a study over the effectiveness of several photoreductants for the photoreduction of FMN, FAD and flavoproteins (FAD-containing) are given. Massey and Palmer (1966) showed that by anaerobic photoirradiation of flavoproteins in the presence of EDTA the semiquinone form (FADH*) of these flavoproteins is formed. However, in their study no other photoreductants were tested.

Some comparative studies with other enzymes were also performed, details of which are given in the corresponding chapters.
2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Enzymes

D-Amino-acid oxidase was prepared from hog kidneys by the method of Massey et al. (1961). This isolation procedure yields a complex of the enzyme and its stabilizer benzoate. The enzyme was freed of benzoate by reduction with an excess of D-alanine followed by precipitation at 50% saturated (NH₄)₂SO₄. This was repeated twice after which the enzyme was dialysed overnight against 0.1 M pyrophosphate (pH 8.3). To be certain that not too much FAD dissociated from the protein, an excess of FAD was added and the enzyme precipitated at half-saturated (NH₄)₂SO₄. The precipitate was washed several times with 50% aq. (NH₄)₂SO₄ and finally dissolved in 0.1 M pyrophosphate (pH 8.3). Spectrophotometric control ensured that the enzyme was free of benzoate.

L-Amino-acid oxidase was isolated from snake venom (Crotalus adamanteus) according to the method of Wellner and Meister (1960b).

Shethna flavoprotein was isolated according to Shethna et al. (1966).

D-Oxynitrilase was isolated according to Becker et al. (1963) and in part was a gift from Prof. Dr. Pfeil, University of Marburg/Lahn (W-Germany).

Glucose oxidase was purchased from Boehringer and Söhne.

Catalase was purchased from Boehringer and Söhne.

2.1.2. Reagents

D-Alanine, D-methionine, dimethylglycine ethyl ester and triethylamine were obtained from Fluka, Switzerland; ATP, GTP, pyruvate from Boehringer and Söhne; FAD and FMN from Sigma Chemical Co.; L-leucine, L-valine, L-methionine, pyrophosphate, benzoic acid, the substituted benzoic acids and EDTA, from British Drug Houses. L-Leucine was recrystallized; some of the substituted benzoic acids (B.D.H.) were first treated with charcoal after which they were recrystallized. EDTP was obtained from K & K Laboratories, California, U.S.A. Nicotine was obtained from Ver. Handel en Industrie Onderneming, The Hague, The Netherlands. NN Dimethylaminopropanol was a gift from Dr. Pandit (Department of Organic Chemistry, University of Amsterdam, The Netherlands).

2.2. METHODS

2.2.1. The enzymatic activity of D-amino-acid oxidase was measured in two ways:

1. With the Gilson Differential Respirometers at the temperatures indicated in the tables and in the legends, under the conditions described by Burton (1955).

2. With the Gilson Oxygraph Model KM. A polarizing voltage of -0.63V was applied to the electrode. The oscillation frequency of the electrode was 120 cycles per sec and the greatest amplitude was used. The amount of oxygen con-
sumed was calculated from the slope of the recorded line by the method of Dixon and Kleppe (1965). The conditions described by Burton (1955) were applied, but without catalase.

Protein estimation was performed spectrophotometrically at 453 nm for D-amino-acid oxidase by assuming a millimolar extinction coefficient of 11.3 cm$^{-1}$ and 2 moles FAD bound per molecule of enzyme. The activity is expressed by $\mu$1 or $\mu$moles O$_2$/min/mg.

The enzymatic activity of L-amino-acid oxidase were measured on the Gilson Oxygraph Model KM under the conditions described by Wellner and Meister (1960b). The enzymatic activity was expressed by $\mu$moles O$_2$/min/A$_{464nm}$. ($A_{464nm}$ is the maximum of the spectral band of FAD bound to the enzyme). The different oxygen concentrations were obtained by mixing nitrogen and oxygen from cylinders.

2.2.2. Titration experiments

Agla micrometer syringes (Burroughs Wellcome and Co, London) were used for the spectrophotometric titration experiments with D-amino-acid oxidase and benzoate. The optical differences were recorded with the 0–0.1 absorbance slide wire of the Cary model 14 recording spectrophotometer. The differences were recorded at 497.5 nm.

2.2.3. Photoreduction

These experiments were performed with Thunberg cuvettes. The deoxygenation was accomplished by repeated evacuation with a vacuum pump followed by readmission of nitrogen. The nitrogen was made oxygen-free by passage through aqueous sodium pyrogallate. The illumination was done with light from a 500 W xenon lamp unit (Zeiss LX501) with waterbath, but without blue filter, and the wavelength was selected with the monochromator (M4 QIII) of a Zeiss spectrophotometer. Generally the band width was 25 nm. To obtain a homogenous photoreduction, the contents of the cuvette was stirred by a plastic-coated iron bar within the cell and the external magnetic rotor of an electric motor. The temperature was maintained, generally at 5°, by circulation of cold water through the cuvette holder. The rate of photoreduction was followed by registering the spectra with a Cary model 14 recording spectrophotometer with a cell holder also controlled at a temperature of 5°.

2.2.4. Optical rotatory dispersion experiments

The ORD data were obtained with the Jasco Model ORD/UV-5. The data were analysed in terms of the empirical Moffitt-Yang equation (Moffitt and Yang, 1963)

$$[m'] = \frac{\alpha_{\lambda}MRW}{cI} = \frac{a_0 \lambda_0^2}{\lambda^3 - \lambda_0^3} + b_0 \frac{\lambda_0^4}{(\lambda^3 - \lambda_0^2)^2}$$

by assuming a mean residue weight (MRW) of 115 (Fasman, 1963); $[m']$ is the
mean residue rotation; \( \alpha_\lambda \) is the rotation at wavelength \( \lambda \); \( \lambda_0, a_0 \) and \( b_0 \) are the Moffitt-Yang parameters, \( \lambda_0 \) was taken to be 212 nm, while \( a_0 \) and \( b_0 \) were determined grafically; \( c \) and \( l \) are respectively the concentration and the light-path (1 cm cells are used).

2.2.5. *Stopped-flow experiments*

These experiments were performed with a Durrum-Gibson stopped-flow spectrophotometer. For the kinetic experiments of Chapter 3 the temperature was maintained at 26° and the optical differences were monitored at 550 nm. The velocity of the reaction was calculated by the method of Chance (1943) and Gibson et al. (1964). In this method the area above the curve may be equated with the amount of oxygen originally present. Thus the amount of oxygen consumed in a given time can be calculated. The binding of benzoate to D-amino-acid oxidase is followed by the increase in absorbance at 497.5 nm. The monitored transmission differences were recalculated to absorbance differences. The temperatures used are mentioned in the legends of the corresponding figures.
3. ON THE CATALYTIC MECHANISM OF D-AMINO-ACID OXIDASE

3.1. INTRODUCTION

D-Amino-acid oxidase catalyses specifically the oxidation of glycine and D-amino acids. In the plot of the reciprocal velocity versus the reciprocal substrate concentration straight lines are obtained. Massey et al. (1961) and Massey and Gibson (1964) obtained parallel lines in the 1/v versus 1/[S] plot at different oxygen concentrations. This was confirmed by Dixon and Kleppe (1965), who proposed that the product dissociates from the enzyme before the reduced enzyme is reoxidised by oxygen. On the other hand the catalytic mechanism proposed by Massey and Gibson (1964) is different; the reduced enzyme is reoxidised before the product dissociates from the enzyme.

This study shows that it is possible to resolve this difference in interpretation. It can be deduced that in a catalytic mechanism such as the one proposed by Massey and Gibson (1964) the lines plotted for reciprocal velocity versus reciprocal substrate at different acceptor concentrations converge in the presence of high concentrations of a competitive inhibitor. In the mechanism proposed by Dixon and Kleppe (1965) the lines are parallel in the presence of a competitive inhibitor. A useful method for distinguishing between the two mechanisms is the method of Slater (1955).

With succinate dehydrogenase (EC 1.3.99.1) a similar problem was solved (DerVartanian et al., 1966; DerVartanian, 1965; Zeylmaeker et al., in press; Zeylemaker, 1967) in the same way. In fact it could be shown that the maximum activity of this enzyme is independent of the nature of the electron acceptors.

3.2. RESULTS

Dixon and Kleppe (1965) proposed the following kinetic reaction mechanism on the basis of their results:

Mechanism I

\[ E + S \xrightleftharpoons[k_{-1}]{k_{+1}} ES \]

\[ ES \xrightleftharpoons[k_{-2}]{k_{+2}} E'P \]

\[ E'P \xrightleftharpoons[k_{-3}]{k_{+3}} E' + P \]

\[ E' + O_2 \xrightarrow{k_{+4}} E + H_2O_2 \]

in which $E$ is the oxidised and $E'$ the reduced enzyme.

For the steady-state with $[P] = 0$, the rate equation becomes (cf. DIXON and KLEPPER, 1965; CLELAND, 1963)

$$v = \frac{V_{\text{max}}}{1 + \frac{K_s}{[S]} + \frac{K_o}{[O_2]}}$$

in which:

$$V_{\text{max}} = \frac{k_{+2} k_{+3} [E_0]}{k_{+2} + k_{-2} + k_{+3}}$$

$$K_s = \frac{k_{-1} k_{-2} + k_{-1} k_{+3} + k_{+2} k_{+3}}{k_{+1} (k_{+2} + k_{-2} + k_{+3})}$$

$$K_o = \frac{k_{+1} k_{+3}}{k_{+4} (k_{+2} + k_{-2} + k_{+3})}$$

$[E_0]$ = total enzyme concentration

$[S]$ = D-alanine concentration

In the presence of a competitive inhibitor ($I$), the rate equation is modified into

$$v = \frac{V_{\text{max}}}{1 + \frac{K_s}{[S]} \left(1 + \frac{[I]}{K_i} \right) + \frac{K_o}{[O_2]}}$$

in which $K_i$ is the dissociation constant of the enzyme-inhibitor complex. It is clear from these formula that in the plot $1/v$ versus $1/[S]$, also in the presence of a competitive inhibitor the lines should be parallel at different oxygen concentrations.

An alternative is the following mechanism, which is related to the mechanism proposed by GIBSON and MASSEY (1964):

**Mechanism II**

$$E + S \xrightleftharpoons[k_{-1}]{k_{+1}} ES$$

$$ES \xrightleftharpoons[k_{-2}]{k_{+2}} E'P$$

$$E'P + O_2 \rightarrow EP + H_2O_2$$

$$EP \xrightleftharpoons[k_{-4}]{k_{+4}} E + P$$
With the restriction that \( [P] = 0 \), the following rate equation is valid for the steady state (DerVartanian et al., 1966; Cleland, 1963):

\[
 v = \frac{V_{\text{max}}}{1 + \frac{K_S}{[S]} + \frac{K_O}{[O_2]} + \frac{K_{OS}}{[S][O_2]}}
\]

in which;

\[
 V_{\text{max}} = \frac{k_{+2}k_{+4}[E_0]}{k_{+2} + k_{+4}} \\
 K_S = \frac{k_{+4}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+4})} \\
 K_O = \frac{k_{+4}(k_{+2} + k_{-2})}{k_{+3}(k_{+2} + k_{+4})} \\
 K_{OS} = \frac{k_{-1}k_{-2}k_{+4}}{k_{+1}k_{+3}(k_{+2} + k_{+4})}
\]

In the presence of a competitive inhibitor (I), the rate equation becomes:

\[
 v = \frac{V_{\text{max}}}{1 + \frac{K_S}{[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{K_O}{[O_2]} + \frac{K_{OS}}{[S][O_2]}\left(1 + \frac{[I]}{K_i}\right)}
\]

From this equation it can be concluded that in the \( 1/v \) versus \( 1/[S] \) plot at different oxygen concentrations the lines normally converge to one point in the third quadrant, in the presence or absence of a competitive inhibitor.

Nevertheless, when \( K_{OS}/[S] \) is small in comparison with \( K_O \), the rate equation becomes similar to that derived for Mechanism I, with parallel lines at different oxygen concentrations. In the presence of the competitive inhibitor however, the term \( K_{OS}/[S] \) is multiplied by the factor \( (1 + [I]/K_i) \) and thus, depending on the ratio \( [I]/K_i \), may make a considerable contribution to the rate equation, which results in converging lines. Therefore it is possible that in the absence of a competitive inhibitor the lines are parallel, but in its presence they converge.

Fig. 1A shows that the obtained results confirm those of Massey et al. (1961), Massey and Gibson (1964) and Dixon and Kleppe (1965). Parallel lines are obtained for different oxygen concentrations in the \( 1/v \) versus \( 1/[D\text{-alanine}] \) plot. Fig. 1B shows the same experiment carried out in the presence of a competitive inhibitor (benzoate, 50 \( \mu \)M). It is clear that under these conditions the lines converge to a point in the third quadrant.

