MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

II. INHIBITORY ACTIONS OF BENZENE DERIVATIVES

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SUMMARY

1. The inhibitory action of several benzene derivatives on D-amino acid oxidase was studied. Three types of inhibition; namely, (a) direct complex formation with FAD, (b) competitive inhibition with FAD, and (c) competitive inhibition with the substrate, could be specifically attributed to the (a) phenylhydroxyl group, (b) phenylamino or phenylnitro group, and (c) phenylcarboxyl group of benzene derivatives, respectively.

2. The type of inhibition is not influenced by other groups, but the degree of inhibition is affected by other groups attached to other positions in the same benzene ring.

3. The relationship between the degree of inhibition and the structure of the inhibitor is discussed. Resonance effects may explain the degree of inhibition.

INTRODUCTION

YAGI AND MATSUOKA[1] found complex formation of riboflavin with phenols and predicted that the latter would inhibit flavin enzymes. In a previous paper[2], it was shown that *p*-aminosalicylic acid inhibits the activity of D-amino acid oxidase not only by forming a complex with FAD, but by other mechanisms. It was suggested that (a) direct complex formation with FAD, (b) competitive inhibition with FAD, and (c) competitive inhibition with the substrate may be attributed to the (a) phenylhydroxyl-, (b) phenylamino-, and (c) phenylcarboxyl groups of *p*-aminosalicylic acid, respectively.

It is especially interesting that a substance forms a complex with FAD resulting in inhibition of the enzyme activity; in other words, a substance combines with FAD in competition with the apo-protein. This inhibition provides a clue as to the binding site of FAD with enzyme protein. WEBER[3] has suggested that the tyrosyl group of the protein may be the binding site with imino NH (3) group of the flavin, and THEORELL[4] reported that such a binding occurred in old yellow enzyme.

This paper deals with the relationship between the radical attached to the benzene nucleus and the type of inhibitory action, especially that due to complex formation between FAD and the benzene derivative.

Abbreviation: FAD, flavin adenine dinucleotide.

References p. 110.

MATERIALS

D-Amino acid oxidase protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[5], as described previously[6].

FAD was prepared by the method of YAGI *et al.*[7]. Its purity was over 92 % and it contained no other flavins, nucleic acids, or metals.

DL-Alanine and benzene derivatives were chemically synthesized samples.

METHODS

The activity of the oxidase was measured as described previously[6]. The dissociation constant of FAD with the oxidase protein (K_f) and that of D-alanine with the oxidase protein (K_s) were calculated to be $1.1 \cdot 10^{-7}$ M and $3.3 \cdot 10^{-3}$ M respectively, by using the equation of MICHAELIS-MENTEN. The benzene derivative was added from the side bulb to the reaction mixture. After temperature equilibrium was reached, readings were taken every 5 min for 30 min.

If a benzene derivative inhibits oxidase activity by competing with the FAD for the protein, the reaction velocity (v) can be represented by equation (1):

$$v = \frac{V f}{K_f (1 + i/K_{ip}) + f} \tag{1}$$

where K_{ip} is the dissociation constant of the inhibitor–protein complex, f and i are the concentrations of FAD and inhibitor respectively, V is the maximum velocity obtained in the presence of a large excess of FAD, and K_f is independent of f or v.

If a benzene derivative combines directly with FAD in competition with the oxidase protein, the reaction velocity is given by Eqn. (2) (*cf.* ref. [6]).

$$v = \frac{V e}{K_f (1 + i/K_{if}) + e} \tag{2}$$

where V is the maximum velocity in the presence of a large excess of oxidase protein, and K_{if} is the dissociation constant of the inhibitor–FAD complex.

In actual measurement, V was 143 μl of O_2 uptake/30 min in the presence of $1.6 \cdot 10^{-7}$ M of FAD and $3 \cdot 10^{-5}$ M of the oxidase protein. The mol. wt. of the oxidase protein was calculated to be $1 \cdot 10^5$ from its sedimentation constant by ultracentrifugation using Spinco type E. The concentration of the oxidase protein in the enzyme solution was estimated by its extinction coefficient at 280 mμ using KALKAR's formula[8].

Rearranging Eqns. (1) and (2) gives

$$\frac{1}{v} = \frac{K_f (1 + i/K_{ip})}{V f} + \frac{1}{V} \tag{3}$$

$$\frac{1}{v} = \frac{K_f (1 + i/K_{if})}{V e} + \frac{1}{V} \tag{4}$$

K_{ip} and K_{if} may be calculated from the slopes of the straight lines obtained by plotting $1/v$ against $1/f$ or $1/e$ in the presence of a constant concentration of the inhibitor (Method I).

