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MECHANISM OF ACTION OF *p*-HYDROXYBENZOATE HYDROXYLASE FROM *Pseudomonas putida*. III.* THE ENZYME-SUBSTRATE COMPLEX**

BY

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FOOTNOTES

*Paper I of this series is reference 1, and Paper II is reference 2.

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ABSTRACT

The mechanism of action of p-hydroxybenzoate hydroxylase from Pseudomonas putida, strain M-6, has been investigated. The aromatic substrate analogues, benzoate, p-fluorobenzoate, p-chlorobenzoate, p-nitrobenzoate, p-aminobenzoate, and 6-hydroxynicotinate, are found to be competitive inhibitors. This finding differs from the previously reported noncompetitive behavior in a different buffer system.

The optical activity of the enzyme-inhibitor complex is studied. From the kinetic and circular dichroism (CD) measurements, we have found that the carboxyl moiety is necessary and sufficient for the enzyme-substrate binding, whereas the hydroxyl group alone will not lead to binding. There are two classes of inhibitory analogues: one causes changes in CD spectra of the enzyme similar to those evoked by the substrate, and the other does not cause significant changes. The results indicate that more than one mode of enzyme-inhibitor interaction is involved. The CD of the enzyme-NADPH complex under anaerobic conditions suggests that the oxidized enzyme and reduced pyridine nucleotide form a complex, both in the absence and presence of the substrate, p-hydroxybenzoate. Furthermore, evidence for a ternary complex is given.

Introduction

Hydroxylation plays an important role in the oxidative metabolism of aromatic compounds by bacteria. As a consequence, the mechanisms of action of those enzymes that mediate these hydroxylation reactions have stimulated profound interest. Two mono-oxygenases, p-hydroxybenzoate hydroxylase and salicylate hydroxylase, have been purified to homogeneity and investigated actively.[1-4] Both monooxygenases are inducible. They are flavoproteins requiring FAD and reduced pyridine nucleotides as cofactors; however, they differ in specificity. Salicylate hydroxylase is specific for NADH, whereas p-hydroxybenzoate hydroxylase is specific for NADPH.[2] The precise role of the flavin prosthetic group and the mechanism of action of the reduced pyridine nucleotide are not clear. Recently, investigation on the nature of the enzyme-substrate ternary complexes of salicylate hydroxylase by fluorometric method have been reported by Takemori *et al.*[5-7] Nakamura, Higashi and co-workers have reported kinetic studies on the reaction mechanism of p-hydroxybenzoate hydroxylase from *Pseudomonas desmolytica*.[8,9]

To elucidate the mechanism of enzymatic catalysis it is pertinent to study the intermediate enzyme-substrate complexes and also the interactions of various analogues. Hesp *et al.*[2] reported that the CD spectrum of p-hydroxybenzoate hydroxylase was perturbed significantly upon addition of the substrate, p-hydroxybenzoate. CD measurements thus provide a sensitive method of studying the enzyme-substrate complexes. This

report presents the results of a kinetic study which indicates the competitive inhibitory behavior of some of the substituted benzoates, and the circular dichroism (CD) which suggests the interaction of the substrate and the bound FAD.

p-Hydroxybenzoate hydroxylase was induced in Pseudomonas putida. The two subspecies, putida and desmolytica, have functionally the same p-hydroxybenzoate hydroxylases; however, they differ in the subsequent oxidation of protocatechuate. The former uses the ortho cleavage pathway, yielding β-carboxy-cis-cis-muconate;[10] the latter uses meta cleavage, yielding α-hydroxy-γ-carboxy-cis-cis-muconic semialdehyde.[11]

Experimental Procedure

Material. Reagents used routinely for enzyme preparation and assays were the purest grade obtainable and purchased from sources described previously.[1] NADPH (grade II), NADH(Sigma grade), $NADP^+$ (grade III), NAD^+ (grade IV), FAD (grade III),p-fluorobenzoate, and p-aminobenzoate were purchased from Sigma. 6-Hydroxynicotinic acid was obtained from Aldrich. Phenol was from Mallinckrodt Chemical, and p-chlorobenzoate, p-nitrobenzoate were from Eastman Kodak. All the benzoate derivatives and phenol were twice crystallized from water before use. All other reagents were used directly without further purification.

Enzyme preparation. p-Hydroxybenzoate hydroxylase from Pseudomonas putida, strain M-6 (ATCC 17428), was prepared and purified by a modification of the method described in paper I of this series. The method is similar to that reported in paper II. The purified enzyme was stored in a stabilizing mixture at -70°C until use, as described previously.[2] The enzyme was found to denature slowly over a period of one year.

The preservation of the enzyme was improved by storing it under an oxygen-free helium atmosphere.

Enzyme assays. p-Hydroxybenzoate hydroxylase was assayed by spectrophotometric measurement of the substrate-dependent oxidation of NADPH. The procedure has been described earlier.[1] The standard assay system is slightly modified from the previous method. The standard system contained in 3.0 ml, 67 mmoles K_2HPO_4-KH_2PO_4 (pH 7.0), 3.3 μmoles of FAD, 0.13 mmoles of NADPH, 0.67 mmoles of p-hydroxybenzoate, enzyme and deionized water. The unit of enzyme activity is defined as that amount of enzyme which oxidized 1.0 μmoles of NADPH/min under the conditions of the spectrophotometric assay.

Kinetic measurements. The kinetics were studied spectrophotometrically by measuring the substrate-dependent oxidation of NADPH at 340 mμ. Various substrate analogues were incubated for 2 min (or longer) before the measurement. All substrate analogues were adjusted to pH 7.5. All experiments were carried out at 20°C ± 1°C. Each experiment was repeated at least twice until consistent results were obtained.

Spectroscopic method. The absorption spectra were measured in a Cary Model 15 spectrometer. The circular dichroism measurements were obtained with a Cary Model 60 spectropolarimeter with a standard Model 6001 CD accessory attachment. A Cary thermostattable temperature cell compartment was used; the temperature for all experiments was maintained at 2°C ± 0.1°C. The slit width program was set for 15 Å resolution and the time constant and scan speed were adjusted for best signal:noise ratio for each individual experiment. In all cases, optical path length of 1.0 cm was used.

Anaerobic experiment. To efficiently achieve the anaerobic condition without denaturing the enzyme in a cuvette suitable for optical activity measurement and still permitting the addition of various reagents have been found to be difficult. We have used the method described previously.[2] The space above the enzyme solution in the cuvette is first flushed with oxygen-free helium for 30 min. The enzyme solution is then bubbled with O_2-free helium at a rate of 1.5 cc/min for 15 min. If the enzyme is noticed to denature during the assay procedure, the result is discarded. We have been able to achieve strict anaerobic condition with negligible amount of denaturation.

