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N^1-Alkylnicotinamide Chloride 유도체에 의한 탈수소 효소의 불활성화에 관한 연구

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Inactivation Study of Pyridine-Linked Dehydrogenases by N^1-Alkylnicotinamide Chlorides

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요 약. Pyridine 관여 탈수소 효소는 N^1-alkylnicotinamide chloride 유도체에 의하여 저해 작용을 받고 있는 바 저해제의 농도 변화에 따른 효소 저해작용이 가역 또는 비가역 불활성화 반응에 기인하는지의 여부를 밝혀 보기 위하여 토끼 근육으로 부터 유리한 L-α-glycerophosphate dehydrogenase를 사용하여 연구하였다.

이 효소의 저해작용은 사용한 저해제 유도체의 농도가 희박했을 경우 가역적인 효소저해 반응을 보여주고 있으나 저해제의 농도가 증가함에 따라 점차 비가역적인 효소 불활성화로서 나타남을 알았으며 이러한 비가역 불활성화 반응은 저해제의 농도가 증가함에 따라 형성될 수 있는 micelle 구조의 미세분자와의 결합에 의한 효소의 변성에 기인 할 것이라고 결론을 얻었다.

ABSTRACT. A series of N^1-alkylnicotinamide chlorides, N^1-methyl- to N^1-dodecylnicotinamides inclusive were studied with rabbit muscle L-α-glycerophosphate dehydrogenase to investigate the possibility of reversible and irreversible inactivation of the pyridine-linked dehydrogenases by the coenzyme-competitive inhibitor derivatives. The inhibition of the enzyme by N^1-alkylnicotinamide chlorides was demonstrated to be reversible at the dilute concentration of the inhibitors but this reversible inhibition was found to be followed by an irreversible time-dependent inactivation measuable at high concentrations of the inhibitors. The properties of this time-dependent inactivation were discussed on the basis of the denaturation of the enzyme by the binding of small micelle-like structures formed at higher concentrations of the inhibitors.

INTRODUCTION

A series of N^1-alkylnicotinamide(ANA) chlorides and N^1-benzyl-3-substituted pyridinium chlorides have been used as the coenzyme-competitive inhibitors in a number of pyridine-linked dehydrogenase systems to investigate the effect of the nicotinamide moiety of nicotinamide adenine dinucleotide (NAD), and the binding studies along with the other series of coenzyme-competitive inhibitors suggested an important role of different functional groups of the coenzyme molecule for the binding to the dehydrogenases[1~7].

Through multiple inhibition analysis of several dehydrogenases including *L*-α-glycerophosphate dehydrogenase (*L*-α-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8), the mode of enzyme inhibiton by ANA chlorides was suggested by the reversible binding of the inhibitors at a pyridinium region of the NAD binding site of the enzyme molecule differing from the region interacting with the other coenzyme-competitive adenosine analogues[5~8]. However, although the non-polar interactions between the non-polar side chain of the inhibitors and a hydrophobic region of the enzymes were demonstrated by several dehydrogenases, the reversibility of the specific inhibition was found only at dilute concentrations of the inhibitors[3,9~11].

With yeast alcohol dehydrogenase the specific binding of ANA chlorides was observed through a reversible inhibition upon dilution, but this reversible binding of the inhibitors to the enzyme was shown to be followed by a slow time dependent irreversible inactivation of the enzyme[9]. With many other pyridine-linked dehydrogenases, i. e., rabbit muscle lactate dehydrogenase, rat liver malic dehydrogenase and yeast glucose-6-phosphate dehydrogenase, however, nonspecific irreversible inhibitions were observed by ANA chlorides at concentrations much higher than those required in the case of the specific binding to yeast alcohol dehydrogenase[12,13].

Since a series of ANA chlorides was found to inhibit the reaction catalyzed by rabbit muscle *L*-α-glycerophosphate dehydrogenase effectively and reversibly at low concentrations of the inhibitors but nonspecifically at higher inhibitor concentrations, it was of interest to demonstrate whether these inhibitors were bound reversibly and to investigate the possibility of time-dependent irreversible inactivation within a reasonable range of the inhibitor concentrations.

EXPEIRMENTAL

Materials. Crystalline rabbit muscle *L*-α-glycerophosphate dehydrogenate (GPDH) was obtained in an ammonium sulfate suspension from the Sigma Chemical Company. The enzyme was diluted in 0.05 *M tris*-HCl buffer, pH 7.85, containing 0.1 percent crystalline bovine serum albumin. NAD, NADH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and α-glycerophosphate were purchased from Sigma. N^1-alkylnicotinamide chlorides(ANA) were kindly supplied by Professor Bruce M. Anderson, Virginia Polytechnique Institute, Blacksburg, Va.