K_{ip} and K_{if} may also be evaluated by the following equations which are derived from Eqns (1) and (2),

References p. 110.

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\frac{i}{K_{ip}} \tag{5}$$

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\frac{i}{K_{if}} \tag{6}$$

where v_0 and v are the reaction velocities in the absence and presence of the inhibitor respectively with a constant concentration of FAD in the formula (5) or with a constant concentration of the protein in the formula (6). K_{ip} and K_{if} are calculated from the slope of the straight lines thus obtained (Method II).

The dissociation constant of the inhibitor with the oxidase protein in competition with the substrate (K_{is}) can also be obtained by Methods I and II, in the presence of a sufficiently large concentration of FAD.

The degree of complex formation of the inhibitor with FAD was measured by the method of YAGI *et al.*[1] using a Beckman spectrophotometer, in which the difference in the absorption of a FAD solution in the presence and absence of the inhibitor was determined at 450 mμ.

The direct complex formation of a phenol derivative with FAD was also measured fluorimetrically using a fluorimeter designed by YAGI *et al.*[9]. The rate of a complex formation was calculated by measuring its quenching action on the fluorescence of FAD using the following equation:

$$\frac{f}{f'} = 1 + \frac{Q}{K_{if}} \tag{7}$$

where f and f' are the intensities of the fluorescence of FAD in the absence and presence of the quencher, respectively, and Q is the concentration of the quencher. K_{if} was calculated from the slope of the straight line obtained by plotting f/f' against Q.

RESULTS

Each of 3 types of inhibition mentioned above was examined with the following typical benzene derivatives: benzoic acid, aniline, phenol, salicylic acid, *m*-aminophenol, *p*-aminobenzoic acid, *p*-nitrobenzoic acid, *p*-nitrophenol, 2,4-dinitrophenol, 2,6-dinitrophenol, and 2,4,6-trinitrophenol.

Complex formation with FAD

Benzene derivatives in aqueous soln. at pH 8.3 and 20° were examined spectrophotometrically for possible complex formation with FAD. It was observed that phenol, salicylic acid, and *m*-aminophenol shifted the absorption of FAD to the longer wavelength region as shown in Fig. 1. The dissociation constants of the complex with FAD were measured at 38° as listed in Table I. These compounds were found to quench the fluorescence of FAD.

The spectrophotometric method could not be used with *p*-nitrophenol, 2,4-dinitrophenol, 2,6-dinitrophenol, or 2,4,6-trinitrophenol, since these compounds absorb in the same region as FAD. Therefore, the interaction between these substances and FAD was measured by their quenching action on the fluorescence of FAD as shown in Fig. 2. Plotting f/f' against the concentration of nitrophenols gave second-order

References p. 110.

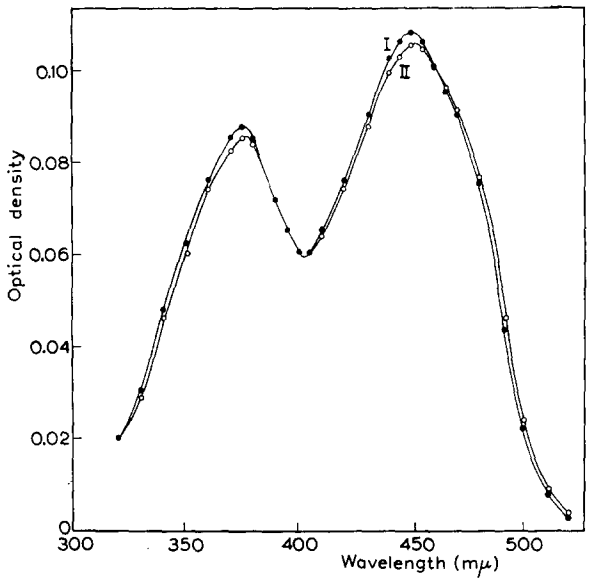


Fig. 1. Shift of absorption spectrum of FAD by phenol. I: absorption spectrum of $9.5 \cdot 10^{-6}$ *M* FAD in pyrophosphate buffer (0.1 *M*, pH 8.3), at 20°. II: I + $5 \cdot 10^{-2}$ *M* phenol.