Results

Chemical structure of substrate analogues. Several aromatic compounds which are structurally related to p-hydroxybenzoate, but cannot serve as a substrate, inhibit the enzyme. The benzoic acid derivatives vary in strength according to the nature of the substituent and its ring position. The relative strength of a substituted benzoic acid is characterized by a substituent constant σ. The more electron-attracting a substituent is, the more positive is its σ value (relative to benzoate as zero). Conversely, the more strongly a substituent donates electrons, the more negative is its σ value. These values for the inhibitory benzoates are given in Table I.

Kinetic study. It was reported previously[1] that benzoate and a series of its analogues were inhibitory to the activity of p-hydroxybenzoate hydroxylase. Using Tris-HCl buffer system for assay, the inhibition was found to be non-competitive. However, enzyme activity drops very quickly during the assay in Tris buffer system (pH 8.0).

Accordingly, there was inaccuracy in determining the initial velocity of the reaction. We could overcome this problem by replacing Tris-HCl buffer with a phosphate buffer system (pH 7.5), in which oxidation of NADPH proceeded almost linearly even at very low concentrations of NADPH and enzyme.

By employing the improved assay system, we have found that benzoate, p-fluorobenzoate, p-chlorobenzoate, p-nitrobenzoate, p-aminobenzoate, and 6-hydroxynicotinate are all competitive inhibitors. Figs. 1-6 show the Lineweaver-Burk plots of the enzyme activity in the presence of these inhibitors. All of them are typical competitive inhibitions. The K_i values were determined by the method described by Wilkinson[13] and Cleland.[14] The results are given in Table II.

A Hammett plot of the action of the substituted benzoate derivatives is given in Fig. 7, where the logarithms of the inhibition constants K_i's are plotted against their σ-values. They fit the Hammett equation very well. The "reaction constant", ρ, which is the slope of the Hammett plot, is estimated to be 1.78. The significance of ρ is that it measures the sensitivity of the reaction to the electrical effects of substituents in the meta and para positions. A positive ρ value suggests that the inhibition is favored by increasing the nucleophilic reactivity of the carboxyl group.

Phenol shows no inhibition at concentrations below 0.01 M. Inhibitory effects occur at concentrations greater than 0.01 M, due presumably to nonspecific binding to the enzyme. The results are given in Table II.

The above findings suggest strongly that the carboxyl group is necessary for the substrate analoge to inhibit the enzyme and possibly is involved in the binding at the active site.

Circular dichroism spectra of the enzyme-inhibitor complexes. The CD spectrum of the holoenzyme of p-hydroxybenzoate hydroxylase has been reported previously.[2] The effect of the substrate, p-hydroxybenzoate, on the holoenzyme manifests itself in the visible region of the FAD absorption. The perturbation of the holoenzyme CD spectrum upon addition of p-hydroxybenzoate was given in paper II. The CD spectra of the enzyme-bound FAD holoenzyme) in the absence and presence of the inhibitors and substrate, p-hydroxybenzoate, are given in Figs. 8 and 9. The effect of benzoate on the CD spectrum of p-hydroxybenzoate hydroxylase was described earlier.[2] A large change was observed in the CD spectrum of p-hydroxybenzoate hydroxylase upon addition of benzoate.

p-Fluorobenzoate. The CD spectrum of the enzyme was changed by the addition of 10^{-3} M p-fluorobenzoate. The 367 nm CD was slightly shifted and decreased. The 455 nm band also decreased. Addition of 10^{-3} M p-hydroxybenzoate to the enzyme p-fluorobenzoate system produced the familiar change of the CD spectrum characteristic of the enzyme p-hydroxybenzoate complex.

6-Hydroxynicotinate. The effect on the CD spectrum of the enzyme caused by addition of 10^{-3} M 6-hydroxynicotinate was significant. Both the 367 nm and 455 nm CD bands decreased markedly. When 10^{-3} M p-hydroxybenzoate was added to the system, enhancement of the 455 nm band was observed. The general appearance of the CD spectrum resembled that of the enzyme p-hydroxybenzoate system.

p-Aminobenzoate. In contrast to the effect produced by p-fluorobenzoate, benzoate, and 6-hydroxynicotinate, the addition of 10^{-3} M p-aminobenzoate caused almost no modification of the CD spectrum of the

enzyme except a slight decrease of the shoulder at 340 mμ. Even after the addition of 10^{-3} M p-hydroxybenzoate, no further change was observed. This experiment was repeated with the order of addition of p-aminobenzoate and p-hydroxybenzoate reversed. The same final CD spectrum was obtained. Since p-aminobenzoate did inhibit the enzyme, this may suggest that the interaction of p-aminobenzoate with the enzyme is such that it does not manifest itself in a CD modification. This result indicates the possibility of a different mode of binding between the enzyme and p-aminobenzoate than that between the enzyme and p-fluorobenzoate.

p-Chlorobenzoate and p-Nitrobenzoate. They have been shown to be the weakest inhibitors (Table I). The CD spectra of the substrate analogue-enzyme mixtures are not significantly modified.

Evidence for the direct binding of NADPH to p-hydroxybenzoate hydroxylase

Since CD spectra of p-hydroxybenzoate hydroxylase served as a sensitive means of detecting the formation of the enzyme-substrate complex, we now extend CD measurements for studying the interaction of NADPH with the enzyme.

Under anaerobic condition, produced by flushing the system with helium, the effect of NADPH on the CD spectra of plain p-hydroxybenzoate hydroxylase was examined. Upon addition of NADPH, there were marked changes, as shown in Fig. 10, in the CD spectra of the enzyme. In the presence of NADPH, both the negative and positive CD bands of the plain enzyme (maxima at 455 nm and 367 nm, respectively) are blue shifted. In addition, a negative contribution to the optical rotation is observed. When p-hydroxybenzoate is added in addition to NADPH under anaerobic condition, the broad negative band of the CD spectrum of the enzyme-NADPH mixture becomes

even more negative. The results suggest that p-hydroxybenzoate hydroxylase forms a complex with NADPH both in the absence and presence of p-hydroxybenzoate.

In order to find whether NADPH or its oxidation product, NADP, is responsible for this binding, the effect of NADP on the CD spectra of the enzyme was examined. Under anaerobic conditions, there are no significant changes in the CD spectra of the enzyme upon addition of NADP. NADP does not affect the familiar substrate-induced changes in CD spectra of the enzyme under aerobic conditions. These facts indicate that there is probably no interaction between NADP and p-hydroxybenzoate hydroxylase or, at least, it does not manifest itself in a CD modification.

NADH does not induce significant changes in CD spectra of plain enzyme solution under anaerobic condition. Therefore, the enzyme-NADPH complex is highly specific, which is consistent with previous reports.[1,2]

From the results described above, we cannot determine the order of the binding of the substrates, NADPH and p-hydroxybenzoate, to the holoenzyme in the ternary enzyme-p-hydroxybenzoate-NADPH complex. We leave it for the future study.

