Electronic, Hydrophobic, and Steric Effects of Binding of Inhibitors to the Horse Liver Alcohol Dehydrogenase–Reduced Pyridine Coenzyme Binary Complex*

Ramaswamy H. Sarma† and Charles L. Woronick

ABSTRACT: Kinetic and thermodynamic considerations of the system containing horse liver alcohol dehydrogenase (LADH, E), reduced diphosphopyridine nucleotide (DPNH, R), and aromatic amides (I) suggest that most likely they form an ERI ternary complex. Proton nuclear magnetic resonance data from such systems are consistent with the thesis that such ternary ERI complexes are formed. Using the fluorescence of the complexes ER and ERI, the dissociation constant $K_{ER,I}$ = ([ER][I]/[ERI]) was determined for a series of meta- and para-substituted benzamides and a few pyridine derivatives. σ, ρ, and π analyses of the $K_{ER,I}$ values indicate that in the case of para-substituted benzamides, the differences in $K_{ER,I}$ are primarily governed by differences in hydrophobic bonding between the para substituent and the enzyme in the ERI complex. In the case of meta-substituted benzamides the differences in $K_{ER,I}$ are primarily electronically controlled. In the case of pyridine derivatives differences in binding are primarily controlled by hydrophobic factors. *N*-Methyl substitution of the amide nitrogen destabilizes the ERI complexes. Complete *N*-methyl substitution as in *N*,*N*-dimethylbenzamide results in a $K_{ER,I}$ value of 49,500 μM as compared to 14,450 μM for *N*-monomethylbenzamide and 536 μM for benzamide. The *N*-methyl groups hinder the binding of the inhibitor, most probably through steric hindrance of the approach of the enzyme to bind to the carbonyl oxygen of the amide. It was observed that *p*-toluamidoxime binds much less tightly than *p*-toluamide in accordance with the proposal that the carbonyl group of the amide is involved in the binding. The fluorescence of the dihydronicotinamide ring of DPNH in the ternary complexes involving aromatic inhibitors such as benzamide is quenched to a very large degree. The explanation offered for this is that the dihydronicotinamide ring of DPNH and the aromatic ring of the inhibitor are stacked in parallel planes.

The binding of an organic molecule to an enzyme is controlled, at least in part by steric, electronic, and hydrophobic interactions and information concerning such interactions can be obtained by studying the dissociation of variously substituted substrate analogs or inhibitors from their enzyme complexes. The horse liver alcohol dehydrogenase system is well suited for such an approach because benzaldehyde is a good substrate for this enzyme (Winer, 1958); hence a series of para- and meta-substituted benzamides can be used as inhibitors to determine $K_{ER,I}$.[1] The $K_{ER,I}$ values can be related to Hammet σ values (McDaniel and Brown, 1958) to understand the electronic control and to the Fujita *et al.* (1964) hydrophobic factor π to understand the hydrophobic control.

This paper deals with the determination of the fluorescent quotient Q_{ERI} and the dissociation constant $K_{ER,I}$ using a number of substituted benzamides and a few pyridine derivatives as inhibitors. It was found that in the ERI complex involving para-substituted benzamides, as well as in the complexes involving pyridine derivatives, differences in the degree of binding are primarily controlled by hydrophobic factors, whereas in the ERI complexes involving meta-substituted benzamides differences in the degree of binding are primarily electronically controlled. It has also been found that methyl substitution of the amide nitrogen of benzamide hinders the binding of inhibitor in the ERI complex as compared with the binding of the unsubstituted compound.

Materials and Methods

Enzyme. Horse LADH was isolated from fresh horse liver and purified as described by Dalziel (1960). The enzyme was then recrystallized six times by dialysis against 0.05 μ sodium phosphate buffer (pH 7.0) containing 10% ethanol. The recrystallized enzyme had an absorbancy ratio of 280 mμ/260 mμ equal to 1.245 and an apparent purity of 103% when assayed by the method of Dalziel (1957) using a value of 0.46 for the absorbancy of 1 mg of protein/ml in a cuvet of 1-cm optical depth as reported by Theorell *et al.* (1966). The concentration[2] of LADH was determined by fluorometric titration of the enzyme with DPNH in the presence of excess isobutyramide as described by Winer and Theorell (1960). The determinations of enzyme concentration by the fluorometric procedure were repeated about five times, and were done every

* From the Department of Chemistry, State University of New York at Albany, Albany, New York 12203 and from the Medical Research Laboratories, Hartford Hospital, Hartford, Connecticut 06115. This research was supported in part by grants from the National Cancer Institute (CA 12462-01), the National Science Foundation (GB 28015), and the Research Foundation of the State University of New York (020-7212-A). Preliminary reports of this work have been presented (Sarma and Woronick, 1967, 1971a).

† To whom correspondence should be addressed at the State University of New York at Albany.

[1] Abbreviations used are: R, DPNH; LADH, horse liver alcohol dehydrogenase; E, LADH; I, inhibitor; ER, enzyme–DPNH binary complex; EI, enzyme–inhibitor binary complex; ERI, enzyme–DPNH–inhibitor ternary complex; Q_{ER} = fluorescence of the ER complex/fluorescence of an equivalent concentration of DPNH used up to form the ER complex; Q_{ERI} = Q_{EIR} = fluorescence of ERI complex/fluorescence of an equivalent concentration of DPNH used up to form the ERI complex; $K_{E,R}$ = [E][R]/[ER]; $K_{ER,I}$ = [ER][I]/[ERI]; $K_{E,I}$ = [E][I]/[EI]; $K_{EI,R}$ = [EI]/[R]/[ERI].

[2] The concentrations of the enzyme are expressed in normality. The enzyme has two binding sites. Hence 1 M enzyme = 2 N enzyme; 1 M DPNH = 1 N DPNH; 1 M inhibitor = 1 N inhibitor.

day in which dissociation constants were determined. Q_{EIsoR}, the fluorescence quotient of the LADH–isobutyramide–DPNH ternary complex, was also determined daily inasmuch as it appeared to change slightly from day to day because of a combination of variations in the instrument and experimental error. Data were processed by an IBM 360, Model 50 computer.

DPNH was purchased from Sigma Chemical Co. and was used without further purification. The percentage of β-DPNH in the commercial product was determined by oxidizing DPNH at pH 7 in the presence of excess acetaldehyde using LADH as the catalyst. The reaction was followed by measuring the change in absorbancy at 340 mμ. Of the total absorbance at 340 mμ, 96.5% was from β-DPNH. DPNH solutions were freshly made every day, and the concentration of DPNH was determined by measuring the absorbancy at 340 mμ (Horecker and Kornberg, 1948).

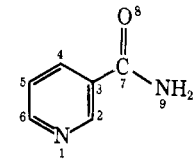
Inhibitors which were bought from commercial sources were purified by recrystallizing from appropriate solvents. If the solution was colored it was decolorized by boiling with activated charcoal. The melting points reported are uncorrected and were determined with a hot stage microscope melting point apparatus (Model 27, The Nalge Co., Rochester, N. Y.). Following are the names of the inhibitors, their melting points, and the solvents used for recrystallization: benzamide, 131°, water; 4-methylbenzamide, 160°, water; 4-nitrobenzamide, 201°, water; *p*-toluamidoxime, 148°, water; *N*-methylbenzamide, 82°, water; *N*,*N*-dimethylbenzamide, 45°, petroleum ether (bp 30–60°); 2-phenylacetamide, 158°, water; 2-picolinamide, 109°, water; nicotinamide, 125°, benzene–pyridine–toluene (4:2:1, v/v); isonitocinamide, 158°, benzene + traces of pyridine; nicotinic acid hydrazide, 163°, ether; nicotinonitrile, 51°, water. The inhibitors listed below were prepared from the corresponding carboxylic acids or acid halides. In those cases where the carboxylic acid was the starting material, the acid was converted to the acid chloride using thionyl chloride, after which the acid chloride was vacuum distilled. The acid halides were then dissolved in anhydrous ether and converted to the amide by treatment with anhydrous ammonia. Following are the names of the inhibitors, their melting points, and the solvents used for recrystallization: 3-bromobenzamide, 156°, water; 3-dimethylaminobenzamide, 153°, water; 4-isopropylbenzamide, 152°, water; 4-chlorobenzamide, 179°, benzene; 3-fluorobenzamide, 133°, water; 3-hydroxybenzamide, 169°, water; 4-methoxybenzamide, 168°, water; 3-nitrobenzamide, 142°, water. The inhibitors listed below were prepared from the corresponding methyl or ethyl carboxylic acid esters by reaction with concentrated ammonium hydroxide (28%) in a sealed flask on a steam bath. Following are the names of the inhibitors, their melting points, and the solvents used for recrystallization: 3-chlorobenzamide, 134°, water; 4-fluorobenzamide, 155°, water; 4-hydroxybenzamide, 162°, water; 3-methylbenzamide, 96°, water; 4-bromobenzamide, 190°, water.