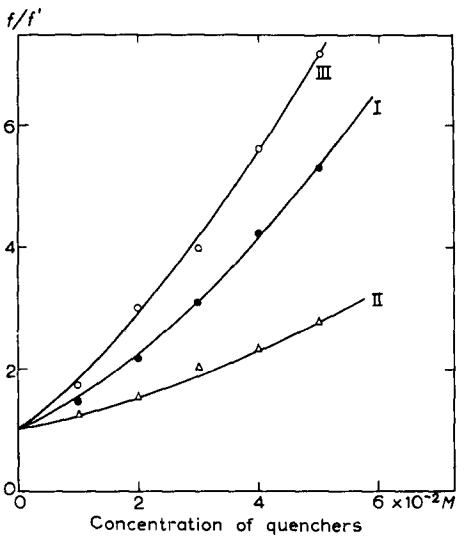


Fig. 2. Quenching action of nitrophenol derivatives on the fluorescence of FAD. *f* and *f'* correspond to the intensity of fluorescence of FAD in the absence and presence of a concentration [Q] of nitrophenol derivatives, at pH 8.3 and 38°. The concn. of FAD was $1.3 \cdot 10^{-6}$ *M*. I: in the presence of 2,4-dinitrophenol. II: in the presence of 2,6-dinitrophenol. III: in the presence of 2,4,6-trinitrophenol.

curves. This may be attributed to complex formation between the quencher and FAD, with a second mechanism involving collision of the quencher with the FAD. The apparent values of K_{if} were calculated by substituting the values of f/f' into Eqn. (7) for each concentration of the quencher, and the largest values obtained are shown in Table II.

From these results, it may be concluded that a phenolic hydroxyl group is essential for the complex formation with FAD.

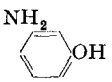
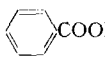
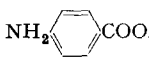
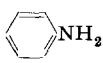
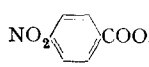
Competitive inhibition with FAD

Among the benzene derivatives examined, aniline, *p*-aminobenzoic acid, phenol, salicylic acid, *m*-aminophenol, *p*-nitrophenol, 2,4-dinitrophenol, 2,6-dinitrophenol,

References p. 110.

TABLE I

DISSOCIATION CONSTANTS OF SEVERAL BENZENE DERIVATIVES WITH FAD OR THE OXIDASE PROTEIN

	Direct complex formation with FAD K_{if} *(M)*	*Competitive inhibition with FAD or the protein* K_{if} *or* K_{ip} *(M)*		*Competitive inhibition with* D*-alanine* K_{is} *(M)*	
	By spectrophotometry	*Method I*	*Method II*	*Method I*	*Method II*
	$3.2 \cdot 10^{-2}$	$5.7 \cdot 10^{-2}$*	$5.6 \cdot 10^{-2}$*		
	$6.5 \cdot 10^{-4}$	$1.0 \cdot 10^{-3}$*	$1.1 \cdot 10^{-3}$*	$3.5 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$
	$1.1 \cdot 10^{-1}$	$2.6 \cdot 10^{-2}$**	$3.0 \cdot 10^{-2}$***		
				$1.4 \cdot 10^{-5}$	$1.3 \cdot 10^{-5}$
		$7.2 \cdot 10^{-3}$§	$7.3 \cdot 10^{-3}$§	$3.1 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$
		$9.2 \cdot 10^{-3}$§	$9.1 \cdot 10^{-3}$§		
		$9.2 \cdot 10^{-4}$§	$7.8 \cdot 10^{-4}$§	$5.2 \cdot 10^{-6}$	$4.1 \cdot 10^{-6}$

* K_{if}.

** K_{ip}, calculated from the slope of the straight line obtained by substituting the value of K_{if} obtained spectrophotometrically into Eqn. (8).

*** K_{ip}, calculated from the slope of curve II, Fig. 3.

§ K_{ip}.

TABLE II

DISSOCIATION CONSTANTS OF PHENOL AND NITROPHENOLS WITH FAD OR THE OXIDASE PROTEIN

	Direct complex formation with FAD K_{if} *(M)*	*Competitive inhibition with FAD or the protein* K *(M)*	*Dissociation constant of hydroxyl group* K_a*
Phenol	$3.2 \cdot 10^{-2}$**	$5.7 \cdot 10^{-2}$***	$1.7 \cdot 10^{-10}$
p-Nitrophenol	$5.0 \cdot 10^{-4}$§	$5.0 \cdot 10^{-3}$§§	$0.96 \cdot 10^{-7}$
2,4-Dinitrophenol	$2.0 \cdot 10^{-5}$§	$3.9 \cdot 10^{-3}$§§	$1.0 \cdot 10^{-4}$
2,6-Dinitrophenol	$4.0 \cdot 10^{-5}$§	$2.3 \cdot 10^{-3}$§§	$2.0 \cdot 10^{-4}$
2,4,6-Trinitrophenol	$1.2 \cdot 10^{-5}$§	$6.4 \cdot 10^{-4}$§§	$1.6 \cdot 10^{-1}$

* See ref. [10].