Discussion

As has been reported, discrepancies were found among the modes of inhibition of p-hydroxybenzoate hydroxylase by a number of substrate analogues. The analogues, such as p-fluorobenzoate, p-aminobenzoate, benzoate and others, inhibited p-hydroxybenzoate hydroxylase from *Ps. putida* A3.12 in a non-competitive manner,[1] whereas these analogues were competitive inhibitors to the p-hydroxybenzoate hydroxylase from *Ps. putida* M-6 (this report) and *Ps. desmolytica*.[8] These discrepancies

may be ascribed to the different buffer systems used. In the Tris-HCl buffer, pH optimum is 8.0 as compared to 7.5[1] and 7.0[2] found in potassium phosphate buffer system, and furthermore, the enzyme activity is found to be inhibited, increasingly and markedly, during the assay due to the presence of chloride ion[(a)]. This inhibitory action of chloride ion made the calculation of enzyme activity inaccurate and resulted earlier in an erroneous interpretation of the mode of inhibition.

The competitive behaviors of the benzoate derivatives indicate strongly that the carboxyl group is essential in the binding. Phenol does not inhibit the enzyme up to a very high concentration where non-specific binding may take place. The above findings, in conjunction with the fact that the enzyme will only catalyze p-hydroxybenzoate and will not catalyze the other analogues to any significant extent,[1] lead to the following hypotheses: the carboxyl moiety participates in binding with the primary site, providing an "anchoring" and, subsequently, the hydroxyl group is bound to a secondary site to facilitate the catalytic activity. This is in agreement with the two binding sites model proposed before.[2,8,9] The positive "reaction constant" (ρ = 1.78) obtained from the Hammett plot (Fig. 7) suggests that the primary binding site is favored by a nucleophilic attack.

Examination of the enzyme-inhibitor complex by CD spectrum showed that there are two classes of substrate analogues which competitively inhibit enzyme activity. One class of substrate analogues causes

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[(a)] Roland G. Kallen and Keiichi Hosokawa, unpublished results.

changes in CD spectra, and the other class shows no significant changes. p-Fluorobenzoate belongs to the former, and p-aminobenzoate is an example of the latter.

p-Fluorobenzoate binds to the enzyme, as evidenced by a remarkable change in CD spectrum upon addition to free-enzyme. If p-hydroxybenzoate is added to the p-fluorobenzoate-enzyme complex, the CD spectrum is converted to the one similar to p-hydroxybenzoate-enzyme complex. This indicates that p-fluorobenzoate binds to the same site as the substrate, and can be displaced by the p-hydroxybenzoate. On the other hand, p-aminobenzoate does not produce significant changes in CD spectrum of the free enzyme, although it inhibits the enzyme activity in a competitive manner. p-Aminobenzoate may bind to a site other than the one binding p-fluorobenzoate, and thus interfere with the overall reaction of p-hydroxybenzoate hydroxylase. Furthermore, the CD spectrum of the p-aminobenzoate-enzyme mixture is not affected significantly upon addition of p-hydroxybenzoate. Conversely, the CD spectrum characteristic of the enzyme-substrate complex is converted to the one similar to free enzyme spectrum by the addition of p-aminobenzoate. The results show that the binding of p-aminobenzoate to the enzyme causes a secondary effect on the substrate binding site so that p-hydroxybenzoate can no longer bind to its site, resulting in the inhibition of activity.

By CD studies, we obtained evidence that NADPH binds to free p-hydroxybenzoate hydroxylase. The binding is so specific that NADH does not become associated with the enzyme. These findings well explain the specific requirement of NADPH for the enzymatic hydroxylation of p-hydroxybenzoate.

Preliminary proton magnetic resonance study of the relaxation times of the NADPH protons in the presence of p-hydroxybenzoate hydroxylase revealed NADPH binding with the enzyme. This result supports the above described CD study that NADPH specifically interacts with p-hydroxybenzoate hydroxylase.

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TABLE I[a]

Summary of Hammett substituent constants, σ

Compound	σ	Estimated limits of uncertainty
p-Aminobenzoate	-0.66	0.1
p-Hydroxybenzoate	-0.37	0.04
Benzoate	0	--
p-Fluorobenzoate	0.062	0.02
p-Chlorobenzoate	0.227	0.02
p-Nitrobenzoate	0.778	0.02

[a] σ values were based on ionization of substituted benzoic acids.[12]

TABLE II

Inhibitory effect of substrate analogues

Substrate analogues	Inhibition (%)[a]	K_i[b]
p-Nitrobenzoate	16	$(4.2 \pm 1.3) \times 10^{-4}$ M
6-Hydroxynicotinate	17	$(3.1 \pm 0.4) \times 10^{-4}$
p-Chlorobenzoate	12	$(1.8 \pm 0.3) \times 10^{-4}$
p-Fluorobenzoate	35	$(7.6 \pm 1.9) \times 10^{-5}$
Benzoate	25	$(5.9 \pm 0.5) \times 10^{-5}$
p-Aminobenzoate	81	$(4.2 \pm 0.9) \times 10^{-6}$

(a) Inhibition was measured by the p-hydroxybenzoate-dependent oxidation of NADPH in the presence of 3.44 μg enzyme, 0.1 mM p-hydroxybenzoate, 0.33 mM substrate analogue and the other conditions were the same as the standard assay system mentioned in the section on experimental procedure.

(b) K_i values were calculated, assuming a completely competitive inhibition, by a linear regression analysis of the equation

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

where S and I denote the substrate, p-hydroxybenzoate, and inhibitory substrate analogue, respectively, v and V_{max} are the reaction velocity and maximal reaction velocity, K_m is the Machelis-Menton constant and K_i is the inhibitor dissociation constant. In our kinetic measurement, the velocity determinations, probably are reasonably homogeneous in variance. If the variance of v is σ^2, the variance of $[S]/v$ can be shown to be $\sigma^2[S]^2/v^4$ (13). Therefore, in fitting the linear form to the above equation, the proper weight $v^4/[S]^2$ is used. The standard error of K_i is estimated by standard procedure of propagation of error. All computations were done on a CDC 6600 computer.

TABLE III

Phenol inhibition[a]

Phenol conc.	% Inhibition
10^{-1} M	78
5×10^{-2} M	51.5
10^{-2} M	0
10^{-3} M	0
8.3×10^{-5} M	0
0	0

[a]The standard enzyme assay system was utilized with 1 mM p-hydroxybenzoate.

TABLE IV

Effect of reduced and oxidized pyridine nucleotides on the CD spectrum of p-hydroxybenzoate hydroxylase

Condition	p-Hydroxy-benzoate	NADPH	NADP	NADH
Aerobic	absent	No changes, but the conclusion cannot be deduced, because NADPH is oxidized in this system.	No changes.	---
	present	NADPH is oxidized quickly.	Familiar changes.	---
Anaerobic	absent	Marked changes.	No effect.	No significant changes.
	present	Further changes, more than above.	---	Slightly affected.

Figure Captions

Fig. 1. Effect of benzoate on the activity of p-hydroxybenzoate hydroxylase: double reciprocal plot. The data are plotted by the method of Lineweaver and Burk.[15] Assay conditions were described under "Experimental Procedure". The reciprocal velocity, 1/v, is in arbitrary units. The concentrations of benzoate in M are indicated on the plot. The same conditions stated above are used in Fig. 2 through Fig. 6.

