Kinetics and Mechanism of the Isoalloxazine (Flavine) Dehydrogenation of Dimethyl Dihydrophthalates†

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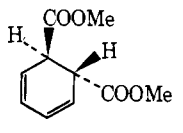
ABSTRACT: The kinetics of the oxidation of dimethyl *trans*-1,2-dihydrophthalate (VIII) and dimethyl 1,4-dihydrophthalate (IX) to dimethyl phthalate by isoalloxazines (I to V) have been investigated. Under the conditions of a large excess of dihydrophthalate over isoalloxazine (flavine) the reaction is initially zero order in flavine. This result is in accord with the trapping of carbanion (formed from carbon acid in a rate-limiting step) by flavine. At very low concentrations of flavine (*e.g.*, 10^{-5} M) and, in particular, in the presence of high concentrations of the protonated component of ethylamine buffer, the kinetic order in flavine changes away from zero order toward first order. Under these conditions, general acid catalyzed reprotonation of carbanion by $EtNH_3^+$ successfully competes with flavine for the carbanion intermediate. Although the logs of the rate constants for reaction of the flavine analogs (I to V) with carbanion do not exhibit a linear dependence upon the reduction potential of flavine, a linear dependence upon the log of the equilibrium constants for complexing by tryptophan was obtained. Thus, as in the previously reported case with NADH, an intermediate face-to-face complex of reactants is suggested. The slope of the plot of log k_r *vs.* log of the equilibrium constant for complexation of I to V with tryptophan is considerably less in the case of the dehydrogenation of VIII than previously found for the dehydrogenation of NADH and *N*-propyldihydronicotinamide. This suggests that the dihydronicotinamides exhibit a higher selectivity to electronic and steric effects in *complexing* with flavine than does the carbanion studied here. The intermediate carbanion involved has been identified kinetically as the one produced in the base-catalyzed isomerization of carbon acid VIII to carbon acid IX. Due to the slower production of carbanion from IX than from VIII, IX is oxidized by flavine at a rate less than one-tenth that noted for VIII. A second carbanion, which would derive easily from IX, is apparently unreactive. It is not possible to differentiate between the three alternative mechanisms of: (1) intracomplex hydride transfer; (2) complex yielding a covalent intermediate which then partitions symmetrically to product and complex; or (3) complex yielding a charge-transfer intermediate which then partitions symmetrically.

Flavoenzymes which catalyze α,β dehydrogenation of carbonyl compounds include fatty acyl-CoA dehydrogenase (Beinert, 1963; Biellman and Hirth, 1970), succinate dehydrogenase (Retey *et al.*, 1970) and Δ^1-, Δ^4-5α-, and Δ^4-5β-3-ketosteroid dehydrogenases (Abul-Hajj, 1971). Furthermore, enzymic aromatization of cyclohexanecarboxyl-CoA to benzoyl-CoA by dehydrogenation *via* cyclohexene-1-carboxyl-CoA involves a flavine coenzyme (Babior and Bloch, 1966).

Recent model studies have revealed flavine-mediated dehydrogenation α,β to carbonyl groups both in the dark (Carr and Metzler, 1970; Weatherby and Carr, 1970a; Brown and Hamilton, 1970) and under illumination (Carr and Metzler, 1970a,b). The base-catalyzed oxidation of dimethyl *trans*-1,2-dihydrophthalate (VIII) to dimethyl phthalate by riboflavine in the dark has been reported by Weatherby and Carr (1970a) to involve a *dicarbanion* derived from the diester by two consecutive ionizations, but several aspects of the mechanisms proposed as well as much of the experimental evidence are not satisfactory.



VIII

As a continuation of our approach to evaluating the significance of preequilibrium complexing and the nature and position of covalent adduct formation as factors in flavine-mediated dehydrogenation reactions (Bruice *et al.*, 1971), we have studied in detail the dark reactions of the isoalloxazines I–V with dimethyl *trans*-1,2-dihydrophthalate (VIII).

Experimental Section

Materials

trans-1,2-Dihydrophthalic acid (VII) was prepared by the method of Baeyer (1892) and was recrystallized from boiling water (mp 208–210°). Baeyer reports a melting point of 210°.

Dimethyl trans-1,2-Dihydrophthalate (VIII). In our hands it was found to be impossible to prepare this ester in pure form using the method of Weatherby and Carr (1970a). The compound was prepared, therefore, by successive additions of diazomethane in ether to a solution of VII in ice-cold methanol. When a faint yellow color persisted, the solution was evaporated and the residue was taken up in carbon tetrachloride and dried ($CaCl_2$). The solution was chromatographed under nitrogen on a short (*ca.* 2.5 cm) column of silica gel using carbon tetrachloride as eluent. Evaporation of the eluting solvent gave the diester as a colorless oil. It is conveniently stored as a dilute solution in carbon tetrachloride under which condition it is quite stable. The only contaminants of the ester when prepared in this manner are small amounts of dimethyl phthalate and dimethyl 1,4-dihydrophthalate (IX). The percentages of these impurities are easily calculated from nuclear magnetic resonance (nmr) spectra and the nmr absorptions listed here are those for

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VIII alone. The nmr spectrum (CCl_4) shows a 8H singlet (δ 3.75) and 4H multiplet (δ 5.8–5.9). In benzene, the 8H singlet is split into two singlets (2 H and 6 H) separated by 44 Hz, showing that in the CCl_4 spectrum, the singlets of the methyl protons and the 1,2 ring hydrogens are superimposed.

Dimethyl 1,4-dihydrophthalate (*IX*) was prepared by chromatography under nitrogen of VIII on a short (*ca.* 1.5 cm) column of alumina (MC/B) using CCl_4 as eluent. It was also prepared by boiling VII in water for several hours, recrystallizing the rearranged acid from water (charcoal) and esterifying it with diazomethane, followed by chromatographic purification on silica gel as for VIII. This ester can be obtained essentially free of VIII but there is always some dimethyl phthalate impurity. The percentage of this impurity is established by nmr analysis. The spectrum recorded here is that for IX alone.

The nmr spectrum (CCl_4) shows a 2H multiplet (δ 2.85–3.2), 3H singlet (δ 3.69), 4H singlet (δ 3.76), 2H multiplet (δ 5.8–5.9), and 1H multiplet (δ 6.98–7.10). In benzene the 4H singlet appears upfield from the 3H singlet.

N-Methylbarbituric acid (*X*) was prepared as for barbituric acid (Dickey and Gray, 1943). It was reacted with benzaldehyde to form *1-methyl-5-benzalbarbituric acid* (*XI*) which was oxidized to *N-methylalloxan* (*XII*) according to the method of Biilmann and Berg (1930).

2-Nitro-N-(2',6'-dimethylphenyl)aniline (*XIII*) was synthesized by heating a stirred mixture of 2,6-dimethylaniline (25 g, 0.2 mole), 2-fluoronitrobenzene (14 g, 0.10 mole), and powdered potassium acetate (12 g, 0.12 mole) at 180° for 12 hr and then at 140–50° for 36 hr. A benzene extract (250 ml) of the product was washed successively with water (two 500-ml portions), 20% HCl (one 400-ml portion and six 100-ml portions) and water (two 500-ml portions). The benzene layer was dried ($CaCl_2$) and evaporated to yield an oil which partially crystallized overnight. Filtering yielded the crude product: 8.5 g, mp 75–95°. Three recrystallizations from methanol gave pure VI: mp 106–107° (4.6 g, 26%); strong ν_{max} (KBr) 743, 772, 1247, 1338, 1485, 1560, 1612, and 3330 cm^{-1}. *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.4; H, 5.82; N, 11.56. Found: C, 69.7; H, 5.96; N, 11.27.

