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The Reactivity of the Electron Donor-Accepter Complex—Catalysis by 1,3-Dimethylalloxazine in an Enzymatic Model Reaction

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The effect of electron donor-accepter(EDA)-complex formation upon the reactivity (or catalytic activity) of 1,3-dimethylalloxazine, a model compound of co-enzyme, FMN, was examined in the redox reaction between methylene blue and ascorbic acid. The 1,3-dimethylalloxazine exhibited a behavior similar to the enzyme, having an optimal pH for the redox reaction. The characteristic behavior of 1,3-dimethylalloxazine was demonstrated in the presence of both 1,3-dimethylalloxazine and pyrene; such behavior was not to be observed upon the addition of either of them. It was accordingly suggested that the EDA complex formation would play an important role in the enzymatic reactions *in vivo*. The influence of various aromatic hydrocarbons upon the enzyme-like catalysis was examined.

It is generally accepted that the electron-transfer process in a living body is frequently associated with the formation of adenosine triphosphate (ATP), thus accumulating energy. As to the mechanism of the electron-transfer process, various hypotheses have been proposed, such as conduction band theories,[1,2] exciton models,[3,4] and charge-transfer models.[5] No crucial evidence, however, has ever been presented to elucidate the reaction mechanism or its reaction intermediate for the redox process catalyzed, for instance, by the flavin enzyme. Especially, the reason why the co-enzymatic action appears, the role of protein in the enzyme, and its reaction site and intermediate still remain important problems to be solved.

The present authors have been working for several years with the problems of the reactivity of electron donor-acceptor (EDA) complexes of

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[1] D. D. Eley, "Horizons in Biochemistry," Academic Press, New York, (1962), p. 341.

[2] M. G. Evans and J. Gergely, *Biochem. Biophys. Acta*, **3**, 188 (1949).

[3] M. Kasha, *Rev. Mod. Phys.*, **31**, 162 (1959).

[4] M. Calvin, *ibid.*, **31**, 16, 147 (1959).

[5] A. Szent-Györgyi, "Introduction to a Submolecular Biology," Academic Press, New York (1960).

aromatic compounds, and they have demonstrated that the reactivity of aromatic compounds, is in general, markedly influenced by the formation of the EDA complexes.[6] Accordingly, in connection with the mechanism of the co-enzyme action, 1,3-dimethylalloxazine[*1] has been employed as a model compound of co-enzyme, flavin mononucleotide (FMN),[*2] and its reactivity or its catalytic activity and the effects of the EDA complex formation thereon have been examined for the redox process between methylene blue and ascorbic acid from the view point of the EDA-complex formation.

It was found that the 1,3-dimethylalloxazine accelerates the redox reaction and that the formation of the EDA-complexes markedly affects the catalytic activity.

Experimental

The 1,3-dimethylalloxazine (DMA)[7] was synthesized from caffeine and *o*-phenylendiamine, while commercial ascorbic acid and methylene blue were employed. The aromatic hydrocarbons, such as naphthacene, perylene, anthracene, pyrene, *p*-terphenyl and phenanthrene, were purified by sublimation. The reactor consisted of a quartz cell for visible spectra and of three glass tubes, containing solutions of ascorbic acid, methylene blue, and DMA or its EDA-complexes, respectively. The reactants were always handled in the absence of air or oxygen. In the oxidation-reduction reaction, ascorbic acid was used as the hydrogen donor, while methylene blue was used as the hydrogen acceptor. The decrease in the absorption peak of methylene blue at 650 mμ was employed as a measure of the extent of the redox reaction to follow the reaction rate. Three different sorts of experiments were carried out as follows: (1) The DMA was used as a catalyst for the redox reaction between ascorbic acid and methylene blue, and the dependence of the rate upon the pH, the reaction temperature, and the addition of electron-donating and -accepting compounds, such as pyrene and tetracyanoethylene (TCNE), was examined.