Fig. 2. Effect of p-nitrobenzoate on the activity of p-hydroxybenzoate hydroxylase.

Fig. 3. Effect of p-chlorobenzoate on the activity of p-hydroxybenzoate hydroxylase.

Fig. 4. Effect of p-fluorobenzoate on the activity of p-hydroxybenzoate hydroxylase.

Fig. 5. Effect of p-aminobenzoate on the activity of p-hydroxybenzoate hydroxylase.

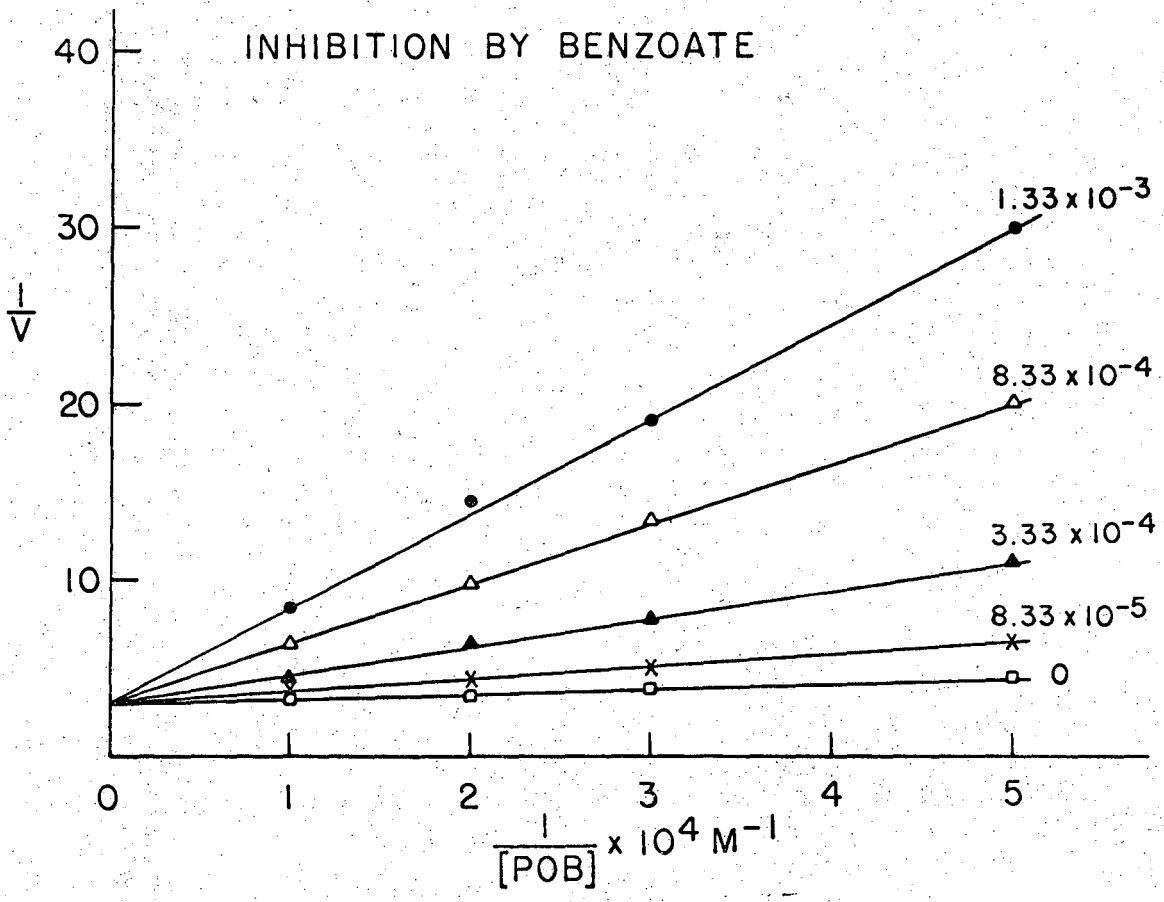
Fig. 6. Effect of 6-hydroxynicotinate on the activity of p-hydroxybenzoate hydroxylase.

Fig. 7. Action of the substituted benzoate derivatives. K_i are the inhibition constants evaluated in Table II. The standard errors in log K_i are indicated by the vertical error bars. The Hammett substituent constants are summarized in Table I. The estimated errors of σ are plotted as horizontal error bars. The slope of the line is 1.78.

Fig. 8. CD spectrum of p-hydroxybenzoate hydroxylase in the presence of 6-hydroxynicotinate and p-hydroxybenzoate. The enzyme (6.7×10^{-5} M) is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 2°C; pathlength, 1.0 cm.

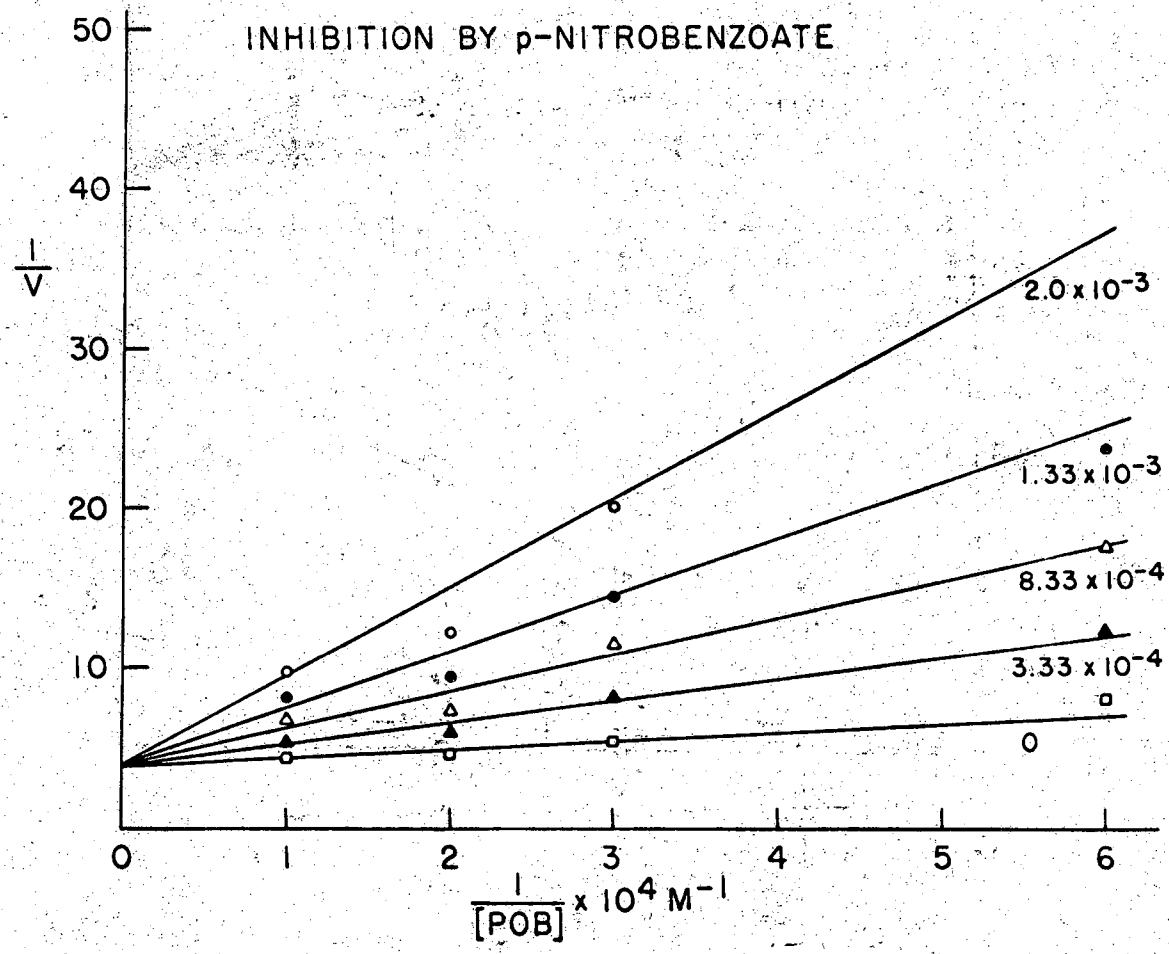
Fig. 9. CD spectrum of p-hydroxybenzoate hydroxylase in the presence of p-fluorobenzoate and p-hydroxybenzoate. The enzyme (6.7×10^{-5} M) is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 2°C; pathlength, 1.0 cm.