10-(2',6'-Dimethylphenyl)-3-methylisoalloxazine (*I*). Compound XIII (3.2 g, 0.013 mole) was dissolved in hot ethanol (100 ml, 95%) and Raney nickel (*ca.* 0.5 g) was added. Hydrazine hydrate (30 ml, 8.5% in ethanol, *ca.* 0.05 mole) was added to the warm (*ca.* 50°) solution. After complete decolorization, the solution was filtered and evaporated (70–80°). The *o*-phenylenediamine (an oil) was immediately taken up in hot glacial acetic acid to prevent air oxidation. To this solution was added a warm solution of *N*-methylalloxan (XII) (2.16 g, 0.014 mole) and boric acid (1 g, 0.016 mole) in glacial acetic acid. The solution turned an intense deep blue color which slowly changed to green-brown. After standing (1 hr at *ca.* 50°) the solvent was evaporated and the residue was taken up in chloroform (150 ml). Hexane (150 ml) was added and after filtration the solution was chromatographed on alumina (MC/B activated) using 1:1 chloroform–hexane as eluting solvent. The fluorescent yellow-green fraction was collected and on evaporation gave I: mp 296–298° (4.1 g, 93%). Recrystallization from methanol gave bright yellow prismic crystals: mp 297–299°. *Anal.* Calcd for $C_{19}H_{16}N_4O_2$: C, 68.66; H, 4.85; N, 16.86. Found: C, 68.45; H, 4.90; N, 16.69.

2-Nitro-N-(2'-methylphenyl)aniline (*XIV*) was synthesized as for XIII using 2-methylaniline but under somewhat less forcing conditions. It had mp 71–72°.

10-(2'-Methylphenyl)-3-methylisoxalloxazine (*II*) was prepared from XIV in 75% yield. It was purified according to the method described for I. Compound II melts at 372°. *Anal.* Calcd for $C_{18}H_{14}N_4O_2$: C, 67.92; H, 4.43; N, 17.60. Found: C, 67.71; H, 4.36; N, 17.30.

2-Nitro-N-phenylaniline (*XV*) was prepared as for XIII but using aniline and 2-fluoronitrobenzene. It had mp 72–74°. Goldberg (1907) reported a melting point of 75°.

10-Phenyl-3-methylisoalloxazine (*III*) was prepared from XV according to the method for I. It was purified by chromatography on alumina using chloroform as eluting solvent and was recrystallized from boiling dimethylformamide as fine yellow needles. Compound III decomposes partially but does not melt below 400°. *Anal.* Calcd for $C_{17}H_{12}N_4O_2$: C, 67.10; H, 3.97; N, 18.41. Found: C, 66.97; H, 3.77; N, 18.25.

2-Nitro-4-chloro-N-methylaniline (*XVI*) was prepared by heating 2,5-dichloronitrobenzene (9.6 g, 0.05 mole), methylamine hydrochloride (10 g, 0.15 mole), and powdered potassium acetate (20 g, 0.2 mole) under reflux at 140° for 3 hr. A benzene extract of the product was washed successively with water (500 ml), sulfuric acid (four 250-ml portions, 1 M), and water (two 500-ml portions) before being dried and evaporated. The residue was dissolved in a chloroform–hexane mixture (40:60) and chromatographed on alumina. The first orange fraction yielded XVI. Recrystallization from ethanol gave orange needles: mp 106°.

7-Chloro-3,10-dimethylisoalloxazine (*IV*) was synthesized from XVI as for I. It precipitated from the reaction solution as a powder. Purification by chromatography on alumina with chloroform as eluting solvent followed by recrystallization from dimethylformamide gave fine orange crystals: mp 295–298°. *Anal.* Calcd for $C_{12}H_9ClN_4O_2$: C, 52.09; H, 3.28; Cl, 12.81; N, 20.25. Found: C, 52.24; H, 3.44; Cl, 12.62; N, 19.97.

3,10-Dimethylisoalloxazine (*V*) was prepared from 2-nitro-*N*-methylaniline and purified according to the method described for IV. It crystallized from dimethylformamide as orange-yellow needles: mp 302–304°. *Anal.* Calcd for $C_{12}H_{10}N_4O_2$: C, 59.50; H, 4.16; N, 23.13. Found: C, 59.39; H, 3.98; N, 23.01.

Apparatus and Methods

Nmr spectra were recorded on a Varian T-60 instrument using an external reference (Me_4Si, $CHCl_3$). A Perkin-Elmer 237 spectrophotometer was used to record *ir spectra*. *Kinetic measurements* were made on a Zeiss-PMQ or a Cary 15 spectrophotometer.

Polarographic half-wave potentials were determined at 30° in borate buffer solution (pH 8.97, μ = 0.20, 5 vol % dimethylformamide) using a Sargent Model XV polarograph equipped with a saturated calomel reference electrode.

Fluorescence quenching studies were carried out at 22° on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer equipped with a Hitachi QPD 33 recorder, using wavelengths of 430–450 mμ (excitation) and *ca.* 510 mμ (maximum emission). β-Resorcylic acid (2,4-dihydroxybenzoic acid) and tryptophan (0–0.002 M) were used as quenchers in phosphate buffer solution (pH 7.85, μ = 2.05, 5 vol % dimethylformamide) containing the isoalloxazines (*ca.* 10^{-5} M).

Kinetic Measurements and Analysis for Dimethyl Dihydrophthalates. Reaction solutions were prepared from deaerated (N_2) solutions of aqueous buffer, isoalloxazines in acetonitrile, and dihydrophthalate esters in 1-butanol in such volumes that the final reaction solution contained 2.5% by volume of each of the organic solvents. The esters were always

present in at least 15-fold excess over isoalloxazine in order that their concentrations be effectively constant during a run.

Nitrogen was bubbled through the buffer solution containing the isoalloxazine in a modified Thunberg cuvet for about 15 min before the nitrogen-flushed cell cap, containing the ester dissolved in 1-butanol in the side arm, was fitted. The whole cell was then evacuated using a water aspirator. Nitrogen was readmitted. This cycle was repeated about 30 times before closing the cell at atmospheric pressure under nitrogen. Oxygen reacts rapidly with reduced isoalloxazines to regenerate the oxidized form, but as no lag period in the reduction of isoalloxazines was observed and reproducible clean zero-order kinetics were obtained the effect of any residual oxygen after the above deaeration procedure is negligible.

After temperature equilibration (29.5°) the contents of the side arm of the Thunberg cuvet were mixed in and changes in optical density at 435 mμ (isoalloxazines) or at 255 mμ (dimethyl *trans*-1,2-dihydrophthalate) were followed. In all cases, admittance of O_2 at t_∞ regenerated oxidized isoalloxazines. First-order rate constants (ester isomerization experiments) were calculated on an Olivetti Underwood Programma 101 using the Guggenheim method of analysis. The disappearance of isoalloxazine was found to be initially of zero order in isoalloxazine (linear optical density *vs.* time plots) but changed toward first order at low concentrations of isoalloxazine. Rates d[F]/dt of zero-order disappearance of isoalloxazines were therefore determined from the slopes d[A]/dt of optical density (D) *vs.* time plots up to the point (*ca.* 60–70% in the reactions under study) where the plots deviated from linearity, making use of the relationship d[A]/dt = ϵ(d[F]/dt), where ϵ is the extinction coefficient of the isoalloxazine (ϵ values × 10^{-4} are for: I, 1.00; II, 1.01; III, 1.04; IV, 1.03; V, 1.01).

