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# THE CHEMISTRY OF FLAVINS AND FLAVOPROTEINS

## III. THE REACTION OF DIHYDROLIPOIC ACID WITH FLAVINS*

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SUMMARY

1. The reaction between dihydrolipoic acid and a number of flavin derivatives has been found to be of first order in both reactants.

2. The rates are strongly pH dependent and are of first order in $OH^-$ concentration as well as being generally base catalysed.

3. The polarographic half-wave potentials of flavin derivatives and their reactivities towards NADH correlate reasonably well with their reactivities towards dihydrolipoic acid.

4. On the basis of these observations and on some electron spin resonance evidence a two-step mechanism is suggested for the reaction, the first being a fast dissociation of one of the –SH groups of dihydrolipoic acid into its anion followed by a second-order reaction between this anion and the flavin, probably by a two-electron transfer.

5. The $pK_a$ of the –SH-groups of the acid has been measured spectrophotometrically, and the value derived (10.7) is in good agreement with the value arrived at from the kinetic studies.

## INTRODUCTION

The reduction of lipoyl dehydrogenase by one of its substrates dihydrolipoic acid gives rise to the formation of a catalytically essential intermediate with a characteristic new absorption band at 530 m$\mu$. This absorption has been attributed to either a stabilised semiquinoid form of the flavin which is in a strong interaction with a sulphur radical[1] or to a charge-transfer complex between enzyme-bound flavine and a vicinal dithiol[2]. The arguments were based mainly on a model reaction between FMN and dihydrolipoic acid. SEARLS AND SANADI[2] found a product with an absorption maximum at 535 m$\mu$ the formation of which, however, was attributed by MASSEY AND ATHERTON[3] to a side-reaction involving light and $H_2O_2$. We now report our detailed investigations of the reaction between dihydrolipoic acid and flavins with a view to elucidating the mechanism of the process[4].

* Pt. II ref. 5.

## MATERIALS AND METHODS

Lipoic acid, FMN, and FAD were purchased from Sigma Chemical Company, riboflavin from BDH, NADH from C. F. Boehringer und Soehne. The origin of the isoalloxazine derivatives has been described previously[5]. All other reagents were commercial products in their purest form. Buffers were made up of previously vacuum-dried salts and in the case of ammediol buffer (2-amino-2-methylpropane-1,3-diol) the reagent was recrystallised from ethanol.

Dihydrolipoic acid was prepared by the method of WAGNER *et al.*[6] and was purified by vacuum distillation (b.p. 178–180° at 1.3 mm) the yield of pure fraction being 59 %. The purity of the preparation was assessed by thin-layer chromatography and by titration with iodine. It was found that in the thin-layer chromatography procedure it was necessary to run two plates for each mixture. One of the silica gel plates was sprayed to wetness with a 100-fold diluted satd. cadmium sulphate solution and dried, the other plate was untreated. Both were run with chloroform–methanol (A.R.) (60:40, v/v) mixture as eluent and the plates were developed with iodine vapour which gave clear, easily visible yellow spots. Dihydrolipoic acid moved very little compared to lipoic acid on the cadmium sulphate-treated plates while the two acids moved together on the untreated plates, which on the other hand were more suitable to detect other unknown impurities in crude preparations. All the studies were carried out with dihydrolipoic acid preparations that were free of impurities as shown by thin-layer chromatography and which gave iodine titres indicating at least 99.9 % purity.

All reactions were carried out anaerobically and were followed by spectrophotometry (usually observing the flavin reduction at 447 m$\mu$). The method of deoxygenation, temperature control and apparatus has already been described[5,7]. EDTA was added to most solutions as some metal ions were found to affect the reaction rates. The reactants were added after deoxygenation in small volumes (NADH dissolved in water and dihydrolipoic acid in acetone). In the case of dihydrolipoic acid NaOH equivalent to the acid concentration was also added to the flavin solution prior to the deoxygenation. Tests were carried out to show that the light beam of the spectrophotometer did not affect the reactions. All kinetic runs were done in at least duplicates.

pH measurements and titrations were made on an EIL pH meter, Type 23A and a Radiometer, Copenhagen, Titrator, Type TTTlb.

