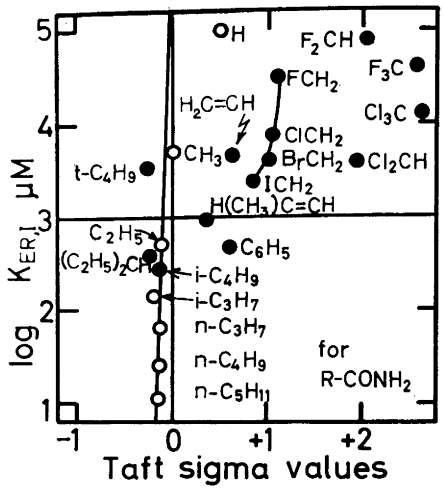

Fig. 2. The values for $K_{ER,I}$ plotted according to the Taft $\varrho^*\sigma^*$ equation. The σ^* values for the n-C_5H_{11} and CF_3 substituents were estimated as described by Taft, [5] while the σ^* value for the CH_2CH substituent was taken from Ref. [10]. The $K_{ER,I}$ values indicated by open circles were taken from Ref. [1], excepting for the value for the i-C_3H_7 substituents which was taken from Ref. [4]. The $K_{ER,I}$ value (270 μM) for the i-C_4H_9 substituent was obtained kinetically by the method described in Ref. [2].

than would be expected if the $\varrho^*\sigma^*$ equation were strictly followed. Similar results were obtained by Drago and coworkers [6,7] when they plotted the equilibrium constants of amide-iodine and amide-phenol complexes according to the Taft $\varrho^*\sigma^*$ equation.

Charton (personal communication) has suggested that a better correlation might be obtained if the values of log $K_{ER,I}$ for the amides were plotted against the Hammett sigma values for *para*-substituted benzoic acids [8]. When this was done it was found that the values for the C_2H_5, CH_3CH = CH, CH_2Cl, $CHCl_2$, CCl_3 and CF_3 substituents fell on a line that follows the equation log $K_{E,RI}$ (μM) = 2.7 σ_{para} + 3.1. The correlation coefficient was 0.99 and the standard deviation [9] was 0.26. The values for the H, CH_3, n-C_3H_7, i-C_3H_7, n-C_4H_9, t-C_4H_9 and C_6H_5 substituents deviated from this line. A more detailed report will be submitted for publication at a later date.

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Spectrophotofluorometric Determination of the Dissociation Constants of Relatively Nonfluorescent Liver Alcohol Dehydrogenase Complexes with Amides and Coenzyme

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In the preceding paper, [1] the dissociation constants, $K_{ER,I}$, for a group of amides that form relatively nonfluorescent ERI

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complexes with liver alcohol dehydrogenase were reported. This report concerns the determination of the dissociation constants $K_{E,I}$, $K_{EI,R}$ and $K_{ER,I}$ for most of the same amides by means of a new fluorometric titration procedure.*

To a cuvette containing 0.1 μ sodium phosphate buffer at pH 7.05 $\pm$ 0.05 was added $\sim 10^{-3}$ M isobutyramide (which forms a highly fluorescent ERI complex [2]), an appropriate concentration (determined by trial and error) of an amide that forms a relatively nonfluorescent ERI complex, and $\sim$ 0.25 μN enzyme. The mixture, at 23.5°, was then titrated with $\sim$0.1 μM aliquots of DPNH solution, and the apparent dissociation constant, D_{app}, and the Q value for the mixture, Q_{obs}, calculated by a modification of the method of Theorell and Winer [2,3]. During the titration, the cuvette was irradiated with monochromatic light at 330 mμ, and the fluorescence produced at 410 mμ was recorded.

The concentrations of the various components in the reaction mixture at the mid-point of the titration were calculated as follows:

$$\mathrm{EIsoR} = \frac{0.5\,(Q_{obs}-Q_{EIR})}{\frac{K_{ER,Iso}}{[Iso_f]}\,(Q_{ER}-Q_{EIR}) + Q_{EIsoR}-Q_{EIR}} \quad (1)$$

$$\mathrm{ER} = \frac{K_{ER,Iso}\,[\mathrm{EIsoR}]}{[\mathrm{Iso}]} \quad (2)$$

$$\mathrm{EIR} = 0.5-\mathrm{EIsoR}-\mathrm{ER} \quad (3)$$

$$\mathrm{E_f} = \frac{K_{E,R}[\mathrm{ER}]}{[\mathrm{R_f}]} \quad (4)$$

$$\mathrm{EIso} = \frac{K_{EIso,R}\,[\mathrm{EIsoR}]}{[\mathrm{R_f}]} \quad (5)$$

$$\mathrm{EI} = 0.5-\mathrm{E_f}-\mathrm{EIso} \quad (6)$$

The values used for the constants were: $K_{ER,Iso}$ = 170 μM[4], $K_{E,R}$ = 0.5 μM**, $K_{EIso,R}$ = 0.003 μM**, $K_{E,Iso}$ = 28 000 μM**,and Q_{ER} = 12.8**. The average value for Q_{ERIso} was 40, but this value was redetermined daily inasmuch as it changed somewhat from day to day because of variations in the instrument. The average value for Q_{ER} was corrected for these variations. It may be noted that $[Iso_f] \approx [Iso_t]$, while $[I_f] \approx [I_t]$. $D_{app} = [R]$ at the mid-point of the titration at which the calculations were performed. The subscripts f and t refer to the free and total concentrations, respectively. In this manner it was possible to calculate the dissociation constants $K_{E,I}$, $K_{ER,I}$ and $K_{EI,R}$ which are presented in Table 1. The values for $K_{ER,I}$ determined by this method agree rather well with the values obtained by direct titration of the ER complex [1], as well as with the values of the Michaelis inhibitor constants obtained kinetically [4].