Pipets and Syringes. The syringes and Lang–Levy micropipets which were used in the course of the experiments were calibrated. The micropipets were calibrated using a solution of adenosine monophosphate as the standard, and measuring at 259 mμ the optical densities of appropriate dilutions. Gastight 50- and 100-μl Hamilton syringes with Teflon-covered plungers and Teflon needles were used together with a Hamilton repeating dispenser that delivered $^1/_{50}$th of the syringe volume (Hamilton Co., Inc., Wittier, Calif.). The syringes, together with the repeating dispenser, were calibrated by making repeated additions of a DPNH solution to pH 7 phosphate buffer in a cuvet and measuring the change in fluorescence after each addition.

Apparatus. Experiments to determine the enzyme concentration and the dissociation constants of enzyme complexes were performed with a Zeiss PMQ II spectrophotofluorometer having two monochromators and a 450-W xenon lamp. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 1.0 mm wide. The contents of the cuvet were excited with light at 328 mμ and the fluorescence at 410 mμ was measured with a 10-in. Varian recorder, Model G-40. Under normal operating procedures, the recorder sensitivity was set at 5-mV full scale, with the output of the Zeiss PMQ II set at 10-mV full scale. All experiments were performed in 0.1 μ sodium phosphate buffer (pH 7.0) at 23.0°.

The proton nuclear magnetic resonance experiments were performed with a Varian HA100D spectrometer using tetramethylammonium chloride as a standard. The signal-to-noise ratio was improved by using a computer of average transients (Varian C-1024) functioning in the internal trigger mode and an external function generator (HP 3310A).

Molecular orbital calculations for 2-, 3-, and 4-pyridine amides were performed by the Hückel LCAO approximation method. In the case of nicotinamide, the molecular skeleton is



comprised of 9 atoms, including 6 carbons, 2 nitrogens, and 1 oxygen. N_1 and O_8 contribute one electron and one atomic orbital each to the π system, while N_9 contributes two electrons of its lone pair, and one atomic orbital. Each carbon atom participates in the π system with one electron and one orbital. The total conjugated system involves 10 electrons and 9 molecular orbitals. The same considerations apply to α-picolinamide and isonicotinamide. Each molecular orbital is considered to be a linear combination of the nine atomic orbitals involved: $\phi = C_1X_1 + C_2X_2 + \ldots + C_9X_9$, using the parameters listed below, a secular determinant was derived and the equations were solved with a IBM 360, Model 50, computer. The parameters and the procedure are taken from Pullman and Pullman (1963).

Bond	η	δ
C=N	1	δ=N— = 0.4
C—N—	0.9	δ—N— = 1
C=O	2	δ=O = 1.2

The dissociation constant, $K_{ER,I}$, for the inhibitors which are relatively soluble in the buffer, was determined by the spectrophotofluorometric titration procedure developed by Winer and Theorell (1960), Theorell and McKinley-McKee (1961a), and Woronick (1963a,b). The fluorescence of approximately 7.5 μM DPNH was determined, after which LADH at a final concentration of approximately 0.8 μN was added to this cuvet to form the highly fluorescent LADH–DPNH complex. Under these conditions about 94% of the added LADH is converted into the LADH–DPNH binary complex. The binary complex was then titrated with appropriate aliquots of inhibitor solution, the fluorescence being recorded after each

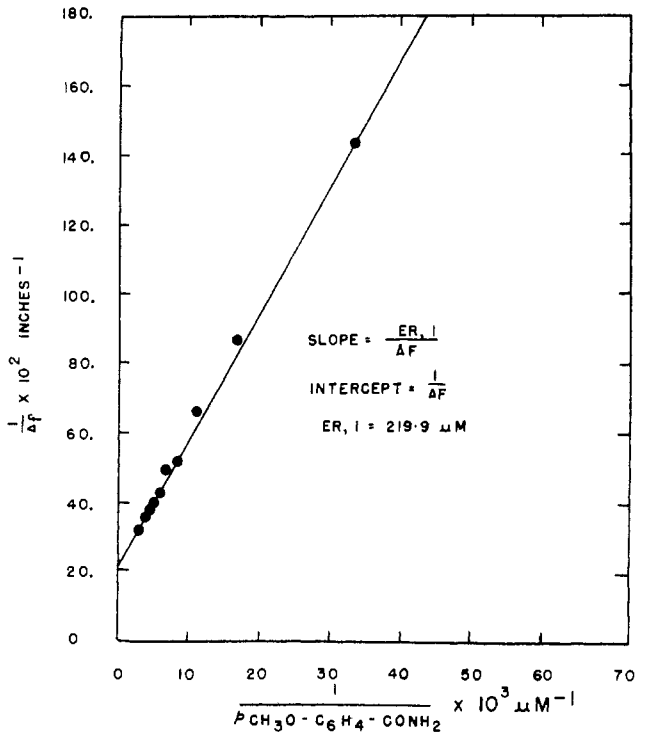


FIGURE 1: Graphical method for determining the values of $K_{ER,I}$. In this case, E–DPNH binary complex was titrated with 30 μM increments of *p*-methoxybenzamide. The symbols used are described in the text.

addition of inhibitor. The change in fluorescence after each addition of inhibitor was calculated as follows

$$\Delta f_1 = f_i - f_1$$
$$\Delta f_2 = f_i - f_2$$
$$\vdots$$
$$\Delta f_n = f_i - f_n$$

where f_i is the total fluorescence in the cuvet after the LADH–DPNH complex was initially formed, and $f_1, f_2, \ldots\ldots f_n$, are the fluorescence values after the 1st, 2nd, ... and *n*th addition of inhibitor. The various fluorescence readings were corrected for fluorescent impurities added with the buffer, LADH, and inhibitor, for dilution after each addition of inhibitor, for quenching of the fluorescence of the free DPNH by inhibitor, and for the reduction of the free DPNH concentration caused by the formation of the DPNH-containing complexes. The quenching corrections were obtained from the titration of free DPNH solution with the inhibitor.

It has been shown (Woronick, 1963a) that a plot of Δf *vs.* [I] will result in a rectangular hyperbola. Therefore, a plot of $1/\Delta f$ *vs.* 1/[I] will give a straight line which will follow

$$\frac{1}{\Delta f} = \left[\frac{K_{ER,I}}{\Delta F}\frac{1}{[I]}\right] + \frac{1}{\Delta F} \qquad (1)$$

where Δf is the absolute value of the total change in fluorescence at each inhibitor concentration, ΔF is the absolute value of the total change in fluorescence at infinite inhibitor concentration, and [I] is the inhibitor concentration. The slope divided by intercept will give $K_{ER,I}$. Table I and Figure 1 illustrate the application of the methods described above to solve for the dissociation constant $K_{ER,I}$ for *p*-methoxybenzamide.