Reversibility and Time Dependence of the Inhibition of *L*-α-Glycerophosphate Dehydrogenase by N^1-Alkylnicotinamide Chlorides. Inhibition of the enzyme was studied in two ways. In the first case, the enzyme was pre-incubated in 0.05 *M tris*-HCl buffer, pH 7.85, and at various time intervals, an aliquot of the pre-incubated enzyme was transferred to a reaction mixture containing NAD, substrate, buffer medium and inhibitor for the enzyme assay. This provides a measure of the instantaneous inhibition since the inhibitor is only present in the final reaction mixture. Concentrations of inhibitor for

these experiments were chosen to give approximately 25 percent inhibition. In a second experiment, the inhibitor and enzyme were preincubated together, both at concentrations ten times those used in the final reaction mixture of the first experiment. Samples of the preincubated mixture were transferred to an assay mixture for initial velocity measurements. Aliquots transferred were chosen to provide a ten-fold dilution so that the resulting reaction mixture contained concentrations of inhibitor and enzyme identical to those employed in the first experiment. Measurement of activity at zero time of preincubation provided a measure of the reversibility of the inhibition upon dilution. Activity measurements of samples taken at various time intervals of pre-incubation provided data concerning the time dependence of inhibition. For the study of ANA chlorides effect on the time-dependent enzyme inactivation, the enzyme was preincubated with varying concentrations of ANA chlorides. A control pre-incubation was performed by incubating 2.0μg of enzyme alone at 15° in 3.0 m*l* of 0.05 *M tris*-HCl buffer, pH 7.85. The temperature was controlled by a circulating constant temperature water bath, Forma Temp Jr. Model 2095. At various time intervals, the enzymatic activity was assayed by introducing 0.3 m*l* of the control preincubation mixture into cuvettes containing 2.7 m*l* of a standard assay mixture. The standard assay mixture used for all of these experiments contained 0.05 *M tris*-HCl buffer, pH 7.85, 4.3×10^{-3} *M* *L*-α-glycerophosphate and 2.04×10^{-5} *M* NAD. Enzyme activities were determined at 25° by measuring the linear increase in the fluorescence intensity of NADH produced for one minute at 460 nm, with excitation at 386 nm using an Aminco-Bowman Spectrophotofluorometer. Measurements of pH were made at 25° with a Radiometer pH meter, type PHM 4b with a G-200-B glass electrode.

Determination of the Critical Micelle Concentration of *N*-Alkylnicotinamide Chlorides. Determinations of critical micelle concentrations were based on the abrupt changes in the molar extinction coefficient of the long chain compounds on the micelle formation[13,14]. Ultraviolet absorption of the ANA-chlorides was measured in 0.05 *M* phosphate buffer, pH 7.0 at 25° under a temperature-controlled cell compartment of a Zeiss PMQ II Spectrophotometer using 1.0 cm light path cells, and the absorbance measured was plotted as a function of ANA-chloride concentration. The concentrations at which abrupt changes in absorbance occurred were taken as the critical micelle concentrations of the compounds[14]. In some cases, critical micelle concentrations were also determined using the light scattering method described by Ford *et al.*[15] Light scattering cells were washed thoroughly with distilled water and ethanol. Solutions of ANA-chlorides in 0.05 *M* phosphate buffer, pH 7.0 were clarified by filtering under pressure through type GS Millipore filters of 1.2 μ pore size. Light scattering measurements were performed at room temperature (25°) using unpolarized incident light from a mercury lamp at 546 nm. Light transmitted at 0° (G_W) and the light scat tered by a solution at 90° (G_S) were measured by the deflections on the galvanometer. The data from these experiments were presented in the form of the scattering ratios, G_S/G_W, against concentration curves and the critical micelle concentrations were obtained by the extrapolation to the zero scattering ratio of the part of the curve which showed a greater slope. The Brice-Phoenix Universal Light Scattering Photometer, Model 2000, connected to a high sensitivity spot light galvanometer was used for these experiments.