Fig. 10. The effect of NADPH on the CD spectrum of p-hydroxybenzoate hydroxylase. The enzyme is in 0.05 M potassium phosphate buffer, pH 7.5; temperature 2°C; pathlength, 1.0 cm; anaerobic condition. ——— enzyme alone (6.9×10^{-5} M); - - - - enzyme in the presence of 10^{-3} M NADPH: — · — · — enzyme in the presence of 10^{-3} M NADPH and 10^{-3} M p-hydroxybenzoate. ..... indicates region of the curve where signal/noise is poor.



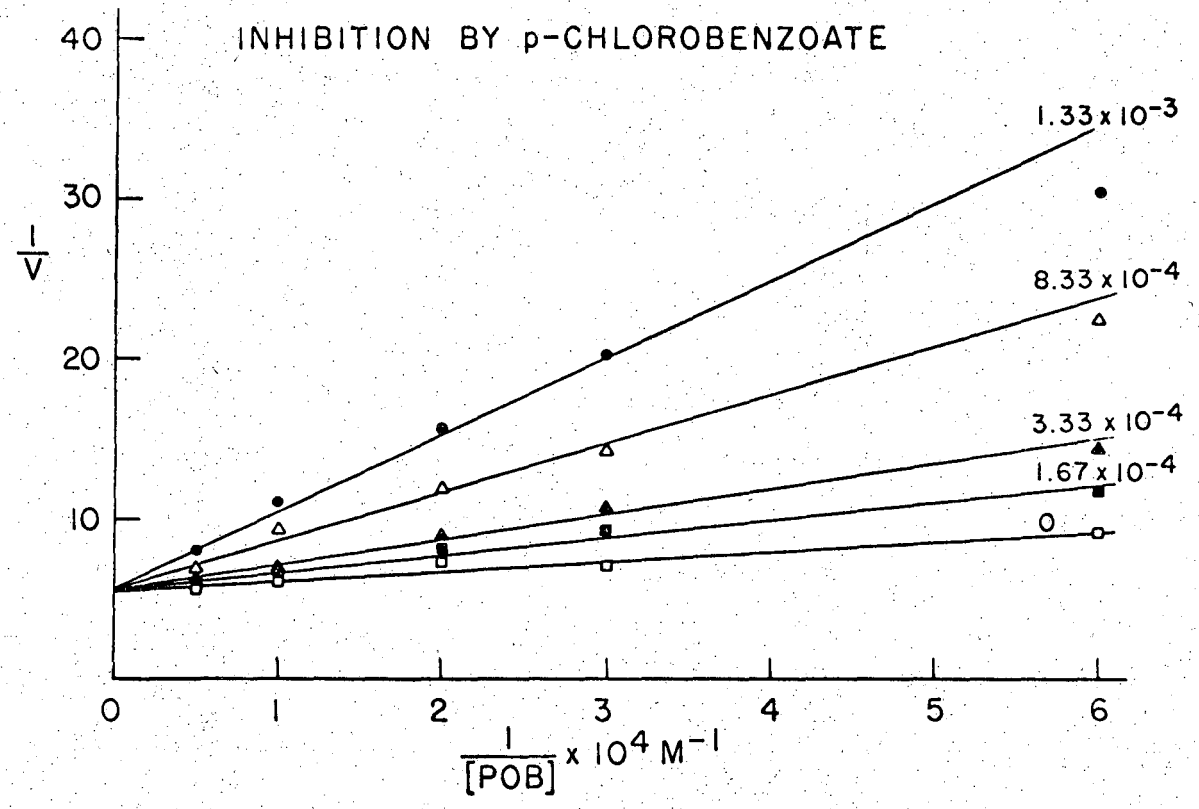
XBL 7010-5463

Fig. 1



XBL 7010-5465