In these spectrophotofluorometric titration procedures developed by Winer and Theorell (1960), Theorell and McKinley-McKee (1961a), and Woronick (1963a,b), it is customary to assume that the total inhibitor added is equal to the amount of free inhibitor present. It was not necessary to estimate the free inhibitor concentration after each addition because in all cases the free inhibitor concentration was almost equal to total inhibitor concentration. In the example given in Table I, no more than 0.868 μM out of a total of 30 μM of the inhibitor could have been bound to the enzyme at the first addition. In actual fact, it can be seen from the data that about 0.2 μM is actually bound. This means that more than 99% of the total inhibitor added remain as free inhibitor at the first addition, and as the titration proceeds, the percentage inhibitor remaining free increases. Therefore, the error introduced from this assumption is negligible compared to other sources of error. (See Table II for standard errors.)

The fluorescence quotients Q_{ERI} and Q_{ER} are defined as $Q_{ERI} = f_{ERI}/f_{Req}$ and $Q_{ER} = f_{ER}/f_R$, where f_{ERI} = fluorescence of the ERI complex, f_{Req} = fluorescence of an equivalent concentration of DPNH used up to form the ERI complex, f_{ER} = fluorescence of the ER complex, and f_R = fluorescence of an equivalent concentration of DPNH used up to form the ER complex. The values of Q_{ERI} and Q_{ER} can easily be calculated from the titration data, the concentration of the enzyme and the value obtained for ΔF in eq 1.

The titration data were also processed by an IBM 360, Model 50 computer. A Fortran program for the nonlinear least-squares treatment of the data was initiated by computing approximate values of $K_{ER,I}$ and of the asymptote using eq 2 and 3 obtained by rearranging the equations for a rectangular hyperbola. The asymptote is the maximum change in fluorescence as [I] → ∞. In the above equations *n* was usually 6. Δf refers to the absolute value of the total change in fluorescence, and [I] refers to the amide concentration. The values of $K_{ER,I}$ and of the asymptote are then used as initial estimates for initiating a nonlinear least-squares treatment, which is a reiterative process and adjusts the values of $K_{ER,I}$ and of the asymptote so that the sum of $(\Delta f_{calcd} - \Delta f_{obsd})^2$ reaches a minimum. The quantity Δf_{obsd} is the observed change in fluorescence, whereas Δf_{calcd} is the change in fluorescence computed by the nonlinear least-squares treatment. Part of the computer output is shown in Table I. The $K_{ER,I}$ value in Table I for manual calculation is 220 μM for *p*-methoxybenzamide; the $K_{ER,I}$ value for the same inhibitor, calculated by the computer in 178 μM. The difference is due to the ability of the computer to initiate a nonlinear least-squares treatment to refine the data.

$$K_{ER,I} = \frac{\Delta f_n - \Delta f_1}{\Delta f_1/[I_1] - \Delta f_n/[I_n]} \qquad (2)$$

$$\text{asymptote} = \left[\frac{K_{ER,I}}{[I_1]} + 1\right]\Delta f_1 \qquad (3)$$

Dissociation constants of inhibitors which are not very soluble in water or which are very loosely bound to the ER complex cannot be determined by the above method. This is

TABLE I: Graphical Solution of the Value of $K_{ER,I}$ for *p*-Methoxybenzamide.[a]

I[b]	II (in.)[c]	III (in.)[d]	IV (in.)[e]	$1/\Delta f$ (in.$^{-1}$)	1/[I] (μM^{-1})	$K_{ER,I}$	Q_{ERI}
0	8.22	8.22	0.00	∞	∞		
1	7.53	7.52	0.70	1.43	0.033		
2	7.07	7.06	1.16	0.862	0.017		
3	6.72	6.70	1.52	0.657	0.011		
4	6.31	6.29	1.93	0.518	0.0086		
5	6.22	6.19	2.03	0.492	0.0069		
6	5.91	5.86	2.35	0.426	0.0058		
7	5.73	5.69	2.53	0.395	0.0050		
8	5.65	5.61	2.61	0.383	0.0044		
9	5.46	5.40	2.81	0.356	0.0040		
10	5.19	5.14	3.08	0.324	0.0036	220	1.22

Reiterative Computer Solution of the Value of K_{ERI} for *p*-Methoxybenzamide

Cycle	Old Asymptote (in.)	Δ Asymptote (in.)	New Asymptote (in.)	Std Error Asymptote (in.)	Old K_{ERI} (μM)	$\Delta K_{ER,I}$ (μM)	New $K_{ER,I}$ (μM)	Std Error $K_{ER,I}$ (μM)	Sum of Δ (Fluorescence)^2 (in.)^2
1	4.83	−0.39	4.44	0.37	204.0	−29.00	175.0	3.02	0.0777
2	4.44	0.04	4.48	0.28	175.0	3.00	178.0	2.31	0.0648
3	4.48	0.00	4.48	0.29	178.0	0.00	178.0	2.36	0.0647

[a] Enzyme concentration in the cuvet = 0.868 μN; initial concentration of DPNH in the titration cuvet = 6.66 μM; inhibitor concentration in stock solution = 3070 μM; initial volume in the cuvet = 2025 μl; volume of inhibitor per addition = 20 μl; pH 7.00 ± 0.05; ionic strength = 0.1; buffer = sodium phosphate. [b] Number of additions of inhibitor. [c] Fluorescence corrected for fluorescence of buffer and for dilution. [d] Data in previous column corrected for quenching and fluorescence impurities in the inhibitor. [e] Δ corrected height, *i.e.*, Δf_1, Δf_2, ...

because a large volume of the stock inhibitor solution (saturated solution of inhibitor in buffer) must be added each time in order to get measurable complex formation. This creates difficulties and uncertainties in making dilution corrections. Therefore, the following method was adopted for these inhibitors. To a cuvet was added a 2-ml buffer solution, and its fluorescence was recorded. To the buffer solution was added about 8 μM DPNH, and again the fluorescence was recorded. Finally, to the same solution in the cuvet was added about 1 μN enzyme, and again the fluorescence was recorded. The cuvet was now washed and filled with 1.8 ml of buffer and 0.2 ml of buffer almost saturated with inhibitor, and the above sequence of fluorescence measurements was repeated. The sequence of measurements was repeated with 1.5 ml of buffer plus 0.5 ml of inhibitor, 1.0 ml of buffer plus 1.0 ml of inhibitor, etc., and finally with 2.0 ml of inhibitor solution. The fluorescence measurements were corrected for differences in sensitivity and quenching, and $K_{ER,I}$ and Q_{ERI} were calculated by plotting $1/\Delta f$ *vs.* 1/[I] according to the procedure of Woronick (1963a). This procedure was used with *m*-dimethylaminobenzamide, *N*-methylbenzamide, *p*-toluamidoxime, 2-picolinamide, isonicotinamide,and nicotinonitrile.

Results and Discussion

The spectrophotofluorometric titration procedure employed in this paper was developed by Winer and Theorell (1960), Theorell and McKinley-McKee (1961a), and Woronick (1963a,b). This procedure can be used to calculate $K_{ER,I}$ only if the inhibitor forms a ternary ERI complex with fluorescence characteristics different from that of the ER binary complex. When the aromatic amides in Table II were used to titrate the fluorescent ER complex, the fluorescence intensity of the solution in the cuvet decreased. This indicates case I, II, or III.

Case I. The inhibitor binds to its specific binding site in the ER complex and forms a ternary ERI complex. In the ERI complex, the inhibitor and the dihydropyridine moiety are juxtaposed to each other and the relative geometry between them is such that the inhibitor quenches the fluorescence of the dihydropyridine chromophore.

