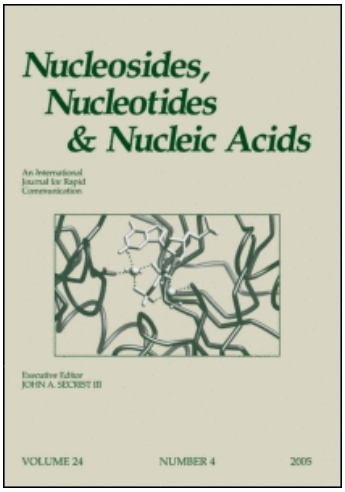

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Oligonucleotides and Nucleotidepeptides. XLIII. Inhibition of Lipoamide Dehydrogenase by Nucleotidyl-(N)-amino Acids and Their Ethyl Esters[1]

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OLIGONUCLEOTIDES AND NUCLEOTIDE-PEPTIDES.XLIII. INHIBITION OF LIPOAMIDE DEHYDROGENASE BY NUCLEOTIDYL-(P→N)-AMINO ACIDS AND THEIR ETHYL ESTERS[1]

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Abstract. It has been shown that some nucleotidyl-(P→N)-amino acids and their esters inhibit the lipoamide dehydrogenase reaction. For EtO-Phe-pU and HO-β-Ala-pU the inhibition mechanism has been proposed.

Lipoamide dehydrogenase (NADH: lipoamide oxidoreductase (EC 1.6.4.3) (LDH*), a constituent of the α-keto-acid dehydrogenases polyenzyme complex, catalyzes the reversible reduction of lipoic acid:

$$LipS_2 + NADH + H^+ \overset{LDH}{\rightleftharpoons} Lip(SH)_2 + NAD^+$$

It has been found that to attain optimal reaction rate NAD^+ should serve as activator[2,3]. Detailed studies have

*Abbreviations: LDH, lipoamide oxidoreductase; $LipS_2$, oxidized lipoic acid; $Lip(SH)_2$, reduced lipoic acid; EtO-Phe-pU, EtO-Leu-pU, EtO-Phe-pG, EtO-Phe-dpU, EtO-Phe-LpU, ethyl esters of uridylyl-(5'→N)-DL-phenylalanine, -DL-leucine, guanylyl-(5'→N)-DL-phenylalanine, deoxyuridylyl-(5'→N)-DL-phenylalanine, L-uridylyl-(5'→N)-DL-phenylalanine, respectively; HO-β-Ala-pU, uridylyl-(5'→N)-β-alanine.

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shown that NAD^+ is necessary for the stabilization of flavin semiquinone and for the prevention of complete reduction of flavin[4-6]. Taking into account that three nucleotides (FAD, NADH and NAD^+) take part in the reaction catalyzed by LDH, a search was made among the nucleotide derivatives for the effectors of the process. Here we report the inhibitory effect of a number of nucleotidyl-(5'→N)-amino acid esters and their analogs with a free carboxyl group on LDH isolated from Saccharomyces cerevisiae.

RESULTS AND DISCUSSION

In the initial part of the work the effect of ethyl esters of uridylyl-(5'→N)-β-alanine, -α-DL-alanine, -DL-phenylalanine, -DL-tyrosine, -DL-serine, -DL-methionine, -DL-leucine and their analogs with a free carboxyl group on the LDH reaction rate was tested. To estimate the influence of the base or monosaccharide on the inhibition of LDH, derivatives of phenylalanine esters and of adenylic, guanylic, cytidylic, deoxyadenylic, thymidylic, deoxyuridylic or L-uridylic acids, thymidylyl-(3'→N)-DL-phenylalanine and corresponding derivatives with a free carboxyl group were analysed. For a preliminary estimation of the effect of a substance and the selection of potential inhibitors all the compounds investigated were introduced into the reaction mixture at 10^{-3} M concentration. This made it possible to find that LDH was not inhibited by nucleotides, free amino acids and their ethyl esters. Among nucleotide derivatives of amino acids only EtO-Phe-pU, EtO-Leu-pU, EtO-Phe-pG, EtO-Phe-dpU, EtO-Phe-L-pU, and HO-β-Ala-pU demonstrated the inhibitory effect. This was most often the case with substances containing phenylalanine. It should be noted that, at equal concentrations of EtO-Phe-pU and the corresponding L-isomer of UMP, the same degree of inhibition was observed.