As an aqueous solution of ascorbic acid (3.27×10^{-4} mol/*l*, 2 m*l*), a solution of methylene blue (3.27×10^{-4} mol/*l*, 1 m*l*), a tetrahydrofuran (THF) solution of DMA (1.64×10^{-4} mol/*l*, 2 m*l*), and a phosphate buffer (20 m*l*) were mixed in the absence of oxygen and the rate of the reduction of methylene blue was studied at various temperatures and at various pH values, as controlled by the phosphate buffer.

(2) The influence of the EDA-complex formation upon the catalytic activity of DMA was studied. In this case, a 1,2-dichloroethane solution of DMA and pyrene (2 to 1 molar ratio; 3.27×10^{-7} mol and 1.64×10^{-7} mol, respectively, in a 4.5 m*l* solution) was added to a redox system of a 1,2-dichloroethane solution of ascorbic acid (3.27×10^{-4} mol/*l*, 1 m*l*) and methylene blue (3.27×10^{-4} mol/*l*, 1 m*l*).

(3) The reduction of methylene blue by ascorbic acid was affected by the addition of various aromatic hydrocarbons, such as naphthacene, perylene, anthracene, pyrene, *p*-terphenyl, and phenanthrene, as electron-donating compounds for the methylene blue, and the influences of these aromatic additives were examined in the 1,2-dichloroethane solution.

Results and Discussion

(1) Catalysis by 1,3-Dimethylalloxazine (DMA). The reduction reaction of methylene blue by ascorbic acid was accelerated to a facter of 50 by the addition of DMA. The reaction was first-order with respect to methylene blue. The higher the temperature of the reaction, the greater the rate of the reaction, when the pH was constant, as is shown in Fig. 1. When the pH was changed at a constant temperature, 13°C, the gradient of the rate against the pH became less steep in the region of pH 10 and approached a saturation value in the region higher than pH 12. At a constant temperature, 0°C, on the other hand, a maximum peak appeared in the reaction rate in the range from pH 9 to pH 10, as is shown in Fig. 2. A minimum activation energy for the reaction was also found in the same region (Table 1). When an electron-donating compound, such as pyrene, or an electron-accepting compound, such as TCNE, for the DMA was added to the system of the redox

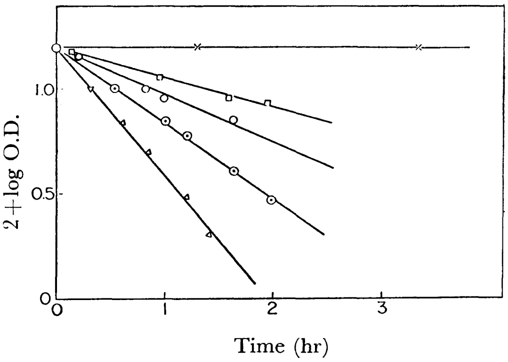

Fig. 1. Dependence of the rate constants *k*, $-d(\mathrm{Mb})/dt=k(\mathrm{Mb})$, upon temperature in the redox reaction between ascorbic acid and methylene blue (Mb) catalyzed by DMA.

O. D. represents the optical density of methylene blue.

—×— at 0°C, pH 10.4, without DMA

—○— at 0°C, pH 10.4

—⊙— at 26°C, pH 10.4

—□— at 0°C, pH 12.0

—△— at 25°C, pH 12.0

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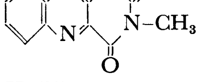
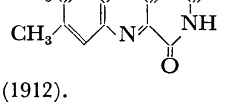
[6] M. Ichikawa, M. Soma, T. Onishi and K. Tamaru, *Trans. Faraday Soc.*, **63**, 997 (1967), S. Tanaka, M. Ichikawa, S. Naito, M. Soma, T. Onishi and K. Tamaru, This Bulletin, **41**, 1278 (1968).

[*1]



[*2]



[7] H. Biltz, *Ber.*, **45**, 3674 (1912).

