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CONTENTS

MECHANISM OF ENZYME ACTION—AN APPROACH THROUGH THE STUDY OF SLOW REACTIONS

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CONTENTS

I. Introduction

Michaelis and Menten (18) assumed that in the molecular interaction between an enzyme and a substrate, the enzyme–substrate complex, E·S, is formed in the initial stage of an enzymic reaction:

$$E + S \rightarrow E \cdot S$$

As the result of this union, E·S turns to E·S′.

$$E + S \rightarrow E \cdot S \rightarrow E \cdot S'$$

The unstable intermediate complex then gives rise to the end product, P, and the free enzyme molecule:

$$E \cdot S' \rightarrow E \cdot P \rightarrow E + P$$

Taking into account the reversibility of the enzymic reaction, the minimum number of steps involved in the reaction could be represented as follows:

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

This equation indicates that the enzymic catalysis consists in the mutual conversions of two neighboring intermediates. An approach to the mechanism of enzyme action could be attained by characterizing these intermediates and arranging them to form the dynamic feature of the process of enzyme action. However, the process shown in reaction 1 takes place, in general, within a very short time. Therefore, the lifetimes of the intermediates are very short, and to observe them, a rapid method is naturally required. For this purpose, the rapid flow technique (7) or rapid scanning spectrophotometry (1,2), etc., have been adopted in the past.

The present author suggests an alternative: Lower the velocity of the enzymic reaction sufficiently to observe these intermediates.

In order to decrease the velocity of the enzymic reaction, it may be brought about under conditions that are other than optimum. Or, a substrate which is attacked only slowly by the enzyme may be selected, e.g., D-lactate for D-amino acid oxidase (see below) (47). By these devices, the intermediates in the enzymic reaction may be more easily demonstrated. Furthermore, the enzymic reaction may be interrupted by a suitable method and accumulation of the intermediate complexes may be expected. As a result of these treatments, the author has succeeded in crystallizing an intermediate complex that is referred to as a "Michaelis complex"* (36,44).

* Concerning the definition of Michaelis constant, it has been considered as pointed out by Neilands and Stumpf (*Outline of Enzyme Chemistry*, Wiley, New York, 1958, p. 96) that the E·S postulated in the theory of Michaelis and Menten is not restricted to a single complex but refers to a series of many intermediates from E·S to E·P: $E + S \rightleftharpoons E \cdot S \rightleftharpoons E \cdot S' \rightleftharpoons E \cdot S'' \cdots E \cdot P'' \rightleftharpoons E \cdot P' \rightleftharpoons E \cdot P \rightleftharpoons E + P$. Therefore, any of these intermediates may be designated as a "Michaelis complex." However, this crystallized intermediate may be more adequately named after its complete characterization is achieved.

This review attempts to present a comprehensive introduction of the results obtained in a series of experiments based on the above-mentioned idea: demonstration and characterization of the reaction intermediates by the study of "slow reactions."

Before entering into the concrete description, it may be indicated to the readers why the author adopted D-amino acid oxidase, a flavoprotein, as a tool.

D-Amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] has been characterized by earlier workers (22,27) as being composed of the apoenzyme and the coenzyme, flavin adenine dinucleotide (FAD). These two components are easily split apart and the holoenzyme can be easily reconstructed by mixing the two. As is well known, the coenzyme, FAD, has characteristic physicochemical properties, viz., an absorption spectrum in the visible region (27,29) and yellow fluorescence (56). These characteristics are mainly due to the conjugated double bonds between N(1) and N(10), viz. N(1)=C—C=N(10), in the isoalloxazine moiety of FAD (see page 5).

The absorption spectrum or the fluorescence of FAD could be changed by the bindings occurring near the chromophore or by direct electron transfer to the chromophore. Therefore, the binding or electron transfer to the isoalloxazine moiety could be easily perceived by the change in the absorption or in the fluorescence. In other words, the most substantial actions involved in the enzymic catalysis, i.e., initial intermolecular binding between the enzyme and the substrate and the successive reaction (e.g., the electron transfer from the substrate to the isoalloxazine moiety), could be demonstrated by the change in the absorption spectrum or in the fluorescence. The conjugated system, N(1)—N(10), therefore, is the most important functional part of this enzyme and serves, at the same time, as an "indicator" for the researcher.