From the rate equations it can be concluded that the inhibitor acts, in Mechanism I uncompetitively, and in Mechanism II noncompetitively towards oxygen. Fig. 2 gives the \( 1/v \) versus \( 1/[O_2] \) plot from which it is clear that the inhibitor acts noncompetitively towards oxygen.
For Mechanism II there are not enough relationships between the parameters to allow the calculation of the individual rate constants from the kinetic data. From the rate equation however, the following relations between the kinetic parameters can be derived in which $V_{\text{max}}^{S}$ is the velocity at an infinite concentration of D-alanine and a finite concentration of oxygen and $K_{S}^{m}$ is the apparent Michaelis constant in the $1/v$ versus $1/[D\text{-alanine}]$ plot at that particular oxygen concentration.

$$K_{S}^{m} = V_{\text{max}}^{S} \left( \frac{k_{-1} + k_{+2}}{k_{+1} k_{+2} [E_{0}]} - \frac{k_{-1} k_{+2} k_{-2} + k_{-1} k_{-2} k_{+4}}{k_{+1} k_{+2} k_{+4} (k_{+2} + k_{-2}) [E_{0}]} \right) + \frac{k_{-1} k_{-2}}{k_{+1} (k_{+2} + k_{-2})}$$
FIG. 2. $1/v$ vs $1/[O_2]$ plots at different concentrations of D-alanine. $\square - \square$, 5 mM D-alanine; $\triangle - \triangle$, 10 mM D-alanine; $\circ - \circ$, at infinite D-alanine concentration. The plots $\blacksquare - \blacksquare$ and $\blacktriangle - \blacktriangle$ are at respectively 5 mM and 10 mM D-alanine, but in the presence of 50 $\mu$M benzoate.

$$
\frac{1}{V_{max}} = \frac{k_{+2} + k_{-2}}{k_{+2} k_{+3} [E_0]} \frac{1}{[O_2]} + \frac{k_{+2} + k_{+4}}{k_{+2} k_{+4} [E_0]}
$$

$$
K_{m}^{S}/V_{max} = \frac{k_{-1} k_{-2}}{k_{+1} k_{+2} k_{+3} [E_0]} \frac{1}{[O_2]} + \frac{k_{-1} + k_{+2}}{k_{+1} k_{+2} [E_0]}
$$

$[E_0] = $ total enzyme concentration.

When the method of Slater (1955) is applied, the plot $K_{m}^{S}$ versus $V_{max}^{S}$ (Fig. 3) gives a straight line which does not pass through the origin, as was calculated by the method of the least squares. The intercept with the ordinate ($V_{max}^{S} = 0$) is equal to $k_{-1} k_{-2}/k_{+1} (k_{+2} + k_{-2})$. In the $1/V_{max}^{S}$ versus $1/[O_2]$ plot (Fig. 4A) the intercept with the ordinate ($1/[O_2] = 0$) is

$$
\frac{k_{+2} + k_{+4}}{k_{+2} k_{+4} [E_0]} \frac{1}{[E_0]}
$$

FIG. 3. The plot $K_{m}^{S}$ vs $V_{max}^{S}$. The values of $K_{m}^{S}$ and $V_{max}^{S}$ are calculated from Fig. 1A. The line is calculated by the method of the least squares.

Fig. 4A. The plot $1/V_s^{max}$ vs $1/[O_2]$. These values are calculated from Fig. 1A. B. The $K_s^{m}/V_s^{max}$ vs $1/[O_2]$. The values of $K_s^{m}/V_s^{max}$ are calculated from Fig. 1A.

From the plot $K_s^{m}/V_s^{max}$ versus $1/[O_2]$ the intercept with the abscissa ($K_s^{m}/V_s^{max} = 0$) is $k_{-3} (k_{-1} + k_{+2})/k_{-1} k_{-2}$ and with the ordinate ($1/[O_2] = 0$) is $(k_{-1} + k_{+2})/k_{+1} k_{+2} [E_0]$.

The relationships between the kinetic parameters are summarised in Table I.

It is possible to calculate the rate constants for the limiting case, in which the enzyme-substrate complex is rapidly converted into either $E'P$ or the free enzyme, e.g. Mechanism II A (cf. DERVARTANIAN et al., 1966; DERVARTANIAN, 1965)

\[
E + S \xrightleftharpoons[k_{-1}]{k_{+1}} E'P
\]

\[
E'P + O_2 \rightarrow EP + H_2O_2
\]

\[
EP \xrightleftharpoons[k_{-4}]{k_{+4}} E + P
\]

For which the rate equation is:

\[
v = \frac{k_{+4} [E_0]}{1 + \frac{k_{+4}}{k_{+1} [S]} + \frac{k_{+4}}{k_{+3} [O_2]} + \frac{k_{-1} k_{+4}}{k_{+1} k_{+3} [S] [O_2]}}
\]
TABLE I. The relations between kinetic parameters calculated for Mechanism II, with D-alanine as substrate at 37°.

Conditions as described in Chapter 2.

\[
\begin{align*}
\frac{k_+ (k_{+2} + k_{-2})}{k_- k_{-2}} &= 5 \cdot 10^9 \text{ mole}^{-1} \text{ l}^{-1} \\
K_S &= 4 \cdot 10^{-3} \text{ mole} \text{ l}^{-1} \\
K_D &= 4 \cdot 10^{-4} \text{ mole} \text{ l}^{-1} \\
\frac{k_{-1} + k_{+2}}{k_{+3} k_{-2}} &= 8 \cdot 10^{-5} \text{ mole} \text{ l}^{-1} \text{ sec}^{-1} \\
K_{DS} &= 8 \cdot 10^{-8} \text{ mole}^2 \text{ l}^{-2} \\
\frac{k_{+3} (k_{-1} + k_{+2})}{k_{-1} k_{-2}} &= 54 \cdot 10^3 \text{ mole}^{-1} \text{ l}^{-1} \\
\frac{k_{+1} k_{+3} k_{-3}}{k_{-1} k_{-2}} &= 7 \cdot 10^8 \text{ mole}^{-2} \text{ l}^{4} \text{ sec}^{-1} \\
\frac{k_{+2} + k_{+4}}{k_{+3} k_{+4}} &= 18 \cdot 10^{-3} \text{ sec}^{-1}
\end{align*}
\]

The following kinetic relationships can be derived

\[
\frac{1}{V_{\text{max}}} = \frac{1}{k_+ [E_0]} \left(1 + \frac{k_{+4}}{k_{+3}} [O_2]\right)
\]

Upon plotting \(1/V_{\text{max}}\) versus \(1/[O_2]\) a straight line (Fig. 4B) is obtained, which does not pass through the origin. The intercept with the ordinate \((1/[O_2] = 0)\) equals \(1/k_{+4} [E_0]\) from which \(k_{+4}\) can be calculated. The intercept on the abscissa \((1/V_{\text{max}} = 0)\) is equal to \(-k_{+3}/k_{+4}\), from which \(k_{+3}\) can be calculated.

\[
K_S = \frac{(k_{-1} + k_{+3} [O_2]) k_{+4}}{(k_{+3} [O_2] + k_{+4}) k_{+1}}
\]

therefore

\[
\frac{K_S}{V_{\text{max}}} = \frac{1}{k_{+1} [E_0]} \left(1 + \frac{k_{-1}}{k_{+3}} [O_2]\right)
\]

The plot \(K_S/V_{\text{max}}\) versus \(1/[O_2]\) gives a straight line (Fig. 4B), which does not pass through the origin, while the intercept with the ordinate is equal to \(1/k_{+1} [E_0]\). On the abscissa \((K_S/V_{\text{max}} = 0)\) the intercept is equal to \(-k_{+3}/k_{-1}\).

Table II summarizes the values of the different constants calculated from Fig. 4A, B.

TABLE II. Rate constants calculated for Mechanism IIA, with D-alanine as substrate at 37°.

Conditions as described in Chapter 2.

\[
\begin{align*}
k_{+1} &= 1.3 \cdot 10^4 \text{ mole}^{-1} \text{ l} \text{ sec}^{-1} \\
k_{-1} &= 5 \text{ sec}^{-1} \\
k_{+3} &= 2.6 \cdot 10^5 \text{ mole}^{-1} \text{ l} \text{ sec}^{-1} \\
k_{+4} &= 55 \text{ sec}^{-1}
\end{align*}
\]
Fig. 5. The plots $1/v$ vs $1/[O_2]$ at different D-alanine concentrations at 25°. These results are obtained with the stopped-flow apparatus by applying the method described by Chance (1943) and Gibson et al. (1964). Benzoate-free D-amino-acid oxidase (15.6 μM enzyme-flavin) is mixed with 0.64 mM $O_2$ at different D-alanine concentrations in the presence of an excess of FAD (0.1 mM). O – O, 10 mM D-alanine; x – x, 5 mM D-alanine; △ – △, 2.5 mM D-alanine. The plots • – • and ■ – ■ are at respectively 10 and 5 mM D-alanine, but in the presence of 1.25 μM benzoate. (All concentrations after mixing).

By the stopped-flow method (Chance, 1943; Gibson et al., 1964) more evidence for Mechanism II is provided. It has been demonstrated that in the catalysis a spectral intermediate is involved (Massey and Gibson, 1964), which can be monitored at 550 nm, a wavelength at which neither the oxidised nor the reduced enzyme shows any absorption. By analysis of the time course of the formation and disappearance of this spectral intermediate upon mixing the enzyme with oxygen and an excess of substrate, it is possible to calculate the rate of oxygen consumption at a number of oxygen concentrations just from one experiment. Fig. 5 shows that in the absence of benzoate a series of parallel lines is obtained (Chance, 1943). On the other hand the addition of benzoate (1.25 μM) changes the slopes of the lines in the plot $1/v$ ($v$ = moles $O_2$ consumed per sec) versus $1/[O_2]$. In agreement with Mechanism II, the inhibition is non-competitive towards $O_2$.

Mechanism II also predicts that the product of the reaction has to be competitive towards the substrate. Fig. 6 shows that pyruvate in the presence of $NH_4^+$ acts according to this prediction. The inhibition constant is 38 mM.

Fig. 7A shows the Lineweaver-Burk plot with D-methionine as substrate at different oxygen concentrations. In contrast to the results with D-alanine these lines converge to a point in the third quadrant. It should be emphasized that in
3.3. DISCUSSION

From the results shown it can be concluded, in agreement with Massey et al. (1961) and Massey and Gibson (1964), that in the catalytic overall reaction of D-amino-acid oxidase, e.g. the oxidation of D-alanine by O₂, after reduction by substrate the enzyme is reoxidised before the product dissociates from the enzyme. This conclusion disagrees with the mechanism proposed by Dixon and Kleppe (1965), in which the product dissociates from the enzyme before the
latter is reoxidised. The reason for this discrepancy lies in the fact that in the rate equation for Mechanism II, the term $K_{OS}/[S]$ is relatively small compared with $K_{0}$, which means that this mechanism apparently changes into Mechanism I. When the term $K_{OS}/[S]$ in the presence of a competitive inhibitor is multiplied by a factor $(1 + [I]/K_{i})$, this will manifest itself in the rate equation.

As has been pointed out by Dixon (1955), the rate equation for a two substrate mechanism which does not contain a term depending on both substrates, will show in the $K_s^m$ versus $V_{s_{max}}$ plot, as introduced by Slater (1955), a straight line which passes through the origin:

$$v = \frac{V_{max}}{1 + \frac{K_1}{[S_1]} + \frac{K_2}{[S_2]}}$$

$$V_{s_{max}} = \frac{V_{max} [S_2]}{([S_2] + K_2)}$$

$$K_{s_{max}} = K_1 [S_2]/([S_2] + K_2)$$

However, in the case of a mechanism which gives a term in the rate equations depending on both substrates, the straight line in the $K_s^m$ versus $V_{s_{max}}$ plot does not pass through the origin:

$$v = \frac{V_{max}}{1 + \frac{K_1}{[S_1]} + \frac{K_2}{[S_2]} + \frac{K_{12}}{[S_1][S_2]}}$$

$$V_{s_{max}} = \frac{V_{max} [S_2]}{([S_2] + K_2)}$$

$$K_{s_{max}} = (K_1[S] + K_{12})/([S_2] + K_2)$$

This is due to the contribution of the term $K_{12}$ in the equation for $K_{s_{max}}$. If this contribution is small, the lines obtained in the $1/v$ versus $1/[S]$ plots at different concentrations of the second acceptor become apparently parallel. The line in the $K_s^m$ versus $V_{s_{max}}$ plot, however, does not pass through the origin, although its point of intersection with the $K_{s_{max}}$ axis may be very close to it. In such a case it is recommended to use a competitive inhibitor to distinguish clearly between the two mechanisms.

The results of Radhakrishnan and Meister (1958) show that the catalytic reaction is reversible under anaerobic conditions. Their results are easily explained by Mechanism I and are interpreted by Dixon and Kleppe (1965) as a support for it. However, these results can also be explained by Mechanism II. It has been shown (Massey et al., 1966; Koster and Veeger, unpublished results) that when the enzyme is mixed with the substrate a broad absorption band above 500 nm appears transiently and disappears upon full reduction of the enzyme-flavin. This band belongs to a catalytically active intermediate. When pyruvate and ammonia are added to the fully reduced enzyme, this absorption band is restored. Since the spectral properties of this band depend on the product of the enzymatic reaction, it is clear that the reaction $E'P \rightleftharpoons E'' + P$ exists as a slow side reaction, which becomes important under anaerobic con-
ditions in static experiments like spectral studies. Thus it is clear that the results of Radhakrishnan and Meister (1958) can also be obtained in the case of Mechanism II.

Further support for this mechanism comes from the observation that pyruvate, in the presence of ammonia and in the process of forming the imino acid (product of the reaction), inhibits competitively the substrate D-alanine. This is in full agreement with Mechanism II, but quite difficult to understand if Mechanism I should be valid. From other studies (Massey and Gantner, 1965; Yagi, 1966; Veege et al., 1966) it is known that the oxidised enzyme in the presence of pyruvate shows the same spectral effect as in the presence of the competitive inhibitor benzoate.