For the quantitative estimation of the inhibitory effect it was decided to determine I_{50}. Fig.1 gives the I_{50} values for the nucleotide derivatives showing the inhibitory effect on LDH.

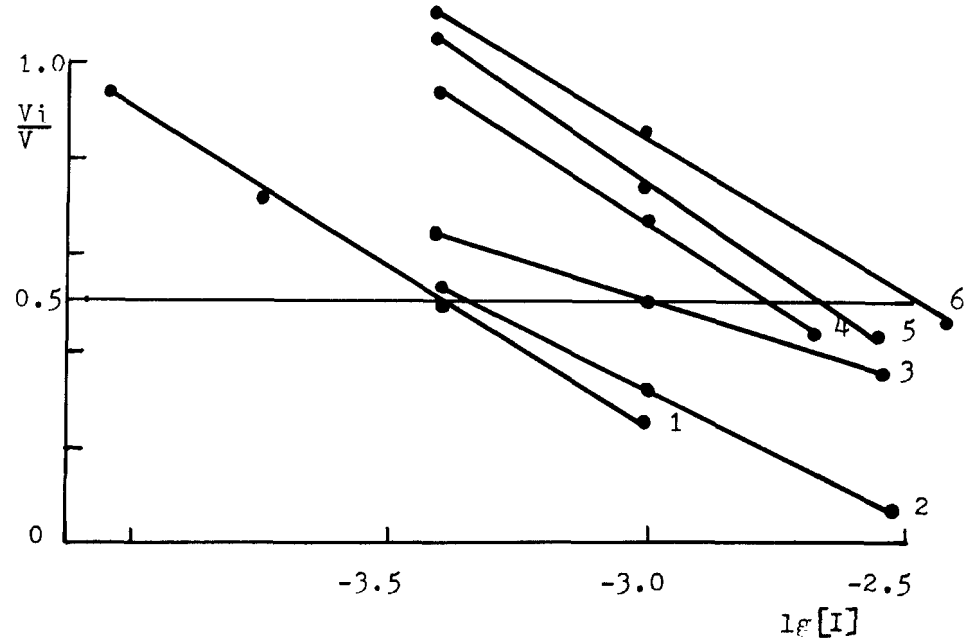

FIG.1. The effect of the concentration of nucleotidyl-(5→N)-amino acids on the LDH reaction rate. The composition of the reaction mixture is as described in the text. To the reaction mixture the following materials were added: 1.EtO-Phe-pU; 2. EtO-Phe-pG; 3. HO-β-Ala-pU; 4. EtO-Phe-dpU; 5. EtO-Leu-pU; 6. EtO-Phe-L-pU.

pI_{50}, estimated from the data presented in Fig.1, turned out to be 3.4, 2.7, 3.4, 3.0, 2.8, 2.5 for EtO-Phe-pU, EtO-Leu-pU, EtO-Phe-pG, HO-β-Ala-pU, EtO-Phe-dpU and EtO-Phe-L-pU, respectively. It follows from the above-mentioned data that EtO-Phe-pU and EtO-Phe-pG are the most effective inhibitors. Obviously, the inhibitory effect of the compounds investigated depends to a great extent on the nature of the heterocyclic base and on the state of the carboxyl groups of the amino acid. We failed, however, to establish a clear-cut correlation between the structure and the inhibitory effect of the substances investigated.

In order to discover possible principles of interaction of nucleotidyl-(5→N)-amino acids with LDH a detailed study of the kinetics of EtO-Phe-pU has been made. The experimental data processed by the Dixon method[7] are presented in Fig.2.