Temperature Dependence of Inactivation of

L-α-Glycerophosphate Dehydrogenase. The effect of temperature on the inactivation of the enzyme was studied with both a heat inactivation process and an inactivation by N^1-dodecylnicotinamide chloride (DNAC). In the studies of heat inactivation, 2.0 μg of GPDH were preincubated in 3.0 m*l* of 0.05 *M tris*-HCl buffer, pH 7.85 at five different temperatures ranging 15 to 35°C. At various time intervals, 0.3 m*l* aliquots of the preincubated mixture were transferred into cuvettes containing 2.7 m*l* of the previously described standard assay mixture. The logarithms of the first order rate constants of inactivation were plotted against the reciprocal of the absolute temperature, and the activation energy for the enzyme inactivation was determined graphically. In a series of inactivation by DNAC, 2.0 μg of the enzyme were preincubated with 5×10^{-4} *M* of DNAC, and the first order rate constants were measured at five different temperatures between 15 to 35°C.

RESULTS

Reversibility and Time Dependence of L-α-Glycerophosphate Dehydrogenase Inhibition by N^1-Alkylnicotinamide Chlorides. Twelve ANA-chlorides were studied as possible irreversible inactivators of this dehydrogenase. The effect of N^1-octylnicotinamide chloride (ONAC) on the catalytic activity of GPDH is shown in *Fig.* 1. Line A represents the changes in the enzyme activity plotted on a logarithmic scale with time of preincubation of the enzyme alone, and line B represents instantaneous inhibition by $3.3\times10^{-3}M$ of ONAC in which the enzyme was preincubated in the absence of inhibitors. Line C shows the changes in the residual enzyme activity with time when the enzyme was preincubated with 3.3×10^{-2} *M* of ONAC and diluted ten-fold for the enzyme assay. As shown in *Fig.* 1, the reactions A and B are essentially

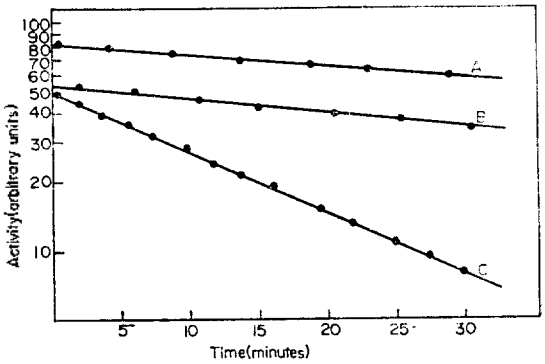

Fig. 1. Time dependent inactivation of *L*-α-glycerophosphate dehydrogenase by N^1-octylicotinamide ohloride.

Line A represents preincubation of enzyme alone; Line B, instantaneous inhibition with 3.3×10^{-3} *M* of ONAC; Line C, preincubation of enzyme with 3.3×10^{-2}*M* of ONAC.

parallel indicating that preincubation of the enzyme does not increase its sensitivity to the instantaneous inhibition by ONAC. The degree of the reversibility for GPDH inhibition by ANA-chlorides was expressed in terms of the difference values between the instantaneous inhibition and the enzyme inhibition at zero time preincubation with inhibitors (*Table* 1). The inhibition observed by adding enzyme to assay mixtures containing inhibitor did not differ greatly from the inhibition obtained by combining enzyme and inhibitor at ten-fold higher concentrations and diluting one-tenth into an assay mixture. From these data, the inhibition by ANA-chlorides is considered to be reversible and the slight irreversibility observed was attributed to a time-dependent inactivation which followed the initial inhibition. The rate constants of the time-dependent enzyme inactivation with twelve ANA-chlorides, N^1-methyl to N^1-dodecylnicotinamide, inclusive, were listed in *Table* 2. The values of the first order rate constants divided by the respective inhibitor concentrations ($k_r/[I]$) will provide an information on apparent second order rate constants or

Table 1. Reversible inhibition of *L*-α-glycerophosphate dehydrogenase by N^1-alkylnicotinamide chlorides.

Alkyl group of nicotinamide	Inhibitor concentration (M.)	Percent inhibition (Instantaneous)	Percent inhibition (Upon Dilution)	Degree of irreversibility (Per Cent)
Methyl	6.67×10^{-3}	17	19	2
Ethyl	1.00×10^{-2}	22	26	4
Propyl	3.30×10^{-2}	22	23	1
Butyl	1.33×10^{-2}	17	24	7
Pentyl	6.67×10^{-3}	22	22	0
Hexyl	6.67×10^{-3}	30	38	8
Heptyl	3.33×10^{-3}	23	29	6
Octyl	3.33×10^{-3}	30	38	8
Nonyl	1.33×10^{-3}	34	47	13
Decyl	3.33×10^{-4}	17	20	3
Undecyl	1.67×10^{-4}	18	31	13
Dodecyl	6.67×10^{-5}	16	27	11

Table 2. Time dependent inactivation of *L*-α-glycerophosphate dehydrogenase by N^1-alkylnicotinamide chlorides.