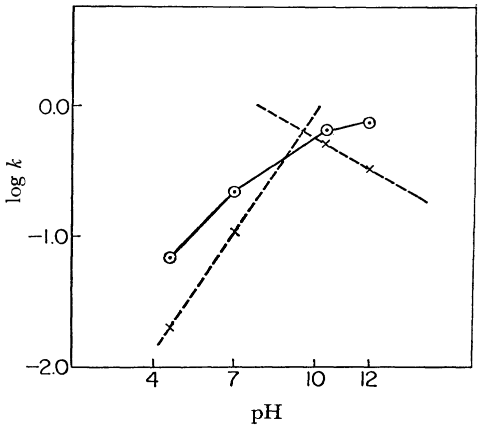


Fig. 2. Dependence of the rate constants k, $-\mathrm{d(Mb)}/\mathrm{d}t=k(\mathrm{Mb})$, upon pH:
—⊙— at 13°C
—×— at 0°C

TABLE 1. THE REDUCTION OF METHYLENE BLUE BY ASCORBIC ACID CATALYZED BY DMA

pH	Temperatures (°C)	Rate constants (hr^{-1})	Activation energies (kcal/mol)
4.6	0	0.020	14.8
	13	0.069	
7.0	0	0.11	8.0
	13	0.22	
10.4	0*	0.011*	—
	0	0.53	2.8
	26	0.84	
12.0	0	0.35	9.2
	25	1.43	

* Without DMA

reaction of ascorbic acid, methylene blue, and DMA, the rates of the reduction reaction of methylene blue decreased to approximately one-third.

The activity of enzymes is, in general, high in weak alkaline solution and has an optimal temperature range to work in. It is interesting to note that the DMA, a model compound of the co-enzyme, FMN, exhibited a catalysis similar to that of the enzyme and that the optimal pH values were demonstrated in the activity of the redox reaction; this suggests that the behavior of the active site of the enzyme is similar to that of the co-enzyme model.

(2) Effects of the EDA Complex Formation between DMA and Pyrene in the Redox Reaction. The 1,2-dichloroethane solutions of ascorbic acid, methylene blue, and a mixture of DMA-pyrene (2 : 1) were mixed at 0°C in the absence of oxygen. For 10 to 15 min the time-concentration relation for methylene blue exhibited a valley-shaped curve; thereafter, the reaction reached an equilibrium, as Fig. 3 shows. This valley curve can be explained not as an equilibrating reaction with a single reaction path, but as a successive reaction to consume the methylene blue, followed by its reproduction.

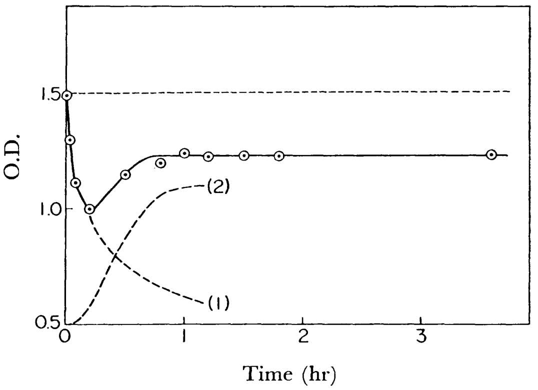


Fig. 3. The reduction of methylene blue by ascorbic acid in the presence of DMA and pyrene at 0°C in 1,2-dichloroethane solution.
——— O.D. of methylene blue at $t=0$.
----(1) forward reaction, Eqs. (b) and (c)+(c′).
----(2) back reaction, Eq. (e).

Such characteristic behavior appeared only in the presence of both DMA and pyrene, was not observed upon the addition of either of them or in a phosphate buffer as has been described in Section (1), where the interaction between them is very weak. Consequently, the following experiment was undertaken: first, ascorbic acid and methylene blue were reacted in the presence of only pyrene, and then DMA was introduced into the system during the course of the redox reaction. The results are given in Fig. 4. The results in Fig. 4 exhibit a behavior similar to those shown in Fig. 3.