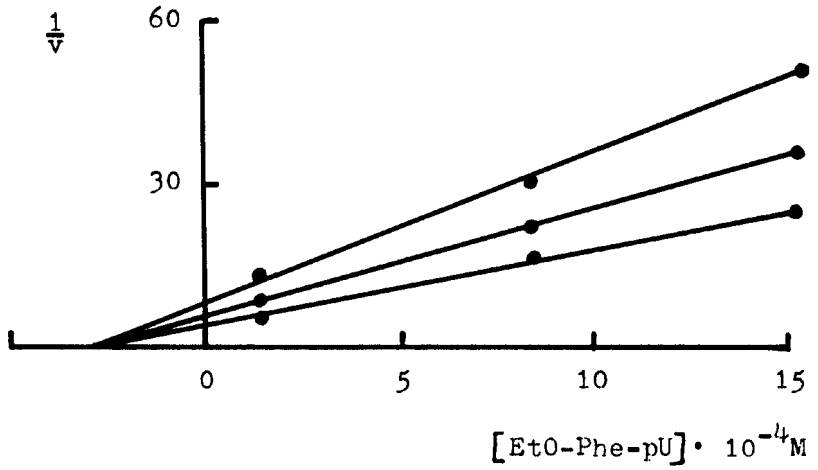

FIG.2. The dependence of the LDH reaction rate on the concentration of EtO-Phe-pU. The composition of the reaction mixture is as described in the text. The concentrations of EtO-Phe-pU are shown in the Figure. The concentrations of lipoic acid are as follows: 1. 3.3×10^{-2} M; 2. 1.3×10^{-2} M; 3. 6.7×10^{-3} M.

Thus, a family of straight lines intersecting on the abscissa axis was obtained, which proves again that here we are dealing with non-competitive inhibition. The K_i (3.0×10^{-4} M), determined as the reciprocal intersection of the abscissa axis, is in good agreement with value ($4.2 \cdot 10^{-4}$M) found in Fig.1. To verify them, the values of partial inhibition were plotted against the concentration of the substrate (Fig.3).

Partial inhibition was found to be practically independent of the concentration of lipoic acid, corresponding to non-competitive inhibition. However, the K_i value plotted on the abscissa axis is $6.7 \cdot 10^{-4}$M, i.e. two times higher than the value obtained by the Dixon method. Since the activator NAD^+ takes part in the reaction, the effect of its concentration on the LDH reaction rate in the presence of EtO-Phe-pU has been studied. It turned out that there was competition between the activator NAD^+ and the inhibitor EtO-Phe-pU (Fig.4).

This enabled us to understand the changes in the value of the apparent K_i, depending on the way of processing