Alkyl substituent	Conc. of inhibitor ([I], *M*)	k_r (minute^{-1})	k_r/[I] (minute^{-1} M^{-1})
Methyl	6.67×10^{-2}	2.11×10^{-1}	3.16
Ethyl	1.00×10^{-1}	7.58×10^{-2}	0.758
Propyl	1.33×10^{-1}	5.24×10^{-3}	0.039
Butyl	1.33×10^{-1}	4.29×10^{-3}	0.032
Pentyl	6.67×10^{-2}	4.07×10^{-3}	0.061
Hexyl	5.00×10^{-2}	5.65×10^{-3}	0.113
Heptyl	3.33×10^{-2}	3.04×10^{-2}	0.912
Octyl	2.33×10^{-2}	5.14×10^{-2}	2.206
Nonyl	1.33×10^{-2}	3.40×10^{-2}	
Decyl	3.33×10^{-3}	4.28×10^{-2}	12.86
Undecyl	1.67×10^{-3}	8.52×10^{-2}	51.00
Dodecyl	6.67×10^{-4}	6.53×10^{-2}	97.98

the degree of enzyme inactivation at unit concentration of the inhibitors. These apparent second order rate constants were found to be increased with increasing chain length of the inhibitors. However, from the study of enzyme inactivation with different inhibitor concentrations, it was demonstrated that these constants were not true second order rate constants. In all cases, the plots of first order rate constants versus inhibitor concentration were not linear. This was exemplified by the inactivation of enzyme by DNAC as shown in *Fig.* 2. In *Fig.* 2, a linear response was observed only at low inhibitor concentrations, but at higher concentration an abrupt deviation from linearity was observed instead.

Determination of Critical Micelle Concentrations of *N*-Alkylnicotinamide Chlorides. In solutions of long chain electrolytes such as the ANA chlorides, there is a tendency for the

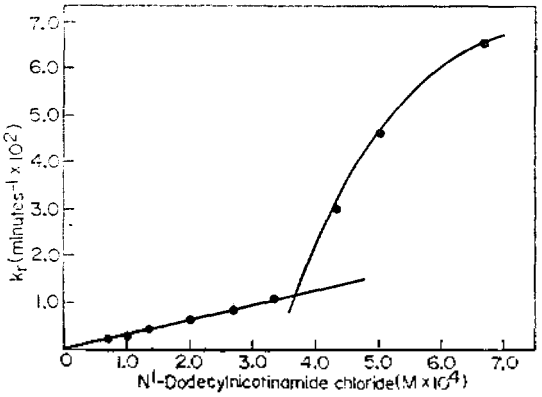

Fig. 2. The effect of N^1-dodecylnicotinamide chloride concentration on the irreversible inactivation of *L*-α-glycerophosphate dehydrogenase.
Enzyme (2.0μg) was preincubated with varing concentration of DNAC at 15°C in 3.0*ml* of 0.05*M* *tris*-*H*Cl buffer, pH 7.85. The residual enzyme activity was expressed by the first order inactivation rate constant (k_r) based on *Fig.* 1.

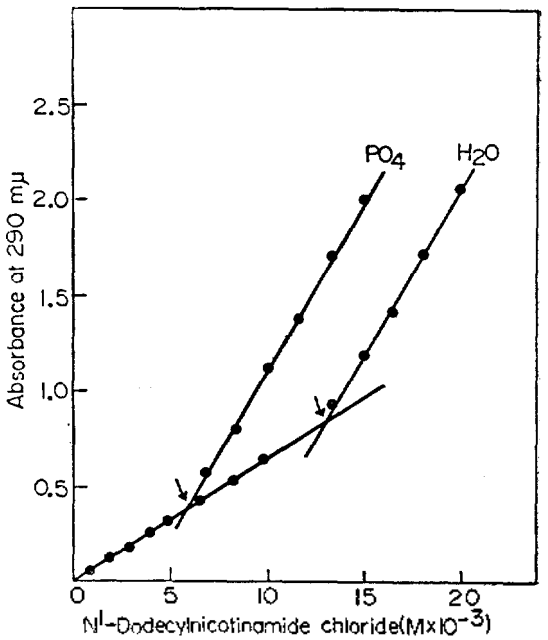

Fig. 3. The spectrophotometric determination of the critical micelle concentration of N^1-dodecylnicotinamide chloride.