ESR measurements were made on a Varian Model V 4500. This instrument has 100 kcycles modulation of the magnetic field and a 3-cm klystron. Solutions were observed in a 0.3-mm thick Varian aqueous sample cell.

A Radiometer, Model PO 4 polarographic equipment was used for recording current–voltage curves for the flavins.

Fluorescence measurements were carried out using a Zeiss spectrofluorimeter.

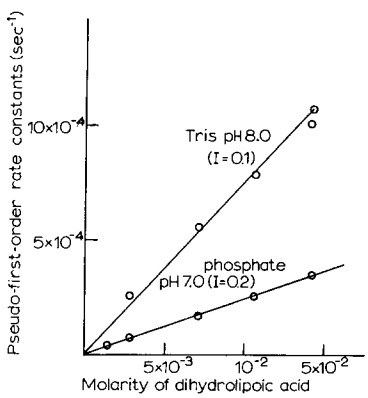
## RESULTS

When a solution of riboflavin ($0.3 \cdot 10^{-4}$ M) is mixed with approx. 40-fold excess of dihydrolipoic acid under anaerobic conditions a slow decrease in the 447-m$\mu$ absorption of riboflavin is observable and after several hours a spectrum characteristic

of reduced riboflavin remains. That this reaction is not in fact a photoreduction induced by the monochromatic light of the spectrophotometer is shown by comparing the rate of the reaction with the spectrophotometer beam off and kept on continuously. No difference was observable. In fact in contrast to other reductions of riboflavin this reaction is not accelerated by even intense visible light. After reoxidation by air the riboflavin spectrum can be completely restored. Lipoic acid has been extracted from the reaction mixture by chloroform and was shown to be the quantitative product of the reaction on spectral analysis. Thin-layer chromatography of the reaction mixture after complete reduction of the flavin only indicated dihydrolipoic and lipoic acids present. No other unknown products were detectable.

*Kinetics of the reaction*

With excess dihydrolipoic acid flavin concentration (calculated from the decrease of the 447-m$\mu$ absorption) as a function of time gave good pseudo-first-order plots under all conditions used in this work up to 60–80 % reaction. When the concentration of dihydrolipoic acid (still in excess) was varied a plot of the pseudo-first-order rate constants against the acid concentration gave good straight lines showing the reaction to be overall second order (Fig. 1). The second-order rate constants then can be derived.



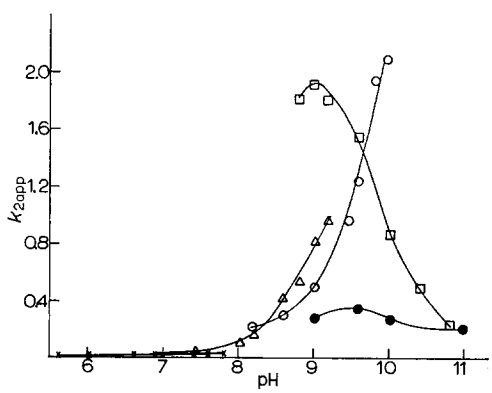




Fig. 1. Dependence of pseudo-first-order rates on dihydrolipoic acid concentration.

Fig. 2. pH dependence of apparent second-order rate constants. ×, phosphate ($I$, 0.2); △, Tris ($I$, 0.1); ○, ammediol ($I$, 0.1); □, glycine ($I$, 0.1); ●, carbonate ($I$, 0.1).

*pH dependence*

The pH dependence of the apparent second-order rate constant ($k_{2\,\mathrm{app.}}$) in phosphate ($I$, 0.2), Tris, ammediol, glycine and carbonate ($I$, 0.1) buffers is shown in Fig. 2. Of the buffers used plots of hydroxyl ion concentration against the rate constants are only linear between pH 7.0 and 9.0 in Tris, and between pH 5.6 and 6.8 in phosphate. The slopes of the log [$OH^-$] *vs.* log $k_{2\,\mathrm{app.}}$ plots are 0.9 and 0.8 for the two buffer systems, respectively, showing nearly first-order dependence in $OH^-$ concentration. A similar graph for ammediol buffer shows deviations from linearity at high pH's.