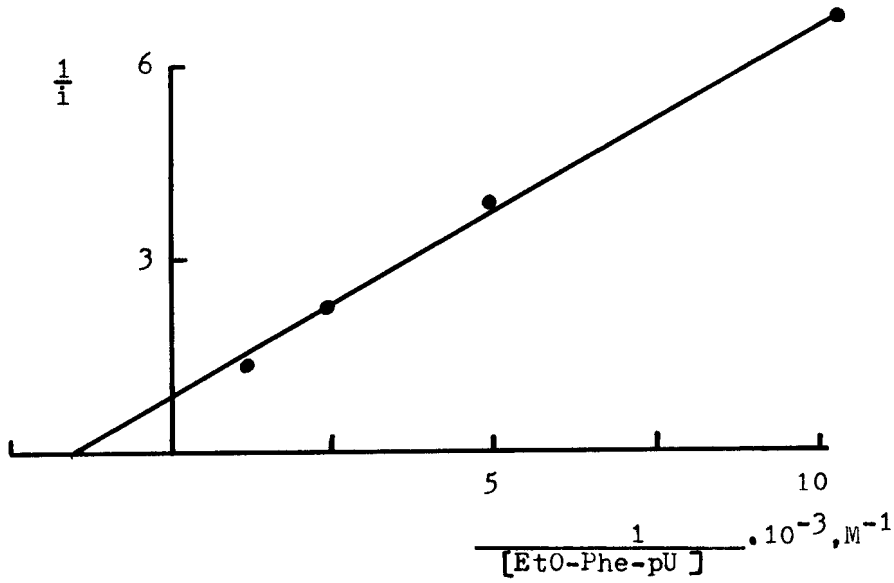

FIG.3. The dependence of partial inhibition on the concentration of EtO-Phe-pU. The concentration of $LipS_2$ was changed in the range from $3.3 \cdot 10^{-3}$ to $3.3 \cdot 10^{-2}$ M.

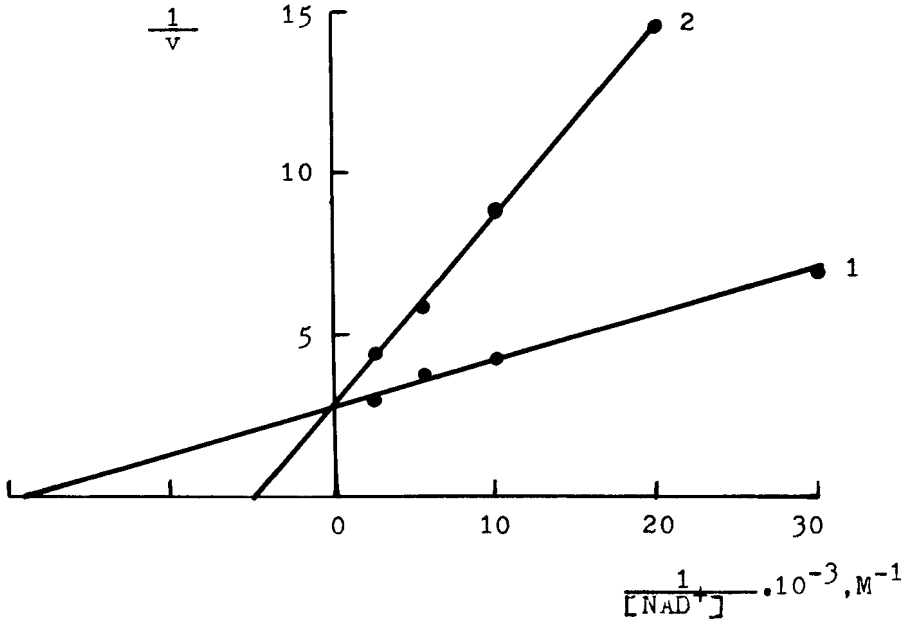

FIG.4. The dependence of the LDH reaction rate on the concentration of NAD^+. 1. EtO-Phe-pU is absent; 2. The concentration of EtO-Phe-pU is 3.4×10^{-3} M.

the experimental data, since the concentrations of the substrates and the activator are of considerable importance to the K_i value. To determine the actual K_i and the activation constant K_{NAD^+}, the data were plotted; $\frac{(1-i)}{i}$ depending on the concentration of NAD^+(Fig.5).

The K_i value was found to be 1.6×10^{-4}M, while K_{NAD^+} was 0.8×10^{-4}M. Using the K_i value obtained by the Dixon method and taking into account that the concentration of NAD^+ was 1.04×10^{-4}M, it was calculated that the actual K_i value was 1.7×10^{-4}M. The K_i values obtained in the two ways coincide. Besides, the K_{NAD^+} value is in full agreement with the data reported in the literature ($0.5-0.6 \times 10^{-4}$M)[6]. Thus the effect of EtO-Phe-pU on LDH most likely manifests itself at the enzyme activation level. It has been shown recently that the adenine ring of NAD^+ interects with phenylalanine[8]. Taking into consideration the role of the adenine ring in the interaction of the nicotine coenzymes and the apoenzyme, it is quite reasonable to regard the breaking of these bonds as a result of the inhibitory effect. The situation is, however, not so simple, since EtO-Phe-pU turned out to be inef-