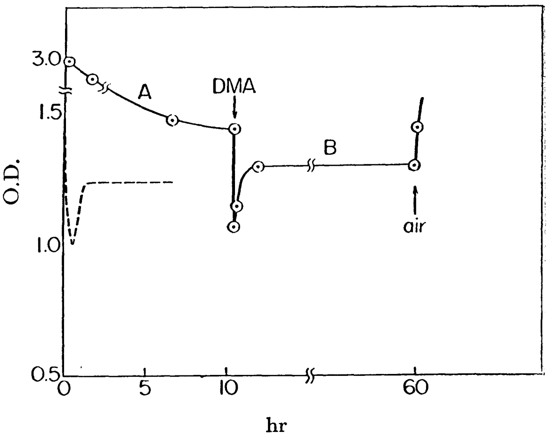


Fig. 4. Effect of the formation of EDA complex in the redox reaction of methylene blue with ascorbic acid at 0°C:
---- in the presence of DMA and pyrene
A: catalyzed by pyrene.
B: DMA addition into A.

When the temperature of the reaction was kept constant at 26°C, the valley in Fig. 3 became more shallow.

The characteristic behavior shown in Fig. 3 was not observed for lower concentrations of both DMA and pyrene in comparison with methylene blue; for instance, when the molar ratios of DMA and pyrene to methylene blue were 1/22.5 and 1/45, respectively or 1/45 and 1/90, respectively, no valley could be detected. When leuco-methylene blue which had been obtained by bubbling hydrogen gas over a platinum catalyst in a dichloroethane solution of methylene blue was introduced into a solution containing both DMA and pyrene, it was oxidized to methylene blue rather rapidly. DMA and pyrene form a solid EDA complex with a molar ratio of 2 : 1 and with a charge-transfer band in the region from 400 to 500 mμ.[8] It was concluded, in accordance to these results, that the appearence of the characteristic curve shown in Fig. 3 is due to the complex formation between DMA and pyrene.

It is also confirmed spectroscopically and theoretically that the enzymes or co-enzymes form EDA complexes.[9-11] When DMA, a model compound of the co-enzyme, FMN, of the flavin enzyme formed an EDA complex with pyrene, there was a controlled reaction; this suggests that the EDA complex formation plays an important role in the enzymatic reaction *in vivo*.

Mechanisms. In the presence of both DMA and pyrene (P), the mechanism of the reduction reaction of methylene blue (Mb) by ascorbic acid ($\mathrm{AsH_2}$) may be as follows:

$$\mathrm{AsH_2} + \mathrm{Mb} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{As} + \mathrm{MbH_2} \qquad \text{(a)}$$

$$\mathrm{AsH_2} + \left(\frac{\mathrm{P}}{2}\cdot\mathrm{Mb}\right) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{As} + \frac{\mathrm{P}}{2} + \mathrm{MbH_2} \qquad \text{(b)}$$

$$\mathrm{AsH_2} + \mathrm{DMA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{As} + \mathrm{DMAH_2} \qquad \text{(c)}$$

$$\mathrm{DMAH_2} + \mathrm{Mb} \underset{k_{-3'}}{\overset{k_{3'}}{\rightleftharpoons}} \mathrm{DMA} + \mathrm{MbH_2} \qquad \text{(c}'\text{)}$$

$$\mathrm{AsH_2} + \left(\frac{\mathrm{P}}{2}\cdot\mathrm{DMA}\right) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{As} + \frac{\mathrm{P}}{2} + \mathrm{DMAH_2} \qquad \text{(d)}$$

$$\mathrm{MbH_2} + \left(\frac{\mathrm{P}}{2}\cdot\mathrm{DMA}\right) \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{Mb} + \frac{\mathrm{P}}{2} + \mathrm{DMAH_2} \qquad \text{(e)}$$

where (P/2·Mb) and (P/2·DMA) represent the complexes between pyrene and methylene blue, and between pyrene and DMA, respectively. The reaction (a) is first-order with respect to ascorbic acid and also to methylene blue, and it is expressed by the following equation:

$$-\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{Mb}) = k_1(\mathrm{AsH_2})(\mathrm{Mb})$$