Case II. The inhibitor displaces the DPNH from the ER complex and forms a binary EI complex and free DPNH. This will cause the fluorescence intensity to decrease because free DPNH is less fluorescent than the ER complex, and the EI complex is nonfluorescent.

Case III. The inhibitor binds to a remote site on the protein of the E–DPNH complex to form a ternary complex and induces a conformational change on the protein so that a quenching amino acid side chain interacts with DPNH. This situation seems to be unlikely because certain organic amides are known to increase the fluorescence intensity of the enzyme–DPNH binary complex.

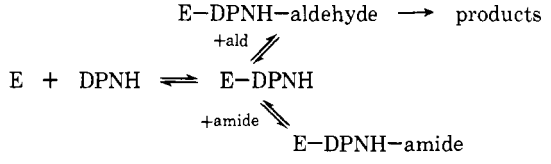
The discussion presented below indicates that most likely a ternary ERI complex of the type described in case I is formed. (1) One could distinguish between cases I and II by performing kinetics, *i.e.*, if *case I* were true, the organic amides will inhibit the LADH system competitively when the concentration of aldehyde is varied and the DPNH concentration is kept constant. In addition, the system will display no competition when DPNH is varied. In fact, it has been shown by Theorell and McKinley-McKee (1961b), Woronick (1961),

TABLE II: $K_{ER,I}$ and Q_{ERI} Values as Determined by the Direct Titration Method in Sodium Phosphate Buffer at pH 7.0, μ = 0.1, 23°.

Inhibitor	No. Determinations	Q_{ERI} [a,b]	$K_{ER,I}$ [a] (μM)
Benzamide	4	0.32 ± 0.12	536 ± 14
N-Methylbenzamide	2	1.27 ± 0.15	14,450 ± 440
N,*N*-Dimethylbenzamide	4	1.7 ± 0.4	49,500 ± 6500
p-Methylbenzamide	6	0.38 ± 0.09	54 ± 4
m-Methylbenzamide	5	2.9 ± 0.4	33 ± 3
p-Isopropylbenzamide	4	0.39 ± 0.08	50 ± 2
p-Hydroxybenzamide	3	5.4 ± 0.4	294 ± 10
m-Hydroxybenzamide	3	2.6 ± 0.2	1,260 ± 70
p-Nitrobenzamide	3	2.3 ± 0.5	420 ± 33
m-Nitrobenzamide	4	1.6 ± 0.6	1,910 ± 160
p-Chlorobenzamide	4	0.6 ± 0.2	87 ± 2
m-Chlorobenzamide	4	0.47 ± 0.24	603 ± 10
p-Fluorobenzamide	5	0.49 ± 0.14	417 ± 7
m-Fluorobenzamide	3	1.4 ± 0.3	770 ± 15
m-Bromobenzamide	1	4.1 ±	650 ±
p-Methoxybenzamide	5	2.2 ± 0.3	160 ± 10
m-Dimethylaminobenzamide	2	0.60 ± 0.28	197 ± 40
Nicotinamide	5	0.41 ± 0.11	24,800 ± 1100
2-Picolinamide	2	0.38 ± 0.05	6,340 ± 730
Isonicotinamide	2	0.38 ± 0.26	14,000 ± 1600
Propionamide	4	26.9 ± 0.5	363 ± 12
n-Valeramide	5	8.5 ± 0.3	40 ± 4
Isobutyramide	16	46.7 ± 0.5	163 ± 13
p-Toluamidoxime	1	3.8	2,690
2-Phenylacetamide	4	6.0 ± 0.2	256 ± 16
Nicotinonitrile	2	0.46 ± 0.19	47,600 ± 3800
Nicotinic acid hydrazide	2	9.6 ± 2.0	15,900 ± 500

[a] The deviations listed are the standard errors. [b] Q_{ER} = 13.2.

and Sarma and Woronick (1971b) that organic amides indeed display competitive kinetics in the LADH system when the concentration of aldehyde is varied and [DPNH] is kept constant. These observations have been accounted for by Theorell and McKinley-McKee (1961b), Woronick (1961), and Sarma and Woronick (1971b), on the basis of the formation of an enzyme–DPNH–amide ternary complex. It has also been



shown by Woronick (1963a,b) that the K_i values obtained kinetically under the above conditions agree with the $K_{ER,I}$ values obtained by two different spectrophotofluorometric titration procedures, even for amides that form ERI complexes having little or no fluorescence.

Theorell and McKinley-McKee (1961b) has further shown that when [DPNH] was varied and [aldehyde] was kept constant the system displayed uncompetitive behavior. This indicates that DPNH and amide do not compete as envisioned in case II. (2) The dissociation constant for the binary LADH–DPNH complex is 0.3 μM (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961a). The dissociation constant for the binary LADH–benzamide complex has been reported to be 110,000 μM by Sigman (1967). The reaction which is postulated in case II is the sum of the following reactions:

$$\begin{array}{ll} ER \rightleftharpoons E + R & K = K_{ER} \\ E + I \rightleftharpoons EI & K = \dfrac{1}{K_{EI}} \\ \hline ER + I \rightleftharpoons EI + R & K = K_{ER}/K_{EI} \end{array} \qquad (4)$$

The constant for the dissociation of the inhibitor is $K_{diss} = K_{EI}/K_{ER}$. Thus, the dissociation constant obtained by our method would be $K_{diss} = K_{EI}/K_{ER}$ if case II were followed. In the case of benzamide our observed dissociation constant should have been about 370,000 μM. Instead we obtained a dissociation constant of 536 μM, which agrees with the value of 470 μM obtained by Woronick (1963a) who used this same procedure.

The arguments presented above show that when the ER binary complex is titrated with the aromatic amides it is unlikely that they form EI and R. Under these circumstances, the observation that addition of the inhibitor to the ER binary complex causes diminution of the fluorescence intensity can best be rationalized on the basis of the formation of a ternary

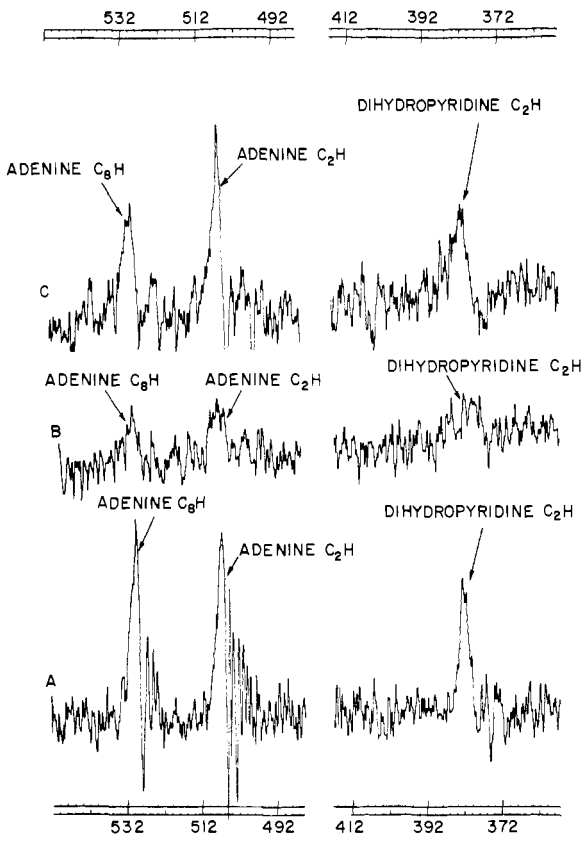


FIGURE 2: Low-field region of the proton nuclear magnetic resonance spectrum of DPNH in the absence and presence of horse LADH. *2A*. 4000 μM DPNH in pH 7.5, 0.1 M sodium phosphate buffer in D_2O. *2B*. 4000 μM DPNH + 500 μN LADH; *2C*. 8000 μM DPNH + 500 μN LADH. The concentration of the enzyme is expressed in normality. The enzyme has two binding sites. Hence 1 N enzyme = 2 N enzyme; also 1 M DPNH = 1 N DPNH.