Fig.2


INHIBITION BY p-CHLOROBENZOATE
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30
20
10
1/V
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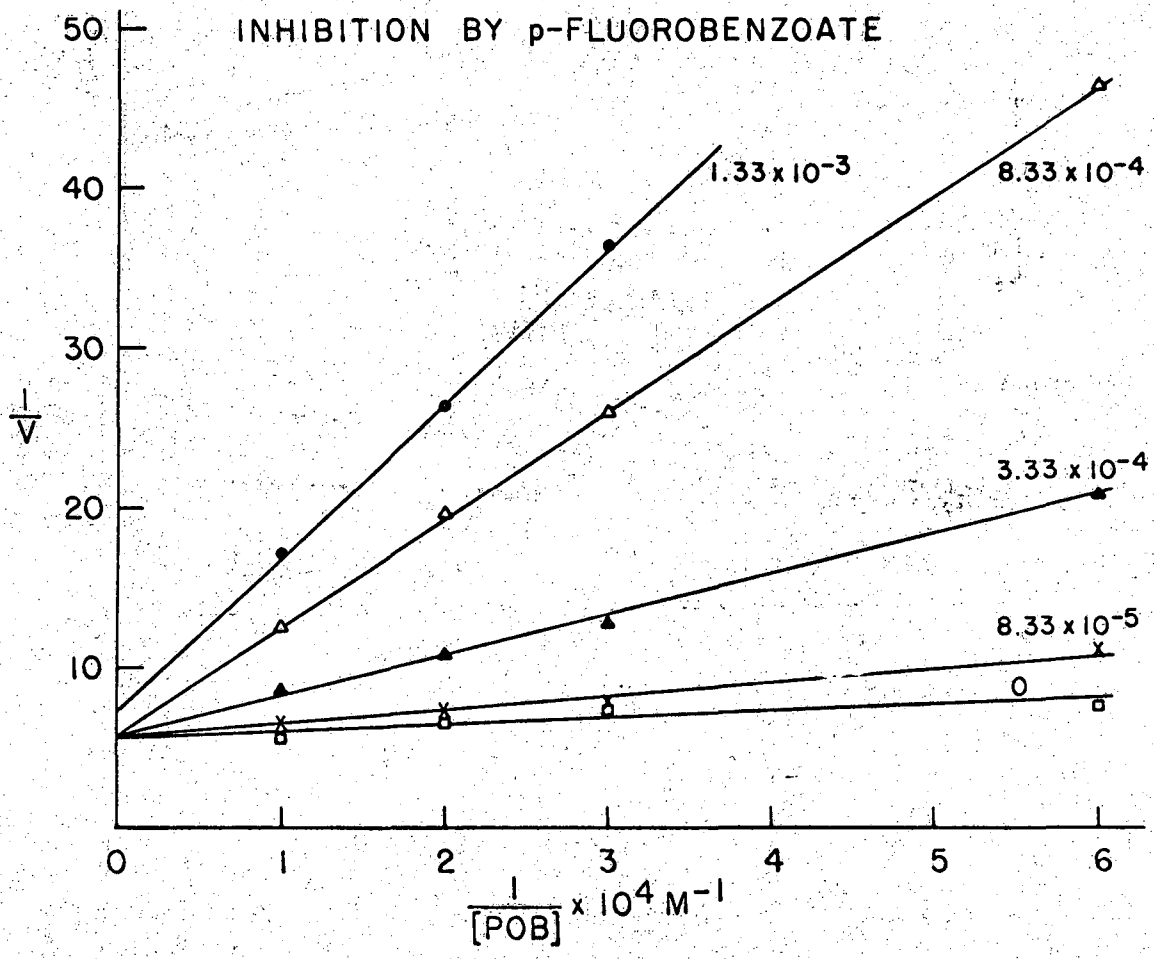
8.33 x 10-4
3.33 x 10-4
1.67 x 10-4
0
0
1
2
3
4
5
6
1/[POB] x 10^4 M^-1
XBL 7010-5464

Fig.3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8.33 x 10-4
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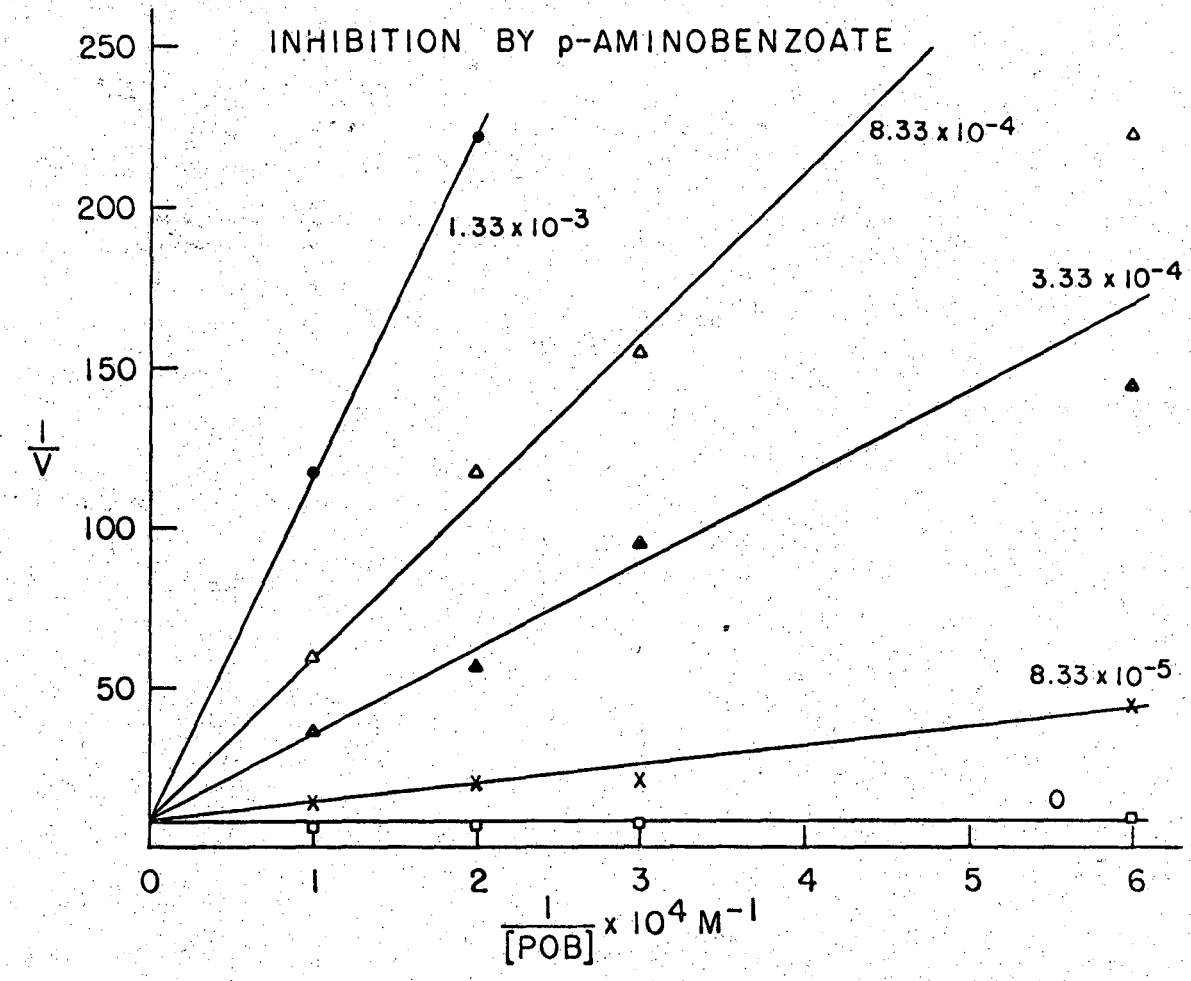
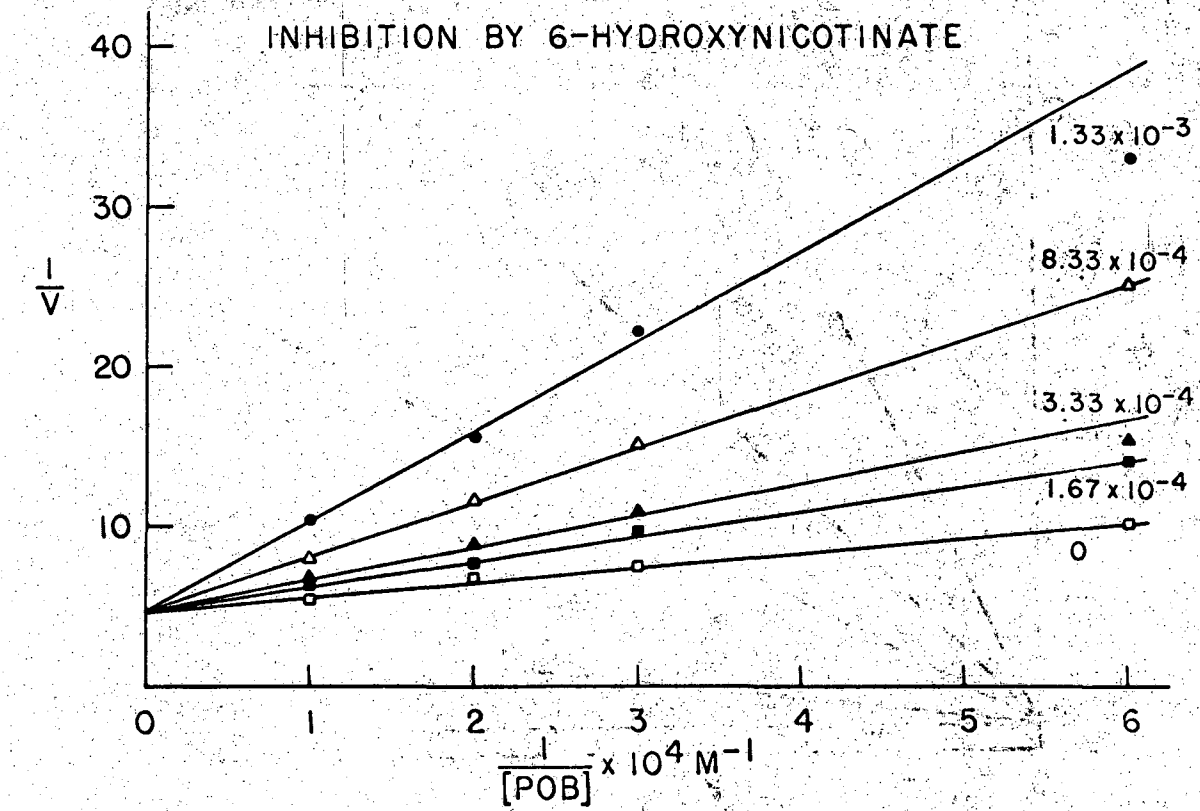
XBL 7010-5462