The results obtained with D-methionine as substrate support the proposed mechanism. With this substrate the contribution of \( K_{OS} \) in the kinetic equation is presumably much more important than in the case of D-alanine.
4. THE RELATION BETWEEN TEMPERATURE-INDUCIBLE ALLOSTERIC EFFECTS AND THE ACTIVATION ENERGIES OF AMINO-ACID OXIDASES

4.1. INTRODUCTION

Neims et al. (1966) investigated with D-amino-acid oxidase the relationship between the logarithm of the maximum velocity and the \( \sigma \) values for \( m- \) and \( p- \) substituted phenylalanine and C-phenylglycine. They obtained an asymmetrical, biphasic plot with a maximum at \( \sigma = +0.04 \) for C-phenylglycine, and at \( \sigma = +0.23 \) for phenylalanine. Although it is known that benzoate (Burton, 1951) and some substituted benzoates (Yagi et al., 1959) are competitive inhibitors of this enzyme, no attempt was made to investigate the correlation between the inhibition constants and the corresponding \( \sigma \) values.

With L-amino-acid oxidase, Radda (1964) investigated the correlation between the velocity and the \( \sigma \) value of ring-substituted phenylglycines as substrates at one concentration. As with D-amino-acid oxidase, benzoate is a competitive inhibitor for L-amino-acid oxidase (Zeller and Moritz, 1945). Zeller and Claus (1966) reported, however, that they could not find a correlation between the \( V_{\text{max}} \), \( K_m \) and \( K_i \) determined for ring-substituted phenylalanines as substrates, the various benzoic acids as inhibitors and the \( \sigma \) values.

Massey et al. (1966) reported that D-amino-acid oxidase shows a discontinuous Arrhenius plot, from which it was concluded that the enzyme exists in two forms, a low-temperature form and a high-temperature form, in equilibrium with each other, having different activation energies. The temperature at which the break in the plot occurs, is dependent on the substrate used. With D-alanine it is at about 14°, while with D-methionine it is at 24°. From fluorescence and ultraviolet spectral studies, it was concluded that D-alanine has equal affinities for both forms, while D-methionine has more affinity for the low-temperature form than for the high-temperature form. With other enzymes a different explanation has been given for this phenomenon, e.g. an equilibrium between two or more forms differing in their catalytic activities (Kistiaikowsky and Lumry, 1949) or a temperature-induced conformational change of the active site (Levy et al., 1962).

This study was undertaken to investigate the nature of the conformational change of the enzyme D-amino-acid oxidase in comparison with L-amino-acid oxidase.

4.2. RESULTS AND DISCUSSION

4.2.1. The relation between the inhibition constants and the \( \sigma \) values of the substituted benzoates

Benzoate and the substituted benzoates inhibit D-amino-acid oxidase competitively. Table I summarizes the inhibition constants of benzoate and the ring-substituted benzoate derivatives for D-amino-acid oxidase, measured at
TABLE I. The inhibition constants (μM) of benzoate and benzoate derivatives in the catalytic overall reaction of D-amino-acid oxidase. The inhibition constants were determined by means of the Lineweaver-Burk plots. These experiments were performed at 37° in 0.1 M pyrophosphate buffer (pH 8.3) with D-alanine as substrate.

| Substituent | \( K_i \) (μM) at 37° with D-alanine as substrate. In general the \( o \)-substituted benzoates give a lower inhibition than the \( m \)- and \( p \)-substituted derivatives. Exceptions are the hydroxy- and amino-substituents, of which the \( o \)-substituted derivatives give the highest inhibition, probably due to the formation of hydrogen bridges. The lower inhibition with the other \( o \)-substituted benzoates can be due to steric hindrance, but \( o \)-fluorobenzoate (fluorine is in size comparable with hydrogen) has an inhibition constant of the same order as the other halogen-substituted benzoates. The differences found between the \( o \)-, \( m \)- and \( p \)-substituents are the same as found by Barlett (1948) and Massey and Ganthier (1965). The last investigators made their conclusions from spectrophotometric experiments.

Fig. 1 shows the logarithm of the inhibition constants of the benzoate-derivatives for D-amino-acid oxidase obtained at 37°, plotted against the \( \sigma \) value. The line drawn is calculated by the method of the least squares. The \( \sigma \) value is experimentally obtained for the individual substituents (Gould, 1962); the \( \sigma \) value of hydrogen is arbitrarily chosen as zero, and a positive value of \( \sigma \) means a stronger electron-attracting character than hydrogen. The plot is biphasic with a minimum at \( \sigma = +0.5 \). The slope of the line of the substituents with a \( \sigma \) value lower than 0.5 has a \( p \) value of \(-2.0\), with a correlation coefficient \( r = -0.6 \).

From the plot it can be concluded that the inhibition of the catalytic reaction increases with increasing electron-attracting character (increasing \( \sigma \) value) of the substituents. The other part of the plot is formed by the \( m \)- and \( p \)-nitrobenzoates. The slope of this part has a \( p \) value of about +4.9; this value is questionable but is only used for comparative reason. It is remarkable that the substituents with a \( \sigma \) value smaller than 0.5 have a -I and +R effect, while the nitro-derivatives have a -I and -R effect (Gould, 1962). Neims et al. (1966), working with ring-substituted substrates, found that increasing \( \sigma \) values lead to...
FIG. 1. Hammett plot of the action of ring-substituted benzoate derivatives with D-amino-acid oxidase. The logarithms of the inhibition constants at 37° are plotted against their σ values. The $K_i$'s were determined from Lineweaver-Burk plots; D-alanine was used as substrate. The line drawn was calculated by the method of the least squares; in this calculation m-dimethylaminobenzoate and m-methoxybenzoate were not taken into account for reasons of steric hindrance.

an increasing maximum velocity up to a σ value depending on the substrate used. At a higher σ value, a decline in activity is observed.

The results of DE KOK and VEEGER (1968) showed that a similar plot made for L-amino-acid oxidase differs in some respects from that of D-amino-acid oxidase, but the behaviour of $m$- and $p$-nitrobenzoate is similar with both enzymes. Other substituted benzoates with negative σ values have approximately equal inhibition constants with L-amino-acid oxidase. The $m$-halogen-substituted derivatives, on the other hand, show a sharp increase in inhibition of L-amino-acid oxidase in comparison with the $p$-substituents. This behaviour at 37° differs only quantitatively from that at 25°; binding of these derivatives induce a shift of the flavin absorption band to shorter wavelengths (DE KOK and VEEGER, 1968). The results show that there is a correlation between σ value and the inhibition constant in agreement with the results of RADDA (1964) and in contrast to the results of ZELLER and co-workers (1945, 1966).
4.2.2. The effect of benzoate, substituted benzoates and ATP on D-amino-acid oxidase

The $1/v$ vs $1/[[D\text{-}\text{alanine}]]$ plot at $10^\circ$ of Fig. 2 shows that the line obtained in the presence of benzoate (similar phenomena are found with substituted benzoates) intersects the line obtained in the absence of inhibitor at a finite concentration of D-alanine. At temperatures of $15^\circ$ and higher, benzoate and derivatives are competitive inhibitors (cf. Chapter 3.2.); on the other hand, $m$- and $p$-nitrobenzoate exhibit strictly competitive behaviour over the temperature range $5-40^\circ$. The same effect of benzoate on the activity is evident at $10^\circ$ with D-methionine as substrate (Fig. 3). In this case the effect disappears as the temperature is raised above $24^\circ$ and the inhibition becomes competitive. The most striking phenomenon in these experiments is the observation that, at high concentrations of both D-alanine and D-methionine, the inhibitory effect of benzoate is replaced by an activating effect. In this respect benzoate and its derivatives behave like allosteric inhibitors (cf. Gerhart and Pardee, 1963; Monod et al., 1965), with the difference that the activating effect is not visible at low substrate concentrations.

The model of Monod et al. (1965), which approaches this phenomenon mathematically, predicts that a concerted transition mechanism will show substrate inhibition in case the substrate has a higher affinity for a catalytically inactive than for a catalytically active state. If the discontinuity of the Arrhenius
plot (Massey et al., 1966) is due to the presence of an inactive form at lower temperature, it can be expected that substrate inhibition will be observed in case the substrate has a higher affinity for the inactive form. When the low-temperature form is less active, rather than inactive as assumed in the allosteric theory, only under certain conditions substrate inhibition can be observed. By assuming that the enzyme concentration is much smaller than the substrate concentration, one can derive that, for the steady-state approximation, the general equation for the initial rate of the reaction becomes:

\[
v = \frac{n \cdot \{(1 + \alpha)^n V_{R_{\text{max}}} [S]/([S] + K_{R_m}) + L (1 + cx)^n V_{T_{\text{max}}} [S]/([S] + K_{T_m})\}}{(1 + \alpha)^n + L (1 + cx)^n}
\]

\[n\] is the number of substrate binding sites; \(L\) is the equilibrium constant between the R-form and T-form free of ligand, defined as \([T] = L [R]; \alpha\) is the ratio of the free substrate concentration and the dissociation constant for the binding to the R-form; \(c\) is the ratio of the dissociation constants for the R- and the T-form, i.e. \(c = K_{R_D}/K_{T_D}; V_{R_{\text{max}}}\) and \(V_{T_{\text{max}}}\) are the maximum velocities for the R- and T-state, respectively, per catalytic site; \(K_{T_m}\) and \(K_{R_m}\) are the Michaelis constants for the T- and the R-state, respectively. It must be pointed out that \(V_{T_{\text{max}}}, V_{R_{\text{max}}}, K_{T_m}\) and \(K_{R_m}\) vary with the concentration of the second substrate (see Chapter 3.2.). Furthermore, it is assumed that this second substrate does not influence the equilibrium between R and T. This equation which is derived from the fraction of protein in the R- and T-states is valid, provided the equilibrium between these forms is established at the time of the first rate measurement. This equation is preferable to the relation of the ligand saturation function with the velocity (cf. Frieden, 1967), since this binding function does not take into consideration other possible intermediates in the conversion of the substrate to the product. The rate is determined by the normal kinetic parameters. Since

FIG. 3. Effect of benzoate on D-amino-acid oxidase with D-methionine as substrate. Conditions as described in Chapter 2. Temp., 10°. •••, control; △−△, [benzoate] = 7.5 μM; x−x, [benzoate] = 25 μM.

the $K_m$ for most enzymes is not related to the dissociation constant of the enzyme-substrate complex, it is better not to use the latter in these equations unless a special mechanism is involved (cf. BLANGY et al., 1968; BUC, 1967).

More correctly DALZIEL (1968) has taken into account the steady-state concentrations of the enzyme-substrate complexes for the two enzyme conformations, which leads to the following rate equation:

$$v = \frac{n \{a \beta V_{R_{\text{max}}} (1 + a)^{n-1} + L V_{T_{\text{max}}} \alpha c (1 + c \alpha)^{n-1}\}}{(1 + a)^n + L (1 + c \alpha)^n}$$

(2)

in which $a$ is the ratio of the substrate concentration and the Michaelis constant of the R-form, while $c$ is the ratio of the Michaelis constants of the R- and T-form, i.e. $c = K^R_m/K^T_m$. This equation is derived for a simple reaction mechanism and is analogous to the saturation function as derived by MONOD et al. (1965), but the dissociation constants are replaced by the Michaelis constants. This equation simplifies for the case that $K^R_m = K^T_m$ and $V^R_{\text{max}} = V^T_{\text{max}}$ into:

$$v = \frac{n[S] (V^R_{\text{max}} + L V^T_{\text{max}})}{(L + 1) ([S] + K_m)}$$

(2A)

which is a Michaelis-Menten type of equation. It represents a 'V-system' of MONOD et al. (1965), e.g. no homotropic or heterotropic interactions occur unless an effector with different affinities for the R- and T-form is present, which by its modifying effect on $L$, affects the maximum rate.

It can be calculated from Eqn. 2 that when the equilibrium constant $L$ is small and the substrate has a smaller $K_m$ with the less active T-form, at high concentration, substrate inhibition will be observed. Fig. 4 shows that this is the case at high concentrations of D-methionine. At higher or lower temperatures, this inhibition disappears. With D-alanine no substrate inhibition is observed at any temperature, indicating that this substrate has about equal $K_m$'s for both forms (cf. MASSEY et al., 1966). The observations (Figs. 2 and 3) that the activating effect of benzoate with D-methionine is more pronounced than with D-alanine is in agreement with the above-mentioned conclusion, provided that benzoate has a higher affinity for the R-configuration. Furthermore these figures show that the plots in the presence of inhibitor remain straight. Extrapolation to infinite substrate concentration gives the same intercept on the $1/v$ axis as obtained with the line in the presence of benzoate. The activating effect on the maximum rate indicates that benzoate has a higher affinity for the high-temperature form than for the low-temperature form.