It is obvious from Fig. 2 that the buffers used also have different specific effects. We have therefore studied the effect of buffer concentration on the rates at a constant

pH (Table I). EDTA which is present in $10^{-3}$ M concentration in all our experiments also has a small effect on the rates but without it the rates were not easily reproducible probably due to metal ion contaminants. The effect of ionic strength of the reaction rate is recorded in Table II.

TABLE I

EFFECT OF BUFFER CONCENTRATION ON REACTION RATES OF RIBOFLAVIN WITH DIHYDROLIPOIC ACID AT 25°

| *Buffer* | *Ionic strength of buffer* | *pH* | $k_{2\ app.}$ $(l \cdot mole^{-1}\ sec^{-1})$ |
|---|---|---|---|
| Phosphate | 0.05 | 7.8 | $1.61 \cdot 10^{-2}$ |
| | 0.1 | 7.8 | $2.22 \cdot 10^{-2}$ |
| | 0.2 | 7.8 | $3.84 \cdot 10^{-2}$ |
| Tris | 0.05 | 7.8 | $4.38 \cdot 10^{-2}$ |
| | 0.1 | 7.8 | $6.97 \cdot 10^{-2}$ |
| | 0.15 | 7.8 | $7.59 \cdot 10^{-2}$ |
| | 0.2 | 7.8 | $10.2 \cdot 10^{-2}$ |

TABLE II

EFFECT OF IONIC STRENGTH ON REACTION RATES IN TRIS BUFFER ($I$, 0.1; pH 8.0)

| *Ionic strength of KCl* | $k_{2\ app.}$ $(l \cdot mole^{-1}\ sec^{-1})$ |
|---|---|
| 0 | $8.54 \cdot 10^{-2}$ |
| 0.1 | $11.2 \cdot 10^{-2}$ |
| 0.2 | $9.91 \cdot 10^{-2}$ |
| 0.3 | $9.79 \cdot 10^{-2}$ |
| 0.4 | $11.2 \cdot 10^{-2}$ |
| 0.6 | $12.0 \cdot 10^{-2}$ |
| 0.8 | $11.6 \cdot 10^{-2}$ |
| 1.0 | $12.1 \cdot 10^{-2}$ |

*Substituent effects*

If the flavin linkage $N_{10}$–$N_1$ which is reduced in the reaction is considered to be a substituent on the benzenoid ring of the isoalloxazine system then the 6-position may be regarded *meta-* and the 7-position *para-* to the reaction centre. Although the flavins studied had varying 9-side-chains it is clear from Table III that 6- and 7-substituents affect the reaction rates. For comparison with a reaction that is thought to involve a two-electron mechanism we studied the rates of reaction with NADH with the same flavins and also measured the polarographic half-wave potentials of these flavins (Table III).

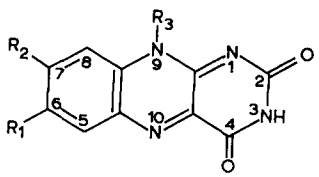
*Electron spin resonance*

To get a comparison with the observed reaction rates we attempted to study the ESR signals during the reaction. MASSEY AND ATHERTON[3] already reported the appearance of a radical signal under their conditions. The most clearly observable ESR signal under our conditions was that with 7-chloro-9-(3'-diethylamino-*n*-propyl)-

