

INHIBITION OF LACTATE DEHYDROGENASE BY HIGH CONCENTRATIONS OF PYRUVATE: THE NATURE AND REMOVAL OF THE INHIBITOR

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1. Introduction

The inhibition of lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC1,1,1,27) by high concentrations of the substrate, pyruvate, has been attributed to the formation of an abortive ternary complex of the enzyme, NAD and pyruvate [1-5]. High concentrations of pyruvate, greater than 0.5 mM, are required to observe the inhibition and demonstrate the formation of a ternary complex. The effects could be due to a contaminant or a form of the substrate present in only trace amounts. We have prepared samples of pyruvate which give no excess substrate inhibition and have obtained suggestive evidence for the chemical nature of the inhibitor present in the specimens of pyruvate normally used for investigations on lactate dehydrogenase.

2. Materials and methods

Sodium pyruvate (Calbiochem, Grade A) was chromatographed by the method of Von Korff [6] and the eluent produced by a linear gradient of HCl monitored at 260 and 330 m μ using a Gilford model 2000 recording spectrophotometer. NAD, NADH, APAD (3-acetylpyridine adenine dinucleotide) were obtained from The Boehringer Corporation (London). Imidazole was obtained from British Drug Houses Ltd. and was recrystallized twice. All other chemicals were AnalaR grade or equivalent. The enzyme was isolated from pig heart muscle [7] and stored as a suspension in 70% ammonium sulphate. The suspension was diluted with phosphate buffer, treated

with activated charcoal (Fison Ltd.) to remove nucleotides [8] and dialysed against the buffer used for assay. The enzyme was assayed at pH 6.90 using NADH (100 μ M) in either of two buffer systems: Phosphate (0.2 M) or imidazole (0.2 M) HCl containing 0.3 M sodium chloride. Pyruvate in acid solution was pipetted into the cuvette containing buffer and allowed to equilibrate for two min. NADH and enzyme were then added to start the reaction. The change in pH produced by the acid was always less than 0.06 of a unit. The reaction was followed at 340 m μ using a Cary 14 recording spectrophotometer and the initial velocity measured. Difference spectra were measured in split compartment cells using the same instrument.

3. Results and discussion

The elution profile of pyruvate is shown in fig. 1. The material of peak 1 is not pyruvic acid since it fails to oxidise NADH in the presence of lactate dehydrogenase. It has an absorption maximum at 260 m μ , unlike pyruvate which has two peaks at 210 and 330 m μ . The nature of this material, which does not inhibit the enzyme, is not known. On standing in solution for a few days at pH 4.0 it produces pyruvate as shown by enzyme assay and absorption spectrum. Peak 2 reacts as pyruvate in all tests and is considered to be highly purified material.

The initial velocity of the oxidation of NADH catalysed by lactate dehydrogenase is the same at high (10 mM) and low (0.25 mM) substrate if this purified pyruvate is used and the assay is carried out in imidazole buffer. If unpurified pyruvate is used the ratio of

