Beef Liver Glucose Dehydrogenase

II. Inhibition Studies with Compounds Related to the Coenzymes

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In 1942, v. Euler¹ observed inhibition of liver glucose dehydrogenase and lactic dehydrogenase by compounds related to nicotinic acid, and in some cases it was shown that the inhibitor competed with diphosphopyridine nucleotide (DPN). Recently, a number of workers have reported the competitive inhibition of various dehydrogenases by nicotinamide^{2,3} and by adenine, adenosine and adenosine triphosphate (ATP)⁴. With the availability of highly purified beef liver glucose dehydrogenase (GDH)⁵, it seemed desirable to make an investigation of the types of compounds related to DPN (and deaminated DPN, which also functions as a coenzyme in this system⁵) which might cause inhibition, and of the qualitative and quantitative nature of the inhibitions. Such findings might be expected to contribute to an understanding of the nature of apoenzyme-coenzyme interactions.

EXPERIMENTAL

The inhibitor to be tested was dissolved in 0.2 *M* phosphate buffer, pH 7.0, and the pH of the resulting solution adjusted to 7.00 by the addition of dilute alkali or mineral acid if necessary; then the solution was diluted to a phosphate concentration of 0.076 *M*. A 6.86 ml volume of the inhibitorbuffer solution was mixed with 3.50 ml of 20 % aqueous glucose solution and 0.14 ml of coenzyme solution of known concentration. Three 3.00 ml aliquots of the reaction mixture were pipetted into 1 cm Beckman cuvettes for triplicate determinations. The reaction was initiated at the Beckman by the addition of 200 units of purified (1 000-2 500 u./mg) glucose dehydrogenase in a volume of 0.02 ml. The absorption of the solution at 340 m μ was read within 5 to 10 seconds after mixing and the increase in absorption at 340 m μ noted after 15 and 30 seconds. All determinations were carried out at temperatures between 21 and 22°.

Calculations *. The initial reaction rate, *v*, was calculated from the observed increase in optical density at 340 m μ per 30 seconds, Δd_{30} , using the expression $v = \frac{2 \Delta d_{30}}{6.23 \times 10^3}$ moles liter⁻¹ minute⁻¹. This was in turn derived from the absorption coefficient of reduced DPN, $E_{1\text{ cm}}^{1\%} = 93.8$ at 340 m μ . When deaminated DPN was the coenzyme, the rate

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Table 1. Inhibition by substituted pyridines.

Inhibitor	Conc. of Inhib., M/L	Coenzyme	Slope	Intercept	K_i , M/L
None	---	DPN	0.420	0.98×10^5	$K_M = 4.28 \times 10^{-6}$
None	---	Deam.-DPN	0.875	»	$K_M = 9.0 \times 10^{-6}$
Pyridine	1.31×10^{-1}	DPN	0.60	»	3×10^{-1}
Nicotinamide	1.00×10^{-1}	DPN	1.985	»	2.7×10^{-2}
»	2.00×10^{-2}	DPN	0.794	»	1.9×10^{-2}
»	5.00×10^{-2}	Deam.-DPN	5.50	»	1.0×10^{-2}
Nicotinic acid	1.31×10^{-1}	DPN	2.83	»	2.3×10^{-2}
»	2.20×10^{-2}	DPN	0.885	»	2.0×10^{-2}
Isonicotinic acid	5.00×10^{-2}	DPN	0.42	»	No inhib.
Isonicotinic acid hydrazide	7.00×10^{-2}	DPN	0.545	»	2×10^{-1}
2-Methyl-nicotinic acid	3.37×10^{-2}	DPN	0.994	»	2.5×10^{-2}
Quinolinic acid	4.00×10^{-2}	DPN	0.42	»	No inhib.
Trigonelline	6.12×10^{-2}	DPN	0.42	»	»
β -Hydroxy-pyridine	1.00×10^{-2}	DPN	0.875	»	0.92×10^{-2}
Pyridoxal*	5.00×10^{-3}	DPN	4.03	»	6×10^{-4}
»	1.00×10^{-3}	DPN	0.830	»	1×10^{-3}
4-Pyridoxic acid *	1.00×10^{-3}	DPN	1.84	»	3×10^{-4}