molecules to assoiate and to form a micellar structure when the concentration reaches a certain point. Since micelles would be expected to affect enzyme activity differently than individual molecules of inhibitors in solution, it is important to determine the critical micelle concentrations of these inhibitors. The critical micelle concentrations for certain ANA chlorides were determined by changes in absorbance and by light-scattering methods. The changes in absorbance of DNAC with increasing concentration in water and in 0.05 *M* phosphate buffer, pH 7.0, is shown in *Fig.* 3. The concentration at the point of the break in the curve was taken as the critical micelle concentration under these conditions. The critical micelle concentration of DNAC was also determined in 0.05 *M* *tris*-HCl buffer, pH 7.8 (ionic strength of buffer was adjuated with NaCl). The same critical micelle concentration was observed in both phosphate and *tris*-HCl buffers. This observation indicates that the critical micelle concentration is affected by ionic strength but not by the kind of buffer used. The lowering of the critical micelle concentrations of similar long chain compounds by increasing ionic strength has been reported previously[15]. For other ANA chlorides, N^1-octyl- to N^1-undecyl-, inclusively, the critical micelle concentrations were determined in 0.05 *M* phosphate buffer and are listed in *Table* 3.

Table 3. Critical micelle concentrations of N^1-alkylnicotinamide chlorides in 0.05*M*. Phosphate buffer, pH 7.0

Alkyl substituent	Critical micelle concentration (*M*) Method 1[a]	Method 2[b]
Octyl	0.117	
Nonyl	0.094	
Decyl	0.039	0.036
Undecyl	0.016	
Dodecyl	0.0058	0.006

[a]Measured by spectrophotometric method using changes in optical density at 300mμ, [b]Measured by the light scattering method.

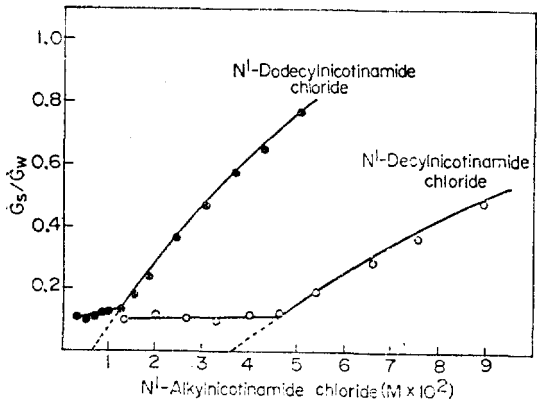

Fig. 4. The determination of the critical micelle concentration of N^1-alkylnicotinamide chloride by the light scattering method.

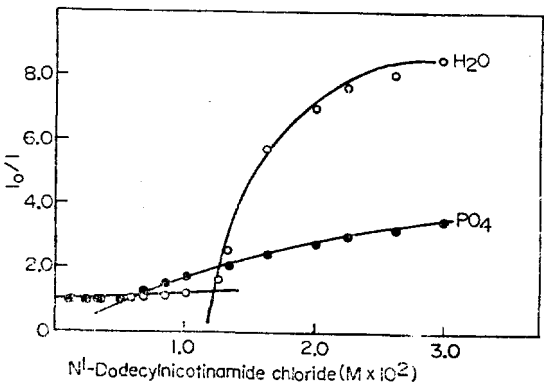

Fig. 5. The effect of N^1-dodecylincotinamide chloride concentration on the fluorescence intensity of NADH.

Micelle formation with N^1-decylicotinamide and N'-dodecylnicotinamide measured by light scattering method is shown in *Fig.* 4. Light scattering measurements were plotted as the scattering ratio (G_S/G_W) versus the concentration of the ANA chlorides in 0.05 *M* phosphate buffer, pH 7.0. Critical micelle concentrations obtained from the extrapolation of the increased slope portion of the curve agreed well with those measured spectrophotometrically (*Table* 3).

Interactions between N^1-Alkylnicotinamide Chlorides and NADH or Flavin Nucleotides. Many compounds have been shown to interact with pyridine nucleotides or flavin nucleotides resulting in a fluorescence quenching or fluorescence enhancement of these nucleotides in solution. Since micellization of ANA chlorides brings many changes in their physical properties, it was of interest to study the effect of the concentration of ANA chlorides on the fluorescence intensity of NADH, FAD and FMN.