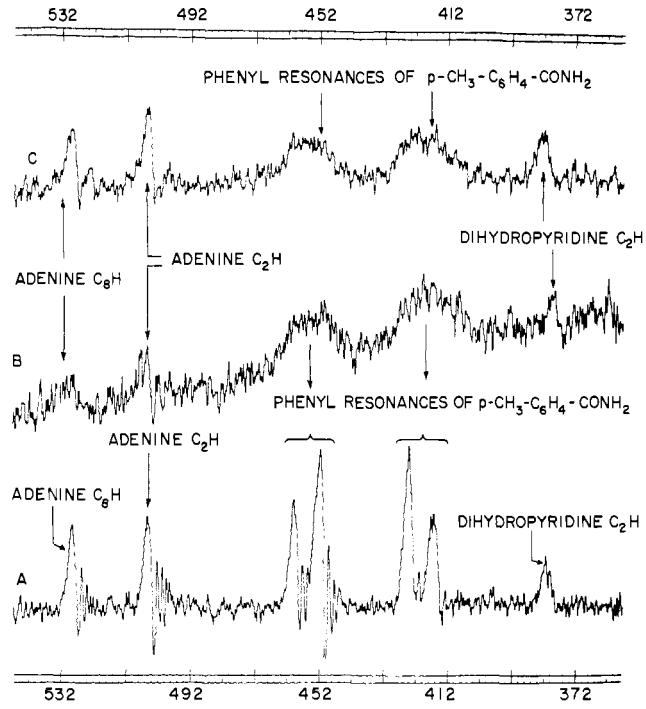


FIGURE 3: Effect of inhibitor on the low-field proton nuclear magnetic resonance spectrum of DPNH–LADH binary complex. *3A*. 4000 μM DPNH + 10000 μM *p*-methylbenzamide; *3B*. 4000 μM DPNH + 10000 μM *p*-methylbenzamide + 500 μN LADH; *3C*. 8000 μM DPNH + 10000 μM *p*-methylbenzamide + 500 μN LADH. Again note that 1 M enzyme = 2 N enzyme, 1 M DPNH = 1 N DPNH and 1 M inhibitor = 1 N inhibitor.

ERI complex with lower fluorescence. The evidence so far presented for the existence of a ternary complex is based on the "conventional wisdom" based largely on the work of Theorell which has accepted such measurements as conclusive evidences for ternary complexes. Below we present direct spectroscopic evidence that the amides form ERI ternary complexes. (3) Mildvan and Weiner (1969) have used proton nuclear magnetic resonance to study the thermodynamic, kinetic, and structural properties of ternary complexes. Our recent application of proton nuclear magnetic resonance to study the conformation of coenzymes in the presence and absence of enzymes (Sarma *et al.*, 1968a, 1970; Sarma and Kaplan, 1969a, 1970a–c) have prompted us to direct the same technique to this problem.

The binding of aromatic amides to the enzyme–DPNH binary complex can be monitored through the chemical shifts and line widths of the phenyl resonances. Inhibitors such as methyl substituted benzamides are particularly amenable to proton nuclear magnetic resonance investigations of their binding because of the sharp singlet resonance from the CH_3 group. The binding of DPNH in the ternary ERI complex can be followed through the singlet resonance of adenine C_8H and C_2H and dihydropyridine C_2H. In the following discussion, the application of proton nuclear magnetic resonance to follow the formation of ternary and binary complexes is illustrated in the case of the inhibitor *p*-methylbenzamide.

It can be seen from Figure 2A–C that the resonances of adenine C_8H, C_2H, and dihydropyridine C_2H of 4000 μM DPNH (Figure 2A) are broadened, in the presence of 500 μN LADH (Figure 2B), to an extent that they are only bearly detectable. In Figure 2, as one goes from 2B (middle) to 2C (top), the concentration of DPNH is increased from 4000 to 8000 μM, while the concentration of enzyme remains the same. Under these conditions virtually all the enzyme is present as the LADH–DPNH binary complex. It may be seen that the width of the lines at half-height has become shorter and the resonances have become sharper, as one goes from Figure 2B–C.

Figure 3A (bottom) shows the low-field proton nuclear magnetic resonance spectrum of 4000 μM DPNH + 10,000 μM *p*-methylbenzamide, the inhibitor. The quartet at the center arises from the four protons of the phenyl ring of the inhibitor. It can be seen that the line width of the dihydropyridine C_2H of DPNH in the presence of the inhibitor has increased slightly (compare Figure 2A with Figure 3A). This broadening probably originates from the quadrupole broadening effects of the adjacent aliphatic nitrogen of the dihydropyridine moiety. Similar effects from the ^{14}N of the dihydropyridine moiety of DPNH were reported earlier (Sarma and Kaplan, 1969b, 1970b). Because of this complication from quadrupole effects, the dihydropyridine C_2H resonance will not be included in the following discussion. Comparison of Figure 3A with Figure 3B shows the effect of introducing the inhibitor, *p*-methylbenzamide to the enzyme–DPNH binary complex. The resonances from the inhibitor, as well as those from adenine C_8H and C_2H, have become clearly broadened in Figure 3B. Furthermore, it may be seen that the adenine C_2H and C_8H resonances in Figure 3B are either as broad or slightly broader than in Figure 2B.

The observed broadening of the DPNH resonances in the presence of the enzyme as well as the broadening of the DPNH

and inhibitor resonances in the presence of the enzyme can originate from any one of the following events: (a) introduction of paramagnetic impurities with the enzyme, (b) nonspecific broadening, (c) a fast exchange, and, (d) an intermediate rate of exchange into an environment with different chemical shift.

In the various spectra accumulated under different conditions, the width of the peak arising from residual HDO in the sample was used as an index of resolution. This width was found to be 1 Hz and was found to be unperturbed by the presence of enzyme. This observation along with the finding that the width of the internal standard (tetramethylammonium chloride) was also unaffected by the enzyme enables us to eliminate any complications due to possible introduction of paramagnetic ions with the enzyme since these would strongly alter the width of the HDO and internal standard peaks (Jardetzky, 1964).

In order to check whether proteins in general can cause nonspecific broadening, the spectrum of DPNH was recorded in the presence of such proteins as lysozyme and bovine serum albumin (Sarma and Kaplan, 1970c). The resonances appeared very sharp thereby indicating that no interactions between these proteins and DPNH occur. Further, if LADH can nonspecifically broaden the resonances of DPNH and inhibitor, it would have affected the signal from the internal standard which was not found to be the case.

The observed broadening of the protons of DPNH, in the presence of enzyme, may indicate that they have a shorter transverse relaxation time, T_2, and a longer correlation time, *i.e.*, the adenine and the dihydropyridine moieties of DPNH have lost their segmental motion and have become immobilized by forming an enzyme–DPNH binary complex. The dynamics of the interactions between LADH and DPNH may be interpreted as one of fast exchange (see below for a chemical shift mechanism for broadening). In the case of a fast exchange the transverse relaxation time is governed by the expression

$$\frac{1}{T_2} = \frac{P_b}{T_{2b}} + \frac{P_f}{T_{2f}} \qquad (5)$$

where T_2 is the observed transverse relaxation time, T_{2b} and T_{2f} are the relaxation times of DPNH in its enzyme complex and free forms, respectively; P_b and P_f are the populations of the bound and free DPNH, respectively. Hence, in the case of a fast exchange, the observed transverse relaxation time will approach that of unbound DPNH, as the concentration of free DPNH is increased. Examination of Figure 2 shows that the width of the lines at half-height has become shorter and the resonances have become sharper, as one goes from Figure 2B to C indicating that the data are consistent with a fast-exchanging situation. Arguing along the same direction, one may conclude that the system containing the inhibitor, DPNH, and enzyme (Figure 3) may also under the control of expression 5, *i.e.*, the broadening of the inhibitor resonances as well as that of DPNH (the noise does not allow us to determine whether the amide alters the broadening of DPNH resonances) in the presence of the enzyme indicates longer correlation times due to lack of segmental motion. If the inhibitor were able to displace DPNH (case II, see above) free DPNH will be liberated into the system and this will cause the DPNH resonances to become sharper and this was not observed. For example, comparison of Figure 3B,C shows that introduction of free DPNH into the system causes sharpening of the DPNH resonances. Nuclear magnetic resonance experiments were also conducted at very high concentration of DPNH (enzyme = 350 μN; DPNH = 70,000 μM) to make sure that all the enzyme was tied up as the ER binary complex (K_{ER} = 0.3 μM from Winer and Theorell, 1960, and Theorell and McKinley-McKee, 1961a). To this system, 7000 μM inhibitor was added, and it was observed that the peak of the inhibitor became considerably broader. The details of this work has appeared in a preliminary communication (Sarma and Woronick, 1971a).