It had been generally assumed that the isolation of the Michaelis complex might be difficult because of its short lifetime until its isolation by crystallization was realized in our laboratory. In fact, our efforts had been devoted to isolation of the model of the enzyme–substrate complex or that of the Michaelis complex. To obtain such a model, it is necessary to let the enzyme combine with a substance in which binding sites duplicate exactly those of the true substrate but which is not oxidized by the enzyme. Such a substance could be

called a "substrate substitute." Naturally, the "substrate substitute" acts as a competitive inhibitor. However, it is well known that all competitive inhibitors do not fill the conditions necessary to be "substrate substitute." It is important to stress that "substrate substitute" and "competitive inhibitor" should be distinguished from each other, for they have different meanings.

Benzoic acid had been found to be suitable as such a "substrate substitute" (10,53), and the complex had already been obtained in crystalline form (35). The complex of enzyme and "substrate substitute" had been referred to as the "enzyme–substrate complex model" or, sometimes, as the "artificial Michaelis complex." Recently, L-lactate was also found to be suitable as a "substrate substitute" (52).

Since crystallization of a Michaelis complex and demonstration of E·S were attained as described in later sections, these model complexes must afford information on the question: why does the electron transfer occur in the true E·S, and not in the E·S model? The proper answer to this question will be a key to the approach to the mechanism of enzyme action.

II. Study of the Enzyme–Substrate Complex by a Slow Reaction

Before the demonstration of E·S, efforts were devoted to obtaining the E·S model, and the results on this model complex offered a basis for the study of E·S. The description will follow the process of the development of these studies.

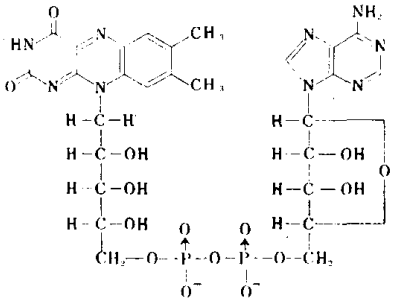
A. FORMATION OF THE TERNARY COMPLEX OF APOENZYME, COENZYME, AND "SUBSTRATE SUBSTITUTE"

Because benzoate inhibits D-amino acid oxidase in competition with substrate (10,53), this substance was considered to be a candidate for "substrate substitute." To examine whether this enzyme–inhibitor complex could be regarded as an E·S model, the elucidation of the bindings involved in this complex was first studied. This study was expected to give some information on the bindings in true E·S as well. From this point of view, bindings in the ternary complex of apoenzyme, coenzyme, and benzoate were examined first by spectroscopy.

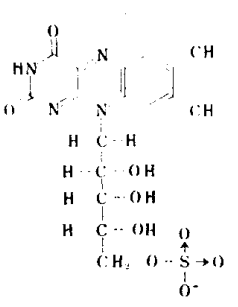
In the ultraviolet region, the following results were obtained (34): When FAD was mixed with the apoenzyme, the peak at 263 mμ

was shifted to 268 mμ and the absorbancy was increased as shown by curve II in Figure 1. When benzoate was further added to this solution, the peak of the spectrum was not shifted; however, the absorbancy was lowered as shown by curve III in Figure 1.

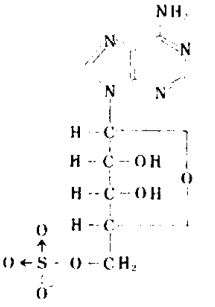
To interpret these phenomena, model substances were adopted, i.e., riboflavin 5′-monosulfate (FMS) (24) and adenosine 5′-monosulfate (AMS) (3), which are specific competitors of the FMN and AMP parts of FAD, respectively (4).



Flavin adenine dinucleotide (FAD)



Riboflavin 5′–monosulfate (FMS)



Adenosine 5′–monosulfate (AMS)

As can be seen in Figure 1, the spectrum of FMS (curve IV) was slightly affected by the addition of the apoenzyme (curve V); the peak was not shifted, and the absorbancy was lowered only a little. Further addition of benzoate caused a more pronounced decrease as shown by curve VI.