TABLE III

REACTIVITIES AND HALF-WAVE POTENTIALS OF SUBSTITUTED FLAVINS IN TRIS BUFFER ($I$, 0.1; pH 8.0)

| *Trivial name or number* | *Substituent* $R_1$ | $R_2$ | $R_3$ | $E_{\frac{1}{2}}$ (*mV*) | $k_2$ *app. for reaction with dihydrolipoic acid* ($l \cdot mole^{-1}\ sec^{-1}$) | $k_2$ *for reaction with NADH* ($l \cdot mole^{-1}\ sec^{-1}$) |
|---|---|---|---|---|---|---|
| FAD | $CH_3$ | $CH_3$ | ribityl-pyrophosphate-adenine | −0.475 | $4.38 \cdot 10^{-2}$ | $1.29 \cdot 10^{-1}$ |
| FMN | $CH_3$ | $CH_3$ | ribityl phosphate | −0.473 | $5.05 \cdot 10^{-2}$ | $1.86 \cdot 10^{-1}$ |
| Riboflavin | $CH_3$ | $CH_3$ | ribityl | −0.460 | $8.54 \cdot 10^{-2}$ | $4.11 \cdot 10^{-1}$ |
| Lumiflavin | $CH_3$ | $CH_3$ | $CH_3$ | −0.460 | $7.98 \cdot 10^{-2}$ | $5.47 \cdot 10^{-1}$ |
| 5 | $CH_3$ | $CH_3$ | $(CH_2)_2OH$ | −0.450 | $6.64 \cdot 10^{-2}$ | $2.68 \cdot 10^{-1}$ |
| 6 | H | H | $CH_3$ | −0.435 | $5.76 \cdot 10^{-1}$ | $8.04 \cdot 10^{-1}$ |
| 7 | Cl | $OCH_3$ | $(CH_2)_2N(C_4H_8)$ | −0.400 | $2.08 \cdot 10^{-1}$ | 1.85 |
| 8 | H | H | $(CH_2)_2N(CH_3)_2$ | −0.385 | 1.30 | 1.70 |
| 9 | $CH_3$ | H | $(CH_2)_2N(C_2H_5)_2$ | −0.360 | 1.62 | 3.69 |
| 10 | Cl | Cl | $CH_3$ | −0.333 | 12.5 | 4.82 |
| 11 | H | Cl | $(CH_2)_3N(C_2H_5)_2$ | −0.315 | 5.59 | 2.36 |
| 12 | Cl | H | $(CH_2)_3N(C_2H_5)_2$ | −0.301 | 33.20 | 4.01 |




isoalloxazine ($7.5 \cdot 10^{-3}$ M) and dihydrolipoic acid ($2.5 \cdot 10^{-2}$ M) in phosphate buffer (pH 6.0; $I$, 0.2). The signal rose to a maximum value at about 60% reduction as calculated from the rate constant. In Tris buffer (pH 8.0) the same compound showed a maximum in 560-m$\mu$ absorption after 1.5 min which is near a calculated extent of reaction of 50%.

*Complex of FMN with dihydrolipoic acid*

An attempt was made to observe complexing between FMN and dihydrolipoic acid since SEARLS AND SANADI[2] attributed the 535-m$\mu$ new absorption band to this interaction. We, however, did not observe any absorption at 535 m$\mu$ and our attempts to observe complexing by spectroscopy were unsuccessful particularly since the high concentrations of dihydrolipoic acid required by such measurements lead to some reaction of the flavin even under aerobic conditions. Since fluorescence measurements require lower concentrations of both flavin and dihydrolipoic acid we studied the quenching of flavin fluorescence by this acid at 20°. The solution used was $1.36 \cdot 10^{-5}$ M riboflavin in phosphate buffer (pH 6.6; $I$, 0.1). Varying amounts of dihydrolipoic acid, in a fixed volume of acetone, were added and fluorescence was immediately measured at 530 m$\mu$. A plot of $(I_0 - I)/I$ against concentration of dihydrolipoic acid shows a distinct curvature, quenching being more significant at higher quencher concentration (Fig. 3).