was calculated on the assumption that reduced deaminated DPN has the same absorption coefficient at 340 $m\mu$ as reduced DPN. A plot of the reciprocal of the velocity against the reciprocal of the molar concentration of coenzyme gave a straight line, for which the slope and the intercept on the $(1/v)$ -axis were noted. In cases of competitive inhibition, the lines representing the reaction in the presence and absence of inhibitor have a common intercept, but different slopes. The inhibition constant, K_i , was calculated from the relationship:

$$\text{Slope} = (K_M/V_{\text{Max.}}) \left(1 + \frac{[I]}{K_i} \right)$$

where K_M is the Michaelis constant for the coenzyme, $1/V_{\text{Max}}$ is the intercept on the $(1/v)$ -axis, and $[I]$ is the molar concentration of the inhibitor.

The results are presented in Tables 1 and 2. The compounds marked with an asterisk had such high absorption at 340 $m\mu$ that it was difficult to obtain accurate readings, and the values recorded should be regarded as approximate.

One compound, *nebularine* ** (9-D-ribosylpurine)⁸, was available only in limited amounts as a 3 mg/ml solution in physiological saline. In this case the inhibitor-buffer solution was prepared by addition of 4.5 M phosphate buffer, pH 7.00, to the proper phosphate concentration before mixing with glucose and DPN. Only a single determination was made at each DPN concentration. A special determination of the Michaelis constant for DPN under the same conditions was made. From the observed intercept of 0.98×10^5 and slope of 0.635, a K_M of 6.48×10^{-6} was calculated. For the reaction in the presence of $1.0 \times 10^{-3} M$ inhibitor, a slope of 0.885 and an intercept of 0.98×10^5 gave a value for K_i of $2.5 \times 10^{-3} M$. This value must of course be regarded as approximate.

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Table 2. Inhibition by substances related to the adenyl moiety of the coenzymes.

Inhibitor	Conc. of Inhib., M/L	Coenzyme	Slope	Intercept	K_i , M/L
Phosphate	1.5	DPN	1.385	0.98×10^5	7.6×10^{-1}
»	1.0	DPN	0.910	»	8.6×10^{-1}
Adenine	1.75×10^{-2}	DPN	1.035	»	1.2×10^{-2}
»	8.75×10^{-3}	DPN	0.720	»	1.2×10^{-2}
Adenosine	4.58×10^{-3}	DPN	2.42	»	9.7×10^{-3}
»	9.2×10^{-3}	DPN	0.857	»	8.8×10^{-3}
3-Adenylic acid	1.0×10^{-2}	DPN	1.646	»	3.3×10^{-3}
»	3.48×10^{-3}	DPN	0.815	»	3.7×10^{-3}
ATP	4.8×10^{-3}	DPN	1.55	»	1.8×10^{-3}
»	2.4×10^{-3}	DPN	1.01	»	1.7×10^{-3}
»	4.0×10^{-3}	Deam.-DPN	1.95	2.49×10^3	Non-competitive
Inosinetriphosphate	1.2×10^{-3}	DPN	0.23	1.65×10^5	Non-competitive
Inosinetriphosphate	9.0×10^{-4}	Deam.-DPN	1.28	0.98×10^5	2.0×10^{-3}

DISCUSSION OF RESULTS

Competition of Substituted Pyridines and DPN. Pyridine-3-sulfonic acid inhibited GDH in competition with DPN, according to v. Euler¹, and it was noted that at a fixed DPN concentration the reaction was also inhibited by nicotinic acid and amide, but not by trigonelline. In the present study, pyridine and ten substituted pyridines have been examined with respect to their ability to inhibit the GDH-DPN system. Of these eleven compounds, six caused fairly strongly inhibition, two inhibited weakly, and no inhibition could be detected with three others. All of the inhibitors operated competitively with DPN. A typical example is represented graphically in Fig. 1. From the competitive nature of the inhibition, it is evident that the inhibitors complex with the enzyme on the same point or points on the protein surface as does DPN. The ratio K_i/K_{DPN} , a measure of the relative tightness of the binding to the protein of DPN and inhibitor, is large for all the compounds tested. Even with the strongest inhibitor of this type tested, 4-pyridoxic acid, the coenzyme was bound seventy times more tightly than the inhibitor.