The step (b) is a reaction catalyzed by pyrene, while the steps (c) and (c′) show that the redox reaction is catalyzed by DMA. When the steady-state method of Birggs and Haldan is applied to this reaction system, the rate equation of the reaction is given as follows:

$$-\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{Mb}) = k_3'(\mathrm{DMAH_2})(\mathrm{Mb})$$

$$= k_3'(\mathrm{DMA})_0(\mathrm{Mb}) \frac{1}{1 + \dfrac{k_{-3}(\mathrm{As})}{k_3(\mathrm{AsH_2})} + \dfrac{k_3'(\mathrm{Mb})}{k_3(\mathrm{AsH_2})}}$$

where $(\mathrm{DMA})_0$ represents the initial concentration of DMA, and under the conditions when $(\mathrm{DMA})_0 \ll (\mathrm{AsH_2})_0$ and $(\mathrm{Mb})_0$, $16(\mathrm{Mb})_0 = (\mathrm{AsH_2})_0$, and $k_3 \approx k_3'$:

$$\frac{1}{1 + \dfrac{k_{-3}(\mathrm{As})}{k_3(\mathrm{AsH_2})} + \dfrac{k_3'(\mathrm{Mb})}{k_3(\mathrm{AsH_2})}}$$

becomes about unity, following equation is thus obtained:

$$-\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{Mb}) = k_3'(\mathrm{DMA})_0(\mathrm{Mb}) = k_3''(\mathrm{Mb})$$

Under these circumstances, the reaction was first-order with respect to methylene blue, and the rate constant, k_3'', is proportional to the initial concentration of DMA, as is shown in Fig. 5. The dihydro-1,3-dimethylalloxazine ($\mathrm{DMAH_2}$) in Eqs.

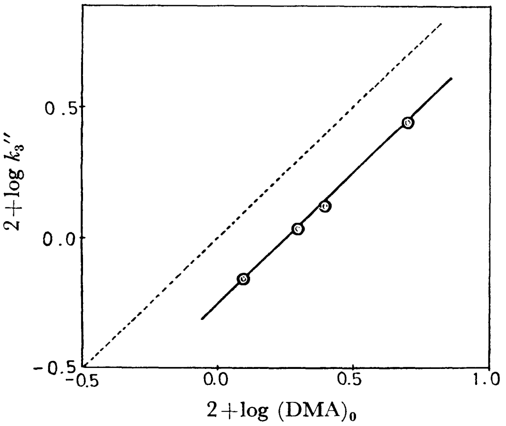


Fig. 5. The dependence of the rate constant k_3'' upon the initial concentration of DMA; $-\mathrm{d}(\mathrm{Mb})/\mathrm{d}t = k_3'(\mathrm{DMA})_0(\mathrm{Mb}) = k_3''(\mathrm{Mb})$, where $(\mathrm{DMA})_0$ and (Mb) represent the concentration of DMA at time zero and methylene blue, respectively. Solid line runs parallel with broken line (slope of 45°).

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[8] U. Matsunaga, *Nature*, **211**, 182 (1966).

[9] A. Pullman and B. Pullman, "Horizons in Biochemistry," Academic Press, New York (1962), p. 553.

[10] R. S. Mulliken, *J. Chem. Phys.*, **61**, 20 (1964).

[11] L. J. Andrews and R. W. Keefer, "Molecular Complexes in Organic Chemistry," Holden-Day, San Franscisco (1964).

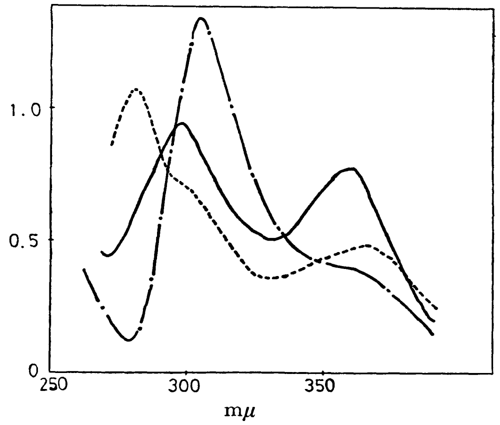

Fig. 6. Spectra of 1,3-dimethylalloxazine (DMA).
—— DMA in THF
--- DMA plus ascorbic acid
---- DMA plus HCl (45°C heating)