On the other hand, the absorption peak of AMS located at 258 mμ (curve VII) was shifted to 263 mμ and the absorbancy decreased by the addition of the apoenzyme as shown by curve VIII. In contrast with the case of FMS, further addition of benzoate did not

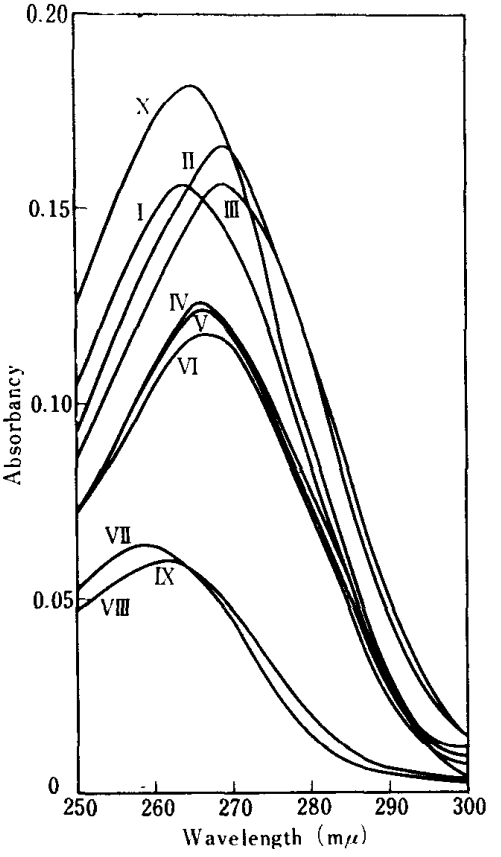


Fig. 1. Absorption spectra of FAD and model substances (FMS and AMS) in the presence of the apoenzyme of D-amino acid oxidase and benzoate in pyrophosphate buffer ($M/60$, pH 8.3). I, FAD ($4.0 \times 10^{-6}M$); II, FAD + apoenzyme ($4.0 \times 10^{-6}M$); III, FAD + apoenzyme + benzoate ($1.0 \times 10^{-4}M$); IV, FMS ($4.0 \times 10^{-6}M$); V, FMS + apoenzyme ($4.0 \times 10^{-6}M$); VI, FMS + apoenzyme + benzoate ($1.0 \times 10^{-4}M$); VII, AMS ($4.0 \times 10^{-6}M$); VIII, AMS + apoenzyme ($4.0 \times 10^{-6}M$); IX, AMS + apoenzyme + benzoate ($1.0 \times 10^{-4}M$); X, sum of spectra of FMN ($4.0 \times 10^{-6}M$) and AMP ($4.0 \times 10^{-6}M$).

cause any further change of the spectrum, as shown by the coincidence of curves VIII and IX.

These results indicate that the apoenzyme readily combines with AMS, and benzoate does not influence the binding between the apoenzyme and AMS, whereas it does influence the interaction between the apoenzyme and FMS.

From the results obtained by the use of FMS and AMS, the effects of the apoenzyme and benzoate on the ultraviolet spectrum of FAD

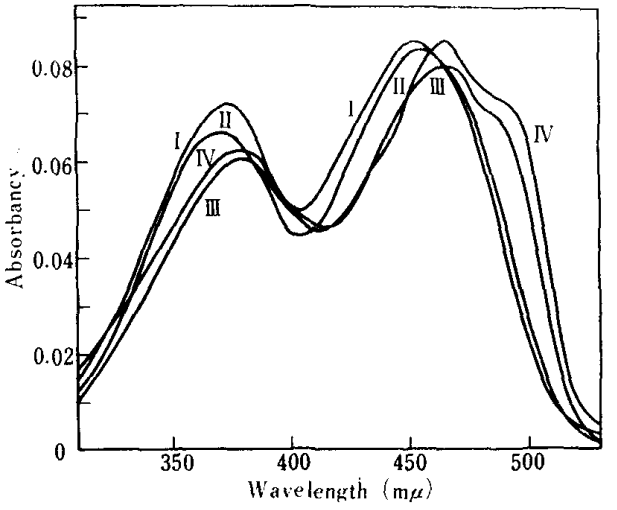


Fig. 2. Absorption spectra of FAD in the presence of the apoenzyme of D-amino acid oxidase and benzoate in pyrophosphate buffer ($M/60$, pH 8.3). I, FAD ($7.6 \times 10^{-6}M$); II, FAD + apoenzyme ($3.8 \times 10^{-6}M$); III, FAD + apoenzyme + benzoate ($1.0 \times 10^{-4}M$); IV, FAD + apoenzyme ($7.6 \times 10^{-6}M$) + benzoate ($1.0 \times 10^{-4}M$).