When ethylamine is used as the buffer, the reaction deviates from zero order sooner and it is possible to obtain rate data from the latter portion of the reaction. A generalized treatment is given here, but A and B can be taken in the present context as representing the ester VIII and the carbanion VIIIA, and F the flavine or isoalloxazine. From steady-state consideration

$$A \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} B \xrightarrow{k_b[F]} \text{product} \qquad (1)$$

one obtains eq 2. From this equation it is evident that the

$$\frac{-d[F]}{dt} = k_b[F][B] = \frac{k_a[A][F]}{k_{-a}/k_b + [F]} \qquad (2)$$

rate is zero and first order in F at high and low [F], respectively. If the optical density of F is D and the extinction coefficient is ϵ then [F_t] is given by eq 3 and eq 2 can be rewritten

$$[F_t] = (D_t - D_\infty)/\epsilon = \Delta D/\epsilon \qquad (3)$$

as eq 4. Equation 4 can be easily programmed on an analog

$$\frac{-d[F]}{dt} = \frac{k_a[A](\Delta D/\epsilon)}{k_{-a}/k_b + (\Delta D/\epsilon)} \qquad (4)$$

computer (EAI TR-20), where k_a[A] and k_{-a}/k_b can be varied until the computer generated ΔD *vs.* time curve coincides with the experimental points. A typical computer generated plot along with computer wiring diagram is shown in Figure 1.

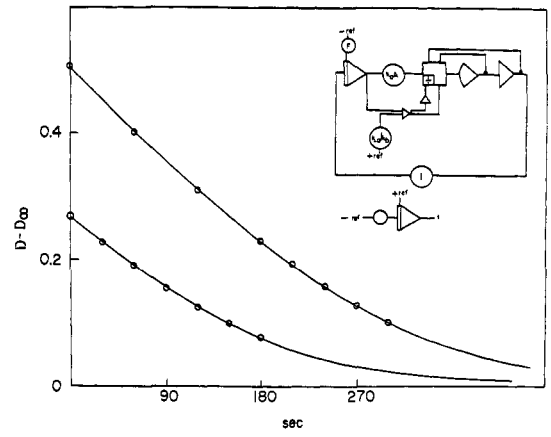


FIGURE 1: Plot of $D - D_\infty$ *vs.* time for the reaction of II (lower) and III (upper) with IX in ethylamine buffer, $[EtNH_2]_T$ = 0.0897 (pH 9.5), μ = 1.0 (KCl). The points are experimental and the line is generated by an analog computer wired as shown in the inset to solve eq 4.

The partition factor k_b/k_{-a} was determined in this manner. Thus, relative rates of reaction of a series of different F's with B are given by the relative values of k_b/k_{-a}, since k_{-a} is the same in each case.

Kinetic Measurements for 1,4-Butanedithiol. Techniques used in kinetic studies of the anaerobic oxidation of 1,4-butanedithiol by isoalloxazines (I–V) (29.9°, pH 8.98) were essentially as described for the dihydrophthalate esters (*vide supra*). The reaction solutions consisted of aqueous borate buffer (μ = 0.20) containing 5 vol % dimethylformamide. Solutions of 1,4-butanedithiol used in preparation of the reaction solutions were standarized by iodometric titration. The reactions were found to be first order in both isoalloxazine and dithiol. Second-order rate constants (k_b) were calculated from pseudo-first-order rate constants (k_{obsd}) by dividing by the concentration of (excess) 1,4-butanedithiol. Mean values of k_b are given (see Figure 4).

Results

Polarography. The half-wave potentials of the isoalloxazines are recorded in Table I.

Fluorescence Quenching. The observed decrease in the fluorescence of an isoalloxazine with increasing concentrations of tryptophan or β-resorcylic acid was interpreted as resulting from the formation (eq 5) of a nonfluorescent complex (*cf.* Weber, 1965)

$$F + Q \rightleftharpoons FQ \qquad (5)$$

The equilibrium constant, K (eq 6), for the complexing

$$K = [FQ]/[F][Q] \qquad (6)$$

TABLE I: Polarographic Half-Wave Potentials at 30°, pH 8.97, with Respect to Saturated Calomel Electrode.

Isoalloxazine	I	II	III	IV	V
$E_{1/2}$ (V)	0.440	0.445	0.450	0.445	0.480

TABLE II: Equilibrium Constants for Complexing of Isoalloxazines with β-Resorcylic Acid and Tryptophan, 22°, pH 7.85.

Isoalloxazine	K_1 (β-Resorcylic Acid) (M^{-1})	K_1 (Tryptophan) (M^{-1})
I	13.6	18
II	33	31
III	38	44
IV	80	112
V	55	82

was calculated from eq 7

$$K = \left(\frac{I_0 - I}{I}\right)\frac{1}{[Q]} \quad (7)$$

where I_0 and I are the recorded fluorescence intensities in the absence and presence, respectively, of quencher at concentration [Q].

Values of K (eq 7) were found not to be constant, however, but to increase slightly with [Q], *e.g.*, for IV, values of K at [β-resorcylic acid] = 4×10^{-3}, 8×10^{-3}, 12×10^{-3}, and 16×10^{-3} M were calculated as 90, 101, 112, and 122 M^{-1}. We interpret this increase in K as due to formation of a second 2:1 complex[1] (eq 8) since this leads to the linear expression

$$FQ + Q \rightleftharpoons FQ_2 \quad (8)$$

(eq 9) consistent with the above data

$$K = K_1 + K_1K_2[Q] \quad (9)$$

where K_1 now replaces K as the equilibrium constant for eq 6 and K_2 applies to eq 8. Values of K_1 were obtained by least-squares analysis based on eq 9 and are recorded in Table II. Values of K_2 (not recorded) are small and subject to considerable experimental error.

Kinetics. A. ZERO-ORDER REDUCTION OF ISOALLOXAZINES. Initial runs showed that rates were insensitive to the concentration of carbonate buffer to 0.12 M at constant pH. In this buffer (μ = 1.0 [KCl], pH 9.72, 29.5°) rates of disappearance of isoalloxazines were found to be linear with time up to about 70% reaction. To show that this portion of the reaction was truly zero order in isoalloxazine, we measured these rates, d[F]/dt, with varying concentrations of ester (VIII). For each isoalloxazine d[F]/dt is proportional to [ester] (Table III). Mean values of k_{obsd} defined by eq 10 were determined

$$k_{obsd} = (d[F]/dt)/[ester] \quad (10)$$

by least-squares analysis and are seen (Table III) to be equal within experimental error for all isoalloxazines.

It is evident, therefore, that the ester (AH_2) forms a reactive intermediate in a slow (mean k_{obsd} = 0.0144 min^{-1}) step and that this intermediate reacts rapidly with isoalloxazines. In eq 11 and 12 this intermediate is written as a carbanion al-

[1] 2:1 complexes of naphthalane-2,3-diol with lumiflavine have recently been reported by Trus *et al.* (1971).

TABLE III: Zero-Order Rates of Isoalloxazine Reduction by Dimethyl *trans*-1,2-Dihydrophthalate;[a] pH 9.72, Carbonate Buffer, 29.5°.

Isoalloxazine	$10^5\left(\frac{d[F]}{dt}\right)$ (M min^{-1}) At [ester]($\times 10^{-4}$ M) 4.41	9.45	17.85	$k_{obsd} = \frac{d[F]/dt}{[ester]}$ (min^{-1})
I	0.564	1.20	2.67	0.0158
II	0.558	1.26	2.45	0.0141
III	0.533	1.36	2.45	0.0141
IV	0.586	1.20	2.43	0.0138
V	0.635	1.32	2.64	0.0144

[a] The ester contained 15% of the rearranged ester IX (see subsequent discussion in kinetics section C.

$$AH_2 \xrightarrow[slow]{-H^+} AH^- \xrightarrow[fast]{F} \text{products} \quad (11)$$

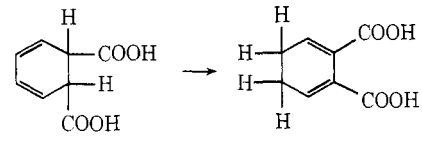
$$-d[F]/dt = +d[AH^-]/dt = -d[AH_2]/dt = k_{obsd}[AH_2] \quad (12)$$

though a study of the effect on the reaction of pH and concentrations of buffer components was necessary to disprove the possibility that the reactive species is not, for instance, an isomerized dihydrophthalate ester.