The results are summarized in Table 3, where the inhibitory abilities of the compounds relative to that of nicotinamide are presented. It would appear that for such a compound to be a reasonably good inhibitor in this system, substitution of the pyridine ring at the 3-position is required. At this position, substitution of a carboxyl group for an amide group did not significantly alter the inhibitory activity, and replacement of the carboxylic acid amide by a hydroxyl group markedly increased it. The largest inhibitions were produced by pyridoxal and 4-pyridoxic acid, compounds containing negative groups in both β -positions (3 and 5), as well as at position 4. It is of interest that when the carboxyl group was moved from position 3 to position 4 of the pyridine ring the ability of the substance to inhibit this enzyme system vanished. The

Fig. 1. Inhibition by nicotinic acid. Curve I: No inhibitor. Curve II: 2.2×10^{-2} M inhibitor. Curve III: 1.31×10^{-1} M inhibitor.

addition of a methyl group to the nicotinic acid structure had no effect on the inhibiting ability, but upon the addition of a second carboxyl group in the 2-position, the inhibitory activity was abolished. In view of the structural similarity between DPN and trigonelline, it is of particular interest that no inhibition was caused by the betaine, a finding reported earlier by v. Euler¹.

Competition of Inorganic Phosphate and Compounds Related to Adenine with DPN. Williams⁴ has described the competitive inhibition of malic dehydrogenase by adenine, adenosine and adenosinetriphosphate (ATP). In this system it appeared that both adenine and adenosine possessed the same K_i , whereas ATP was probably but not certainly a stronger inhibitor. It was concluded that one point of attachment of DPN to the enzyme surface is at the adenine moiety, and perhaps another through the phosphate groups,

Table 3. Relative Inhibitory Activity of Pyridine Derivatives.

Compound	Relative Inhibitory Activity
Pyridine	0.08
Nicotinamide	1.0
Nicotinic acid	1.1
Isonicotinic acid	0
Isonicotinic acid hydrazide	0.1
2-Methylnicotinic acid	0.9
Quinolinic acid	0
Trigonelline	0
β -Hydroxypyridine	2.5
Pyridoxal	29
4-Pyridoxic acid	77

Table 4. Relative Inhibitory Activity of Compounds Relative to Adenine

Compound	Relative Inhibitory Activity
Adenine	1.0
Adenosine	1.3
3-Adenylic Acid	3.4
ATP	6.9
9-D-Ribosylpurine	0.5

but with the ribose portion of DPN not involved. We have examined the same three metabolites in the glucose dehydrogenase system (Tables 2 and 4). It can be seen that here ATP was clearly a much stronger inhibitor than adenine, and it is probable that adenosine was somewhat more tightly bound to the protein than was the simple purine. 3-Adenylic acid was intermediate in inhibitory activity, falling in this respect between adenosine and ATP. The inhibitions by adenine and ATP are shown in Fig. 2.

The recent isolation and structure determination of *nebularine*, 9-D-ribosylpurine⁸, made available an ideal compound for investigating the role of the 6-amino group of the adenine nucleus in the protein-coenzyme binding, since *nebularine* differs from adenosine only in the absence of this amino group. It was found that GDH was competitively inhibited by *nebularine*, but less than half as strongly as it was by adenosine.

Glucose dehydrogenase was also inhibited in competition with DPN by relatively high concentrations of inorganic phosphate; at a phosphate concentration of 1.0 M, a K_i of 0.86 was observed, and one of 0.76 at 1.0 M phosphate. This appears to be much weaker inhibition by phosphate than was observed by Theorell in 1935⁹ in the case of the Zwischenferment of Warburg and Christian.

Inhibitors Tested Against Two Coenzymes. It was of interest to examine the behavior of inosinetriphosphate (ITP) in the GDH system, since in this com-

Fig. 2. Inhibition by adenine and ATP. Curve I: No inhibitor. Curve II: Adenine, $8.75 \times 10^{-3} \text{ M}$. Curve III: ATP, $4.8 \times 10^{-3} \text{ M}$.