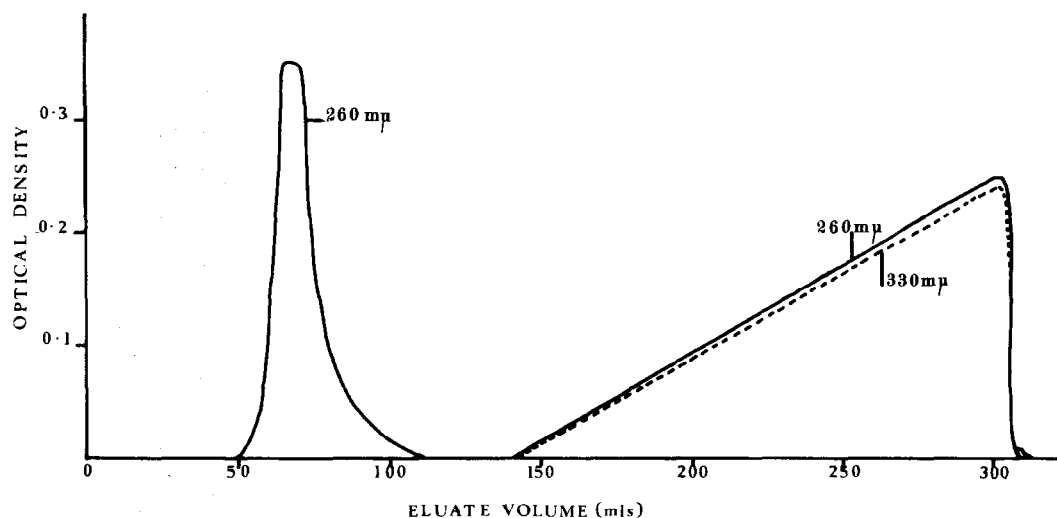


Fig. 1. Purification of pyruvate. 0.1 gm Sodium pyruvate was loaded on a column 15 X 1 cm. Gradient elution was carried out using 0.1 N HCl and a 250 ml water reservoir with a flow-rate of 20 ml/hr.

initial rates at high and low substrate concentrations is 0.8 in the same buffer and even lower, 0.4, in phosphate or tris buffers [9,10]. The material which causes inhibition by high concentrations of pyruvate can clearly be removed from the substrate and the inhibition is not, therefore, caused by excess substrate per se.

If the purified pyruvate is neutralised in imidazole buffer, allowed to stand for five min and then used for assay, the ratio of rates obtained for high and low substrate concentrations is 0.8, the same as unpurified pyruvate. The inhibitor is thus formed rapidly at neutral pH values. Indeed it is formed so rapidly in the presence of phosphate that purified and unpurified pyruvate behave identically in assays with the enzyme if phosphate buffers are used. Pyruvate can be quantitatively assayed with lactate dehydrogenase in the presence of excess NADH. By this means we have shown that purified pyruvate is stable at pH 1.0 for several hr at 0°C.

When pyruvate is added to a mixture of lactate dehydrogenase and NAD the absorption spectrum changes and a peak at 325 mμ appears [1-5]. The rate of appearance of this peak can be measured in a Cary Model 14 spectrophotometer. The kinetic data reported in table 1 show that the rate of formation of this peak in imidazole buffer is reduced if purified pyruvate is used.

Table 1
Rate of appearance of 325 mμ peak.

	Phosphate Buffer (units: increase in absorbance per min)	Imidazole Buffer
Purified pyruvate	0.027	0.016
Unpurified pyruvate	0.03	0.023

Pyruvate (10 mM final concentration) was added directly to the enzyme (14.4 μN) in buffer in the cuvette and allowed two min to equilibrate. NAD (0.1 mM, final concentration) was added to start the reaction. pH 6.90, 25°.

The evidence we have presented established that excess pyruvate inhibition is due to a substance which can be removed from pyruvate solutions and which is in rapid equilibrium with pyruvate at neutral or alkaline pH values. The equilibration of the inhibitor with pyruvate seems to be subject to catalysis by buffer components, especially phosphates. It has been shown [11-13] by N.M.R. and infrared spectroscopy that the hydrate (2,2-dihydroxypropionic acid) is present in acid solutions of pyruvate. This compound cannot be the inhibitor as it cannot be observed at neutral pH values. The enol form of pyruvate would seem to be the only remaining possibility. Further data supporting this idea is shown in figs. 2 and 3. Glyoxalate, which cannot enolise, shows neither substrate inhibi-