B. ISOMERIZATION OF DIMETHYL *trans*-1,2-DIHYDROPHTHALATE. Weatherby and Carr (1970a) reported a base-catalyzed rearrangement of this ester in competition with its reaction with riboflavine; on standing in alkaline solution (anaerobic) the optical density at 255 nm (λ_{max} for the ester) decreases. Using carbonate buffer solutions (μ = 1.0, KCl) we measured the rate of this reaction initially to determine whether blank corrections might be necessary for the reactions in the presence of isoalloxazines. Rates were found to be first order in ester over the pH range 9.9–11.2, and there was no measurable buffer catalysis. A plot of k_{obsd} *vs.* [OH^-] is linear and this finding eliminates the possibility that an associated ionization (*cf.* eq 15) has a pK in this pH range, in spite of suggestions by Weatherby and Carr (1970a) to the contrary. Least-squares analysis gives a second-order rate constant (eq 13) of k_{2nd} = 233 M^{-1} min^{-1}.

$$-d[AH_2]/dt = k_{obsd}[AH_2] = k_{2nd}[OH^-][AH_2] \quad (13)$$

By analogy with the reported rearrangement in boiling water of *trans*-1,2-dihydrophthalic acid to 4,5-dihydrophthalic acid (eq 14) (Baeyer, 1892) we anticipated the corresponding



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rearrangement for the diester. The nmr spectrum (see Experimental Section) of the ester in CCl_4 after extraction from anaerobic basic solution after ten rearrangement half-lives was indeed consistent with a 1:1 mixture of the corresponding

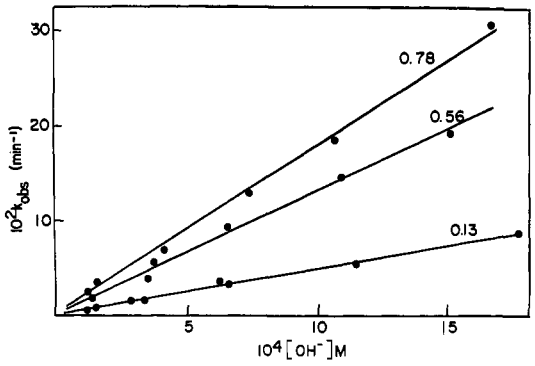


FIGURE 2: k_{obsd} *vs.* [OH^-] for reaction of III with mixtures of the esters VIII and IX containing the shown fractions (f^{VIII}) of VIII. Carbonate buffer, 29.5°.

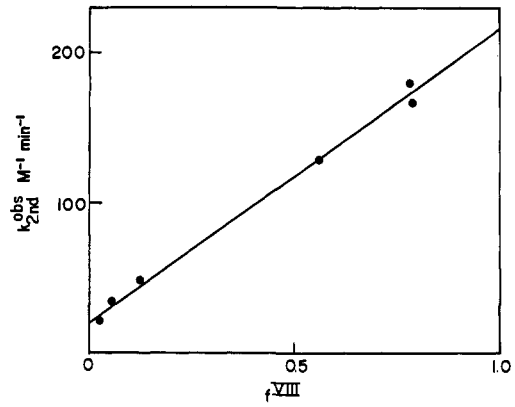
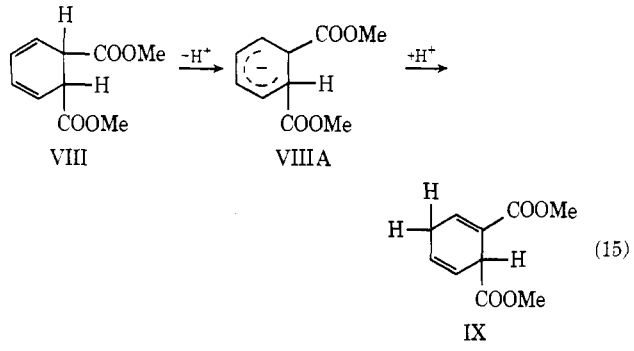


FIGURE 3: k_{2nd}^{obsd} *vs.* f^{VIII} for reaction of mixtures of esters VIII and IX with V. Intercepts: k_{2nd}^{obsd} = 19 M^{-1} min^{-1} at f^{VIII} = 0; k_{2nd}^{obsd} = 216 M^{-1} min^{-1} at f^{VIII} = 1.0.

esters. The nmr spectrum in benzene of the ester rearrangement product proved the absence of dimethyl *trans*-1,2-dihydrophthalate (VIII), and we therefore conclude that VIII rearranges completely (eq 15) to dimethyl 1,4-dihydrophthal-



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ate. Not only is IX consistent with the nmr spectrum, but the decrease in uv absorption at 255 nm (λ_{max} for a cyclic conjugated diene) when VIII rearranges shows that a nonconjugated diene is formed.

Since analogous ultraviolet (uv) spectral changes are observed when 1,2-dihydrophthalic acid rearranges upon boiling in water, and since the ester IX can be prepared by reaction of diazomethane with the rearranged acid, we prefer to formulate the latter as 1,4-dihydrophthalic acid rather than 4,5-dihydrophthalic acid as previously reported (Baeyer, 1892; eq 14).

C. ZERO-ORDER RATES WITH MIXTURES OF ESTERS VIII AND IX. The zero-order rate of reduction of isoalloxazines in the presence of VIII implies the formation of a reactive species from VIII in a slow step. The possibility that this reactive species is the carbanion VIIIA must be considered. If VIIIA reacts to form IX (by protonation) in the absence of isoalloxazine, and to form dimethyl phthalate in the presence of isoalloxazines, then it is obvious that the rate of conversion of VIII to IX and the rate of reduction of isoalloxazines by VIII should be equal. The rate of rearrangement (eq 15) is given by $k_{2nd} = k_{obsd}/[OH^-]$ = 223 M^{-1} min^{-1} (see section B). The rate of zero-order reduction of isoalloxazine, *i.e.*, the rate of formation of the isoalloxazine reactive species from VIII (85% pure, Table III), is given by k_{obsd} = 0.0144 min^{-1} at pH 9.72, *i.e.*, $k_{2nd} = k_{obsd}/[OH^-]$ = 182 M^{-1} min^{-1} using pK_w = 13.83. It therefore appears quite possible that VIIIA is responsible for reduction of isoalloxazines.

To further test this hypothesis more closely, rates of reduction of isoalloxazines by mixtures of VIII and IX were determined. As mentioned in the Experimental Section, it was not possible to obtain VIII free from IX or free from dimethyl phthalate. Mixtures of synthetic VIII and IX were therefore prepared and the compositions of the mixtures in terms of VIII, IX, and dimethyl phthalate were determined by nmr analysis of samples of each mixture dissolved in carbon tetrachloride. A known weight of each ester mixture was then dissolved in a known volume of 1-butanol and the concentration of VIII and IX in the resulting solution was calculated using the nmr percentage composition data.

The zero-order rates of reduction of V by each mixture were determined in carbonate buffer solution (pH 9.8–11.3) and values of k_{obsd} (eq 10) were calculated in terms of total ester (VIII + IX). In Figure 2 is plotted k_{obsd} *vs.* [OH^-] for three of a series of ester mixtures examined. Values of k_{2nd}^{obsd} were obtained from slopes defined by eq 16 and are given

$$k_{obsd} = k_{2nd}^{obsd}[HO^-] \quad (16)$$

in Table IV along with the fractions of ester VIII defined by eq 17. Figure 3 shows that k_{2nd}^{obsd} is linear in f^{VIII}. The inter-

$$f^{VIII} = [VIII]/([VIII] + [IX]) \quad (17)$$

cept at f^{VIII} = 1.0 (k_{2nd}^{obsd} = 216 M^{-1} min^{-1}) is the rate constant for formation of the isoalloxazine-reactive species from pure VIII and it is, in fact, equal to the rate constant for rearrangement of VIII to IX (eq 15) in the absence of isoalloxazine (k_{2nd} = 223 M^{-1} min^{-1}). This is compelling evidence that the formation of the carbanion VIIIA (eq 21) is rate limiting for both the ester rearrangement and (under the zero-order conditions) the isoalloxazine reduction and that VIIIA is

TABLE IV: Values of k_{2nd}^{obsd} for Ester Mixtures Containing Fraction f^{VIII} of VIII: Carbonate Buffer (μ = 1.0, KCl), 29.5°, K_w = 13.83.[a]

f^{VIII}	0.03	0.06	0.13	0.56	0.78	0.79
k_2^{obsd} (M^{-1} min^{-1})	21.6	32.4	47	126	180	168

[a] Robinson and Stokes (1959).