The presence of a non-reacting ligand binding to both states with different affinities modifies Eqn. 2 into:

$$v = \frac{n \{a \beta (1 + \alpha)^n (1 + \beta)^n V^R_{\text{max}} + L \alpha c (1 + c \alpha)^n (1 + d \beta)^n V^T_{\text{max}}\}}{(1 + a)^n (1 + \beta)^n + L (1 + c \alpha)^n (1 + d \beta)^n}$$

(3)

In case of a competitive inhibitor equation 3 changes into:

\[ v = \frac{n \left\{ \alpha (1 + \alpha + \beta)^{n-1} V_{\text{max}}^R + L(1 + \alpha + \beta)^{n-1} V_{\text{max}}^T \right\}}{(1 + \alpha + \beta)^n + L(1 + \alpha + d\beta)^n} \]  

(4)

In these equations \( \beta \) is the ratio of the free ligand concentration and the dissociation constant of the R-form; \( d \) is the ratio of the dissociation constants of the binding of the ligand to the R- and T-form.

The main factors which determine the effects to be observed are \( L, c \) and \( d \). Thus it can be expected that the substrate inhibition observed without benzoate (Fig. 4) is abolished upon benzoate addition. At the temperatures used in these experiments, \( L \) is small and \( c \approx 1 \), which exerts its influence only at very high D-methionine concentrations. By adding benzoate \( (d < 1) \), this effect is diminished, because according to the equation the main influence is on the R-form \( (\beta) \) and not the T-form \( (d\beta) \). As a consequence the substrate inhibition is apparently abolished. The results obtained are those for true competitive inhibition.

Nitrobenzoates \( (m- \) and \( p- \) ) act as true competitive inhibitors over the temperature range 5°–40° with D-alanine as substrate. With D-methionine as substrate however, although extrapolation of the initial slope indicates true competitive inhibition (Fig. 4), at high concentration of substrate, deviation from the linearity and substrate inhibition occur. It must be pointed out, how-
ever, that this only occurs around 25°. At higher or lower temperatures the inhibition is competitive. In connection with the ideas developed, it is clear that these inhibitors have about equal affinities for both forms (Δ=1). With D-alanine as substrate, ATP is a non-competitive inhibitor. The concentration of ATP which inhibits the activity is strongly temperature-dependent, for instance at 5° hardly any inhibition is observed in the presence of 200 μM ATP and a small inhibition (< 8%) with 20 mM ATP; at 37°, 300 μM ATP gives an inhibition of 26% (Figs. 5A, B). Furthermore it must be remarked that GTP in about the same concentration gives similar effects. Fig. 4 shows that, in the presence of a large concentration of ATP, no substrate inhibition is obtained with high D-methionine concentrations. ATP inhibits the enzyme only partially, since a 100-fold increase in concentration does not affect the extent of inhibition very much (Fig. 6). Furthermore at high ATP concentration, in contrast with the results at low concentrations, substrate inhibition is observed. Although only at very high concentration of D-methionine is a deviation from linearity observed, it must be remarked that three different experiments show this effect.

These results indicate that ATP has a higher affinity for the low-temperature conformation than for the high-temperature form. This non-competitive inhibition is due to a shift of the equilibrium between the two states. This equilibrium lies at 37° towards the R-form, at 5° towards the T-form. It is the cooperation between D-methionine and ATP, the latter having higher affinity to the T-form, which at 37°, where the allosteric constant is small, causes substrate inhibition only at very high concentration of both compounds.

Figs. 7 and 8 provide further evidence for the idea that benzoate and ATP have their highest affinity for different forms of the enzyme. Fig. 7 shows that
Fig. 6. Effect of ATP on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions as described in Chapter 2. ○ - ○, control; △ - △, [ATP] = 200 μM; x - x, [ATP] = 20 mM.

Fig. 7. Effect of ATP and benzoate on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions as described in Chapter 2. ○ - ○, control; △ - △, [ATP] = 200 μM + 10 μM benzoate; x - x, [ATP] = 200 μM.
the non-competitive inhibition of ATP at 37° is abolished by the addition of benzoate. The fact that the curve in the presence of benzoate is convex towards the abscissa is in agreement with the ideas outlined here. At low concentrations of D-methionine the activating effect of benzoate is larger than at higher concentrations, since it can more easily overcome the synergistic action of ATP and the substrate. However, Dalziel and Engel (1968) showed that with the model of Monod et al. (1965) no convex downwards curve (Fig. 7) can be expected. They suggested that in case of a convex downwards curve the model of Koshland et al. (1966) with negative interactions provides a feasible explanation.

Further evidence for the presence of two conformations with different velocities and affinities comes from the following experiments. The Arrhenius plot (Fig. 8) of the maximum velocity with D-methionine shows a break point at 24° (cf. Massey et al., 1969). In the presence of benzoate however, the break becomes less sharp, when the extrapolation of the linear part of the Lineweaver-Burk plot is taken as maximum velocity (cf. Figs. 3–5). In the presence of ATP, a fairly good straight-line relationship is obtained, but with lower activities at temperatures higher than 5°. The plot in the presence of ATP (ΔE = 9900 cal mole⁻¹) is almost parallel with the upper part of the Arrhenius plot without any effector present (ΔE = 9300 cal mole⁻¹). It can be argued that the difference is

Fig. 8A. Effect of effectors on the Arrhenius plot of D-amino-acid oxidase. The log \( V_{\text{max}} \) (μ moles O₂ min⁻¹mg⁻¹) with D-methionine as substrate, obtained by extrapolating the linear part of the Lineweaver-Burk plot, is plotted against 1/T. \( \triangle - \triangle \), control; • – •, in the presence of 25 μM benzoate; ○ – ○, in the presence of 20 mM ATP.

B. Van 't Hoff plot of the temperature-dependent equilibrium between the low- and high-temperature forms of D-amino-acid oxidase. The equilibrium constant \( L \) was calculated as described in this Chapter.

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within the experimental error. On the other hand, the fact that the plots are not completely parallel is partly due to the fact that the ATP concentration is not saturating. This gives a slightly higher maximum velocity due to the presence of some R-form; the result is a small increase of the activation energy. A close examination of the experimental points reveals that the tendency of a break is still present. Affinity for both conformations in the case of benzoate binding can lead to the Arrhenius plot not being completely straight. On the other hand, the inaccuracy of the extrapolation is large especially at the lower temperature where the differences are the greatest. The presence of the T-form with lower activity leads to a smaller value of the maximum velocity. Therefore it is concluded that the two forms have the same activation energy but differ in maximum rate (25%), indicating that the identical transition states have different transition probabilities. From the difference in rates it can be calculated that the difference in entropy of activation between the R- and T-forms is $\Delta S^* = 0.7$ e.u.

If for a certain condition $V_{max}$ is the experimentally obtained maximum velocity, $V^T_{max}$ the maximum velocity of the T-form, $V^R_{max}$ the maximum velocity of the R-form, $f_R$ the molar fraction of the R-form, and $1-f_R$ the molar fraction of the T-form, then for the equilibrium between the two forms the following relations are valid:

$$V_{max} = f_R V^R_{max} + (1 - f_R) V^T_{max}$$

$$f_R = \frac{V_{max} - V^T_{max}}{V^R_{max} - V^T_{max}}$$

and

$$L = \frac{[T]}{[R]} = \frac{1 - f_R}{f_R}$$

Since the activation energies are the same, the $f_R$ value can be calculated for the temperature range where the transition occurs and connected with this, the allosteric constant $L$. From the van 't Hoff plot of the temperature dependent variation of $L$ (Fig. 8B), it can be calculated that $\Delta H = 55000$ cal mole$^{-1}$ and $\Delta S = 185$ e.u.

From tryptophan fluorescence studies MASSEY et al. (1966) found that the fluorescence remained constant between 2° and 9° and between 23° and 28°, but at intermediate temperatures a marked decrease in fluorescence intensity was found with a midpoint of about 14.5°. From this experiment an enthalpy difference of 78000 cal mole$^{-1}$ ($\Delta S = 270$ e.u.) was calculated, by applying the method used with D-methionine in the Arrhenius plot. This value was much higher than the above-mentioned value of 55000 cal mole$^{-1}$. For D-methionine, it was assumed that this substrate exhibited a different affinity for the two conformations. It was therefore of interest, to investigate the Arrhenius plot with D-alanine as substrate, for which equal affinity for both forms was postulated by MASSEY et al. (1966) in order to investigate any influence of the substrate on the thermodynamic parameters calculated from the Arrhenius plot.
Fig. 9A. Effect of ATP on the Arrhenius plot of D-amino-acid oxidase. The log $V_{\text{max}}$ (µmoles $\text{O}_{2} \text{~min}^{-1} \text{mg}^{-1}$) with D-alanine as substrate, obtained from Lineweaver-Burk plots, plotted against $1/T$. $x-x$, control and $O-O$, in the presence of 20 mM ATP.

B. Van 't Hoff plot of the temperature-dependent equilibrium between the low- and high-temperature forms of the enzyme. The equilibrium constant $L$ was calculated as described in this Chapter.

Fig. 9A shows the Arrhenius plot with D-alanine as substrate. This plot confirms the results of MASSEY et al. (1966). In the same Fig. is shown the effect of ATP. In the presence of ATP a linear plot is obtained, which is parallel with the upper part of the plot in the absence of ATP. These results are in agreement with those of D-methionine as substrate in the presence and absence of ATP. From the van 't Hoff plot constructed in the same way as with D-methionine a $\Delta H$ of 67500 cal mole$^{-1}$ and a $\Delta S = 240$ e.u. can be calculated. Although this value is larger than that of D-methionine it is still considerably less than the value obtained from the fluorescence study.

The conclusion that can be drawn is that the substrate has a marked influence on the thermodynamic parameters of the conformational change $R \rightleftharpoons T$.

**Scheme I**

$\begin{align*}
R & \rightleftharpoons T \quad 37^\circ \\
R & \rightleftharpoons T \quad 5^\circ \\
R & \rightleftharpoons T \\
\downarrow & \quad \downarrow & \quad \downarrow \\
R_I & R_S & T_S & T_{\text{ATP}} \\
I & = \text{benzoate} & S & = \text{substrate} & V_{T_{\text{max}}} < V_{R_{\text{max}}} \\
\end{align*}$

4.2.3. Comparison of the affinities of the substrates for the different forms of D-amino-acid oxidase

In case of Mechanism II suggested for the reaction of D-amino-acid oxidase (see Chapter 3) it is not possible to calculate the dissociation constant ($k_{-1}/k_{+1}$) of the enzyme-substrate complex from the kinetic data. However, it can be
derived (ZEYLEMAKER et al., in press) that the $K'_D \left(= \frac{k_{-1} k_2}{k_{+1} (k_{+2} + k_{-2})} \right)$ of the first two equilibria equals the intercept on the ordinate of the plot $K_{sm}^S$ vs $V_{S_{\text{max}}}$ Thus this intercept might be taken as a measure of the affinity of the substrate for the enzyme. For the substrate D-methionine one should expect that the $K'_D^R$, the dissociation constant of the complex with the high-temperature form is larger than the $K'_D^T$, the dissociation constant of the complex with the low-temperature form. For the substrate D-alanine equal dissociation constants are to be expected.

With D-methionine as substrate at 30° the enzyme is totally in the R-form and it is thus possible to calculate the $K'_D^R$ from the normal $K_{sm}^S$ vs $V_{S_{\text{max}}}$ plot. ATP shifts the equilibrium between the two forms towards the T-form. Comparison of the intercept on the ordinate in the plot $K_{sm}^S$ vs $V_{S_{\text{max}}}$ in the absence and presence of ATP enables one to calculate the difference between the affinities for both forms. Fig. 10 shows the $K_{sm}^S$ vs $V_{S_{\text{max}}}$ plot with D-methionine as substrate. A difference of 14% is found between the intercepts in the absence and presence of ATP (Table II).

When D-alanine is used as substrate, equal intercepts on the ordinate are obtained in the $K_{sm}^S$ vs $V_{S_{\text{max}}}$ plot in the absence and presence of ATP (Fig. 11), indicating equal affinities of this substrate for both conformations (Table II).

![Fig. 10. The plot $K_{sm}^S$ vs $V_{S_{\text{max}}}$ with D-methionine as substrate at 30°. Further conditions as described in Chapter 2. The values of $K_{sm}^S$ and $K_{5\text{max}}$ are obtained from Lineweaver-Burk plot at different oxygen concentrations. $\circ \circ$, no ATP; $x \cdot x$, $[\text{ATP}] = 200 \mu\text{M}$.]

**TABLE II.** The dissociation constants $K'_D$ (µM) and the ratios for D-methionine at 30° and D-alanine at 25° in the absence and presence of ATP (200 µM). The $K'_D$'s are obtained from the $K_{sm}^S$ vs. $V_{S_{\text{max}}}$ plots.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K'_D^R$ (µM)</th>
<th>$K'_D^T$ (+ ATP)</th>
<th>$K'_D^R/K'_D^T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Methionine</td>
<td>1600</td>
<td>1400</td>
<td>1.14</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>250</td>
<td>250</td>
<td>1.00</td>
</tr>
</tbody>
</table>
FIG. 11. The plot $K_{m}^S$ vs $V_{\text{max}}^S$ for D-alanine at 25°. The values are obtained from Line-eweaver-Burk plots at different oxygen concentrations. $\circ - \circ$, no ATP; $\times - \times$, [ATP] = 200 $\mu$M.