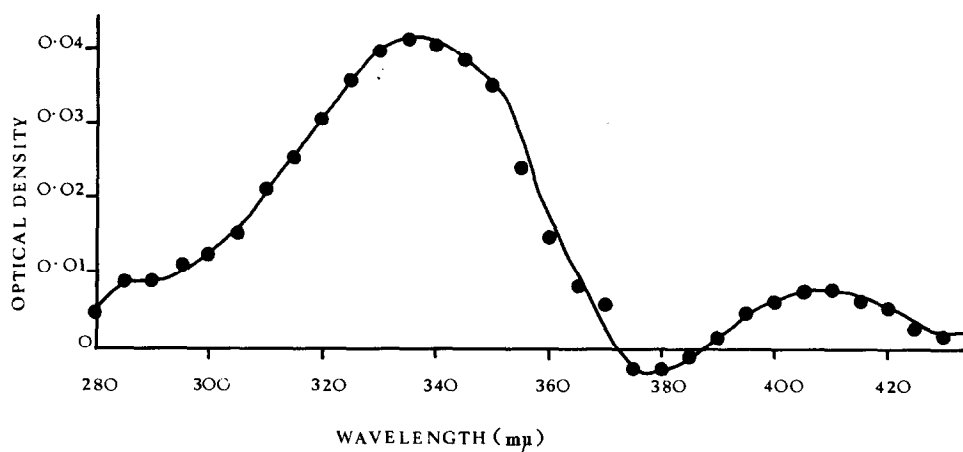


Fig. 2. Difference spectrum on combination of sodium 2-oxobutyrate with APAD and lactate dehydrogenase. Split compartment cells were used and the measurements were in phosphate buffer 0.025 M pH 6.90 at 25°. Contents of compartments: (1) APAD (100 μ M) + enzyme (14.4 μ N); (2) Sodium 2-oxobutyrate (10 mM).

tion nor ternary complex formation. In contrast, 2-oxobutyrate, which can enolise, forms a ternary complex with the enzyme and APAD with a peak at 335 $m\mu$ and shows inhibition at high substrate concentrations.

In view of the fact that complexes are formed between the enol forms of aromatic keto acids and boric acid [18,19] the effects of boric acid on ternary complex formation and substrate inhibition have been investigated and the results are given in table 2. These results, which are preliminary, are consistent with the idea that boric acid sequesters the inhibitor and thus reduces the rate of ternary complex formation.

We propose that the abortive ternary complex is formed by the addition of carbon-3 of the enol of pyruvate to the 4-position of the pyridine ring of NAD, producing a substituted 1,4-dihydronicotinamide adduct. The reaction is analogous to the transfer of a hydride ion from lactate as illustrated in fig. 4, which shows only the overall process. Similar enol additions to NAD have been shown with acetone [17] and pyruvate [14–16] in chemical model systems. Addition of nucleophiles to the 4-position of the nicotinamide ring of NAD is catalysed by the enzyme [20,21] and the addition of the enol of pyruvate would be particularly favoured as it is a substrate analogue. We have shown that semicarbazide reacts with NAD in the presence, but not the absence of the

enzyme, at pH values around 9.0 to give a material with an absorption peak at 338 $m\mu$. The adduct produced is a very powerful inhibitor of the enzyme and this could explain the different values for the pH optimum of lactate oxidation reported in the literature. Winer and Schwert [22] used semicarbazide as a pyruvate trap and obtained a value of 8.8 whereas Sawaki and co-workers [23] report a value of 10.0 in the absence of semicarbazide.

As a test of the hypothesis, the pyruvate adducts of NAD and APAD were prepared by addition of pyruvate to the coenzymes in alkali [14–16] and examined as enzyme inhibitors. The adduct with APAD was found to be a very powerful inhibitor and 50% inhibition was obtained with this compound at 0.01 μ M concentration. It is of interest that this extremely powerful inhibitor can be pictured as an analogue of the transition state of the hydride transfer reaction. The adduct is unstable at pH 6.90 and the characteristic 362 $m\mu$ peak disappears at this pH with a half-life of about six min. In contrast the adduct with NAD is stable in neutral solution but inhibits no more powerfully than does NAD. However, in this compound the keto group of the pyruvate is probably condensed with the amide group of the nicotinamide ring of NAD to give a reduced naphthyridone [15–17,24,25] which would not be a transition state analogue.

Table 2
Effects of borate on lactate dehydrogenase.

	Ternary complex formation (change in absorbance at 325 mμ/min)	Substrate inhibition Rate at 10 mM Rate at 0.25 mM
No Boric Acid	0.023	0.80
With Boric Acid	0.006	1.18

Where indicated boric acid (0.1 M) incubated with pyruvate for two min before addition of enzyme and NAD or NADH. Conditions for assay as for Methods section and for ternary complex formation as table 1. Imidazole was the buffer.