TABLE 2. THE REDUCTION OF METHYLENE BLUE BY ASCORBIC ACID

Steps		Rate constant
(a)*	k_1	$2.0\times10^2\ l\cdot\text{mol}^{-1}\cdot\text{hr}^{-1}$
(b)*	k	$0.0058\ \text{hr}^{-1}$
(c)(c′)*	k_3'	$1.0\times10^4\ l\cdot\text{mol}^{-1}\cdot\text{hr}^{-1}$
(e)*	k'	$\sim2\ \text{hr}^{-1}$

* (a): $-\text{d(Mb)}/\text{d}t = k_1(\text{AsH}_2)(\text{Mb})$
(b): $-\text{d(Mb)}/\text{d}t = k_2'(\text{Ar})_0{}^{1/2}(\text{Mb}) = k(\text{Mb})$
(c)(c′): $-\text{d(Mb)}/\text{d}t = k_3'(\text{DMA})_0(\text{Mb}) = k_3''(\text{Mb})$
(e): $-\text{d(MbH}_2)/\text{d}t = k'(\text{Mb})$, when $(\text{DMA})_0 \gg (\text{Mb})_0$, where (Mb), (MbH_2), (AsH_2) and $(\text{DMA})_0$ represent the concentrations of methylene blue, leuco-methylene blue, ascorbic acid and the DMA at $t=0$, respectively.

(c) and (c′) was detected by UV spectroscopy, as is shown in Fig. 6.[12]

The steps (d) and (e) form another reaction path resulting from the formation of the EDA complex of DMA with pyrene, while the back reaction in Fig. 3 is due to the step (e).

The rate constants of all the steps, (a), (b), (c), (c′), and (e), are given in Table 2.

(3) Influences of Various Aromatic Hydrocarbons in the Redox Reaction. Various aromatic hydrocarbons, such as naphthacene, perylene, anthracene, pyrene, *p*-terphenyl, and phenanthrene, all electron donors for methylene blue but with different ionization potentials, accelerated the reduction reaction of methylene blue by ascorbic acid, though they are generally considered to be inert in the reaction.

When the aromatic hydrocarbons form EDA complexes with methylene blue of a molar ratio of 1 : 2, the reaction equation is given as follows:

$$\tfrac{1}{2}(\text{Ar}) + \text{Mb} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\tfrac{1}{2}\text{Ar}\cdot\text{Mb}) \qquad (\text{g})$$

$$(\tfrac{1}{2}\text{Ar}\cdot\text{Mb}) + \text{AsH}_2 \xrightarrow{k_2} \text{MbH}_2 + \tfrac{1}{2}\text{Ar} + \text{As} \qquad (\text{h})$$

where (Ar) represents aromatic hydrocarbons:

$$K = \frac{k_1}{k_{-1}} = \frac{(\tfrac{1}{2}\text{Ar}\cdot\text{Mb})}{(\text{Ar})^{1/2}(\text{Mb})}$$

Accordingly,

$$\frac{\text{d}}{\text{d}t}(\text{MbH}_2) = k_2(\tfrac{1}{2}\text{Ar}\cdot\text{Mb})(\text{AsH}_2)$$
$$= k_2K(\text{AsH}_2)(\text{Ar})^{1/2}(\text{Mb}).$$

When $(\text{AsH}_2)_0 \gg (\text{Mb})_0$:

$$-\frac{\text{d}}{\text{d}t}(\text{Mb}) = k_2K(\text{AsH}_2)_0(\text{Ar})_0{}^{1/2}(\text{Mb})$$
$$= k_2'(\text{Ar})_0{}^{1/2}(\text{Mb})$$
$$= k(\text{Mb})$$

where $(\text{Ar})_0$ is the initial concentration of the aromatic hydrocarbons.