The fluorescence intensity of 8.52×10^{-6} *M* NADH in 0.05 *M* phosphate buffer, pH 7.0 and in water was measured in the presence of DNAC. Fluorescence of NADH was quenched by DNAC as shown in *Fig.* 5, where the data were plotted as the ratio of fluorescence intensity of NADH in the absence of inhibitor (I_0) over the fluorescence intensity of NADH in the presence of inhibitor (I). The concentrations of DNAC at which an abrupt change in quenching occurred correlate well with the critical micelle concentrations observed under these conditions (*Fig.* 3). The effects of DNAC on the fluorescence intensity of FAD and FMN are illustrated in *Fig.* 6. Data obtained from these experiments were plotted as the ratio, I/I_0, versus the concentration of DNAC. Concentrations of FAD and FMN used were 1.67×10^{-5}*M* and 3.3×10^{-6}*M* in 0.05*M* phosphate buffer, pH 7.0, respectively. DNAC caused an abrupt enhancement of fluorescence of FAD in the region of the critical micelle concentration but no such effect was observed with FMN.

Temperature Effect on the *L*-α-Glycerophosphate Dehydrogenase Inactivation Reactions. *L*-α-Glycereophosphate dehydrogenase (GPDH) was preincubated at five different temperatures between 15 and 35° and at various times the residual activity was measured. First order rate constants obtained for two different types of inactivation reactions were plotted on a

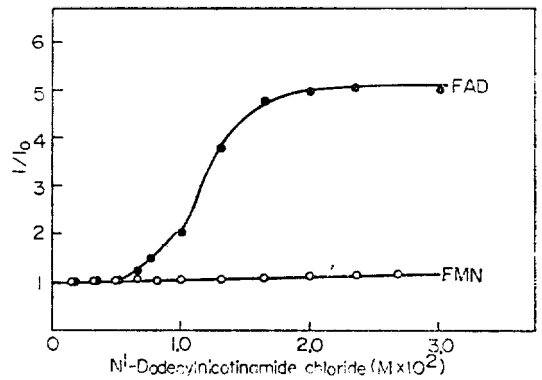

Fig. 6. The effect of N^1-dodecylnicotinamide chloride concentration on the fluorescence intensity of FAD and FMN.

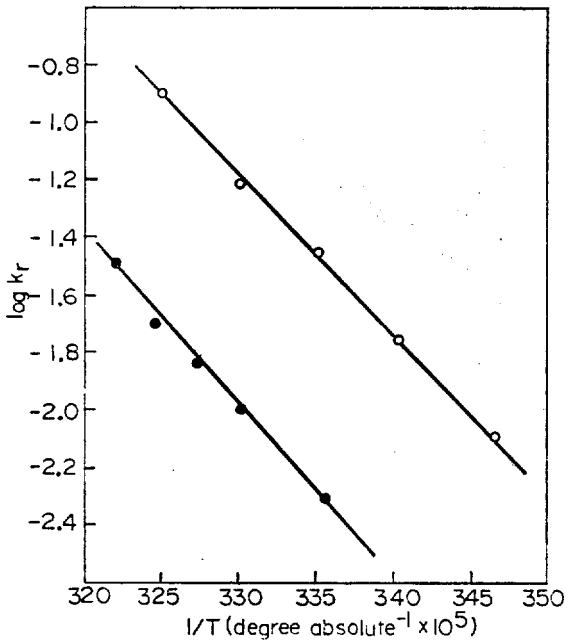

Fig. 7. The Arrhenius plot showing the temperature dependence of *L*-α-glycerophosphate dehydrogenase inactivation reactions.
Closed circle represents heat inactivation and open circle indicated DNAC inactivation at the concentration of $5\times10^{-4}M$.

logarithmic scale against the reciprocal of the absolute temperature (*Fig.* 7). The closed circles represent the heat inactivation of the enzyme in 0.05 *M tris*-HCl buffer, pH 7.85. The inactivation reactions of GPDH in the presence of DNAC in 0.05 *M tris*-HCl buffer, pH 7.85 are represented as open circles in *Fig.* 7, after correction for the rate of enzyme inactivation in the absence of inhibitor. The Arrhenius activation energy (E_a) for these two inactivation processes was calculated from these plots. Equilibrium constants for the formation of the transition states of these reactions were calculated from the equation,

$$k_r=\frac{kT}{h}K^*$$

where k_r=the first order rate constant, k=Boltzman constant, h=Planck's constant, T=absolute temperature and K^*=equilibrium constant for the formation of the transition state. The thermodynamic factors, ΔF^*, ΔH^* and ΔS^* calculated from these data are listed in *Table* 4 for the two inactivation processes studied.