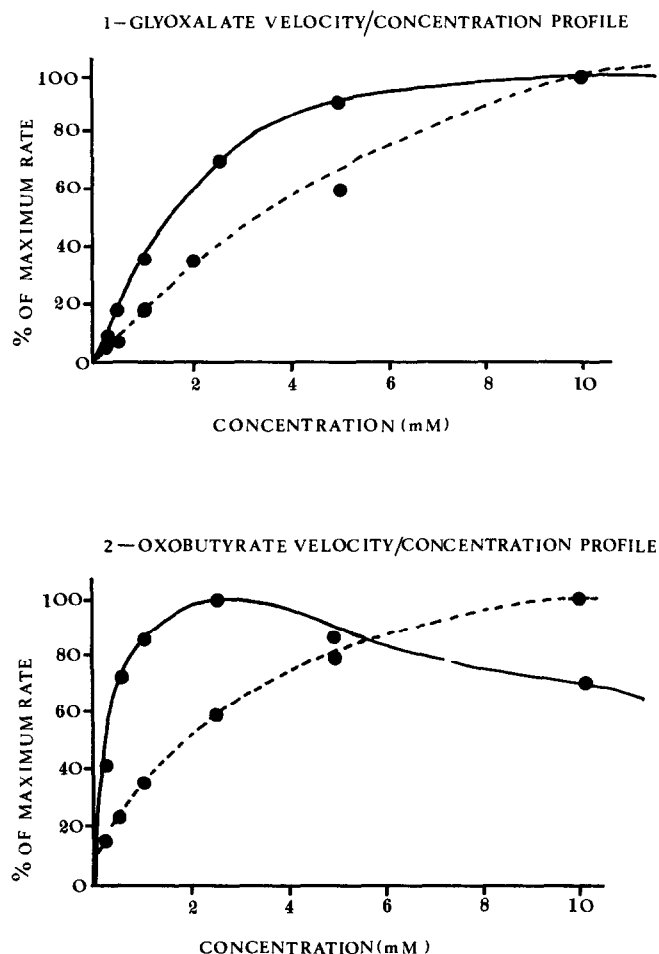


Fig. 3. Effects of concentration on initial velocity for glyoxalate [1] and 2-oxobutyrates [2]. Substrate incubated with NADH (0.1 mM) in buffer pH 6.90, 25°C. LDH (3.15 ng/ml) added to start the reaction. Phosphate (0.1 M) – solid line. Imidazole (0.1 M) containing 0.15 M NaCl – dashed line.

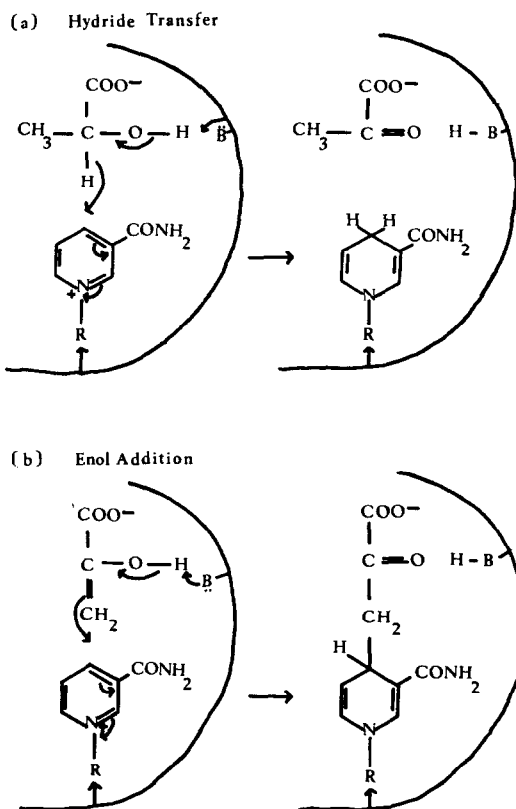


Fig. 4. Comparison of hydride transfer reaction from substrate with adduct formation from enol pyruvate.

To summarise it has been demonstrated that pyruvate can be prepared which is free of inhibitor for lactate dehydrogenase. All the evidence strongly indicates that the enol form of pyruvate is responsible for excess substrate inhibition. We have suggested a molecular basis for the inhibition and we have presented evidence to show that the suggested adduct is indeed a powerful inhibitor.

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