The small difference in $K'_{D}$ with D-methionine as substrate is not expected and in fact cannot explain the difference in thermodynamic parameters and thus the shift in the temperature of the break, e.g. the point where 100 % high-temperature form is present. In the Arrhenius plots presented here the oxygen concentration is finite. However, the same temperatures for the breaks were found by Massey et al. (1966), working at infinite oxygen concentrations. Therefore it can be concluded that the second substrate oxygen has no influence on the conformational changes. According to Chapter 3 the maximum rate at finite $[O_2]$ but infinite [D-amino acid] is

$$1/V_{\text{max}}^S = \frac{k_{+2} + k_{-2}}{k_{+2} k_{+3} [E_0]} + \frac{k_{12} + k_{+4}}{k_{+2} k_{+4} [E_0]}$$

It follows from the lack of influence of the oxygen concentration on the temperature of the break, that the main factor determining the shape of the Arrhenius plot is $k_{+2}k_{+4}/(k_{+2} + k_{+4})$. From the results of Massey and Gibson (1964) it is known that $k_{+2} \gg k_{+4}$ thus the rate limiting step in the Arrhenius plot at infinite $[O_2]$ is the dissociation of the product from the reoxidised enzyme. Since it was already concluded that the affinities for both conformations of D-methionine differed too little to account for the shift in the $T_{\text{break}}$, the most logical explanation is that the product by being bound to the protein is responsible for the shift. Scheme II provides a possible explanation. Assume that all intermediates in the two forms are in equilibrium with each other. The rate limiting step is $k_{+4}$. The free enzyme forms (R and T) try to establish their equilibrium, but

**Scheme II**

$$ER + S \rightleftharpoons ER \quad O_2 \quad k_{+4}$$

$$\downarrow \quad \downarrow$$

$$ER + S \rightleftharpoons ET$$

$$ER \rightarrow ER \quad P$$

$$\uparrow \quad \uparrow$$

$$ET + S \rightleftharpoons ET$$

$$ET \rightarrow ET \quad + \quad P$$

the rate of conversion into each other is slower (cf. section 4.2.4.) than the rate of the reaction with the substrate. This means that the distribution between the $E^R$ and $E^T$ is mainly determined by the equilibrium $E^R P \rightleftharpoons E^T P$. Thus the shift in the Arrhenius plot represents the shift in this equilibrium ($k_{T^A}^R < k_{T^A}^E$). The explanation is derived for a simplified scheme, but in fact is applicable to the rate limiting step of more complicated mechanisms.

From the strict non-competitive inhibition by ATP with D-alanine as substrate it can be concluded that we are dealing with the 'V-system' of Monod et al. (1965) (see section 4.2.2. E$qn.$ 2A). This explains the straight Lineweaver-Burk plots at all temperatures with this substrate. With D-methionine $K_{T,m} = 1.1 K_{R,m}$ (see Chapter 3, Fig. 7), this difference in $K_m$ is sufficiently small to give straight Lineweaver-Burk plots according to the 'V-system' kinetics.

4.2.4. On the binding of benzoic acid to D-amino-acid oxidase

The binding of benzoic acid to the enzyme induces a red shift in the flavin absorbance. The maximum at 453 nm shifts to 462 nm and a shoulder appears at 485 nm. The difference spectrum shows a maximum at 497.5 nm (Yagi and Ozawa, 1962a; Massey and Palmer, 1965; Veeger et al., 1965; Massey et al., 1965). Yagi and Ozawa (1962a) reported that one molecule of benzoate is bound per molecule of flavin. From extrapolation of the initial part of the titration curve Massey et al. (1965) concluded that one molecule of benzoate is bound per molecule of flavin, e.g. 2 molecules of benzoate per molecule of protein. A closer analyses of the curve shows that the line is linear to a benzoate flavin ratio of 0.5, indicating very tight binding of benzoate; at higher ratios the curve starts to deviate from a rectangular hyperbola type of saturation curve.

Fig. 12. Determination of the composition of the complex between the enzyme and benzoate by Job's method (1928) of continuous variation. • - •, enzyme dialysed 7 times against 5 l of 0.05 M phosphate buffer (pH 6.3) followed by dialysis against 200 μM FAD, precipitated with solid (NH$_4$)$_2$SO$_4$ and washed twice with 50% saturated aq. (NH$_4$)$_2$SO$_4$ in 0.1 M pyrophosphate buffer (pH 8.3). The sum of the concentrations of enzyme and benzoate was 73 μM. The medium was 0.1 M pyrophosphate buffer (pH 8.3). $\triangle - \triangle$, the enzyme made benzoate-free by excess of D-alanine according to Yagi and Ozawa (1963). Sum of enzyme and benzoate concentration is 65 μM in 0.1 M pyrophosphate buffer (pH 8.3). $x_B$ represents the mol fraction of benzoate.
Another attempt to determine the number of molecules of benzoate bound per molecule of flavin, by using the continuous variation method of Job (1928), is shown in Fig. 12. From the mole fraction of the maximum in this experiment the number of benzoate molecules bound to the enzyme (which contains two molecules of flavin) is between 1 and 2. The same number is found regardless of whether benzoate-free enzyme is prepared by excess of substrate or by prolonged dialysis. A serious limitation of the Job method is that it is only useful if identical complexes are involved. It is known that by dialysis of the holoenzyme, half of the flavin is quickly removed while the other part is hardly removable even by prolonged dialysis (Veezer et al., 1966). This can mean that the two flavin molecules are bound differently one more firmly than the other. It is possible that the same holds for the binding of benzoate: the first molecule of benzoate is firmly bound and is responsible for the linear part of the titration curve and the other less firmly bound and responsible for the deviation, which means negative homotropic interaction. It is clear that in this case, application of the continuous variation method of Job (1928) does not give the exact number of benzoate molecules bound to the protein.

From the kinetic experiments it was concluded that ATP counteracts the benzoate binding. The non-competitive inhibition by ATP at 37° and the small inhibition at 5° indicates that the counteraction of the benzoate binding is not due to binding of the nucleotide at the active centre. When the enzyme is titrated with benzoate, a 2–3 fold concentration of benzoate is needed for 50% saturation in the presence of ATP. It should be mentioned that ATP itself does not induce spectral changes. Fig. 13 illustrates on a logarithmic scale the titration of D-amino-acid oxidase with benzoate in the presence and absence of ATP. Koshland et al. (1966) have introduced the ratio \[ R_a = \frac{(S_{0.9})(S_{0.1})}{(S_{0.5})^2} \] as a measure for the symmetry of the saturation curve around the midpoint (\(S_{0.5} = 50\%\); \(S_{0.9} = 90\%\); \(S_{0.1} = 10\%\) saturation). The \(R_a\) value without ATP is 0.6 and with ATP 2.5. At a value of 1 the curve is symmetrical around the midpoint, so both curves are asymmetrical around the 50% saturation point. Another important conclusion from the titration curves is the strong cooperative binding as derived from the relation \(S_{0.9}/S_{0.1} = 81 \times S_{0.5}\) for a true Michaelis-Menten saturation curve (Koshland et al., 1966). In case of cooperation a value smaller than 81 is found. (In the presence of ATP 4 and in the absence 1). The presence of ATP diminishes this cooperation some extent.

By using a somewhat modified equation of Stockwell (1959) it is possible to determine the number of molecules bound per molecule of protein:

\[
\frac{[B]}{\Delta A/\Delta A_S} = \frac{K}{1 - \Delta A/\Delta A_S} + [E_0]n
\]

in which \([B]\) is the total benzoate concentration, \(\Delta A\) the optical difference observed at that particular benzoate concentration, \(\Delta A_S\) the optical difference at saturation, \([E_0]\) the total enzyme concentration, \(K\) the dissociation constant and
n the number of binding sites. In the plot \( \frac{[B]}{\Delta A/\Delta A_S} \) versus \( \frac{1}{1 - \Delta A/\Delta A_S} \) (Fig. 14) the slope represents the dissociation constant. The results show that the first part of the line has a somewhat smaller slope than the second part, indicating that the first molecule of benzoate bound to the enzyme has a smaller dissociation constant than the second molecule of benzoate. Extrapolation of the second part of the line gives for the number of binding sites \( n \) a value of 1.8 molecules of benzoate per molecule protein, in good agreement with the number of flavin molecules bound to the enzyme. The results obtained in the presence of ATP indicate that the negative cooperative interactions in the benzoate binding process are amplified by this nucleotide.

In order to obtain more data about the complex formation with benzoate in relation to the temperature-induced allosteric behaviour of the enzyme, the rate of formation of the spectral complex at 497.5 nm was followed by the stopped-flow method. Fig. 15 shows the spectral changes at 497.5 nm after rapid mixing of benzoic acid and the enzyme at 5 °C. From this plot a first-order reaction curve was calculated as shown in Fig. 16. According to the results, the binding of benzoate shows an initial rapid phase in which the time (t₁/₂) necessary for 50% of the reaction to be completed, is dependent on the benzoate concen-
Fig. 14. Evaluation of spectrophotometric titration data according to Stockwell (1959). For conditions see Fig. 13. The values on the ordinate and abscissa are explained in the text. + -- +, no ATP; O -- O, in the presence of 20 mM.

Fig. 15. The rate of formation of the enzyme-benzoate complex by mixing benzoate-free enzyme (19.5 μM enzyme-flavin) with different benzoate concentrations in 0.1 M pyrophosphate (pH 8.3) at 5°C. The changes in absorbance (Δ A₄₉₇.₅ nm) were followed by the stopped-flow method. • -- •, 5 μM benzoate; O -- O, 10 μM benzoate; Δ -- Δ, 20 μM benzoate and □ -- □, 30 μM benzoate. All concentrations after mixing. Lightpath 2 cm.
FIG. 16. The first-order reaction plot of the results shown in Fig. 15. O – O, 5 μM benzoate; x – x, 10 μM benzoate; △ – △, 20 μM benzoate and □ – □, 30 μM benzoate.

Fig. 18A shows the influence of temperature with fixed concentrations of enzyme (20 μM enzyme-flavin) and benzoate (30 μM). As a measure of velocity is taken the reciprocal of $t_{1/2}$ of the total reaction. A plot of the logarithms of these velocities against the corresponding reciprocal absolute temperatures is shown in Fig. 18B. The Arrhenius plot obtained is discontinuous. The break can be explained as due to the first-order reaction, which becomes more important
FIG. 17. The first-order reaction plot of the rate of formation of the enzyme-benzoate complex at 19.5°. Further conditions as in Fig. 15. x – x, 5 μM benzoate; • – •, 10 μM benzoate; △ – △, 20 μM benzoate and □ – □, 30 μM benzoate. [Enzyme-flavin] = 20 μM. All concentrations after mixing.

FIG. 18A. The effect of temperature on the change of absorbance at 497.5 nm on mixing 20 μM enzyme-flavin with 30 μM benzoate in 0.1 M pyrophosphate buffer (pH 8.3), obtained by the stopped-flow method. • – •, 4.4°; △ – △, 6.2°; □ – □, 9.9°; x – x, 14° and ○ – ○, 19.5°. For reasons of clarity the intermediate temperatures were left away. The concentrations are after mixing. Lightpath 2 cm.

B. The Arrhenius plot from the results shown in Fig. 18A. As a measure of velocity is taken the reciprocal of the half-time of the reaction. The points indicated by + are the logarithms of the velocity of the rapid phase.
Table III. The amount of R-form present at the indicated temperatures. The amount is calculated by extrapolation of the first-order plot and by assuming that the rapid phase represents the R-form and the first-order reaction the conversion of the T- to the R-form. The values are calculated relative to the total amount of enzyme-benzoate complex formed in the presence of 30 μM benzoate.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>% R-form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>50%</td>
</tr>
<tr>
<td>6.6</td>
<td>60%</td>
</tr>
<tr>
<td>8.1</td>
<td>70%</td>
</tr>
</tbody>
</table>

at decreasing temperatures, resulting in a slower overall reaction. This conclusion is supported by the fact that if the velocities of the rapid phase at low temperatures is taken as the velocity of the overall reaction, a linear Arrhenius plot is obtained.

Table III shows the amount of the R-form present at the different temperatures. These percentages are calculated by extrapolation of the first-order plot at different temperatures. Although these data are far from accurate, the tendency of an increase in the amount of R-form at increasing temperatures is evident. On the other hand, the amount of R-form present is considerably larger than would be expected from the Arrhenius plots of Fig. 8 and 9 and is independent of the benzoate concentration. This can only be due to the fact that benzoate does not bind exclusively to the R-form, but reacts with the T-form at a similar rate. The difference in dissociation constant $K^{R_D} < K^{T_D}$ leads to a change in equilibrium between the R- and T-forms, resulting in the first-order reaction. This assumption also explains why no S-shaped saturation curve is found in the presence or absence of ATP at 5°C.

4.2.5. General discussion

The phenomenon of non-linear Arrhenius plots is well known in enzymology. Massey et al. (1966) have given a list of enzymes showing these effects. Many explanations have been given, the majority rejected on theoretical grounds (cf. Kistiaikowsky and Lumry, 1949; Levy et al., 1962). The recent explanations deal with the existence of different protein conformations, either each with similar activity, but differing in energy of activation (Massey et al., 1966; Dixon and Webb, 1964), or with different activities (Kistiaikowsky and Lumry 1949; Levy et al., 1962).