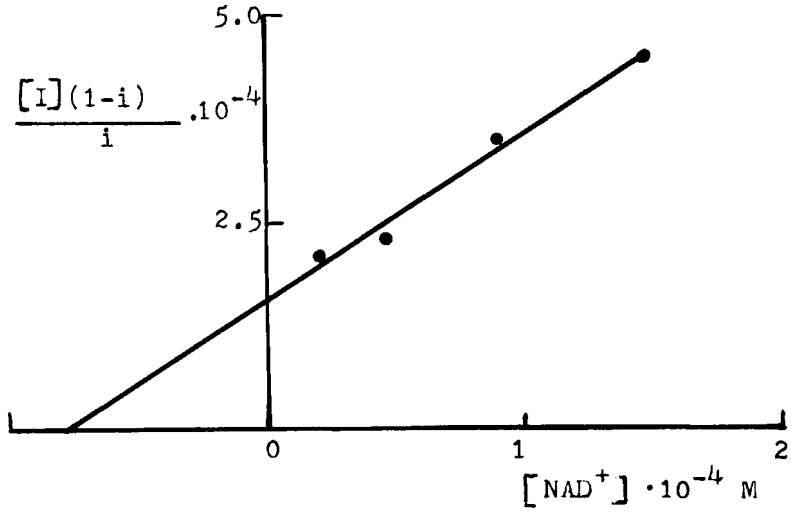

FIG.5. The dependence of partial inhibition on the concentration of NAD^+. Conditions were as described in the legend to Fig.4.

fective with alcohol dehydrogenase and glucose 6-phosphate dehydrogenase, the K_{NAD^+} and K_{NADP^+} of which are comparable to K_{NAD^+} of LDH[9]. Obviously, in the case of LDH the complexity of the structure of the enzyme itself should be taken into account.

Preliminary data obtained on studying HO-β-Ala-pU indicate that in the present case the inhibitory effect manifests itself in the competition between the inhibitor and lipoic acid.

Thus, a study of the kinetics of the inhibition of the lipoamide dehydrogenase reaction by EtO-Phe-pU and HO-β-Ala-pU has shown that the mechanisms of their action are different. EtO-Phe-pU apparently exerts its inhibitory effect at the level of interaction of the enzyme and the activator NAD^+, whereas HO-β-Ala-pU shows its inhibitory effect at the level of interaction of the enzyme and the substrate lipoic acid.

EXPERIMENTAL

The following reagents were used: UMP, CMP, AMP, GMP, NAD^+, NADH, amino acids, alcoholdehydrogenase (Reanal, Hungary), dAMP, TMP (Sigma, USA), thymidine, deoxyuridine, dicyclohexyl carbodiimide, glucose 6-phosphate dehydrogenase (Ferak Berlin), L-uridine was kindly provided by Dr. A. Holy (Czechoslovakia), 3'-TMP was synthesized as in[10], dUMP, L-UMP were synthesized as in[11]. The synthesis of nucleotidyl-(P→N)-amino acid ethyl esters has been described earlier[12]. LDH was isolated and purified to the homogeneous state from yeast Saccharomyces cerevisiae[13]. The LDH reaction rate was determined by recording the changes in the optical density of the reaction mixture every 15 s over 3 minutes at 340 nm. The reaction mixture (3ml) contained 0.08M phosphate buffer, pH 6.0, 1.86×10^{-3} M lipoic acid or its amide, 1.25×10^{-4} M NAD^+, 1.16×10^{-4} M NADH, 2 mg human serum albumin, 5-10 μg LDH. The reaction was started by adding the enzyme. From the linear part of the kinetic curve the reaction rate expressed in μmol/min/mg protein was calculated. When studying the dependence of the reaction rate on the concentration of the subs-

trates, activators or inhibitors, appropriate materials were added to the reaction mixture in amounts indicated in the legends to the Figures.

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