*$pK_a$ of dihydrolipoic acid*

A full interpretation of the rate data requires knowledge of the equilibrium constant for the –SH ionisations. In an anaerobic titration of dihydrolipoic acid with

NaOH only one inflection is observable, that corresponding to the ionisation of the carboxyl group giving a $pK_a$ of 4.85. The –SH ionisation therefore was followed by spectrophotometry by observing the $-S^-$ concentration at 240 m$\mu$ (*cf.* KREEVOY *et al.*[8]). The dependence of 240-m$\mu$ absorption on pH is shown in Fig. 4 for a solution of $1.98 \cdot 10^{-4}$ M dihydrolipoic acid. (The measurements were done anaerobically to avoid autoxidation.) No separate –SH ionisations are distinguishable from the spectrophotometric titration curve. Knowing the total dihydrolipoic acid concentration (either by weight, iodine titration or acid–base titration utilising the carboxyl ionisation for detecting the end-point) the extinction coefficient of the $-S^-$ can be derived from the readings at high limiting pH's. If, as the absence of two ionisations indicates the two –SH ionisations constants are experimentally indistinguishable the extinction coefficient per –SH is $5.9 \cdot 10^3$ mole$\cdot l^{-1} \cdot cm^{-1}$. This is in good agreement with the value of $4–6 \cdot 10^3$ quoted by BENESCH AND BENESCH[9] for aliphatic mercaptide anions. We can therefore conclude that the $pK_a$ for the two –SH groups are equal at 10.7.

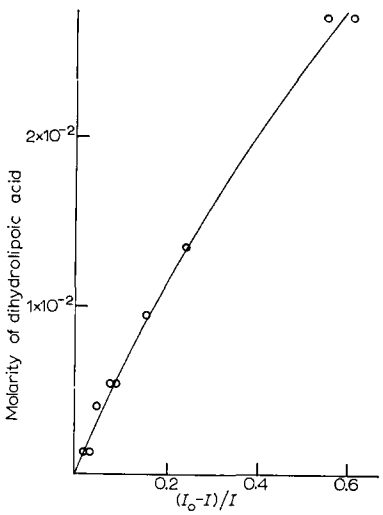



Fig. 3. Quenching of riboflavine fluorescence by dihydrolipoic acid.

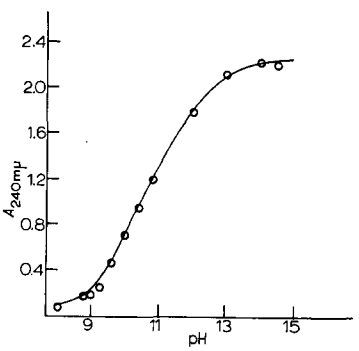



Fig. 4. Spectrophotometric titration of dihydrolipoic acid ($1.98 \cdot 10^{-4}$ M) by NaOH.

## DISCUSSION

Our work leaves no doubt that dihydrolipoic acid does react with flavin nucleotides and a number of other isoalloxazine derivatives under anaerobic conditions at room temperature. We find no evidence for charge-transfer interaction between the two reactants although the fluorescence of riboflavin is quenched by dihydrolipoic acid under aerobic conditions. The fact that quenching becomes more significant at higher dihydrolipoic acid concentrations than expected on the basis of the Stern–Volmer equation strongly suggests that quenching is by a competitive process and is similar to that brought about by KI or thioglycolic acid and not by a static mechanism involving the formation of a flavin–dihydrolipoic acid complex[10].

The observed reaction is of first order in flavin concentration, and at a given pH, of first order in reduced lipoic acid concentration. The rate is strongly dependent on pH (indicating $OH^-$ catalysis) as well as on the nature and concentration of the buffers used. At a given pH the rate is directly proportional to the concentration of the basic component of Tris and phosphate buffers (Table I) showing that the reaction is also susceptible to general base catalysis. The catalytic ability of the basic buffer components may be expressed by a catalytic constant which for Tris and phosphate buffers is obtained from the slopes of $k_{2\,app.}$ *vs.* buffer base component plots and for ammediol and glycine buffers by comparing the rates at pH 9.0 in the buffers to the calculated rates in the absence of general base catalysis. This can be derived from the rate in Tris at pH 9.0 and the catalytic constant for this buffer (Table IV).