** measured spectrophotometrically.

*** from Table I.

§ measured fluorimetrically.

§§ Calculated from the slope of the straight line obtained by plotting $1/v$ against $1/f$ in the presence of the inhibitor and rate-limiting concentrations of FAD using Eqn. (3) or (4). Thus, it is derived from both the dissociation constant of the inhibitor–protein compound and that of the inhibitor–FAD complex.

References p. 110.

and 2,4,6-trinitrophenol were demonstrated to be the inhibitors of the oxidase activity in the presence of a rate-limiting concentration of FAD and a large excess of DL-alanine. The inhibition was reversed by the addition of a large excess of FAD.

Three possible inhibitory mechanisms were considered, namely, (a) competition with FAD, (b) complex formation with FAD, and (c) both competition with and complex formation with FAD. These possibilities were checked for each inhibitor by comparing the results obtained by methods I and II, and by spectrophotometric or fluorimetric measurement of the complex formation.

In the case of aniline, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid, every plot of $1/v$ against $1/f$ was on a straight line with the intercept $1/V$ (Eqn. (3)), and also each value of v_0/v plotted against i was on a straight line with the intercept 1 (Eqn. (5)). Since spectrophotometric and fluorimetric measurements showed that these compounds do not form a complex with FAD, it was concluded that they combine with the oxidase protein in competition with FAD. Values of K_{ip} calculated by methods I and II were in good agreement (Table I).

In the case of phenol and salicylic acid, it was also found that a straight line was obtained in method I (Eqn. (4)) with the intercept $1/V$, and with the intercept 1 in the case of method II (Eqn. (6)), which indicates that they also inhibit with respect to FAD by a single mechanism. In these cases, however, complex formation between phenol or salicylic acid and FAD was demonstrated as mentioned above and K_{if} obtained by spectrophotometry agreed approximately with that obtained by the enzymic reaction. From these results, it may be concluded that phenol or salicylic acid inhibits the oxidase activity by combining with FAD in competition with the protein.

In the case of *m*-aminophenol, each plot of v_0/v against i was on a second-order curve as shown in Fig. 3, curve I, which indicates that there may exist 2 or more inhibitory mechanisms with respect to FAD, *e.g.*, complex formation and competitive inhibition with FAD, as shown previously for *p*-aminosalicylic acid[2].

Assuming that the inhibitor competes with the protein for the FAD and, at the same time, competes with FAD for the oxidase protein, the reaction velocity may be expressed by Eqn. (8)

$$\frac{1}{v} = \frac{K_f (1 + i/K_{ip}) (1 + i/K_{if})}{V f} + \frac{1}{V} \tag{8}$$

from which it follows that

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right) \left\{\frac{K_{ip} + K_{if} + i}{K_{ip} K_{if}}\right\} i \tag{9}$$

Eqn. (9) shows that the values of v_0/v plotted against the concentration of the inhibitor will fall on a second-order curve. Thus, when a straight line is obtained by plotting v_0/v against i, it indicates that there can be only one inhibitory mechanism, as in Eqns. (5) or (6).

On the assumption that there are only two inhibitory factors, K_{ip} was calculated by substituting the value of K_{if} obtained by spectrophotometry into the Eqn. (8). If this assumption is correct, the correction factor to be applied to curve I in Fig. 3 to eliminate the influence of complex formation with FAD can be obtained from Eqns. (3) and (8); this is

References p. 110.

$$\frac{(V - v_0)(1 + i/K_{ip}) + v_0}{(V - v_0)(1 + i/K_{ip})(1 + i/K_{if}) + v_0} \qquad (10)$$

Substituting the values of K_{ip} obtained above and of K_{if} obtained by spectrophotometry into (10), the correction factor was calculated for each point of curve I in Fig. 3. When these points were multiplied by the appropriate correction factor, curve II, a straight line, was obtained, showing that there are only 2 inhibitory mechanisms in the case of *m*-aminophenol. The values of K_{ip} obtained from Eqn. (8) and calculated from the slope of the line II in Fig. 3 are given in Table I.