Fig. 3. Inhibition by ITP in the GDH-deaminated DPN system. Curve I: No inhibitor. Curve II: ITP, 9.0×10^{-4} M.

pound the 6-amino group of the purine nucleus has been replaced by a hydroxyl group. Thus, ITP bears the same structural relationship to ATP that deaminated DPN does to DPN. As can be seen from Table 2 and Figs. 3 and 4, the GDH system was inhibited by ITP; the substance competed with deaminated DPN, but the inhibition was non-competitive when DPN was the coenzyme. In view of this result, ATP was examined in the GDH-deaminated DPN system. The resulting inhibition was non-competitive in nature (Table 2). Finally, nicotinamide was tested, and found to inhibit the action of the enzyme in competition with deaminated DPN as well as with DPN itself.

Conclusion. From the degree and nature of the interactions between the apoenzyme and inhibitors structurally related to the coenzyme, one can draw certain conclusions regarding the structural features of the coenzyme molecule which are involved in the linkages in the enzyme-coenzyme complex.

It appears that DPN is attached to the GDH surface through linkages involving several parts of the coenzyme molecule. It is quite evident that the nicotinamide moiety is involved, with a key structural feature being the carboxylic acid amide group at the 3-position of the pyridine ring. While there is no direct evidence for participation by the ribosyl group attached to the nicotinamide ring, the failure of trigonelline to inhibit perhaps suggests some importance for this carbohydrate portion of the molecule. The adenine

Fig. 4. Inhibition by ITP in the GDH-DPN system. Curve I: No inhibitor. Curve II: ITP, 1.2×10^{-3} M.

moiety clearly contributes to the GDH-DPN binding and the probable increased inhibition by adenosine makes it likely that the ribose structure of this portion of the molecule is also involved. 9-D-Ribosylpurine inhibited the GDH-DPN system about as strongly as did nicotinamide, but less than half as strongly as did adenosine; and from these results it may be concluded that both the purine ring structure and the 6-amino group of the adenyl moiety of DPN are important in the protein-coenzyme complex. The participation of the phosphate portion of the structure is clearly demonstrated by the magnitude of the inhibition produced by ATP, as well as by the weak inhibition caused by inorganic phosphate.

The Michaelis constants for DPN and deaminated DPN (Table 1) indicate that DPN is bound to the apoenzyme more tightly than is deaminated DPN, and it would hence appear that the 6-hydroxyl group contributes less to the binding than does the corresponding amino group. However, the hydroxyl group is involved, and a qualitatively different type, or site, of binding is suggested by the results obtained when ATP and ITP were each studied against both coenzymes.

Deamination causes no change in the pyridine portion of DPN and, as expected, nicotinamide inhibition was competitive with respect to both coenzymes. The smaller value observed for the K_i against deaminated DPN is in accordance with the larger Michaelis constant for the deaminated coenzyme.

There is reason to believe that the oxidation of glucose is not the true physiological role of liver glucose dehydrogenase^{5,10}. In this connection, it is of interest that of the various substituted pyridines studied as inhibitors, pyridoxal and the closely related 4-pyridoxic acid were bound to GDH many times more tightly than were the simpler compounds of this class (Table 3). It seems at least conceivable that GDH might be able to function as a pyridoxal phosphate-linked transaminase. However, this has not been subjected to experimental verification.

It is evident that the conclusions presented here could be strengthened and broadened by the examination of additional compounds for inhibitory activity. Further, it should be emphasized that these ideas may be valid only for the particular enzyme system studied here; differences are to be expected with other enzymes.

SUMMARY

1. Eighteen compounds structurally related to DPN have been examined for their ability to inhibit beef liver glucose dehydrogenase activity.
2. From the nature and intensity of the inhibitions observed, some conclusions have been drawn concerning the portions of the coenzyme molecules which are involved in the binding of the apoenzyme-coenzyme complexes.
3. It appears that most moieties of the coenzyme molecules contribute to the linkage with the protein surface. In the DPN molecule, the pyridine ring and its 3-amide group, the phosphoric acid residues, the ribose moiety attached to adenine, and the adenine nucleus and its 6-amino group all participate. The substitution of a hydroxyl group for the amino group in the deamination of DPN results in a weaker and qualitatively different type of binding in the altered coenzyme.

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