were interpreted as follows: The shift of the absorbancy of FAD at 263 to 268 mμ by the addition of the apoenzyme could be provoked by the binding with the AMP part of FAD, and the increase in the absorbancy may be explained by considering the intramolecular binding of FAD. According to Weber (28) and Whitby (29), the binding between the isoalloxazine moiety and the adenosine moiety decreases the absorbancy of FAD as compared with a spectrum obtained by summation of the spectra of FMN and AMP (see curve X in Fig. 1). Therefore, the increase of the absorbancy of FAD at 268 mμ upon addition of the apoenzyme may be attributed to the cleavage of the intramolecular binding of FAD by its binding with the apoenzyme. Its decrease to the original level of FAD absorbancy by further addition of benzoate indicates that the binding of benzoate affects the resonance of the FAD to the same level as in the case of its intramolecular complex formation. Since benzoate interacts with the FMN-apoenzyme binding, it is believed that the apoenzyme combines with both the FMN and AMP parts of FAD via bonds 2 and 1 in Figure 4, respectively.

The spectrum at visible wavelengths was then examined. The absorption peak of FAD at 375 mμ was lowered by the apoenzyme (curve

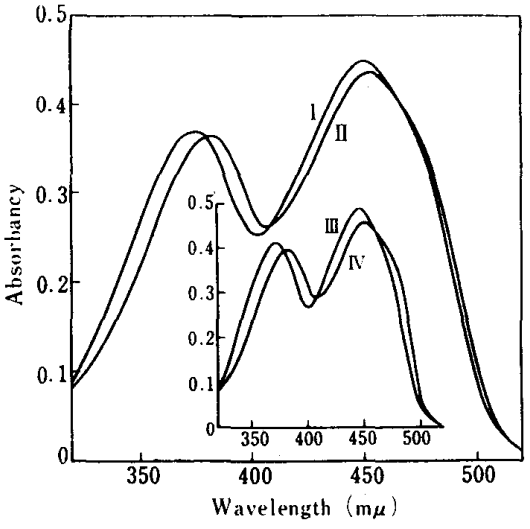


Fig. 3. Absorption spectra of FAD and riboflavin in the presence of excess benzoate. I, FAD (4.0×10^{-5} *M*) in pyrophosphate buffer (*M*/60, pH 8.3); II, FAD + benzoate (1.0*M*); III, riboflavin (4.0×10^{-5} *M*) in pyrophosphate buffer (*M*/60, pH 8.3); IV, riboflavin + benzoate (1.0*M*).

II in Fig. 2) and was shifted from 375 to 380 mμ by further addition of benzoate (curves III and IV in Fig. 2). To clarify the cause of this red shift, the model experiment using FAD– or riboflavin–benzoate mixture was carried out. The changes in visible spectra of flavins are shown in Figure 3, which indicates that the above-mentioned red shift of the 375 mμ peak is due to the binding of the riboflavin part of FAD with benzoate (bond 3 in Fig. 4). Considering the possible charge rearrangements of the isoalloxazine moiety of FAD by light absorption described later (see p. 15), this shift may be interpreted as provoked by the binding between benzoate and 4-CO of the isoalloxazine moiety of FAD. The absorption peak of FAD at 450 mμ was shifted to longer wavelength by the apoenzyme. This shift was also observed in the case of the FAD–phenol complex (32). Such similarity in spectral change may indicate that the isoalloxazine nucleus of FAD probably combines with a phenolic group of tyrosine residue of the protein molecule (bond 2 in Fig. 4). It may be noted that the apoenzyme or phenol fails to affect the 375 mμ peak of FAD.