TABLE IV

CATALYTIC CONSTANTS FOR THE VARIOUS BUFFERS

| *Buffer* | $k_{cat.}$ | $pK_a$ *of relevent species* |
|---|---|---|
| Glycine | 262 | 9.6 |
| Ammediol | 1.25 | 8.67 |
| Tris | 0.85 | 8.08 |
| Phosphate | 0.4 | 6.7 |

The catalytic constants increase with increasing $pK_a$ of the four buffers as might be expected from the Brönsted relation although because of the different charge types involved the correlation is limited as usual[11]. Variation in the ionic strength of the solution affects the rates to a much smaller extent (Table II). The effect of EDTA can again be understood in terms of general base catalysis.

From the log $[OH^-]$ *vs.* log $k_{2\,app.}$ plots in Tris and phosphate buffers the respective slopes of 0.9 and 0.8 suggest that the reaction is also first order in $OH^-$ concentration. If the apparent second-order rate constants are corrected for the Tris buffer effect using the catalytic constant and knowing the amine concentration at any one pH we obtain the corrected apparent second-order rate constants shown in Table V. These values also give a slope of 0.85 in the log $[OH^-]$ *vs.* log $k_2'_{app.}$ plots.

TABLE V

CORRECTED $k_{2\,app.}$ IN TRIS BUFFER ($I$, 0.1) FOR THE REACTION OF RIBOFLAVIN WITH DIHYDROLIPOIC ACID

| *pH* | *Corrected* $k_{2\,app.} = k_2'_{app.}$ $(l \cdot mole^{-1}\ sec^{-1})$ |
|---|---|
| 7.0 | $0.73 \cdot 10^{-2}$ |
| 7.4 | $2.23 \cdot 10^{-2}$ |
| 7.8 | $2.90 \cdot 10^{-2}$ |
| 8.0 | $3.70 \cdot 10^{-2}$ |
| 8.2 | $6.0 \cdot 10^{-2}$ |
| 8.6 | $18.6 \cdot 10^{-2}$ |
| 9.0 | $29.3 \cdot 10^{-2}$ |

The variation of these rate constants with pH is a measure of the true pH effect and should be related to the measured acid dissociation constant of dihydrolipoic acid –SH groups and the true second-order rate constant according to the equation:

$$k_2'_{app.} = \frac{K_a \cdot k_2}{[H^+] + K_a}$$

From a plot of $[H^+]$ *vs.* $1/k_2'_{app.}$ (Fig. 5) the intercept will give $1/k_2$ and the slope/intercept $= 1/K_a$. The values derived are $k_2 = 2.0 \cdot 10^{-2}$ l·mole$^{-1}$ sec$^{-1}$ and $pK_a = 10.6$. The $pK_a$ derived from the rate measurements is in remarkably good agreement with the spectroscopically measured value of 10.7 for the –SH ionisation. If on the other hand no correction is made for the buffer effect the apparent $pK_a$ derived is 10.

The observations so far described are consistent with a two-step mechanism, the first being the general base-catalysed fast dissociation of one of the –SH groups of dihydrolipoic acid into its anion followed by a second-order reaction between this anion and the flavin. The second step may involve the transfer of two electrons step by step leading to the formation of free radicals as intermediates (as suggested for the enzymic process[1]) or may involve a two-electron reduction step. We favour the second (ionic) mechanism on the following grounds. (1) The rates of the reactions of a number of flavins substituted in the benzenoid ring are considerably dependent on the nature of the substituent. The reaction is speeded up by electron-withdrawing substituents and slowed down by electron-donating groups. There is a limited correlation with Hammett $\sigma$-values if the 6-substituent is considered to be in the *meta*-position to the reaction centre and the 7-substituent *para*- to it. The lack of precise correlation arises from the different nature of the 9-side-chains (see below). This can be shown by a comparison of the reactivity of lumiflavin, riboflavin and FMN or by comparing Compounds 6 and 8 in Table III. (2) The polarographic half-wave potentials of these flavins determined under the same conditions as the reaction rates, apparently measure the ease of reduction by a two-electron process as can be shown from the shape of the current–voltage curves and by the lack of two separately observable waves[12]. These half-wave potentials show a reasonable linear correlation (correlation coefficient 0.955) with the logarithms of the rates (Fig. 6). The slope of