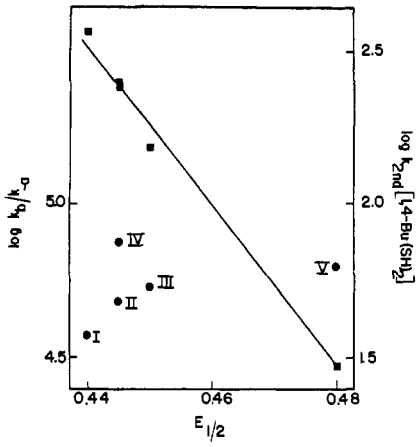


FIGURE 4: Log (k_b/k_{-a}) *vs.* $E_{1/2}$ for the reaction of the carbanion with isoalloxazines I-V (●). (■) Linear relationship observed for a reagent (1,4-butanedithiol; Bruice *et al.*, 1971) whose attack on the isoalloxazine nucleus is unhindered by the 10-phenyl substituents of I, II, and III.

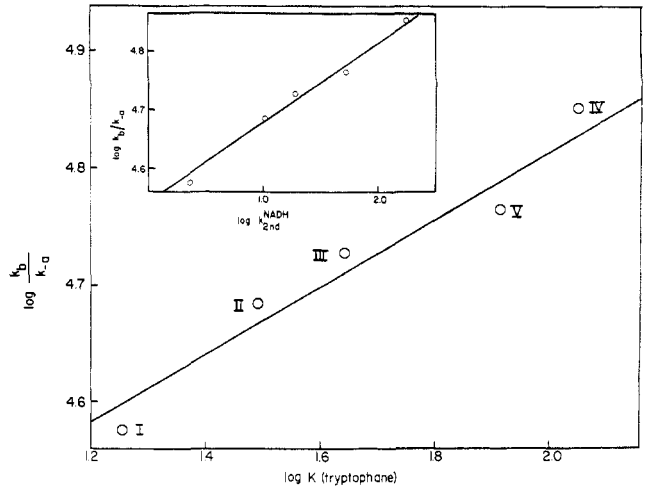


FIGURE 5: Log (k_b/k_{-a}) *vs.* log K_1 for equilibrium complexing of the isoalloxazines with tryptophan. The slope is 0.29 which is less than that recorded for NADH (2.25 for I–V) by Bruice *et al.* (1971). The inset shows log (k_b/k_{-a}) *vs.* log k_{2nd}^{NADH} (slope 0.14).

a common intermediate in these reactions. There is a positive intercept $(k_{2nd}^{obsd} = 19\ M^{-1}min^{-1})$ at $f^{VIII} = 0$ showing that the rearranged ester IX yields carbanion which reduces isoalloxazines but only at less than one-tenth the rate of VIII. IX may yield two carbanions since it possesses two ionizable protons; a discussion of which carbanion, if not both, is reactive with isoalloxazines is deferred to the Discussion section.

D. RELATIVE RATE CONSTANTS FOR REACTION OF THE CARBANION(S) WITH ISOALLOXAZINES I TO V. It was evident from optical density–time curves that different isoalloxazines reacted with the carbanion(s) at different rates. For instance, the deviation away from zero-order kinetics occurred earlier with I than with V. The value of k_b/k_{-a} for each isoalloxazine was therefore determined (by analog computer) under fixed conditions [ethylamine buffer, μ = 1.0 (KCl), [total ester] = 1.2×10^{-3} M, range of measured pH 9.47–9.54]. Values of k_b/k_{-a} were determined for a mixture of VIII (75%) and IX (25%) [$(k_b/k_{-a})_A$ values] and also for 100% IX [$(k_b/k_{-a})_B$ values]. It is evident from Table V that the k_b/k_{-a} values are similar for both esters, thus eliminating the possibility that leakage of VIII to IX is important. Since under the constant reaction conditions the rate constant for reprotonation of the carbanion (k_{-a}) is the same irrespective of the isoalloxazine, the mean values of log (k_b/k_{-a}) given in the table can be taken as relative values of the rate constant for reaction of the carbanion with the various isoalloxazines (k_b).

TABLE V: Relative Rates of Reaction of Isoalloxazines with the Carbanion(s) Derived from Esters VIII and IX.

Isoalloxazine	$10^{-4}\cdot(k_b/k_{-a})_A$[a]	$10^{-4}\cdot(k_b/k_{-a})_B$[b]	Mean $10^{-4}\cdot(k_b/k_{-a})$	Log (k_b/k_{-a})
I	3.76	3.77	3.76	4.58
II	3.96	5.82	4.82	4.68
III	5.88	4.83	5.35	4.73
IV	6.94	7.30	7.12	4.85
V	6.40	5.24	5.82	4.77

[a] Total ester concentration 1.08×10^{-3} M (75% VIII) and initial flavine concentration 3.5 to 5.3×10^{-5} M. [b] Ester concentration 1.11×10^{-3} M and initial flavine concentration 3.6×10^{-5} to 5.88×10^{-5}.

In Figure 4 is plotted log (k_b/k_{-a}) *vs.* the polarographic half-wave potential for each isoalloxazine ($E_{1/2}$) and, as is the case of NADH (Bruice *et al.*, 1971), there is evidently no linear correlation such as that observed for 1,4-butanedithiol (Figure 4) (Bruice *et al.*, 1971). A linear correlation of positive slope in a plot of log (k_b/k_{-a}) *vs.* log K_1, the equilibrium constant for 1:1 complexing of isoalloxazines with tryptophan, is, however, obtained (Figure 5). As in the case of our studies with NADH we take this to imply that face-to-face complexing of isoalloxazines with reductant (in this case the carbanion) precedes hydrogen transfer. The slope for the linear correlation (0.29) is, however, considerably less than that for NADH (2.25) showing that the latter reagent exhibits a higher selectivity to the steric and electronic effects which contribute to complex formation. This is also evident from the slope (0.14) of the correlation of relative rate constants (Figure 5, inset). Since there is only a slight difference in rate of reaction of IV and V with the carbanion as compared to 1,4-butanedithiol, electronic effects evidently have little bearing on the selectivity shown by the carbanion for isoalloxazines. Steric factors evidently predominate. That complex formation occurs along the reaction pathway is supported by the fact that the rate *increases* as K_1 *increases*.