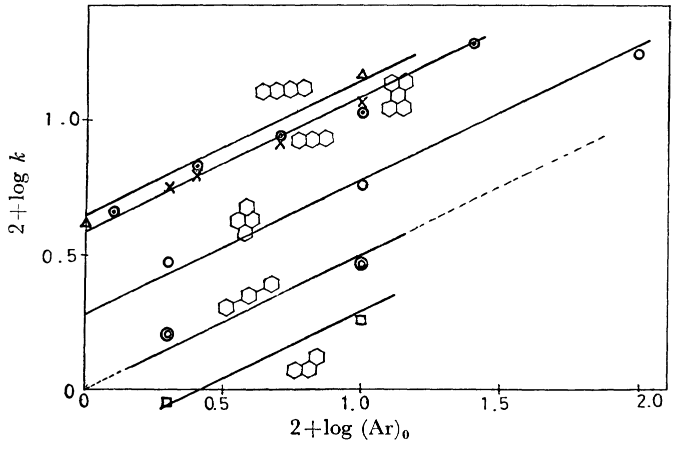

Fig. 7. The linear dependence of rate constants k upon the initial concentrations of aromatic hydrocarbons $(\text{Ar})_0$: $-\text{d(Mb)}/\text{d}t=k_2'(\text{Ar})_0{}^{1/2}(\text{Mb})=k(\text{Mb})$.

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[12] P. Hemmerich, C. Veeger and H. C. S. Wood, *Angew. Chem.*, **77**, 699 (1965).

Table 3. THE REDUCTION OF METHYLENE BLUE BY ASCORBIC ACID, CATALYZED BY AROMATIC HYDROCARBONS

k*	Molar ratios+						
Catalysts	1/4	1/10	1/20	1/40	1/50	1/80	1/100
Naphthacene		0.15					0.043
Perylene		0.12	0.083	0.064	0.058		0.034
Anthracene	0.19	0.11	0.090	0.069		0.038	0.030
Pyrene		0.058			0.030		
p-Terphenyl		0.030			0.017		
Phenanthrene		0.018			0.009		

* $-\mathrm{d(Mb)}/\mathrm{d}t = k_2'(\mathrm{Ar})_0^{1/2}(\mathrm{Mb}) = k(\mathrm{Mb})$
where, $(\mathrm{Ar})_0$ represents the initial concentrations of aromatic hydrocarbons.

+ Molar ratios of aromatic hydrocarbons to methylene blue (1.64×10^{-4} mol/l).

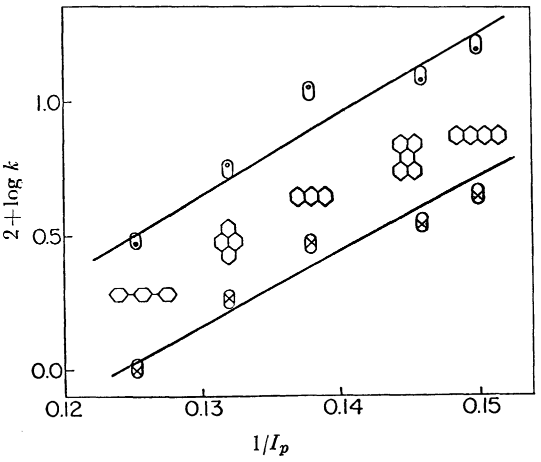

Fig. 8. The relation between the rate constants k, $-\mathrm{d(Mb)}/\mathrm{d}t = k(\mathrm{Mb})$, and ionization potentials (I_p) of aromatic hydrocarbons (Ar):
—⊙— $(\mathrm{Ar})_0$, $1/10 \times 5.45 \times 10^{-5}$ mol/l
—⊗— $(\mathrm{Ar})_0$, $1/100 \times 5.45 \times 10^{-5}$ mol/l

It can be seen in Fig. 7 that the first-order rate constant, k, is proportional to the square root of the initial concentration of aromatic hydrocarbons.

The rate constants of the redox reaction catalyzed by various aromatic hydrocarbons are given in Table 3.

The stabilization energies for the formation of the EDA complexes of aromatic hydrocarbons with methylene blue have been obtained by the second-order perturbation theory,[13] but if the EDA complex formation makes a contribution to a decrease in the activated state of the redox reaction, some correlation must be found between the stabilization energies and the activation energies. In fact, the plots of the logarithms of the rate constant, a measure of the activation energy, against the reciprocal of the ionization potential, a measure of the stabilization energy, gave a good linear relation, as is shown in Fig. 8.

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13) R. S. Mulliken, *J. Amer. Chem. Soc.*, **74**, 811 (1952).