The data have been so far discussed in terms of a fast-exchanging situation. However, the same data are totally consistent with a situation in which broadening results from an intermediate exchange into an environment with different chemical shifts. Lee and Chan (1971) have reported a similar case in the association of uridine 3′-monophosphate to ribonuclease A. In the present case one may attempt to distinguish between the two possibilities by calculating the rate of exchange from the T_2 data and then comparing it to the data obtained from steady-state and stopped-flow kinetics. However, we hesitate to do this because the precision of the T_2 data and hence the exchange rate obtained from line-broadening measurements may not be the same as those obtained by steady-state and stopped-flow kinetics.

Whether the mechanism involved is one of fast exchange or one of intermediate chemical exchange, the data are consistent with the thesis that DPNH forms a binary complex with LADH and that amides form a ternary complex with LADH and DPNH, a fact which has also been shown by Mildvan and Weiner (1969) using nuclear magnetic resonance. The purpose of employing proton nuclear magnetic resonance in the present case is to show that the series of amides which are used in the present study also form ternary ERI complexes.

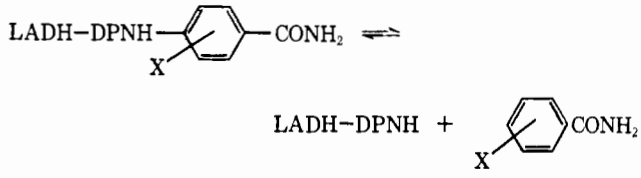
Table II contains the $K_{ER,I}$ and Q_{ERI} values obtained by direct titration of the ER complex with inhibitors. In most cases the values are the average of several determinations, except in those cases where the cuvet contents were changed for each point on the titration curve. The $Q_{ER,I}$ and $K_{ER,I}$ values for propionamide, *n*-valeramide, and isobutyramide generally agree with values reported by one or more other workers (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961; Woronick, 1963a, 1961). The Q_{ERI} and $K_{ER,I}$ values for benzamide and nicotinamide are in general agreement with the values reported by Woronick (1963a,b). In agreement with the empirical rules of Woronick (1963a) the Q_{ERI} values for the substituted benzene and pyridine inhibitors are much smaller than Q_{ER} (which we found to be 13.2).

Table III contains the values of the constants used for the analysis of the influence of the structures of the inhibitors on their interactions with the LADH–DPNH complex. The $K_{ER,I}$ values are those obtained by the direct titration method unless otherwise stated. The Hammett σ values were taken from the compilation of McDaniel and Brown (1958) unless otherwise indicated. The σ values for the para substituents, except for the *p*-OH and *p*-NO_2 groups, are σ^+ values (Brown and Okanoto, 1958). Other σ values for para substituents did not improve the linear relationship between the σ values and log $K_{ER,I}$. π constants are hydrophobic constants invented by Fujita *et al.* (1964), where $\pi = \log P_x - \log P_H$. In this equation, P_H is the partition coefficient of a parent molecule between two immiscible solvents (the above authors used 1-octanol–water as the reference system), and P_x is the partition coefficient of the X-substituted compound. Since π is derived from equilibria, it, like σ, is a free-energy-related constant. These constants do not vary over a large range for a given substituent attached to different carriers, especially

TABLE III: Constants Used for the ρ, σ, and π Analysis of the Dissociation of Horse Liver Alcohol Dehydrogenase Complexes.

X	Log $K_{ER,I}$ [a]	σ[b]	π[c]
H	2.724	0.0	0.0
m-NO_2	3.301	0.710	−0.36
m-Cl	2.903[d]	0.373	0.61
m-Br	2.991[e]	0.391	0.79
m-F	2.887	0.337	
m-OH	2.903[d]	0.121	0.15
m-$(CH_3)_2N$	2.301	−0.211[f]	
m-CH_3	1.477	−0.069	0.57
p-NO_2	2.623	0.778	−0.39
p-Cl	1.929	0.114	0.54
p-F	2.623	−0.073	
p-OH	2.477	−0.37	0.11
p-CH_3	1.778	−0.311	0.52
p-$(CH_3)_2CH$	1.699	−0.256	
p-OCH_3	2.204	−0.788	0.18

[a] The $K_{ER,I}$ values are those obtained by direct titration unless otherwise stated and are for the following reaction:



These logarithms were obtained from the $K_{ER,I}$ values expressed in micromolar units. [b] The σ_{meta} constants are taken from McDaniel and Brown (1958). The σ_{para} constants, excepting for the *p*-OH and *p*-NO_2 substituents, are σ^+ constants from Brown and Okamoto (1958). The σ constants for the *p*-OH and *p*-NO_2 groups are σ constants from McDaniel and Brown (1958). [c] From Fujita *et al.* (1964). [d] This value, obtained by the indirect method (Woronick, 1963b), was used because it gives a better correlation. [e] This value is the average value obtained by the indirect method (Woronick, 1963b) because there was only one value available from the direct titration. [f] From Hine (1962).

for carriers of a similar electronic nature. The π constants in Table III, taken from Fujita *et al.* (1964), were obtained using nitrobenzene and its derivatives, and may reasonably be applied to benzamide derivatives because of the electronic similarities of nitrobenzene and benzamide.

Figures 4 and 5 show the plot of log $K_{ER,I}$ *vs.* σ_{meta} and π_{para}. Least-squares treatment of the points gave eqs 6–9, where n is the number of points taken, s is the standard error of the slope, and r is the correlation coefficient.

	n	s	r	
Para Substituents				
$\log K_{ER,I} = 0.325\sigma - 2.30$	8	0.304	0.36	6
$\log K_{ER,I} = -0.968\pi + 2.45$	6	0.205	0.88	7
Meta Substituents (Excluding *m*-Methyl)				
$\log K_{ER,I} = 0.921\sigma + 2.63$	7	0.130	0.94	8
$\log K_{ER,I} = 0.156\pi + 3.00$	5	0.187	0.36	9

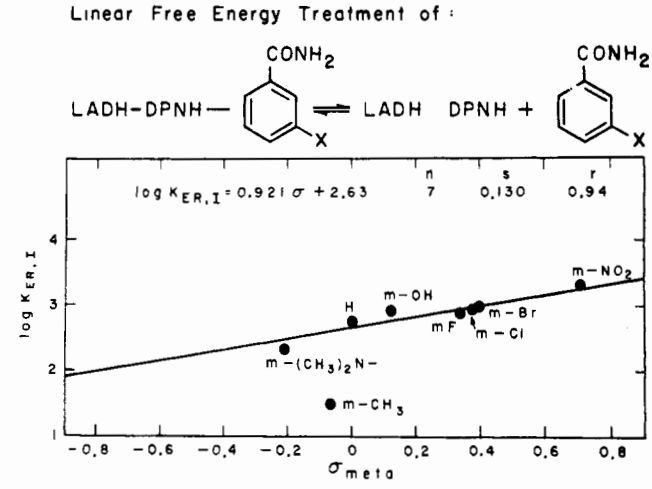


FIGURE 4: Log $K_{ER,I}$ *vs.* σ_{meta}. The value for the *m*-CH_3 substituent was not included in the least-squares calculation because it deviated significantly from the other points.