Table 4. Properties of *L*-α-glycerophosphate dehydrogenase inactivation reactions.

Method of inactivation	K^*	E_a Kcal/mole	ΔF^* Kcal/mole	ΔH^* Kcal/mole	ΔS^* Cal/deg.mole
Heat	3.485×10^{-17}	27.42	22.92	26.83	+12.8
N^1-Dodecylnicotinamide chloride	9.36×10^{-16}	23.85	21.81	23.26	+4.87

DISCUSSION

The instantaneous inhibition by all of the ANA chlorides studied was demonstrated to be reversible upon dilution. As shown in *Fig.* 1, however, this reversible binding to the enzyme is followed by a time-dependent irreversbile inactivation. The slight deviation from complete reversibility observed with these compounds may be attributed to this time-dependent process. The rate of the time dependent irreversible inactivation, corrected for inhibition concentration ($k_r/[I]$) vailed with the chain length of

the inhibitor. This would not be anticipated in the simple equilibrium situation between enzyme and inhibitor, such as

$$\text{Enzyme} + \text{Inhibitor} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{[Enzyme-Inhibitor]} \xrightarrow{k_3} \text{[Enzyme-Inhibitor]}'$$

In this scheme, [Enzyme-Inhibitor] represents the enzyme-inhibitor complex which is dissociable and [Enzyme-Inhibitor]′ represents the irreversiblly inactivated enzyme. In such a reaction sequence, the rate of inactivation would depend on k_3 and the concentration of the enzyme-inhibitor complex. In the present study, the concentration of enzyme-inhibitor complex ([Enzyme-I]) was approximately the same for all ANA chlorides used, since the inhibitor concentrations selected were all ten times of those necessary to produce 25 percent instantaneous inhibition. The chain length effect observed in the rates of inactivation (*Table* 2) does not therefore reflect increasing concentrations of enzyme-inhibitor complex with increasing chain length of inhibitor. The inactivation scheme depicted above would predict a saturation effect at high inhibitor concentration, resulting in identical rates of inactivation for all ANA chlorides, regardless of chain length. When the rates of inactivation of the enzyme were studied as function of inhibitor concentration (*Fig.* 2), saturation effects were not observed. Instead, abrupt changes in the rates of inactivation were obtained at concentrations of inhibitors that varied with the chain length of the compounds studied. The abrupt changes in the rates of inactivation by these compounds were similar to the abrupt changes in other properties of these compounds attributed to micelle formation. The concentrations at which abrupt changes in rates of inactivation occur with the different ANA chlorides are approximately one-tenth the critical micelle concentrations determined for these compounds (*Table* 3). The concentrations of ANA chlorides where these anomalous rates of inactivation occur decrease with increasing chain length of these compounds. This chain length effect on the rates of inactivation of the enzyme is illustrated in *Fig.* 8, line C. Line represents the logarithm of the reciprocal of the concentration required for anomalous inactivation plotted against the number of carbons of the alkyl chain of the ANA chloride used. For comparison, the $k_r/[I]$ values from *Table* 2, were plotted logarithmically against the number of carbons of the inhibitors (line A). Line B of Figure 8 represents the relationship between the logarithm of the reciprocal of the critical micelle concentrations and the chain lengths of the nicotinamide derivatives. The three linear

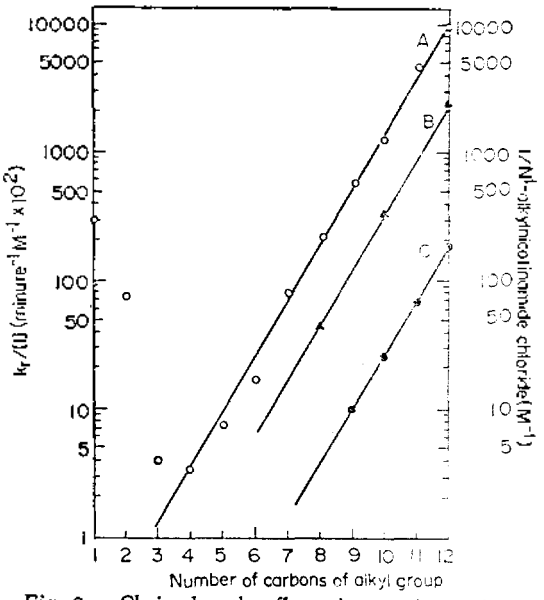

Fig. 8. Chain length effects in reactions of N^1-alky lnicotinamide chlorides. Line A represents the values of $k_r/(I)$ agenist the number of carbons of the inhibitors (see *Table* 2); Line B, the relationship between the critical micelle concentrations and the chain lengths of the nicoti namide derivative; Line C, the chain length effect on the rates of enzyme inactivation (k_r).