It may also be noted that the spectral change of FAD provoked by mixing the three components, FAD, apoenzyme, and benzoate, is somewhat larger than the sum of the changes provoked by mixing two

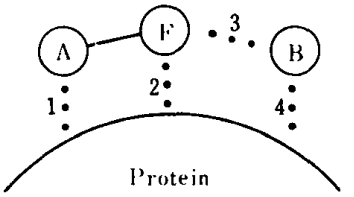


Fig. 4. Scheme of the bindings between the apoenzyme, FAD, and benzoate. The adenine nucleus of FAD is designated by A, the isoalloxazine nucleus of FAD by F, benzoate by B, and the apoenzyme by Protein.

components, i.e., FAD and apoenzyme or FAD and benzoate (compare curve IV in Fig. 2 with curve II in Fig. 2 and curve II in Fig. 3). A marked shoulder at 490 mμ is considered to be characteristic for the mixture of these three components. These spectroscopic observations may indicate that the formation of a ternary complex is brought about by the combination of the apoenzyme, the isoalloxazine moiety of FAD, and benzoate. This is supported by still other evidence: the yellow fluorescence, which is specific for the isoalloxazine nucleus of FAD, is somewhat quenched by combining with the apoenzyme as reported by Walaas et al. (26) and by Yagi et al. (51), but the quenching is further pronounced by the addition of benzoate.

Finally, a complex composed of these three components was isolated in crystalline form as shown in Figure 5 (35). Chemical analyses revealed that the crystalline complex was composed of 2 moles each of FAD and benzoate and 1 mole of the apoenzyme on the molecular basis of 115,000 (see Section IV). When it is dissolved in the buffer, the resulting solution again possesses a characteristic shoulder at 490 mμ as shown in Figure 6, curve I.

When an excess of D-alanine is added to the solution, the yellow color of the enzyme changes to pale yellow, which indicates the reduction of the enzyme (Fig. 6, curve II). This indicates that the benzoate is replaced on the enzyme surface by D-alanine, which reduces the enzyme. In an experiment with a solution containing 1.65×10^{-7} moles of FAD, the enzyme was precipitated by ammonium sulfate at pH 5.1 after the reduction by D-alanine. It was found that the supernatant contained 1.56×10^{-7} moles of benzoate, which indicates that the D-alanine added completely expels the benzoate from the enzyme.

To confirm the meaning of the occurrence of the above-mentioned characteristic shoulder at 490 mμ, the difference spectrum between the

Fig. 5. Crystals of the ternary complex of the apoenzyme, coenzyme, and benzoate, an E·S model (× 1000).

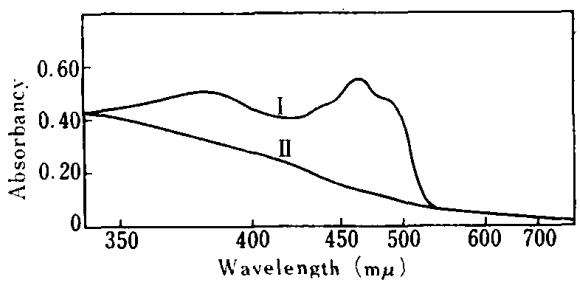


Fig. 6. Absorption spectra of the crystalline ternary complex of apoenzyme, coenzyme, and benzoate, an E·S model, dissolved in 0.05*M* phosphate buffer, pH 6.3. I, oxidized form. II, reduced by adding D-alanine.

ternary complex and the holoenzyme was recorded (41). The positive difference spectrum was found at wavelengths longer than 458 mμ, with a peak at 497.5 mμ. In the presence of sufficient concentration of benzoate and a limiting concentration of FAD, the difference of the absorbancy at 497.5 mμ ($\Delta A_{497.5}$) was found to depend on the concentration of the apoenzyme as shown in Figure 7.