Equations 7 and 8 have a correlation coefficient of 0.88 and 0.94, respectively, and relatively small standard errors for the slope. Thus, the data show a good linear correlation between log $K_{ER,I}$ and π_{para} as well as between log $K_{ER,I}$ and σ_{meta}. This suggests that hydrophobic bonding plays an important role in controlling differences in binding of para-substituted benzamides in the ternary complex, whereas in the case of meta-substituted benzamides, electronic factors are more important in determining relative binding. It is known that inductive rather than resonance electronic effects operate at the meta position. Furthermore, the large standard error in the slope (slope = 0.325, error = 0.304!) and the poor value of r in eq 6 indicates that a poor correlation exists between log $K_{ER,I}$ and σ for para-substituted benzamides. This may be interpreted to mean that the dissociation of para-substituted benzamides from the ternary ERI complex is poorly controlled by electronic factors. Similar consideration of eq 9 suggests that hydrophobic control plays little or no role in controlling the binding of meta-substituted benzamides in their ERI ternary complexes. It may be concluded that the geometric orientation of the substrate analog in the ERI complex is such that the hydrophobic site is accessible only to the

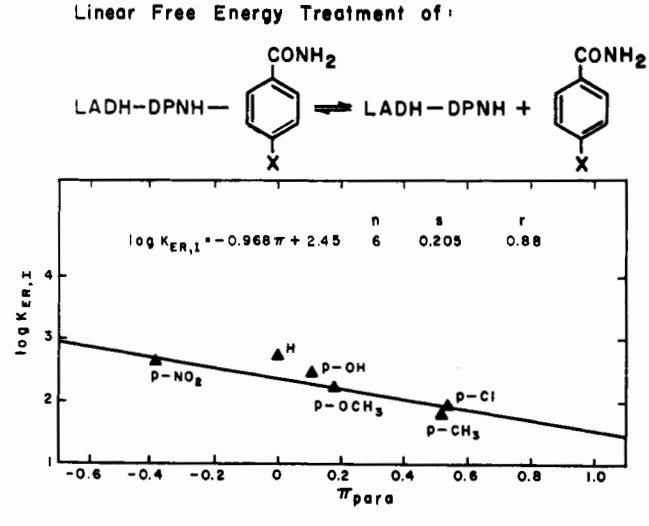


FIGURE 5: Log $K_{ER,I}$ *vs.* π_{para}.

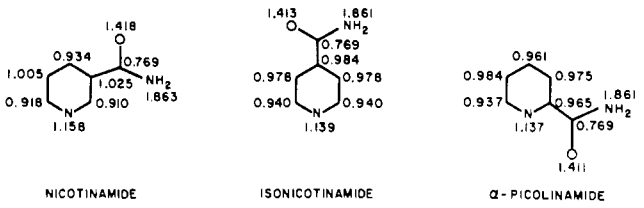
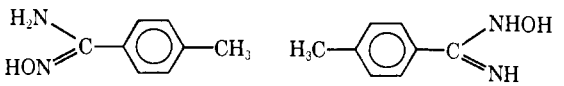


FIGURE 6: π-Electron densities at the different atoms of the pyridine amides calculated by the LCAO approximation method.

para substituents, and that the meta substituents are located in the ERI complex in such a way that they cannot significantly interact with the hydrophobic binding site on the enzyme surface. This information is of great importance for the complete mapping of the electronic and structural features of the active site of LADH. It is interesting to note that similar results were obtained by Hanch *et al.* (1965) when they made a ρ, σ, π analysis of the data of Nath and Rydon (1954) for the enzymatic hydrolysis of substituted phenyl β-D-glucosides by emulsin. It is further interesting to note that comparison of the $K_{ER,I}$ values for the same para and meta substituents (Table II, excluding *p*- and *m*-methylbenzamides) reveal that the para-substituted inhibitor has the smaller dissociation constant. This may be interpreted to mean that hydrophobic factors play a stronger role in stabilizing the inhibitor complexes than do electronic factors.

The fact that dissociation of meta-substituted benzamides follows Hammett's equation with a positive ρ value indicates that this dissociation is favored by electron withdrawal from the amide group. It is highly probable that the carbonyl oxygen is involved in the binding of amides in the ERI complex. Some evidence for this may be obtained by comparing the $K_{ER,I}$ values for *p*-toluamide and *p*-toluamidoxime. The incorporation of an amidoxime group in place of an amide group destabilizes the inhibitor in the ERI complex by 2.3 kcal/mole. The ERI complex formed with *p*-toluamidoxime is therefore more dissociated ($K_{ER,I}$ = 2690 μM). This may indicate the importance of the carbonyl oxygen in binding to the enzyme, although the possible contribution of the *N*-hydroxyl group to binding or to steric hindrance cannot be evaluated from the present data. *p*-Toluamidoxime may actually exist to some extent in a tautomeric equilibrium. The amidoxime



may bind through the oxygen of the hydroxyl group, but more probably binds through a doubly bonded nitrogen. If both tautomeric forms exist to any significant extent in solution, both forms may bind through a doubly bonded nitrogen. The observation that *N*-methyl substitution of benzamide destabilizes the ERI complex (*vide infra*) as compared with the complex formed with the unsubstituted compound is consistent with the view that the amide binds to the enzyme through the carbonyl oxygen, although other interpretations cannot be excluded. It seems likely that the carbonyl oxygen is bound as a monodentate ligand to the catalytically active zinc atoms of LADH (Drum and Vallee, 1970a; Drum *et al.*, 1969a,b). Recent success in Vallee's laboratory in replacing zinc with cobalt in LADH (Drum and Vallee, 1970b) should enable us to settle this problem because cobalt, being paramagnetic with short electronic relaxation time, would be expected to perturb the proton nuclear magnetic resonances of its ligands by contact interaction.

Ring alkyl substitution and *N*-alkyl substitution of benzamide have rather different effects on the stability of the ERI complexes. *p*- and *m*-alkyl substitution of benzamide stabilizes the inhibitor in the ERI complexes as compared with the complex formed with the unsubstituted compound. In contrast, monomethyl substitution of the nitrogen of benzamide destabilizes the inhibitor in the ERI complex by 1.94 kcal/mole ($\Delta G^{\circ\prime} = -RT \ln K_{ER,I}$) whereas dimethyl substitution of the nitrogen in benzamide destabilizes the ERI complex by 2.66 kcal/mole. It may be noted that the ERI complex formed from *N*,*N*-dimethylbenzamide is 0.72 kcal/mole less stable than the ERI complex formed with *N*-methylbenzamide. Thus, the first *N*-methyl group to be introduced into benzamide produces the greater part of the destabilizing effect with respect to dissociation of the amide from the ERI complex. Since it is proposed that the inhibitor binds in the ERI complex through its carbonyl oxygen, the presence of bulky substituents on the adjacent nitrogen may produce steric hindrance between the inhibitor and DPNH or between the inhibitor and the protein, or both.