E. EFFECTS OF pH AND BUFFER CONCENTRATION ON THE RATE OF REACTION OF THE CARBANION WITH ISOALLOXAZINES. The complex of carbanion and isoalloxazine might yield products in a single stage (*e.g.*, by hydride ion transfer) or alternatively, it might give rise to a second discrete intermediate such as a covalent adduct or a charge-transfer complex. As a probe for such an intermediate, the effects on k_b/k_{-a} of varying pH and concentration of buffer components ($EtNH_2$ and $EtNH_3^+$) were examined in an attempt to detect rate-limiting base-catalyzed decomposition of a possible intermediate. For kinetic analysis a general scheme (eq 18)

$$AH_2 \underset{k_{-1}[H_2O]\,/\,k_{-2}[RNH_3^-]}{\overset{k_1[HO^-]\,/\,k_2[RNH_2]}{\rightleftharpoons}} AH^- \overset{Keq[F]}{\rightleftharpoons} C \underset{k_{-3}[HO^-]\,/\,k_{-4}[RNH_2]}{\overset{k_3[H_2O]\,/\,k_4[RNH_3^+]}{\rightleftharpoons}} D \xrightarrow{k_5[HO^-]\,/\,k_6[RNH_2]} \text{product} \qquad (18)$$

assuming general acid and base catalysis at each state was considered. C represents the initial complex of carbanion and isoalloxazine and D is the intermediate succeeding C on the reaction path. Steady-state treatments in AH^-, C, and D lead to a rate equation which can be conveniently rearranged to give eq 19

$$\frac{d[F]}{dt} = \frac{C[A][F]}{W(1/K_{eq})Z + [F]} \quad (19)$$

where

$$C = (k_1 + k_2[RNH_3^+]K_b)[OH^-]$$

$$W = \frac{k_{-1}[H_2O] + k_{-2}[RNH_3^+]}{k_3[H_2O] + k_4[RNH_3^+]}$$

$$Z = 1 + \frac{k_{-3} + k_{-4}[RNH_3^+]K_b}{k_5 + k_6[RNH_3^+]K_b}$$

and K_b is the basicity constant of the amine. Comparing eq 19 to eq 2, it is evident that $[W(1/K_{eq})Z] = k_{-a}/k_b$. Experimentally we have found k_{-a}/k_b to be linear in $[RNH_3^+]$ over a wide range of pH, as shown in Figure 6 for the isoalloxazine V. To obtain linearity of k_{-a}/k_b *vs.* $[RNH_3^+]$ either term W must be linear in $[RNH_3^+]$ and term Z independent of $[RNH_3^+]$ or W must be independent of $[RNH_3^+]$ and Z linear in $[RNH_3^+]$. For W to be linear in $[RNH_3^+]$ requires that $k_3[H_2O] \gg k_4[RNH_3^+]$, which on the basis of the Brønsted concept, the pK_a's of H_2O and RNH_3^+ and the maximum $[RNH_3^+] \cong 0.5$ M is unreasonable. We are, therefore, restricted to schemes dependent on term Z of eq 19 being α to $[RNH_3^+]$. For term Z to be linear in $[RNH_3^+]$ requires that $k_5[OH^-] \gg k_6[RNH_2]$, and for term W to be independent of RNH_3^+ either $k_{-1}[H_2O] \gg k_{-2}[RNH_3^+]$ and $k_3[H_2O] \gg k_4[RNH_3^+]$, which is not reasonable (*vide supra*), or $k_{-1}[H_2O] \ll k_{-2}[RNH_3^+]$ and $k_3[H_2O] \ll k_4 \cdot [RNH_3^+]$, which is acceptable, and gives rise to eq 20.

$$AH_2 \underset{k_{-2}[RNH_3^+]}{\overset{k_1[HO^-]}{\underset{k_2[RNH_2]}{\rightleftharpoons}}} AH^- \overset{K_{eq}[F]}{\rightleftharpoons} C \underset{k_{-4}[RNH_2]}{\overset{k_4[RNH_3^+]}{\underset{k_{-3}[HO^-]}{\rightleftharpoons}}} D \xrightarrow{k_5[HO^-]} \text{product} \quad (20)$$

Under the experimental conditions ($[RNH_2]_{max} \cong 0.05$ M) catalysis by RNH_2 in D → P might not have been detected. Also, it is possible that H_2O catalysis of $AH^- \rightarrow AH_2$ and C → D would not have been detected. In any event, HO^- catalysis of $AH_2 \rightarrow AH^-$ and D → C requires, by microscopic reversibility, H_2O catalysis of $AH^- \rightarrow AH_2$ and C → D. These considerations suggest the scheme of eq 18.

Two alternative kinetic schemes fit the kinetic data under the special condition that the step C → D is spontaneous (eq 21 and 22). Equation 22 can be rejected as it implies gen-

$$AH_2 \underset{k_{-2}[RNH_3^+]}{\overset{k_1[HO^-]}{\underset{k_2[RNH_2]}{\rightleftharpoons}}} AH^- \overset{K_e[F]}{\rightleftharpoons} C \underset{k_{-4}[HO^-]}{\overset{k_3}{\rightleftharpoons}} D \xrightarrow{k_5[HO^-]} \text{product} \quad (21)$$

$$AH_2 \underset{k_{-2}[RNH_3^+]}{\overset{k_1[HO^-]}{\underset{k_2[RNH_2]}{\rightleftharpoons}}} AH^- \overset{K_e[F]}{\rightleftharpoons} C \underset{k_{-4}[RNH_2]}{\overset{k_3}{\rightleftharpoons}} D \xrightarrow{k_6[RNH_2]} \text{product} \quad (22)$$

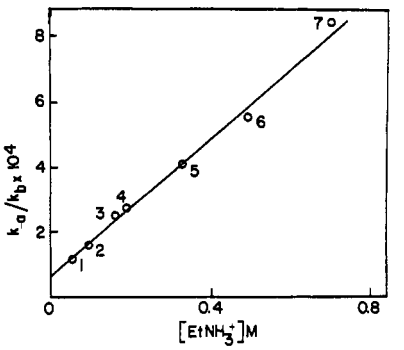


FIGURE 6: k_{-a}/k_b *vs.* $[EtNH_3^+]$ for 3,10-dimethylisoalloxazine (V) with IX. pH values for points: 1, 10.53; 2, 10.07; 3, 9.56; 4, 10.10; 5, 9.55; 6, 9.57; 7, 9.04.

eral base catalysis without catalysis by $[HO^-]$. A major contribution by HO^- would be expected in view of the low concentrations of free amine used experimentally (*vide supra*). Equation 21 implies specific base-catalyzed breakdown of D. This possibility cannot be ruled out. The C → D process would necessarily be a two-stage one, with rapid protonation by H_2O after the rate-limiting formation of an ionized D species. There is thus no contradiction of the principle of microscopic reversibility in the k_5 steps as superficial consideration of eq 21 might suggest.

A fourth scheme (eq 23), which also fits the kinetic data,

$$AH_2 \underset{k_{-2}[RNH_3^+]}{\overset{k_1[HO^-]}{\underset{k_2[RNH_2]}{\rightleftharpoons}}} AH^- \overset{K_e[F]}{\rightleftharpoons} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D \xrightarrow{k_5} \text{product} \quad (23)$$

is one in which not only the C → D but also steps D → P and D → C are spontaneous. Under these conditions, k_{-a}/k_b is represented by eq 24 and it is evident that k_{-a}/k_b will

$$\frac{k_{-a}}{k_b} = \left(\frac{k_{-1}[H_2O] + k_{-2}[RNH_3^+]}{k_5}\right)\left(\frac{1}{K_{eq}}\right)\left(1 + \frac{k_{-3}}{k_5}\right) \quad (24)$$

be linear in $[RNH_3^+]$.

In summary, four kinetic schemes (eq 18, 21, 22, and 23) are consistent with the kinetic data. Of these, only one (eq 22) can be excluded so far, though 21 will be shown (see Discussion) to be unlikely. Among the acceptable schemes, the first (eq 18) involves general base catalysis of the decomposition of D to products; the second (eq 21) involves specific base catalysis; and in the third (eq 24) the decomposition is spontaneous. The three schemes, do, however, have one characteristic in common: if complex of carbanion and isoalloxazine proceed through an intermediate to product then partitioning of intermediate (D) between products (P) and complex (C) is symmetrical with regard to participation by acid and base catalytic species.

These kinetic studies establish the important findings that (i) a complex (C) of carbanion with isoalloxazine is formed, and (ii) if an intermediate (D) succeeding C on the reaction path is formed then it symmetrically partitions between C and products.