In Fig. 1 the values for $K_{E,I}$ from this paper, as well as the values reported by Winer and Theorell, [2] are plotted according to the Taft $\varrho^*\sigma^*$ equation [5]. The pattern obtained is remarkably similar to the pattern obtained when the values for $K_{ER,I}$ were plotted in this manner [1]. The two plots are displaced relative to each other

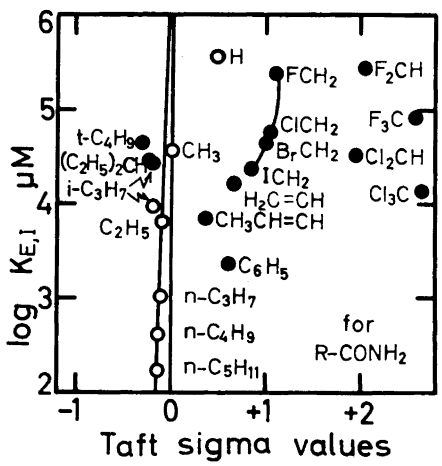

Fig. 1. Taft $\varrho^*\sigma^*$ plot of $K_{E,I}$. The σ^* values of the n-C_5H_{11} and CF_3 substituents were estimated as described by Taft [5], while the σ^* value for the CH_2CH substituent was taken from Ref. [7]. The points indicated by open circles were taken from Ref. [1], excepting for one of the i-C_3H_7 points which was taken from Ref. [6]. The $K_{E,I}$ value for the second i-C_3H_7 point (solid circle) is the value obtained when this constant was redetermined by the author.

* Abbreviations used: E = Enzyme; Iso = Isobutyramide; I = Inhibitor = amide; R = DPNH = diphosphopyridine nucleotide; ER, EIsoR, etc. = Enzyme-DPNH complex, Enzyme-Isobutyramide-DPNH complex, etc.; Q_{ER}, Q_{EIsoR}, *etc.* = Fluorescence of ER/Fluorescence of DPNH, Fluorescence of EIsoR/Fluorescence of DPNH, etc.; $K_{EI,R}$, $K_{ER,I}$, *etc.* = [EI][R]/[EIR], [ER][I]/[EIR], *etc.*

** These values were redetermined by the author.

Table 1. Values of the dissociation constants.

Amide	$K_{EI,R}$ μM	$K_{E,I}$ μM	$K_{ER,I}$ μM
Fluoroacetamide	0.081	240 000	39 000
Difluoroacetamide	0.13	280 000	76 000
Trifluoroacetamide	0.29	81 000	47 000
Chloroacetamide	0.066	58 000	7 600
Dichloroacetamide	0.097	33 000	6 400
Trichloroacetamide	0.31	14 000	8 500
Bromoacetamide	0.066	44 000	5 800
Dibromoacetamide	0.096	17 000	3 200
Iodoacetamide	0.040	24 000	1 900
a,a-Diethylacetamide	0.0068[a]	29 000[a]	390[b]
Trimethylacetamide	0.040[a]	43 000[a]	3 500[b]
Acrylamide	0.23	17 000	7 700
a-Methylacrylamide	0.055	8 700	950
Crotonamide	0.15	6 900	1 280
Tiglamide	0.091	3 900	710
Benzamide	0.13	2 400	610
Isobutyramide	0.003[c]	28 000[c]	170[d]

a These constants were determined by performing the titrations in the absence of isobutyramide and were calculated by a different method.
b From Ref. [1].
c These values were redetermined by the author.
d From Ref. [4].

along the *y*-axis because the addition of DPNH to the enzyme makes a change in the amide binding site so that the dissociation constant for most amides becomes about 9 times smaller (a difference in free energy of 1.3 kcal/mole), with most of the individual ratios of $K_{E,I}/K_{ER,I}$ ranging from 2 to 16. The presence of DPNH in the complex does not cause increased interference with the binding of amides with bulky substituents, as can be seen by the fact that the tertiarybutyl and trichloromethyl substituents occupy the same relative positions on both the $K_{E,I}$ and the $K_{ER,I}$ [1] plots. The amides with a single *a*-alkyl branch behave very differently, however. The presence of DPNH in the complex causes an extra stabilization so that the EIR complex is 2.4-3 kcal/mole more stable than the EI complex.

A plot of log $K_{E,I}$ *versus* σ_{para} resulted in a greater scatter of points than was observed when log $K_{ER,I}$ was plotted in this manner [1]. Also, similar plots of $K_{EI,R}$ resulted in a rather scattered distribution of points. A more detailed report will be submitted for publication at a later date.

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