The $K_{ER,I}$ values for 2-picolinamide, nicotinamide, and isonicotinamide, the three pyridine amides, present some interesting problems (Table II). The large differences in $K_{ER,I}$ values between benzamide and these three isomers are, it appears at first sight, probably caused by the electron-withdrawing effect of nitrogen, which affects the electron density at the carbonyl oxygen of the amide, and thus affects the binding. However, this is probably only one of the reasons for the differences in $K_{ER,I}$ values between benzamide and the pyridine derivatives. Molecular orbital calculations show that the π electron densities on the carbonyl oxygens of the three pyridine amides are practically identical (Figure 6). Although the amide probably binds through the carbonyl oxygen for all three pyridine amides, the differences in binding of the three inhibitors are not caused by differences in electron densities on the carbonyl oxygen. On the other hand, a linear free-energy plot was obtained when the logarithm of $K_{ER,I}$ was plotted against the logarithm of the molar solubilities of these isomers in water. This indicates that the differences in binding among the three isomers are related to differences in the lipophilic characters of the pyridine derivatives, with the least soluble compound being most tightly bound. Comparison of the $K_{ER,I}$ and Q_{ERI} values for nicotinonitrile with the values of these constants obtained for the other pyridine derivatives shows that nicotinonitrile behaves more like nicotinamide rather than like any other pyridine derivative. The binding of nicotinonitrile most probably occurs through the nitrile nitrogen. However, one would expect some differences in the behavior of nicotinonitrile as compared with the behavior of nicotinamide because of differences in the electronegativity and polarizability of oxygen as compared with nitrogen. Because the CC≡N group is linear, whereas the CC(=O)NH_2 group is nonlinear, the geometries of the two different ERI complexes are probably somewhat different. These various differences will undoubtedly be reflected in the values of the dissociation constants, as occurs in this case. The fact that the pyridine derivatives, except for nicotinic acid hydrazide, have practically the same Q_{ERI} values suggests that the mechanism of quenching of the fluorescence of the complexes is the same.

The Q_{ERI} values for all the aromatic and pyridine amides are small as compared to the value of Q_{ER}. This indicates that the fluorescence of the dihydronicotinamide ring in the binary

complex is quenched when inhibitor binds to form the ternary complex. This quenching may be interpreted to result from charge transfer between the dihydronicotinamide ring of DPNH and the aromatic ring of the amide. Fluorescence quenching is a general property of charge-transfer complexes involving molecules capable of fluorescence (Orgel and Phil, 1954). Kosower (1956) has suggested that DPNH might serve as a donor of electrons in a charge-transfer complex, but no charge transfer band has been observed. However, quenching of fluorescence could occur even if there is no charge transfer between the dihydropyridine ring and the aromatic ring of the inhibitor. The dihydropyridine ring of DPNH and the aromatic ring of the inhibitor may be stacked in parallel planes. Such an arrangement could result in the quenching of the fluorescence of the dihydropyridine ring. Recent nuclear magnetic resonance studies by Sarma *et al.* (1968b) have shown that in flavin dinucleotides, the aromatic base pairs are stacked in parallel planes. The quenching of the flavin fluorescence of FAD is caused by an adenine ring which lies in a plane parallel to the isoalloxazine ring. In this case no charge-transfer band was observed. In view of the above, it may be concluded that in the ERI complexes with very low fluorescence, the dihydropyridine ring of DPNH and the aromatic ring of the inhibitor are stacked in parallel planes, and that such a geometry quenches the fluorescence of the DPNH contained in the complex. Furthermore, it has been found that when a CH_2 group is interposed between the phenyl ring and the $CONH_2$ group of benzamide, as in 2-phenylacetamide, the fluorescence of the ERI complex increases. Thus Q_{ERI} is 0.32 for benzamide and 6.0 for 2-phenylacetamide. This increased fluorescence is probably due to less overlap between the π orbitals of the phenyl ring of 2-phenylacetamide and the π orbitals of the dihydropyridine ring of DPNH, as compared to the overlap that may occur between benzamide and DPNH.

Acknowledgment

The authors thank Professor J. O. Edwards for his very helpful discussions. We thank Professor B. Laurenzi and Professor F. Walz for private reviews of this manuscript.

References

Brown, H. C., and Okamoto, Y. (1958), *J. Amer. Chem. Soc. 80*, 4979.
Dalziel, K. (1957), *Acta Chem. Scand. 11*, 397.
Dalziel, K. (1960), *Biochem. J. 80*, 440.
Drum, D. E., Li, T. K., and Vallee, B. L. (1969a), *Biochemistry 8*, 3783.
Drum, D. E., Li, T. L., and Vallee, B. L. (1969b), *Biochemistry 8*, 3792.
Drum, D. E., and Vallee, B. L. (1970a), *Biochemistry 9*, 4078.
Drum, D. E., and Vallee, B. L. (1970b), *Biochem. Biophys. Res. Commun. 41*, 33.
Fujita, T., Iwasa, J., and Hansch, C. (1964), *J. Amer. Chem. Soc. 86*, 5175.
Hanch, C., Deutsch, E. W., and Smith, R. N. (1965), *J. Amer. Chem. Soc. 87*, 2738.
Hine, J. (1962), Physical Organic Chemistry, New York, N. Y., McGraw-Hill, p 87.
Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.
Jardetzky, O. (1964), *Advan. Chem. Phys. 7*, 499.
Kosower, E. M. (1956), *J. Amer. Chem. Soc. 78*, 3497.
Lee, G. C. Y., and Chan, S. I. (1971), *Biochem. Biophys. Res. Commun. 43*, 142.
McDaniel, D. H., and Brown, H. C. (1958), *J. Org. Chem. 23*, 420.
Mildvan, A. S., and Weiner, H. (1969), *J. Biol. Chem. 244*, 2465.
Nath, R. L., and Rydon, H. N. (1954), *Biochem. J. 57*, 1.
Orgel, L. E., and Phil, O. (1954), *Quart. Rev. (London) 8*, 422.
Pullman, B., and Pullman, A. (1963), Quantum Biochemistry, New York, N. Y., Interscience.
Sarma, R. H., Dannies, P., and Kaplan, N. O. (1968a), *Biochemistry 7*, 4359.
Sarma, R. H., and Kaplan, N. O. (1969a), *Biochem. Biophys. Res. Commun. 36*, 780.
Sarma, R. H., and Kaplan, N. O. (1969b), *J. Biol. Chem. 244*, 771.
Sarma, R. H., and Kaplan, N. O. (1970a), *Biochemistry 9*, 539.
Sarma, R. H., and Kaplan, N. O. (1970b), *Biochemistry 9*, 557.
Sarma, R. H., and Kaplan, N. O. (1970c), *Proc. Natl. Acad. Sci. U. S. 67*, 1375.
Sarma, R. H., Moore, M. C., Kaplan, N. O. (1970), *Biochemistry 9*, 549.
Sarma, R. H., Ross, V., and Kaplan, N. O. (1968b), *Biochemistry 7*, 3052.
Sarma, R. H., and Woronick, C. L. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 10–15, Abstr. C-113.
Sarma, R. H., and Woronick, C. L. (1971a), *Res. Commun. Chem. Pathol. Pharmacol. 2*, 117.
Sarma, R. H., and Woronick, C. L. (1971b), *Res. Commun. Chem. Pathol. Pharmacol. 2*, 601.
Sigman, D. S. (1967), *J. Biol. Chem. 242*, 3815.
Theorell, H., and McKinley-McKee, J. S. (1961a), *Acta Chem. Scand. 15*, 1811.
Theorell, H. and McKinley-McKee, J. S. (1961b), *Acta Chem. Scand. 15*, 1834.
Theorell, H., Taniguchi, S., Åkeson, Å., and Skyrsky, L. (1966), *Biochem. Biophys. Res. Commun. 24*, 603.
Winer, A. D. (1958), *Acta Chem. Scand. 12*, 1695.
Winer, A. D., and Theorell, H. (1960), *Acta Chem. Scand. 14*, 1729.
Woronick, C. L. (1961), *Acta Chem. Scand. 15*, 2062.
Woronick, C. L. (1963a), *Acta Chem. Scand. 17*, 1789.
Woronick, C. L. (1963b), *Acta Chem. Scand. 17*, 1791.