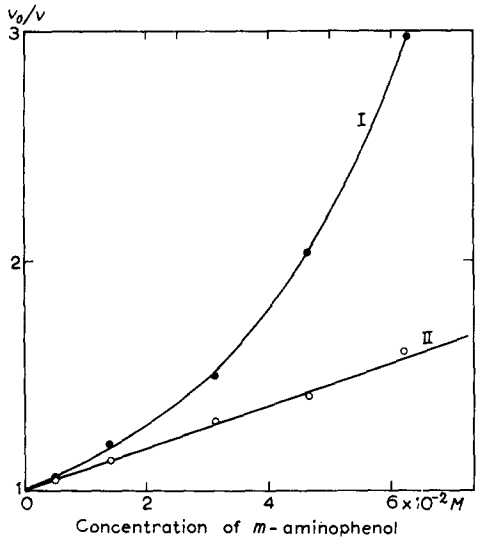


Fig. 3.

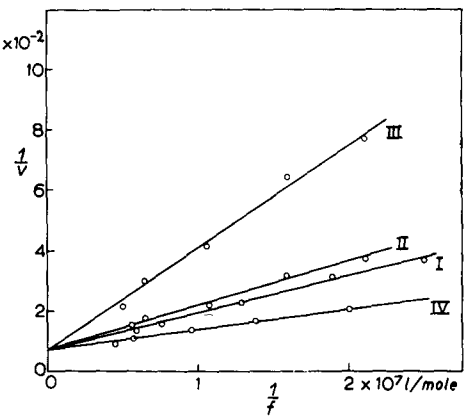


Fig. 4.

Fig. 3. Inhibitory action of *m*-aminophenol on D-amino acid oxidase activity. v_0 and v are the reaction velocities of the oxidase in the absence and presence of a concentration i of *m*-aminophenol, respectively. I: measured values of v_0/v. II: obtained by correcting curve I as described in the text. The reaction mixture contained 15 μg oxidase protein, 0.125 *M* DL-alanine, $2.9 \cdot 10^{-7}$ *M* FAD. V = 132 μl/30 min. v_0 = 96 μl/30 min.

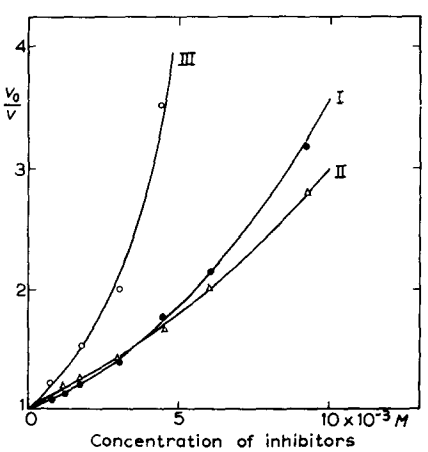


Fig. 4. Inhibitory action of nitrophenol derivatives on D-amino acid oxidase activity. v is μl O_2 uptake/30 min. In the case of I, II, and III, the reaction mixture contained 15 μg oxidase protein, 0.125 *M* DL-alanine, a concn. f of FAD and $2.2 \cdot 10^{-3}$ *M* of the nitrophenol derivative. I: in the presence of 2,4-dinitrophenol. II: in the presence of 2,6-dinitrophenol. III: in the presence of 2,4,6-trinitrophenol. IV: no inhibitor.

Fig. 5. Inhibitory action of nitrophenol derivatives on D-amino acid oxidase activity. v_0 and v are the reaction velocities of the oxidase in the absence and presence of a concentration i of nitrophenol derivatives, respectively. I: in the presence of 2,4-dinitrophenol. II: in the presence of 2,6-dinitrophenol. III: in the presence of 2,4,6-trinitrophenol. The reaction mixture contained 15 μg oxidase protein, 0.125 *M* DL-alanine, $1.3 \cdot 10^{-7}$ *M* FAD, and various concentrations of the nitrophenol derivative. V = 132 μl/30 min. v_0 = 72 μl/30 min.

References p. 110.