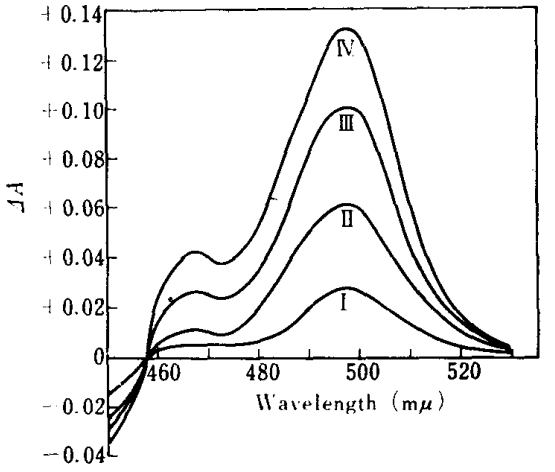


Fig. 7. Difference spectra of the E·S model against the holoenzyme in pyrophosphate buffer ($M/60$, pH 8.3), 25°C. The curves were obtained with solutions of the apoenzyme, FAD (3.4×10^{-5} M), and benzoate (1.0×10^{-4} M) as test samples, solutions of the apoenzyme and FAD (3.4×10^{-5} M) as references. Concentration of the apoenzyme; I, 0.4×10^{-5} M; II, 0.8×10^{-5} M; III, 1.3×10^{-5} M; IV, 2.6×10^{-5} M.

Plots of $\Delta A_{497.5}$ against concentration of the apoenzyme gave a sigmoid curve.

The same positive peak was also observed in the difference spectrum of the mixture of the apoenzyme, coenzyme, and benzoate relative to the holoenzyme in the two cases: (i) a sufficient concentration of benzoate, a limiting concentration of the apoenzyme, and varying concentration of FAD; and (ii) a sufficient concentration of the apoenzyme, a limiting concentration of FAD, and varying concentration of benzoate. In both cases, $\Delta A_{497.5}$ depended on the concentration of the varying component. Plots of $\Delta A_{497.5}$ against FAD concentration or against benzoate concentration also gave a sigmoid curve.

The maximum height of the peak at 497.5 mμ, $(\Delta A_{497.5})_{max}$, increased in direct proportion to the concentration of restricted component, i.e., the concentration of ternary complex formed in the mixture, as shown in Figure 8.

Considering that the appearance of $\Delta A_{497.5}$ is due to perturbations of the chromophore in the isoalloxazine moiety of FAD, it may be concluded that upon formation of the ternary complex, a change takes place in the charge of some ionizable groups near the chromophore

The results suggest the occurrence of hydrogen bonding or charge transfer between the isoalloxazine moiety of the holoenzyme and the protein-bound benzoate (bond 3 in Fig. 4).

B. POSSIBLE BINDINGS IN THE TERNARY COMPLEX OF APOENZYME, COENZYME, AND SUBSTRATE (OR SUBSTRATE SUBSTITUTE)

Considering the above-mentioned results in conjunction with the results by kinetic analyses using competitive inhibitors and chemically modified coenzyme analog (31,33,53) and by fluorimetry (51), possible bindings involved in the ternary complex may be schematically presented (see Fig. 9).

As shown by these schemata, benzoate is considered to be a substrate substitute and the enzyme–benzoate complex to be an E·S model.

Details of the study on the possible bindings involved in the ternary complex have been reviewed (30).

C. DEMONSTRATION OF ENZYME-SUBSTRATE COMPLEX BY A SLOW REACTION

As described above, the most characteristic feature of the absorption spectrum for the ternary complex apoenzyme–coenzyme–benzoate, an E·S model, is a shoulder observed at 490 mμ. Therefore, the appearance

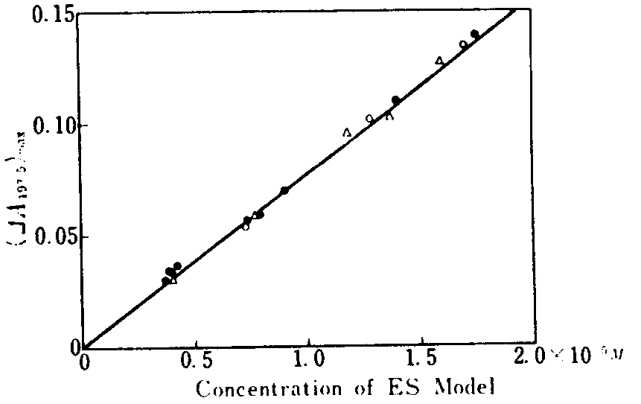


Fig. 8. $(\Delta A_{497.5})_{max}$ plotted against concentration of the E·S model. The E·S model was constituted from a restricted concentration of the component indicated (●, the apoenzyme; O, FAD; Δ, benzoate) and sufficient amounts of other two components ($1 \times 10^{-4} M$).