Discussion

The present study establishes that the isoalloxazines (I to V) mediate dehydrogenation (aromatization) of dimethyl-

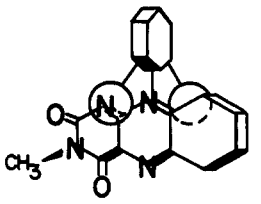
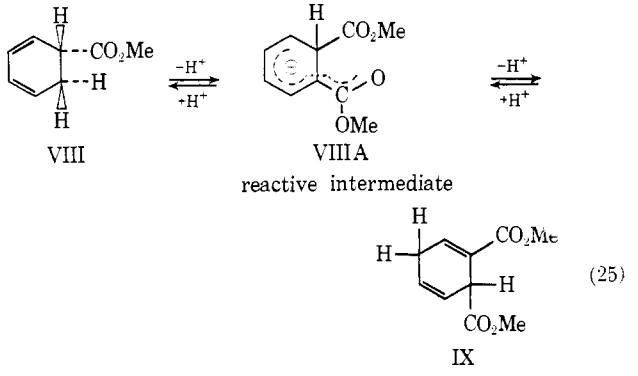
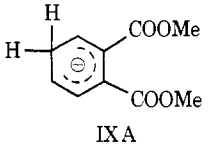


FIGURE 7: Stereochemical representation of 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine (I) showing steric crowding of the 10a position by the methyl groups.

trans-1,2-dihydrophthalate (VIII) and dimethyl 1,4-dihydrophthalate (IX) *via* reaction of isoalloxazine with a single intermediate formed from both VIII and IX in a base-catalyzed step. At high concentrations of isoalloxazine the formation of the intermediate is rate limiting and the reactions exhibit zero-order dependence upon isoalloxazine concentration. At its low concentrations isoalloxazine is not able fully to trap the intermediate which reverts to esters VIII and IX *via* proton capture. Under these conditions the reactions are no longer zero order in isoalloxazine but approach first order. Catalysis of the formation of the intermediate by base and of its reversion to the esters VIII and IX by $C_2H_5NH_3^+$ provides compelling evidence that it is a carbanion rather than, for instance, a reactive isomeric dimethyl dihydrophthalate. Since VIII rearranges to IX at the same rate as zero-order reduction of isoalloxazines by VIII, it is evident that the reactive intermediate in both reactions is the carbanionic species VIIIA. The possibility of some contribution from a second carbanion (IXA) derived from ester IX appears unlikely for the following reason. As is evident in Table V, the reactive species derived from VIII is partitioned (k_b/k_{-a} of eq 2) between reaction with isoalloxazine and reprotonation in the same ratio as the reactive species from IX. This equality is most readily explained if both esters react through the *same* carbanion (VIIIA), for then k_b and k_{-a} are, in fact, the *same* rate constants for any given isoalloxazine. It would be somewhat coincidental if IXA had an equal ratio (k_b/k_{-a}) for a different pair of rate constants. In addition, it should be noted that the required rate ratio for the formation of VIIIA from VIII and from IX of about 10:1 appears to be reasonable.



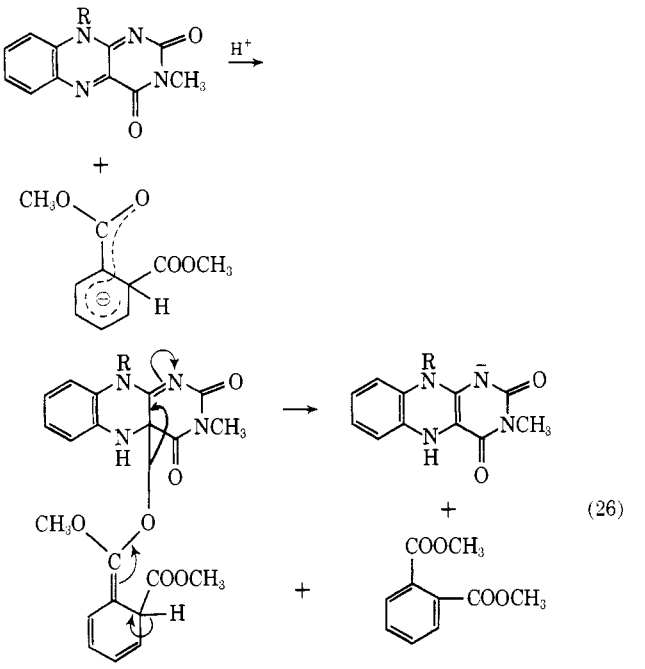
VIII VIIIA reactive intermediate IX (25)



IXA

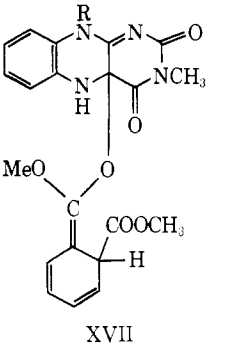
Employing linear free-energy relationships it is found that the rate of reaction of VIIIA with isoalloxazines is not a function of the electron density of I to V (not related to $E_{1/2}$) but of the ability of the isoalloxazine molecule to form a face-to-face complex (related to equilibria of tryptophan complexation). This result strongly suggests that preequilibrium complexation of VIIIA with I to V precedes the redox reaction. *Our data do not differentiate between a complex yielding: (1) a covalent adduct; (2) a complex proceeding to product by direct electron transfer to flavine (vide infra); and (3) intracomplex hydride (or equivalent) transfer.* Whatever the process, however, it can be stated that the 10a position is not involved since the stereochemistry of I does not allow approach to the 10a position (Figure 7).

Explanation of our results by assumption of either an intermediate covalent adduct (eq 26) or hydride transfer therefore

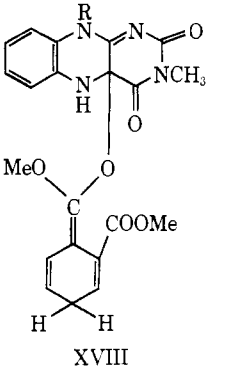


(26)

requires that these processes occur at a position other than the 10a, possibly the 4a or 5 positions (eq 26). The possibility that many flavine-catalyzed dehydrogenation reactions proceed through such covalent adducts, thereby allowing transfer of *both* hydrogen atoms as protons, has recently received considerable attention (Brown and Hamilton, 1970; Hamilton, 1971). In terms of such a mechanism, the difference in reactivity of VIIIA and IXA might be reasonably rationalized in terms of the acidity of the proton to be removed from the corresponding covalent adducts (*e.g.*, XVII and XVIII). It is



XVII

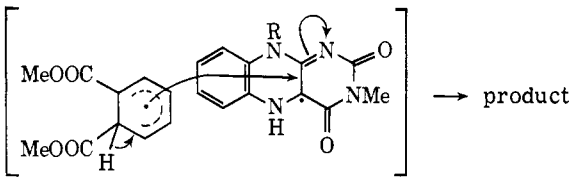


XVIII

evident that the more acidic proton in the adduct derived from VIIIA (XVII) would be more readily lost than that of the adduct from IXA (XVIII).[2]

Thus, referring to eq 18, k_5 and k_6 would be greater for XVII than for XVIII. Consideration of the overall rate equation (eq 19) shows that the rate would be greater for XVII than for XVIII. For the second acceptable kinetic scheme (eq 21) similar arguments would apply, as this is a special case of eq 18. For the third acceptable scheme (eq 23) the same arguments again apply as long as in the spontaneous stage (D → product) proton abstraction from the dihydrophthalate moiety of XVII or XVIII is not catalyzed by an external base.