With nitrophenol and its derivatives, each plot of $1/v$ against $1/f$ gives a straight line as shown in Fig. 4. However, each plot of v_0/v against i was on a second-order curve as shown in Fig. 5. Even the largest value of the apparent K_{if} obtained fluorimetrically was too small to correct this second-order curve to a straight line. If the collision effect was included in the quenching action of nitrophenols as described above, the dissociation constant calculated from the quenching would be smaller than its true value. Therefore, the fact that the apparent K_{if} is too small to correct the second-order curve to a straight line indicates that there are 2 inhibitory mechanisms. Thus, it may be concluded that the inhibitory mechanism of nitrophenol or its derivatives is the same as in the case of *m*-aminophenol, namely, direct complex formation with FAD and competition with FAD.

Competitive inhibition with the substrate

Among the benzene derivatives examined, benzoic acid, salicylic acid, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid were found to be inhibitors of the oxidase in the presence of the rate-limiting concentrations of the substrate and excess FAD.

Plotting $1/v$ against $1/s$, a straight line with the intercept $1/V$ was obtained, and plotting v_0/v against i, a straight line with the intercept 1 was also obtained.

The 2 values of K_{is} calculated by methods I and II were in agreement. The fact that each plot of v_0/v was on a straight line suggested that each inhibitor inhibits with respect to the substrate by a single mechanism. Furthermore, it was observed by spectrophotometry that none of the benzene derivatives examined form a complex with the substrate.

From these results, it was concluded that benzoic acid, salicylic acid, *p*-aminobenzoic acid, or *p*-nitrobenzoic acid inhibits the oxidase activity competitively with the substrate. The dissociation constants are given in Table I. It should be noted that only benzoic acid derivatives inhibit the oxidase activity competitively with respect to the substrate.

DISCUSSION

Group specificity of type of inhibition

Complex formation with FAD was observed only with phenol and its derivatives and not with other benzene derivatives. Therefore, it is concluded that the phenolic hydroxyl group is responsible for the complex formation with FAD. The phenol combines with FAD in competition with the apo-protein, suggesting that the tyrosyl group of the oxidase protein is the binding site with FAD. This type of inhibition might be expected with other enzymes containing a dissociable flavin as a prosthetic group.

It was observed that aniline and nitrobenzene derivatives combine with the oxidase protein competitively with FAD. It may therefore be considered that both phenylamino and phenylnitro groups have some effects on the binding site of the oxidase protein with FAD.

Competitive inhibition with the substrate was demonstrated only with benzoic acid and its derivatives, and it may be supposed that phenylcarboxyl group may have some effects on the binding site of the oxidase protein with the substrate.

It is interesting that there is a group specificity corresponding to the type of

References p. 110.

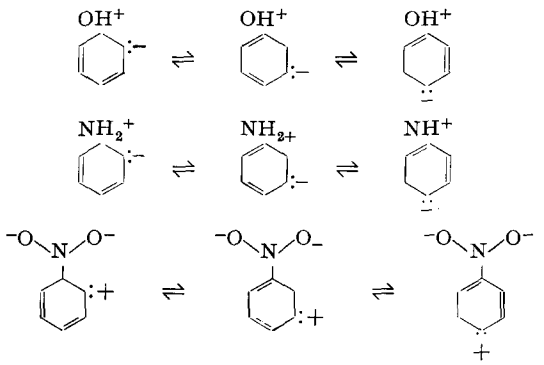
inhibition: namely, phenylhydroxyl for complex formation with FAD, phenylamino and phenylnitro for competition with FAD, and phenylcarboxyl group for competition with the substrate.

It was also interesting that the type of inhibition is independent of other groups attached to the benzene ring.

Degree of inhibition

Although the type of inhibition is determined by the corresponding group attached to the benzene ring and is not influenced by other radicals, the degree of inhibition may be influenced by other groups attached to other parts of the benzene ring.

For example, the dissociation constant of *m*-aminophenol with FAD is larger than that of phenol, and that of *p*-nitrophenol is smaller than that of phenol. This may be explained by the resonance effect. In the case of phenol, aniline, and nitrobenzene, their resonance effects may be shown as follows:



The direction of electron current of phenol is the same as that of aniline and is opposite to that of nitrophenol. Accordingly, the charge of the phenylhydroxyl group is decreased in the case of *m*-aminophenol, and the inductive effect in the case of *p*-nitrophenol increases its dipole moment.

In the case of dinitrophenol and trinitrophenol, a greatly increased dipole moment can be expected. In fact, the K_{if}'s of dinitrophenol and trinitrophenol were smaller than that of mononitrophenol, as shown in Table II. This supports the conception that phenylhydroxyl group actually combines with FAD.

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