The results presented here strongly supports the latter idea. They show that a non-linear Arrhenius plot is to be expected in case the enzyme can undergo a reversible temperature-dependent transition between two conformations with the same activation energies (e.g., the same active complex) but differing in the probabilities of the transitions. Such a mechanism can only be explained in terms of conformational changes around the active site of the enzyme (cf. Levy et al., 1962). The results show clearly that cooperative effects (Monod et al., 1965) are observable in such case, which leads to the question whether similar phenomena occur in other so-called allosteric enzymes. This is especially
important in relation to very complex enzymes like glutamate dehydrogenase. Although explanations have been given for the marked substrate inhibition by the assumption of multiple binding sites for NADH (Frieden, 1963), the results presented here show that other explanations are possible (cf. Eisenkraft and Veejer, 1968). Furthermore, it must be considered that in two-substrate reactions, in the absence or presence of modifiers, all reagents can have different affinities for two or more conformations. This leads to a role of one substrate as an allosteric effector on the kinetic parameters of the second substrate. Variation of the concentrations of both substrates can lead to apparent contradictions. All these phenomena will be dependent on the kinetic mechanism, e.g. the case of either a modified enzyme mechanism, an ordered or a random mechanism.

These and other studies (Kirschner et al., 1966) show the importance of varying the temperature in testing involvement of different conformations. Furthermore, the results show that it is possible to observe two breaks, which according to these hypotheses are to be expected. Even the observation of a linear Arrhenius plot does not allow the conclusion that one conformation is involved. The results also provide an explanation for the exchange in the Arrhenius plot upon the addition of effectors to the enzyme-catalysed reaction (Kistiakowsky and Lumry, 1949; Levy et al., 1962).

It is quite clear from these data that the slope in the Arrhenius plot does not always represent the activation energy of the reaction. For determining the activation energy, the largest possible temperature range should be used, because by using a limited temperature range there is a possibility, even upon finding a straight-line relationship, that one is dealing with a transition between two conformations. In the transition range the slope represents, in addition to the activation energy, the change in activation entropy of the reaction due to the conversion of the low-temperature conformation into the high-temperature one, both having different activities. In fact it is possible to distinguish two cases:

1. The high-temperature form has a higher activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a larger slope than the parts of the plot representing either the pure high-temperature or the pure low-temperature conformation. Examples are the presented here.

2. The high-temperature form has a lower activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a smaller slope than the parts of the plot representing either the pure high-temperature or pure low-temperature conformation. An example might be the results obtained with fumarase (cf. Dixon and Webb, 1964).

Whether these ideas are applicable to those with other enzymes is an open question. However, many of the interpretations in enzyme catalysis based on activation energies have become doubtful in the light of these results and this interpretation.

Although a more physical explanation has been given for cooperative binding phenomena (Koshland et al., 1966) in terms of the relation between sequential changes and ligand binding, the interpretations presented here are more
related to the concerted transition model of MONOD et al. (1965). However, it must be emphasized that an involvement of the induced-fit mechanism cannot be ruled out by these experiments.

It is difficult to compare the kinetic results with the results obtained from the binding studies of benzoate to the enzyme D-amino-acid oxidase. The major difference between these different types of experiments is the enzyme concentration (in the kinetic experiments the concentration is 1/50 of the concentration used in the binding experiments). Although the occurrence of the first-order reaction strongly supports the model of MONOD et al. (1965), other possibilities cannot be excluded completely. It should be mentioned that in the stopped-flow experiments the enzyme undergoes a rapid two fold-dilution (from about 2 mg/ml to 1 mg/ml). This dilution increases the free flavin concentration by about 3%, which is negligible. From the study of ANTONINI et al. (1966) it is known that D-amino-acid oxidase polymerizes at increasing protein concentration, while the enzyme-benzoate complex does so to a lesser extent. From this work it can be seen that at a concentration of 2 mg/ml the degrees of polymerization of the holoenzyme and enzyme-benzoate complex are different, but that a concentration of 1 mg/ml protein practically no difference exists. These data are valid for a temperature of 20°. Remarkable is the Z-shaped curve in the plot representing the effect of temperature on the association-dissociation behaviour of the holoenzyme and enzyme-benzoate complex (ANTONINI et al., 1966). The holoenzyme polymerizes more than the enzyme-benzoate complex at increasing temperature. If the second reaction is due to a depolymerization, occurring upon dilution, this reaction should become more important at higher temperatures. However, the disappearance of the first-order reaction at increasing temperatures, shows that the polymerization decreases at increasing temperatures. This is in contrast to the results of ANTONINI et al. (1966). The most likely explanation for the first-order reaction is found in the model of MONOD et al. (1965).

4.2.6. Temperature-dependent conformational changes of L-amino-acid oxidase and the effect of pyrophosphate and ATP

Comparison of the kinetic properties of L-amino-acid oxidase with those of D-amino-acid oxidase (cf. MASSEY and CURTI, 1967) indicates the possibility that the large substrate inhibition observed with L-leucine as substrate and the fact that hardly any substrate inhibition is found with L-valine (cf. WELLNER and MEISTER, 1961) might be due to an equilibrium between two forms of the enzyme. Similarly these two forms might have different maximum rates and different affinities for the substrate. From the large inhibition with L-leucine, it could thus be concluded that this substrate must have a higher affinity for the less active low-temperature form; therefore L-valine must have about equal affinities for both forms.

The application of the interpretations of the results obtained with D-amino-acid oxidase to the Arrhenius plots of L-amino-acid oxidase provides evidence that the latter enzyme also exists in two temperature-dependent conformations.
with different activities. In Fig. 19 where the Arrhenius plot with L-leucine as substrate is presented, a straight line is obtained. In the presence of 6.7 mM pyrophosphate (Fig. 20A) the Arrhenius plot changes considerably; the plot becomes Z-shaped by showing two breaks at 26° and 10°. The high-temperature part is parallel to the low-temperature part, the three points at the lowest temperatures being averages of two independent determinations. From this plot it can be concluded that L-amino-acid oxidase exists in two temperature-dependent forms. The appearance of two breaks indicates that pyrophosphate shifts the equilibrium between the two forms towards the high-temperature form. Furthermore, it is clear that L-leucine must have a high affinity to the less active form, the conclusion strengthened by the fact that substrate inhibition occurs over the whole temperature range (5°–45°). On the other hand, at low
TABLE IV. The activation energies $\Delta E$ calculated from the Arrhenius plots of the catalytic reaction of L-amino-acid oxidase with L-leucine and L-valine as substrates and the influence of ATP (20 mM) and pyrophosphate (6.7 mM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Addition</th>
<th>$\Delta E$ (cal mole$^{-1}$)</th>
<th>Temp. range ($^\circ$C)</th>
</tr>
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<tbody>
<tr>
<td>L-Leucine</td>
<td>No</td>
<td>13300</td>
<td>5–45</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>Pyrophosphate</td>
<td>2760</td>
<td>&gt;26</td>
</tr>
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<td></td>
<td></td>
<td>13800</td>
<td>10–26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2760</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-Valine</td>
<td>No</td>
<td>2300</td>
<td>&gt;40</td>
</tr>
<tr>
<td>L-Valine</td>
<td>Pyrophosphate</td>
<td>10600</td>
<td>20–40</td>
</tr>
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<td></td>
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<td>2300</td>
<td>&lt;20</td>
</tr>
<tr>
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<td>2300</td>
<td>&gt;33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11500</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2300</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

temperature in the presence of pyrophosphate there is practically no substrate inhibition. The activation energy calculated from the plot of Fig. 19 is about the same as the activation energy calculated from the transition range of Fig. 20A in the presence of pyrophosphate (Table IV).

This indicates that the linear plot in the absence of pyrophosphate represents the transition trajectory. These results are a warning against the conclusion that a linear Arrhenius plot is the reflection of one enzyme conformation.

With D-amino-acid oxidase the Arrhenius plot was dependent on the substrate used; the same is the case with L-amino-acid oxidase. With L-valine as substrate, in contrast with L-leucine, a discontinuous plot with two breaks (at 40° and 20°) is obtained (Fig. 21A). In the presence of 6.7 mM pyrophosphate just one break occurs at 33°, the second one at low temperature has disappeared.

![Fig. 21A](image)

**Fig. 21A.** The Arrhenius plot of L-amino-acid oxidase with L-valine as substrate in the absence and presence of pyrophosphate. Conditions as in Fig. 19. • – •, no pyrophosphate; O – O, with 6.7 mM pyrophosphate (pH 7.4). $V_{max}$ was obtained as described in Fig. 8A.

**B.** Van 't Hoff plot of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase. The equilibrium constant $L$ was calculated as described in this Chapter.
FIG. 22A. Effect of ATP on the Arrhenius plot of L-amino-acid oxidase with L-valine as substrate. Conditions as in Fig. 19. • - •, no ATP; x-x, with 20 mM ATP. $V_{\text{max}}$ was obtained as described in Fig. 8A.

B. Van 't Hoff plots of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase in the presence and absence of ATP. The equilibrium constant $L$ was calculated as described in this Chapter. • - •, no ATP; x-x, [ATP] = 20 mM.

Again the shift of the break towards a lower temperature is an indication that pyrophosphate shifts the equilibrium towards the high-temperature form (cf. L-leucine with 6.7 mM pyrophosphate).

With D-amino-acid oxidase ATP has a higher affinity to the low-temperature form; in the case of L-amino-acid oxidase ATP shifts the equilibrium slightly to the high-temperature form with little influence on the temperature of the break (Fig. 22A). Table IV summarizes the values of the activation energies for the different temperature ranges with L-leucine and L-valine as substrates in the presence and absence of pyrophosphate and ATP. The enzyme used in the experiments of Figs. 21A and 22A are from different preparations; every experimental point is the average of two independent determinations.

In the same way as has been done with D-amino-acid oxidase, the enthalpy and entropy changes of the conformational transition can be calculated from Figs. 20B, 21B and 22B. For L-leucine in the presence of 6.7 mM pyrophosphate the value is 46000 cal mole$^{-1}$; L-valine alone 47000 cal mole$^{-1}$ and L-valine with 20 mM ATP 46000 cal mole$^{-1}$; $\Delta S$ varies from 140-150 e.u. From these values it seems that the $\Delta H$ is rather independent of the substrate and additions made, indicating that the differences between the two protein conformations are the main factor involved. Another conclusion is that conformational changes which occur upon substrate binding induce about the same changes in thermodynamic parameters of both the low- and high-temperature forms.

From the differences in rates the difference in entropy of activation for both forms under each condition is calculated; $\Delta S^* = 2.3$ e.u. for L-leucine with 6.7 mM pyrophosphate; $\Delta S^* = 1.8$ e.u. for L-valine and for L-valine with 20mM ATP $\Delta S^* = 2.1$ e.u.

The great difference between the two amino-acid oxidases is the substrate inhibition, occurring with L-amino-acid oxidase. In the literature different explanations are given for this inhibition (WELLNER and MEISTER, 1961; MASSEY and CURTI, 1967). It was assumed by WELLNER and MEISTER (1961) that
the half reduced enzyme is reoxidised faster than the total reduced enzyme. This explanation was ruled out by the experiments of Massey and Curti (1967). They proved that the reoxidation of the total reduced enzyme cannot be the rate-limiting step in the catalytic overall reaction and showed that the reaction mechanism was quite similar to that of D-amino-acid oxidase. (cf. Chapter 3). To explain the inhibition they assumed the formation of a complex between the fully reduced enzyme and the substrate. This complex should be unreactive or at least react slowly with oxygen. Our explanation of the substrate inhibition is similar to the explanation of the substrate inhibition occurring with D-amino-acid oxidase with D-methionine as substrate. The results shown above indicate that the behaviour of the two oxidases is basically the same in spite of the apparent differences.
5. ON THE MECHANISM OF PHOTOCHEMICAL REDUCTIONS OF FAD AND FLAVOPROTEINS

5.1. INTRODUCTION

The photochemical reduction of free flavins by numerous potential electron donors has been listed by Beinert (1960) and several of the amine type have been studied by Frisell et al. (1959), but the rates at which flavin coenzymes were reduced by the more efficient photoreductants were not reported. Massey and Palmer (1966) demonstrated that several flavoproteins could be photoreduced with EDTA, but other potential photoreductants were not examined.

This study was made to ascertain the rates and relative efficiencies for photoreduction of flavins and flavoproteins, especially FAD systems. Moreover, the effects which free coenzyme, denaturation, and inhibitor have upon the photoreduction rates of flavoproteins have been examined.

5.2. RESULTS

The rates for photoreduction of FMN and especially FAD alone are quite slow and require several hours of exposure for completion under the conditions employed herein. This process primarily involved an intramolecular abstraction of hydrogen, usually from position 2' of the ribityl chain (Yang and McCormick, 1965). The slower rate for photoreduction of FAD, compared to FMN or riboflavin, reflects the quenching effect of the adenine portion which decreases the photodynamic action of the flavin either upon itself (Bessy et al., 1949) or with other oxidizable compounds (Frisell et al., 1959). When oxygen is readmitted to pure photobleached flavin, extensive cleavage of the side chain occurs and is reflected by the incomplete restoration of absorbance at 450 nm and considerable increase in the near ultraviolet due to the formation of lumichrome. However, if the photoreduction of flavin is accomplished by including excess of a compound such as EDTA which is particularly susceptible to photooxidation with flavin (Merkel and Nickerson, 1954), the rate of the reduction is much more rapid, as shown by the spectra in Fig. 1. Photoreduction of the flavin goes to the hydroquinone level and is approximately four times faster with FMN (or riboflavin) than with FAD. The photoreduction of FAD with EDTA in 6 M urea is faster and more nearly like FMN with this photoreductant. Oxygenation of these rapidly photoreduced flavin solutions gives spectra essentially identical to those obtained before light exposure and thereby indicates that no significant photodecomposition of the flavins has occurred.