Fig.4



XBL 7010-5461

Fig.5


INHIBITION BY 6-HYDROXYNICOTINATE
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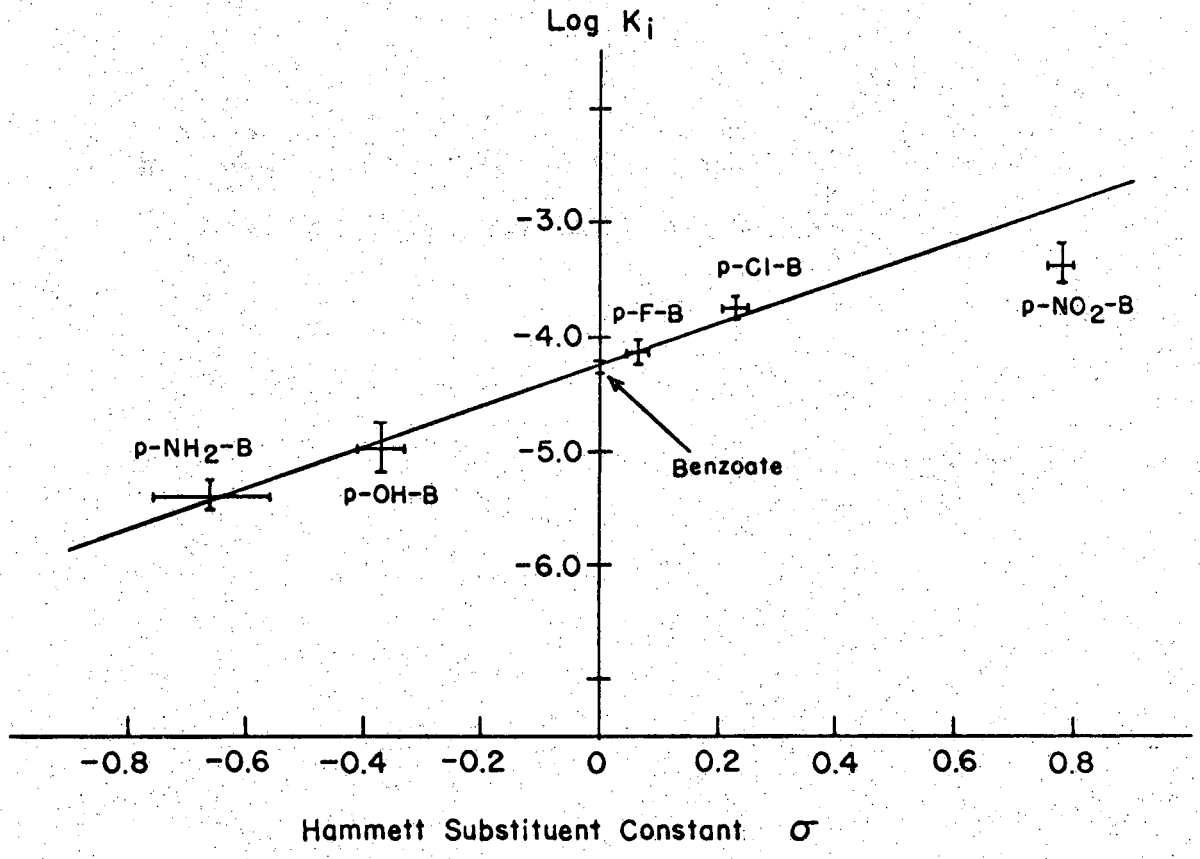
1/[POB] x 10^4 M^-1
XBL 7010-5460