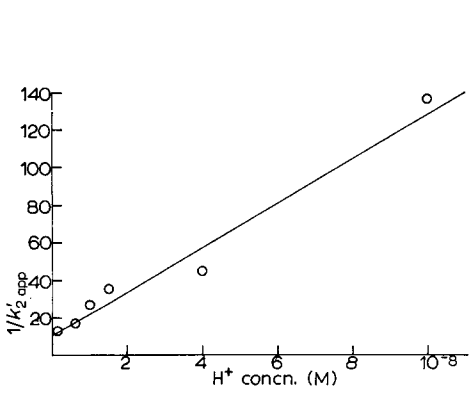



Fig. 5. Plot of $[H^+]$ *vs.* $1/k_2'_{app.}$

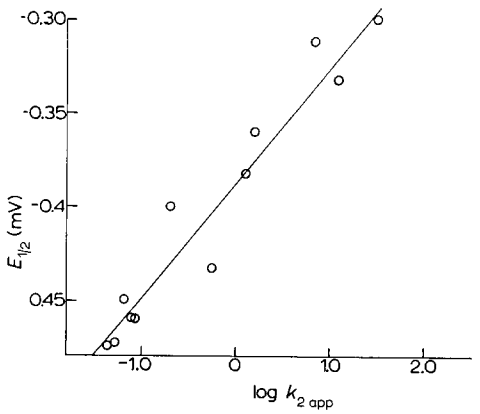



Fig. 6. Correlation of polarographic half-wave potentials with log $k_{2\,app.}$

the line is 14.9 $mV^{-1}$. (3) A similar correlation is observed between the rates of reactions of the substituted flavins with NADH and their half-wave potentials (correlation coefficient 0.914, slope 7.3 $mV^{-1}$, or if compounds with the bulkiest 9-side-chains are left out the correlation coefficient is 0.965). The reaction of NADH is thought to proceed by a hydride ion transfer[7,13]. Its similar susceptibility to substituent effects to that of the dihydrolipoic acid reaction argues in favour of a two-electron process in the latter. The greater susceptibility of the reaction of dihydrolipoic acid than of the pyridine nucleotide system reflects the larger polarisability of the electrons in the sulphydryl groups than in the NADH system. This means that in the former system a greater degree of charge transfer is achieved in the transition state. (4) As for the reaction of NADH with FMN[7] our ESR experiments show that the free radicals produced are at a maximal concentration at approx. 50 % reduction. This is again consistent with (although does not provide proof for) the interpretation that the radicals are produced as a result of a disproportionation equilibrium between the reduced and oxidised forms of the flavin[7].

It has been suggested[14] that all the observations on the reaction of NADH with flavins can be explained by a mechanism involving a fast reversible one-electron transfer followed by a rate-determining hydrogen atom abstraction. Recently FOX AND TOLLIN[15] have excluded this mechanism on the basis of kinetic studies. A similar mechanism may still apply to the reaction of dihydrolipoic acid, although we have as yet no indication that –SH radicals are involved in the process particularly since we can quantitatively account for lipoic acid as the only product of the reaction.

Finally it is notable that the 9-side-chains do affect both the reaction of NADH and of dihydrolipoic acid. Thus FAD is less reactive than FMN in both reactions while compared to a methyl group the ribityl side-chain accelerates the dihydrolipoic acid reaction and slightly retards the NADH reaction. The introduction of a phosphate group in the ribityl side-chain hinders both reactions in contrast to the observation that it accelerates the reduction by *N*-propyl-1,4-dihydronicotinamide[13]. This may be due to electrostatic repulsion between the negative charges on NADH or dihydrolipoic acid and FMN that is not present with the reduced *N*-alkyl-nicotinamide molecule. The low reactivity of FAD is normally attributed to the folded structure of the molecule but we have evidence that this is not the case*. We shall discuss this in a subsequent paper.

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