The rates for photoreduction of FAD with several different types of photoreductants are given by the data in Table I. It is known that EDTA gives glyoxylic acid during flavin reduction (Enns and Burgess, 1965). Some of these compounds, i.e. EDTA and dimethylglycine, have been shown to yield formaldehyde during their photooxidation in the presence of O2 (Frisell et al., 1959). Also EDTA probably decomposes to an aldehyde plus CO2 and the
FIG. 1. Spectra of FMN and FAD during photoreduction with EDTA. Before removal of O₂, 4 ml of solution contained 25 μM flavin and 25 mM EDTA in 0.1 M sodium pyrophosphate buffer at pH 8.3. After removal of O₂, the slightly more concentrated solution was illuminated at 450 nm for the minutes indicated at 5°C.

fragmented amine. The good efficiency of methionine is due mainly to methional (ENNS and BURGESS, 1965), since a primary amine, e.g. glycine, is a very poor photoreductant. The degradation products from nicotine are not known. The sensitivity of dimethylaminopropanol and triethylamine to the difference between pH 7 and 8 reflects the necessity for function of the non-protonated tertiary amine which is present in sufficient amounts only at the higher pH.

Spectra obtained during photoreduction of different flavoproteins are ex-

<table>
<thead>
<tr>
<th>Photoreductant</th>
<th>Time for half reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1</td>
</tr>
<tr>
<td>EDTTP</td>
<td>2</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3</td>
</tr>
<tr>
<td>Nicotine</td>
<td>4</td>
</tr>
<tr>
<td>Dimethylaminopropanol</td>
<td>5</td>
</tr>
<tr>
<td>Dimethylglycine ethyl ester</td>
<td>7</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>60a</td>
</tr>
</tbody>
</table>

a Significant photodecomposition occurs during the prolonged times necessary for photoreduction.

TABLE I. Photoreduction of FAD. Anaerobic solutions contained 25μM FAD and 25 mM photoreductant in 0.1 M sodium phosphate (pH 7) or pyrophosphate (pH 8) buffer at 5°C. Illumination was at 450 nm.
Emplified in Fig. 2. As reported for D-amino-acid oxidase with EDTA (Massey and Palmer, 1961), photoreduction of this enzyme at slightly alkaline pH with this or the other photoreductants studied herein proceeds to the semiquinone level, but not beyond. Glucose oxidase, however, cannot be reduced past the semiquinone at pH 10.3, is only very slowly further reduced at pH 8.3, but is much more rapidly and extensively reduced at pH 6. The reoxidised spectra of both these enzymes shows that little denaturation occurs in the time necessary to effect half reduction at 5°. Also in agreement with Massey and Palmer (1966), L-amino-acid oxidase is photoreduced with EDTA at pH 8.3 more slowly than D-amino-acid oxidase and faster than glucose oxidase at this pH. Oxynitrilase is quite slow, and the Shethna flavoprotein does not bleach within hours of exposure under the conditions applied. The presence of any residual O₂ during illumination of the flavoproteins, especially those most slowly photoreduced, leads to a considerable lag period in the photoreduction. Reoxidation with O₂ goes much faster than the reduction.

The rates for photoreduction of D-amino-acid oxidase and glucose oxidase with the several photoreductants employed are given by the data in Table II. In all cases, photoreduction of the flavoproteins proceeds more slowly than with free FAD (cf. Table I). There appears to be a gross correlation between the...
rates of free flavin reduction and the effectiveness of the compounds as photoreductants for D-amino-acid oxidase. EDTA, EDTP, nicotine, and L-methionine are quite effective; dimethylglycine ethyl ester and dimethylaminopropanol are less so, and triethylamine is rather ineffective. The photoreduction of glucose oxidase is always slower than D-amino-acid oxidase and again best with those compounds most effective with the latter enzyme. However, the order for effectiveness is somewhat different.

The rate of photoreduction of D-amino-acid oxidase is markedly enhanced by the addition of catalytic amounts of free flavin. This is illustrated in Fig. 3 where it can be seen that maximum photoreduction to the semiquinoid flavoprotein requires 10 minutes with EDTA alone, but only 2 minutes with FAD or 1 minute with FMN added in amounts approximately equimolar to flavoprotein. Similarly, the rate for photoreduction of L-amino-acid oxidase is about five times faster with added FAD, but the reduction proceeds towards completion. Addition of these flavins to glucose oxidase or oxynitrilase has little or no effect upon their rates of photoreduction. Since FMN does not bind and function well with the amino-acid oxidases (SINGER and KEARNEY, 1950; WELLNER and MEISTER, 1960b), but can serve at least as effectively as FAD in enhancing the rate of photoreduction of these enzymes, it is unlikely that usual binding of the additional flavin at the coenzyme site is prerequisite for photoreduction of these flavoproteins. The main sequence of reactions in these cases must be photoreduction of free flavin by EDTA followed by reduction of flavoprotein by the photoreduced flavin. Whether the free flavin, which directly reduces the flavoprotein, is the half-reduced semiquinone or the fully reduced hydroquinone could not determined, as illumination was done with all reactants together in solution. Reduction of flavins to the hydroquinone level proceeds via the semiquinone which disproportionates rapidly and nearly completely to oxidised and hydroquinone species at slightly alkaline pH (EHRENBERG, 1962) used for the photoreduction of the amino-acid oxidases. Thus, if reduction of flavoprotein is induced by flavin hydroquinone, the free flavin could be separ-

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**TABLE II.** Photoreduction of D-amino-acid oxidase and glucose oxidase. Anaerobic solutions contained 4 to 5 mg of enzyme and 25 mM photoreductant in 0.1 M sodium pyrophosphate buffer (pH 8.3) in 4 ml at 5°.

<table>
<thead>
<tr>
<th>Photoreductant</th>
<th>D-amino-acid oxidase</th>
<th>glucose oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time for half reduction (min)</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>EDTP</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Nicotine</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>8</td>
<td>60</td>
</tr>
<tr>
<td>Dimethylaminopropanol</td>
<td>25</td>
<td>90</td>
</tr>
<tr>
<td>Dimethylglycine ethyl ester</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>30</td>
<td>180</td>
</tr>
</tbody>
</table>
Fig. 3. Rates of photoreduction of D-amino-acid oxidase with EDTA in the presence and absence of FMN or FAD. Before removal of O₂, 4 ml of solution contained 4 mg of enzyme, 25 μM flavin when added and 25 mM EDTA in 0.1 M sodium pyrophosphate buffer at pH 8.3. After removal of O₂, the slightly more concentrated solution was illuminated at 450 nm for the time shown at 5°.

Fig. 4. The influence of photoreduced FAD on D-amino-acid oxidase. Spectra of FAD photoreduced with EDTA (●); after anaerobic addition of D-amino-acid oxidase (▲); and upon reoxidation of the FAD-oxidase mixture (○). Final concentrations of reactants in 4 ml were 25 μM FAD, 25 mM EDTA, and 3 mg oxidase in 0.1 M sodium pyrophosphate buffer (pH 8.3). Temp. 5°.
ately photoreduced with EDTA and then added to the oxidised flavin-enzyme to generate bound flavin semiquinone. The separate addition of oxidised D-amino-acid oxidase, but not the addition of apoenzyme to reduced FAD (Fig. 4) or reduced FMN, leads to formation of a spectrum characteristic of the flavin semiquinone-enzyme. Thus, it seems that the latter is produced by binding the flavin semiquinone produced by disproportionation between the dissociated oxidised enzyme-flavin and the free hydroquinone. However, the direct transfer of 1 equivalent by an aspecifically bound hydroquinone to the oxidised enzyme-flavin cannot be completely excluded. Similar results were obtained with L-amino-acid oxidase.

When D-amino-acid oxidase is photoreduced in 6M urea with EDTA or another photoreductant such as nicotine, the rate becomes the same as for free FAD in the corresponding situation. The effect of urea concentration on the rate of photoreduction of this oxidase is shown in Fig. 5. A relatively sharp transition is seen near 2.5M urea above which the enzyme is more rapidly and extensively photoreduced than at lower concentration of the denaturation solute. A similar but less easily effected change occurs with glucose oxidase in strong urea solutions. The rate of photoreduction of this enzyme in 6M urea

![Figure 5](image)

**Fig. 5.** Effects of urea concentration on the rate of photoreduction of D-amino-acid oxidase with EDTA. Before removal of O₂, 4 ml of solution contained 4 mg of enzyme and 25 mM EDTA with urea as indicated in 0.1 M sodium pyrophosphate buffer at pH 8.3. After removal of O₂, the solution was illuminated at 455 nm for the time required to half reduction of the flavin at 5°.

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with EDTA is shown in Fig. 6 and is 7 to 8 times faster than without the solute. However, this rate is about 4 times slower than the photoreduction of FAD or D-amino-acid oxidase with EDTA in 6 M urea. Oxynitrilase and especially the Shethna flavoprotein remain difficult to photoreduce even in 6 M urea.

The effect of urea in enhancing the rate of photoreduction of D-amino-acid oxidase could be due to disruption of subunit structure and unfolding or to additional uncoiling of the α-helix. If the concentration of urea needed to break the α-helical structure of this protein is greater than that which allows the marked increase in photoreduction rate, the changes in optical rotation at a wavelength characteristic of α-helix should not occur at the lower concentration of urea. The effect of urea on the reduced mean residue rotation of D-amino-acid oxidase is shown in Fig. 7. More than 4 M urea is required to elicit a large change in the values and an approach to that reported for random coil (Fasman, 1963). As concentrations lower than this are quite effective in increasing the rate of photoreduction of the oxidase with EDTA, it would seem that such enhancement may be due to an unfolding of the enzyme with greater exposure to the photoreductant. This is supported by the finding that the $a_o$ value increases at low urea concentrations. The $b_o$ value declines above 4 M urea concentrations.

An increase in temperature does not appear to alter markedly the rates of photoreduction of the flavoproteins, but the more extensive denaturation which occurs upon illumination at higher temperature, particularly with D-amino-acid oxidase, makes the measurements less meaningful.

The inhibitor benzoate has a marked effect on the photoreduction of D-amino-acid oxidase. If the pure oxidase is converted to the benzoate complex and then thoroughly dialysed, the partially benzoated enzyme obtained requires nearly an hour for photoreduction with EDTA to the semiquinone form. This can be contrasted to the 10 minutes required for the pure oxidase. Addition of FAD to the partially benzoated enzyme shortens the photoreduction with EDTA to 5 minutes. This can be contrasted to about 1 to 2 minutes required for pure oxidase plus FAD with this photoreductant. When an amount of benzoate is added sufficient to convert the enzyme into the enzyme-benzoate complex, no significant photoreduction is seen after two hours. Also a large excess of benzoate considerably lengthens the time for photoreduction of L-amino-acid oxidase at pH 8.3 and glucose oxidase at pH 6.

5.3. DISCUSSION

The reduction of free flavins upon illumination with readily oxidizable compounds proceeds rather quickly, but the similar treatment of flavin which is bound into enzyme results in a slower photoreduction. The lag periods observed when small amounts of O$_2$ are present during photoreduction also emphasize the difference in rates of photoreduction of free flavin versus flavoprotein. The rate of photoreduction of flavin is much slower than its rate of reoxidation by O$_2$. This means that almost all the flavin will be in the oxidised or quinone form as long as traces of O$_2$ are present. The lag period is then de-
terminated by the rate of photoreduction. Since this rate is slower with the flavoprotein than with the free flavin, the lag period in the former is extended.

Photoreduction of free flavins goes via rapid dismutation of the semiquinone to the hydroquinone, but the oxidation-reduction stage achieved with a flavoprotein depends upon the additional stabilization conferred by the protein. As examples, the 2 FAD molecules of D-amino-acid oxidase (Massey et al., 1961) are photoreduced only to the semiquinone level, whereas the 2 in L-amino-acid oxidase (Wellner and Meister, 1960a) can be fully reduced.

The marked stimulation of the rate of photoreduction of both D- and L-amino-acid oxidases by catalytic amounts of external flavin can be readily explained in the former by the presence of an easily dissociable flavin. This has already been indicated by the facile removal of one but not both FAD's upon prolonged dialysis (Veejer et al., 1966). The dissociable flavin is in equilibrium with the added flavin which is rapidly photoreduced. The finding that added FMN is even more effective than added FAD follows from the faster rate of photoreduction of the former and indicates that most of the flavin is reduced when free rather than reassociated with this oxidase which binds FMN much less strongly than its natural coenzyme (Singer and Kearney, 1950). Since the spectrum of photoreduced D-amino-acid oxidase shows that both FAD's are in a semiquinoid form, a subsequent reaction must occur the entering reduced FAD and the oxidised one which tightly bound. Although FAD of L-amino-acid oxidase is not readily removed by dialysis, an explanation for its FAD-enhanced photoreduction may also be found in the small but significant dissociation of coenzyme from this flavoprotein. As expected, the slower photoreduction of glucose oxidase, wherein the 2 FAD's are tightly bound (Keilin and Hartree, 1948), is not enhanced by the addition of free flavin. The same lack of stimulation is found with oxynitrilase which bears 1 tightly bound FAD per molecule of enzyme (Becker, 1964). In such cases as these where extraneous FAD has little or no effect, photoreduction of the flavin must happen predominantly in situ, namely to the flavoprotein per se. Although the direct transfer of 1 equivalent by an aspecifically bound hydroquinone to the oxidised enzyme-flavin cannot be completely excluded, it seems that the flavin semiquinone-enzyme is produced by binding the flavin semiquinone produced by disproportionation between dissociated oxidised enzyme-flavin and the free hydroquinone.