Fig.6



XBL 712-5036

Fig.7

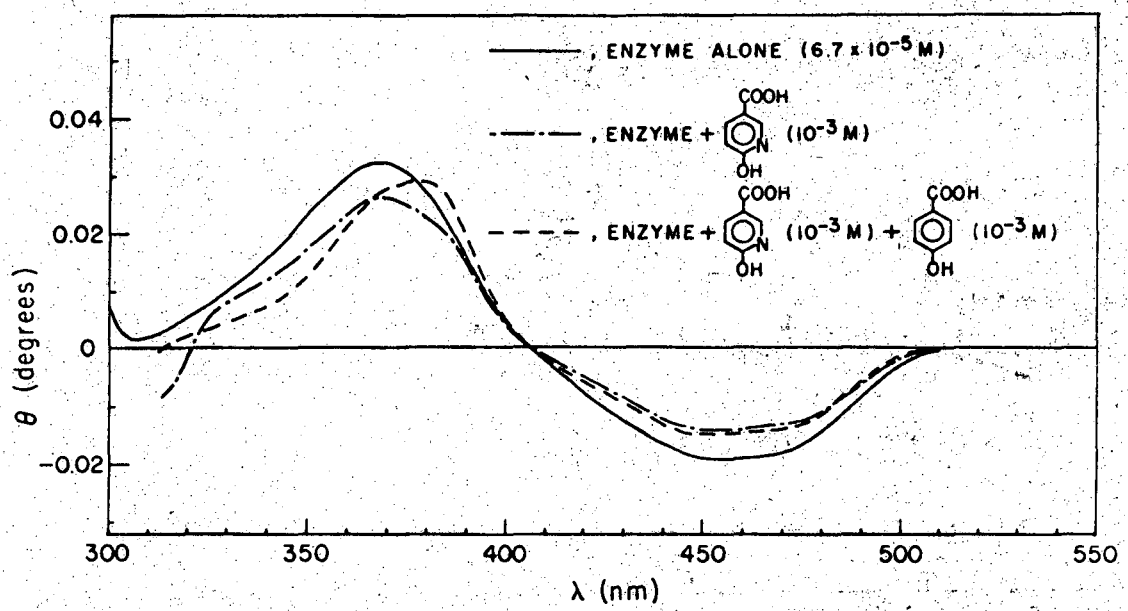

, ENZYME ALONE (6.7 x 10⁻⁵ M)
, ENZYME + (10⁻³ M)
, ENZYME + (10⁻³ M) + (10⁻³ M)
COOH
OH
0.04
0.02
0
-0.02
θ (degrees)
300
350
400
450
500
550
λ (nm)
XBL6912-5385

Fig.8

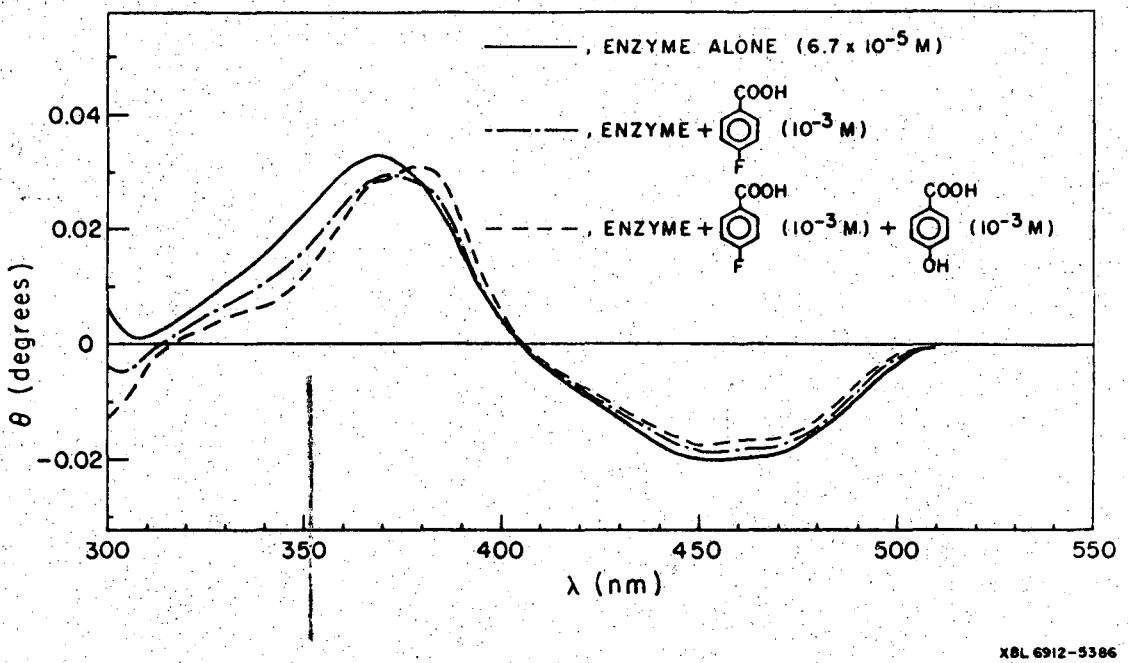

, ENZYME ALONE (6.7 x 10⁻⁵ M)
, ENZYME + (10⁻³ M)
, ENZYME + (10⁻³ M.) + (10⁻³ M)
COOH
F
OH
0.04
0.02
0
-0.02
θ (degrees)
300
350
400
450
500
550
λ (nm)
XBL 6912-5386

Fig. 9

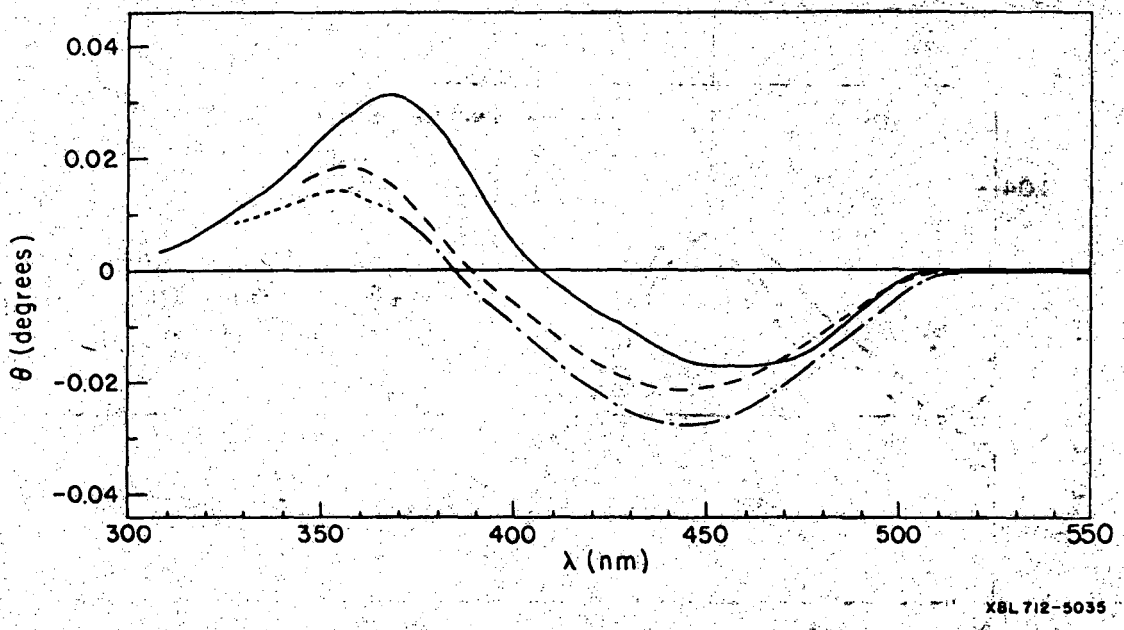

0.04
0.02
0
-0.02
-0.04
θ (degrees)
300
350
400
450
500
550
λ (nm)
XBL 712-5035

Fig. 10

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