Exactly the same rationale employed for XVII and XVIII will explain the difference in reactivity of VIIIA and IXA in terms of an alternative mechanism involving formation from the carbanion–flavine complex of a charge-transfer complex (*e.g.*, XIX) which yields products on transfer of a second elec-

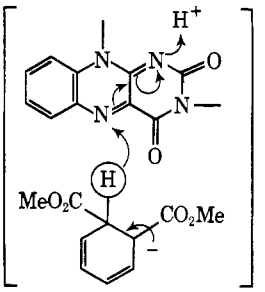


XIX

tron to flavine accompanied by proton abstraction from the dihydrophthalate moiety of the complex. Proton abstraction would be easier for the more acidic proton in the charge transfer complex derived from VIIIA (XIX) than for that from IXA. There may be precedent for such a mechanism. Isotope distribution studies have been interpreted to provide evidence that the reduction of trifluoroacetophenone by dihydronicotinamide is a two-step reaction involving an intermediate noncovalent charge-transfer complex formed in a step with some degree of electron transfer and converted to products in a second step with completion of the electron plus intracomplex proton transfer (Steffens and Chipman, 1971). Since dihydronicotinamides (Bruice *et al.*, 1971) as well as VIIIA form complexes with I–V prior to oxidation, a similar process may occur for the reactions of this investigation and if so the process would be equivalent to a intracomplex hydride ion transfer (to the 5 position).

We are unable to distinguish between the above two possible mechanisms as can be seen in what follows. Our finding of a linear relationship between k_{-a}/k_b and $[RNH_3^+]$ (Results section E) led to eq 18, 21, and 23 as the most reasonable kinetic schemes. At this point we can exclude eq 21, in which the intermediate D yields products in a specific base-catalyzed step, for the following reason. D has been formulated as XVII or XIX and product formation from either of these intermediates would involve proton abstraction from a carbon acid. From what is known of carbon acid ionization, this should involve general base catalysis rather than, as eq 21 would require, specific base catalysis. Equations 18 and 24 remain as acceptable schemes. This is so if covalent addition or charge transfer is involved but the simple scheme of eq 27 suffices for hydride ion transfer without charge transfer.

In summary, kinetic schemes eq 18 and 23 are consistent

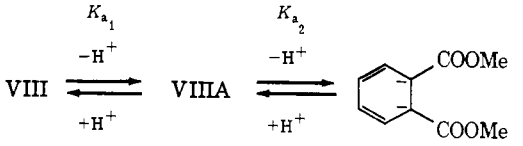


(27)

intracomplex

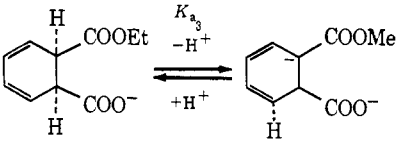
with our kinetic data, but it is impossible to distinguish between an adduct (XVII) or a charge transfer complex (XIX) as the intermediate D. Intracomplex hydride ion transfer from the carbanion moiety (eq 27) is also consistent with our kinetic findings.

Since our experimental results and conclusions concerning mechanism are so strikingly different from those of Weatherby and Carr (1970a), a few comments concerning the latter study are required. In the first place, under comparable reaction concentrations, they report first-order dependence upon flavine (riboflavine) concentration as opposed to our findings with the isoalloxazines (I to V) of initial zero-order dependence. For riboflavine itself reacting with VIII (pH 10, carbonate buffer), we find zero-order dependence upon flavine (8×10^{-5} M) to 75% completion of reaction. Secondly, the contention by Weatherby and Carr (1970a) that the reactive species of VIII is a dicarbanion (eq 28) and that of the cis



(28)

half-ester (eq 29) a monocarbanion, where all ionization



(29)

constants are equal to *ca.* $pK_a = 11$ (*i.e.*, $K_{a1} \approx K_{a2} \approx K_{a3} \approx 10^{-11}$), finds no support either in their experimental findings or in ours. For example, the rate of specific base-catalyzed rearrangement of VIII to IX *via* VIIIA (eq 15) has been found to be linear in hydroxide ion concentration in the region of pH 11, providing no evidence of a pK_a in this range. We have furthermore shown that it is the same ionization (of VIII to VIIIA) which is rate limiting in the reaction of VIII with isoalloxazines I–V, and no pK in the region of 11 can therefore be relevant to this reaction. Also, on replotting the data of Figure 2 of Carr and Weatherby (1970a) in the form k *vs.* $[HO^-]$ we find all but one point to lie close to a straight line which passes through the origin. This finding would be in accord with HO^- catalysis and does not support a pK_a value of ≈ 11. To continue, it must be stated that *all* mechanistic rationalizations of Carr and Weatherby (1970a) are based on the fallacious concept that dimethyl *trans*-1,2-dihydrophthalate (VIII) and dimethyl *cis*-1,2-dihydrophthalate yield *different* monocarbanions which retain their stereochemistry

[2] The same argument applies for covalent adducts formed through ring carbon atoms of VIIIA and IXA rather than the ester oxygen atoms as shown.

in further step(s) and the resultant requirement of the consideration of the relative ease of cis and trans elimination reactions.

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Chemical Nature of the Receptor Site for Various Phytomitogens†

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ABSTRACT: In the inhibition assays of [^{3}H]thymidine incorporation into human peripheral lymphocytes, the stimulatory activity of four kinds of phytomitogens (concanavalin A, *Lens culinaris* hemagglutinin, PHA-M, and *Wistaria floribunda* mitogen) were found to be inhibited by glycopeptides from porcine thyroglobulin, in spite of the fact that these mitogens could be classified into two groups by the inhibition assays using simple sugars as inhibitors. In order to clarify the detailed structure with which these mitogens actually bind, sequential enzymic degradation of one of the glycopeptides with purified glycosidases was carried out, and the inhibitory activity of the residual glycopeptide at each stage of the degradation against the mitogenic as well as the hemagglutinating activities of these mitogens was tested. On the basis of the results obtained, a hypothesis that these mitogens bind to the same core region of the glycopeptide in the inhibition of lymphocyte transformation is proposed.

The phytomitogens, which are plant proteins having mitogenic activity against human peripheral lymphocytes, can generally be classified into two groups on the basis of their specificities, as shown by the inhibition assays of their mitogenic activity with simple sugars. One group, which includes *Phaseolus vulgaris* hemagglutinin, is inhibited by *N*-acetyl-D-galactosamine (Borberg *et al.*, 1968), the other, which includes concanavalin A, is inhibited most by D-mannose (Powell and Leon, 1970; Young *et al.*, 1971). These observations seem to indicate that at least two kinds of receptor sites on the lymphocyte cell surface are involved for the triggering of lymphocyte transformation. In preceding papers (Toyoshima *et al.*, 1970, 1971), we have reported the purification and the characterization of phytomitogens from *Lens culinaris* seeds and from *Wistaria floribunda* seeds. The inhibition assays of these mitogens with simple sugars have indicated the difference of their specificities (Toyoshima *et al.*, 1971). The former belongs to the same group of phytomitogens as concanavalin A, whereas the latter belongs to that including *P. vulgaris* hemagglutinin. However, we have isolated a glycopeptide from human erythrocyte stroma (Akiyama and Osawa, 1971) which has been found to exert a potent inhibitory activity against both groups of phytomitogen (Toyoshima *et al.*, 1971). Recently, Kornfeld *et al.* (1971a,b) isolated a glycopeptide from human γG-myeloma proteins which was found to be a strong hapten inhibitor for both *P. vulgaris* and *L. culinaris* hemagglutinins, and demonstrated that these phytohemagglutinins might bind to different portions of the same glycopeptide.

In this paper, we will report the strong inhibitory activities, for the hemagglutinating and mitogenic activities of both groups of phytomitogen, of a glycopeptide from porcine thyroglobulin (Fukuda and Egami, 1971a,b), and will present evidence that both groups of phytomitogen might bind to the same core region of the glycopeptide in the inhibition of lymphocyte transformation, but bind to the different sugar units in the hemagglutination inhibition reactions.

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