relationships shown in *Fig.* 8 parallel one another. Lines A and C both represent the chain length effect of inhibitors on rates of inactivation of the enzyme. The chain length effect on this property of the inhibitors is essentially the same as that observed with micelle formation (line B). The change in these properties of the inhibitors, per methylene group is larger than that calculated from the linear relationship obtained in the plot of log $1/k_1$ versus the number of carbons of alkyl chain of these inhibitors ofr the enzyme binding process[3]. These observations suggest that the anomalous inactivation of the enzyme involves the interactions similar to those of importance in micellization rather than in simple binding to the enzyme. The interaction of identical alkyl chains which occurs in micellization would be expected to be less affected by steric requirements and therefore to be a more efficient process than the binding of these alkyl chains to a hydrophobic region of an enzyme. The possibility that micellization may be catalytically induced on the enzyme surface was ruled out by a simple experiment. When the absorbance at 290 mμ of the ANA chlorides was measured in the presence of the same concentration of enzyme used for the inactivation experiments, the critical micelle concentration observed did not differ from that measured in the absence of enzyme. These data indicate that the presence of enzyme does not cause any catalyzing effect on the micellization. But these observations do not exclude the alternate possibility that inhibitor molecules begin to aggregate on the enzyme surface, and thereby initiate a denaturing process that inactivates the enzyme. The binding of the molecule of an N^1-ANA chloride to the pyridinium ring region of the enzyme would involve a neutralizing effect of a negatively-charged group of the enzyme on the positively charged inhibitor molecule. Thus, the interaction between the nonpolar groups of the bound inhibitor and a second inhibitor molecule would be more favorable than that between two free inhibitor molecules both of which carry positive charges. In this manner, the enzyme may induce the association of several molecules of inhibitor which could promote conformational changes resulting in the loss of catalytic activity.

A second possibility to be considered in these inactivation reactions is the binding of small aggregates of inhibitor molecules that formed in solution. There is strong evidence for the reversible formation of dimers of long chain alkyl compounds below the critical micelle concentration either as the only aggregation step prior to micelle formation[16,17] or as one of the stages toward micelle formation[18]. Thus the binding of dimers or other small aggregates of inhibitor molecules could promote conformational changes resulting in the loss of catalytic activity. There is no evidence at this time to distinguish between these two possibilities.

In both cases, a conformational change in the enzyme active site was proposed as a direct cause of the loss of enzyme activity. It appeared worthwhile to look at the effects of temperature on the rates of inactivation of GPDH in the presence and absence of ANA chlorides. As shown in *Fig.* 7 and *Table* 4, however, thermodynamic parameters calculated for the two inactivation processes by heat and DNAC are very similar. This would indicate that mechanistically, the enzyme inactivation by heat and by ANA chlorides could occur by a similar process. In many proteins, heat inactivation been shown to be accompanied by a large positive change in enthalpy of activation (ΔH^*) and in entropy of activation (ΔS^*).[19] The high value of ΔH^* may be attributed to the breaking of a large number of weak secondary bonds and the

high value of ΔS^* probably is due to an increased disorganization of the molecular structure from the native form to a more disorganized transition state. The low positive ΔS^* values observed in the inactivation reactions of GPDH suggest that these reactions may not be accompanied by a high degree of disorganization of the enzyme molecule. Perhaps a more subtle change in conformation can result in the loss of catalytic activity, at least this could hold true for the rate-limiting step of this process.

Micelle formation with DNAC has been shown to cause an abrupt change in fluorescence quenching and fluorescence enhancement of NADH and FAD, respectively, but to have no effect on the fluorescence of FMN (*Fig.* 5 and 6). These results suggest that NADH and FAD interact with the DNAC micelles through the adenylic acid moieties of these dinucleotides. This would be consistent with previous observations that ANA chlorides form complexes with a variety of adenine derivatives[5]. The interaction of NADH with micelles of the dodecyl derivative, although not a factor in the normal assay of GPDH due to the high concentrations of inhibitor required, should however be kept in mind as a possible contributory factor in dehydrogenase reactions only affected by micellar concentrations of this inhibitor.

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