The different efficiencies for the several photoreductants with free FAD, D-amino-acid oxidase, and glucose oxidase also points to the role of protein and is especially sizeable in the case of the relatively tight binding conferred by glucose oxidase. With this last-named enzyme, not only are the rates for photoreduction much slower than with free FAD, but also the order for the rates of individual photoreductants is changed in some cases. For instance, the greater efficacy of nicotine compared to EDTA and especially EDTP with glucose oxidase suggests some repulsion of the latter two compounds from the catalytic site of the protein. This may be due to the four negative charges on EDTA and EDTP as contrasted to the positive quaternary nitrogen of nicotine.

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The rates for photoreduction of flavoproteins in strong urea solutions affords another index as to how buried the flavin is within the protein. Urea, a well-known helix-breaking solute, seems to enhance the rate for photoreduction of a flavoprotein in rough proportion to the ease of its photoreduction in absence of the denaturing reagent. Thus, the FAD of D-amino-acid oxidase is quickly photoreduced when the enzyme is in 6 M urea; that of glucose oxidase is less affected, and the Shethna flavoprotein is remarkably stable. From the fact that the \( a_0 \) value already changes at low urea concentration, while the \( b_0 \) value declines above 4M urea, it seems possible that the enhancement of the photoreduction is rather due to unfolding of the enzyme than to \( \alpha \)-helix breaking.

At least with D-amino-acid oxidase, the effects of temperature denaturation are somewhat opposite to those caused by urea. This oxidase tends to coagulate upon heating and thus does not further expose the FAD to the photoreductant. Also a change in temperature has but a small effect on the photochemical process which has a \( Q_{10} \) considerably less than 2 (FRISSELL et al., 1959).

The prevention by benzoate of the photoreduction of D-amino-acid oxidase parallels the protection from heat denaturation caused by complexing with this competitive inhibitor (BURTON, 1951) which is used in stabilizing the enzyme during purification (MASSEY et al., 1961). Both effects seem to point to a tighter configuration of the enzyme-benzoate complex wherein the FAD's are less exposed to solvent and, hence, photoreductants. Similarly, the impedance of L-amino-acid oxidase photoreduction by benzoate must relate to the complexing of this enzyme with benzoate (VEEGER et al., 1966) which also protects L-amino-acid oxidase from alkaline denaturation (PAIK and KIM, 1967). Decrease in catalytic activities of these enzymes with high concentrations of benzoate as well as the decrease in rates of photoreductions must be explained by reaction of flavin, substrate, benzoate, and photoreductant at the same general site of the respective flavoprotein.
SUMMARY

In Chapter 3 it is shown that upon varying the oxygen concentration, a series of parallel lines is obtained in the plot of the reciprocal velocity of the reaction catalysed by D-amino-acid oxidase versus the reciprocal D-alanine concentration. In the presence of a competitive inhibitor, however, these lines do converge to a point in the third quadrant. It is concluded that the substrate-reduced enzyme is reoxidised before the product dissociates from the enzyme. This is supported by the fact that the product of the reaction is a competitive inhibitor towards the substrate. Several relations between the rate constants are derived and for a limiting case it is possible to calculate some rate constants.

In Chapter 4 it is shown that in the catalytic reaction at 37° of D- and L-amino-acid oxidases with D-alanine and L-leucine, respectively, as substrate, benzoate and its derivatives act as competitive inhibitors. The Hammett plots show no straight-line relationship between the logarithm of the $K_i$ and the $\sigma$ values of the different substituents. At 10° with D-amino-acid oxidase in the $1/v$ versus $1/[D$-alanine] plot, the lines obtained in the presence and in the absence of inhibitor (benzoate or its derivatives, with the exception of the nitro-substituents), intercept at finite [D-alanine]. This is also the case with D-methionine as substrate. At 25° with D-methionine substrate inhibition is found at relatively high concentrations, which is not observed with D-alanine. This substrate inhibition can be abolished by benzoate and ATP, but not by m- and p-nitrobenzoate. ATP acts as noncompetitive inhibitor, but only inhibits the reaction partially. At 37° the inhibition of ATP is abolished by benzoate. Titration of D-amino-acid oxidase with benzoate showed that, to obtain 50% saturation, 2–3 times more benzoate is necessary in the presence of an excess of ATP than in its absence.

For D-amino-acid oxidase it is concluded that this enzyme exists in two forms, a low-temperature and a high-temperature conformation, which have about the same activation energies, but differ in activity, due to different transition probabilities ($\Delta S^* = 0.7$ e.u.). $\Delta H$ for this conformational change is 55000 cal.mole$^{-1}$; $\Delta S = 185$ e.u. with D-methionine as substrate. With D-alanine these values are respectively 67500 cal.mole$^{-1}$ and 240 e.u. It can be shown that benzoate and its derivatives have more affinity for the high-temperature conformation, while ATP has more affinity for the low-temperature form; m- and p-nitrobenzoate have equal affinity for both forms.

Furthermore, the formation of the complex between benzoic acid and D-amino-acid oxidase is studied. At low temperature the initial rapid binding of benzoate to the enzyme is followed by a much slower first-order reaction. This reaction has disappeared above a certain temperature ($t > 11^\circ$).

The general phenomenon of non-linear Arrhenius plots of enzyme-catalyzed reaction is discussed in connection with the obtained results.

With L-amino-acid oxidase it is found that ATP and pyrophosphate influence the catalytic oxidation reaction. The linear Arrhenius plot obtained with
L-leucine as substrate is converted by the addition of pyrophosphate into a Z-shaped curve. The substrate inhibition in the presence of L-leucine is abolished by pyrophosphate at low temperature. In the presence of L-valine as substrate a Z-shaped curve is obtained, of which, as in the case of L-leucine, the high-temperature and low-temperature parts are parallel. In the presence of pyrophosphate the curve shows one break. As in the case with D-amino-acid oxidase, this enzyme also exists in two conformations with different activities and entropies of activation ($\Delta S^* = 1.8 - 2.3$ e.u.), but with the same activation energies. From the transition it can be calculated that $\Delta H = 46000$ cal. mole$^{-1}$ and $\Delta S = 145$ e.u. for the conformational transition from the low- to the high-temperature form.

In Chapter 5 data are presented on the photoreduction of FAD and flavoproteins. The rate for photoreduction of FMN is faster than FAD which can be effectively photoreduced to the hydroquinone level by: ethylenediaminetetraacetate > ethylenediaminetetrapropionate > L-methionine > nicotine > dimethylaminopropanol > dimethylglycine ethyl ester > triethylamine. The same compounds can be used to photoreduce flavoproteins, the rates for which are: D-amino-acid oxidase > L-amino-acid oxidase > glucose oxidase > oxynitri-lase > Shethna flavoprotein. The oxidation, reduction stage achieved is characteristic of the protein. Addition of free flavin markedly enhances the photoreduction of those flavoproteins which have a readily dissociable FAD. Urea enhances the photoreduction rate of flavoproteins in approximate correspondence to their ease of photoreduction in the absence of this reagent. Increase in temperature does not greatly enhance the rate of photoreduction of these flavoproteins. Benzoate decreases the rate of photoreduction of certain of the oxidases.
SAMENVATTING

In Hoofdstuk 3 wordt het reactiemechanisme van D-aminozuuroxidase besproken. Door de zuurstof concentratie te variëren wordt in de grafiek, waarin is uitgezet de reciprope snelheid van de reactie tegen de reciprope [D-alanine], een serie van parallele lijnen verkregen. Echter toevoeging van een competitieve remmer doet deze lijnen convergeren naar een punt in het derde kwadrant. Er wordt aangetoond dat het complex substraat-gereduceerd enzym eerst terugoxideerd wordt voordat het product van het enzym dissociërt. Dit wordt ondersteund door het feit dat het reactieproduct t.o.v. het substraat zich als competitieve remmer gedraagt. Verscheidene relaties tussen de snelheidsconstanten worden afgeleid en voor een gelimiteerd geval is het mogelijk om enkele snelheidsconstanten te berekenen.

In Hoofdstuk 4 wordt beschreven dat in de katalytische reactie van D- en L-aminozuuroxidase resp. D-alanine en L-leucine als substraat bij 37°, benzoëzuur en de derivaten hiervan zich als competitieve remmers gedragen. De Hammett grafiek geeft geen lineaire relatie te zien tussen de logarithmen van de $K_i$ en de $\sigma$ waarden van de verschillende substituenten. Bij 10° met D-aminozuuroxidase in de $1/v$ versus $1/[D-alanine]$ grafiek, snijden de lijnen verkregen in aan- en afwezigheid van een competitieve remmer elkaar bij eindige D-alanine concentratie. Dit is ook het geval wanneer D-methionine als substraat gebruikt wordt. Bij 25° met D-methionine als substraat wordt bij relatief hoge D-methionine concentraties substraatremming gevonden, dit wordt niet waargenomen als D-alanine gebruikt wordt. Deze substraatremming wordt door benzoëzuur en ATP teniet gedaan, maar niet door m- en p-nitrobenzoëzuur. ATP gedraagt zich als een noncompetitieve remmer, maar remt de reactie slechts gedeeltelijk. Bij 37° doet benzoëzuur de remming van ATP teniet. Titratie van D-aminozuuroxidase met benzoëzuur toont dat om 50% verzadiging te verkrijgen, 2-3 keer zoveel meer benzoëzuur nodig is in de aanwezigheid dan in de afwezigheid van overmaat ATP. Voor D-aminozuuroxidase wordt geconcludeerd dat dit enzym in twee vormen kan voorkomen, een lage- en een hoge-temperatuur conformatie, die ongeveer gelijke activeringsenergie hebben, maar verschillend zijn in activiteit hetgeen veroorzaakt wordt door verschil in overgangswaarschijnlijkheid ($\Delta S^* = 0.7$ e.u.). $\Delta H$ voor deze conformatieovergang is 55000 cal mol$^{-1}$; $\Delta S = 185$ e.u. met D-methionine als substraat. Als D-alanine gebruikt wordt dan bedragen deze waarden resp. 67500 cal mol$^{-1}$ en 240 e.u. Verder wordt aangetoond dat benzoëzuur en de derivaten ervan meer affiniteit hebben tot de hoge- dan tot de lage-temperatuur conformatie; echter m- en p-nitrobenzoëzuur hebben gelijke affiniteit voor beide vormen.

Voorts is de complexvorming tussen benzoëzuur en D-aminozuuroxidase be-studeerd. Bij lage temperatuur wordt de initiële snelle binding van benzoëzuur aan het eiwit gevolgd door een langzamere 1e-orde reactie. Boven een bepaalde temperatuur ($t > 11^\circ$) verdwijnt deze volgreactie.

Ook wordt in dit Hoofdstuk het algemene verschijnsel van niet lineaire rela-
ties in de Arrhenius grafieken van door enzym gekatalyseerde reactie in verband met de verkregen resultaten besproken.

Voor L-aminozuuroxidase wordt waargenomen dat ATP en pyrophosfaat de katalytische oxidaties lagere reactie beïnvloeden. De lineaire Arrhenius grafiek verkregen met L-leucine als substraat verandert door toevoeging van pyrophosfaat in een Z-vormige curve. De substraatremming met L-leucine verdwijnt bij lage temperatuur door toevoeging van pyrophosfaat. Met L-valine als substraat wordt een Z-vormige curve verkregen, waarvan evenals met L-leucine, het hoge- en lage-temperatuur gedeelte parallel lopen. In aanwezigheid van pyrophosfaat wordt slechts één knik waargenomen. Evenals met D-aminozuuroxidase, komt dit enzym ook in twee conformaties voor, met verschillende activiteit en activeringsentropie \((\Delta S^* = 1,8 - 2,3 \text{ e.u.})\) maar gelijke activeringsenergie. Voor de overgang van lage- naar hoge-temperatuur conformatie bedragen \(\Delta H\) en \(\Delta S\) resp. 46000 cal mol\(^{-1}\) en 145 e.u.

In Hoofdstuk 5 wordt de fotoreductie van FAD en flavoproteinen beschreven. Voor FMN is de fotoreductie snelheid groter dan voor FAD. Beide worden effectief tot de gereduceerde vorm gereduceerd door: ethyleendiaminetetraacetetaat > ethyleendiaminonetetrapropionaat > L-methionine > nicotine > dimethylaminopropanol > dimethylglycine ethyl ester > triethylamine. Deze zelfde verbindingen kunnen worden gebruikt om flavoproteinen te reduceren, de snelheden daarvoor zijn: D-aminozuuroxidase > L-aminozuuroxidase > glucose oxidase > oxynitrilase > Shethna flavoprotein. Het bereikte oxidatiedecarboxylering niveau is karakteristiek voor het eiwit. Toevoeging van vrij flavine verhoogt aanmerkelijk de fotoreductie snelheid van die flavoproteinen, waarvan de prosthetische groep dissociëerbaar is. Ureum versnelt de fotoreductie snelheid van de flavoproteinen in ongeveer dezelfde volgorde als dat deze enzymen zonder ureum te fotoreduceren zijn. Verhogen van temperatuur heeft weinig invloed op de snelheid van fotoreductie. Voor sommige flavoproteinen verlaagt benzoëzuur de snelheid van fotoreductie.
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