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# Kinetics of Rabbit Muscle D-Glyceraldehyde 3-Phosphate Dehydrogenase: Inhibition by Adenine Nucleotides

by

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Adenine nucleotides are known to be effectors of several glycolytic and gluconeogenic enzymes (8, 9, 14, 18, 20). In many instances it appears that the adenine nucleotides play a dominant role in the overall regulation of the major pathways of carbohydrate metabolism (1-3).

GPDH ** may, in certain metabolic states, become of primary significance in the regulation of hexose utilization and synthesis (3, 13, 21).

In view of the structural similarities between the pyridine nucleotide substrates of GPDH and ATP, ADP and AMP, the effects of these latter compounds on the rabbit muscle enzyme have been investigated. In this context it has previously been shown that GPDH from both pea cotyledons (11) and yeast (22) is markedly inhibited by ATP. STANCEL and DEAL (17) have further shown that yeast GPDH undergoes dissociation into subunits in the presence of ATP. In a separate publication (16) we have described the effects of ATP on the biphasic kinetics observed during assay of $NAD^+$-free rabbit muscle GPDH.

The results here reported show that ATP inhibits rabbit muscle GPDH competitively with respect to G3P and noncompetitively with respect to $NAD^+$. Similar effects are noted with ADP and AMP. The metabolic and enzymological significance of these data is discussed.

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** Abbreviations:
GPDH, D-Glyceraldehyde 3-P-dehydrogenase (EC 1.2.1.12);
G3P, D-Glyceraldehyde 3-phosphate.

## Materials and Methods

CHEMICALS. ATP, ADP, G3P (Ba salt of the diethylacetal), phosphoenolpyruvate (tricyclohexylammonium salt), 3-phosphoglycerate (sodium salt), $NAD^+$, NADH and triethanolamine hydrochloride were obtained from the Boehringer Corp. (London) Ltd. Tris (as Trizma Base) was obtained from the Sigma Chemical Co., St. Louis, Mo. (USA). 2-Mercaptoethanol was from Koch-Light Laboratories Ltd., Colnbrook. Bucks. (England). All other chemicals were of A.R. grade or were the purest available.

ENZYMES. Pyruvate kinase, lactate dehydrogenase, phosphoglycerate kinase and adenylate kinase were obtained from the Boehringer Corp. (London) Ltd. GPDH was prepared from rabbit skeletal muscle using the method of AMELUNXEN and CARR (4). The final specific activity of GPDH, after several recrystallizations, was 80 $\mu$moles NADH produced/min/mg enzyme using a modification of Ferdinand's method (7) described elsewhere (16) at pH 8.8. The $E^{1\%}_{1\,cm}$ of the enzyme was taken as 0.92 at 280 m$\mu$.

SPECIAL PREPARATIONS. Solutions of G3P were prepared by the method of RACKER, KLYBAS and SCHRAMM (15). G3P and $NAD^+$ were assayed and standardized spectrophotometrically with GPDH. ATP solutions (pH 7.4) were assayed and standardized enzymically with 3-phosphoglycerate kinase and GPDH and assayed also for contamination by ADP and AMP. ADP and AMP solutions (pH 7.4) were assayed and standardized enzymically with pyruvate kinase, lactate dehydrogenase and adenylate kinase. ADP solutions were also assayed for AMP content (due allowance being made for this in subsequent experiments). Full details of assay procedures are given in NEWSHOLME and RANDLE (12). All adenine nucleotides, both solid and solutions, were kept at —15°. Solutions were never kept for more than one week.

PROCEDURE FOR OBTAINING KINETIC DATA. All experiments were carried out using a Hilger-Gilford recording spectrophotometer at 25°, pH 7.4, with a cuvette of 1 cm light path, final volume either 1 or 3 ml. The change of absorption at 340 m$\mu$ was recorded between 15 and 45 seconds following the initiation of the reaction. The assay medium contained $4.25 \times 10^{-2}$ M Tris, $12.75 \times 10^{-3}$ M $Na_2HAsO_4$, $1.3 \times 10^{-3}$ M EDTA with appropriate concentrations of $NAD^+$ and G3P, pH 7.4. Reactions were initiated by addition of either enzyme or substrate. Control experiments showed that results were independent of the nature of the initiator.

## Results

The reduction of $NAD^+$ catalysed in the forward reaction of GPDH is inhi-

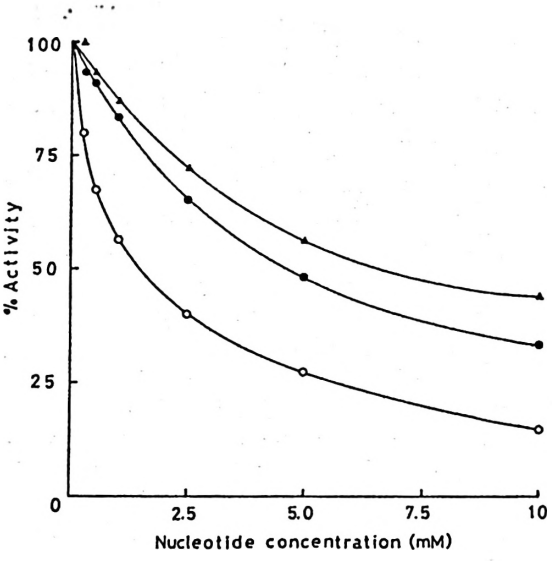


FIG. 1. Effect of adenine nucleotides on GPDH activity at pH 7.4. The assay medium was as described in the text, with $2 \times 10^{-4}$ M $NAD^+$ and $4.5 \times 10^{-4}$ M G3P. The activity in the absence of added nucleotide was assigned the value 100 %.

▲ AMP; ● ADP; ○ ATP

bited by all three adenine nucleotides tested, ATP, ADP and AMP (Fig. 1). This inhibition is most marked with ATP and is least so with AMP. Concentrations for 50 % inhibition at $2 \times 10^{-4}$ M $NAD^+$ and $4.5 \times 10^{-4}$ M G3P were determined as $1.5 \times 10^{-3}$ M, $4.6 \times 10^{-3}$ M and $6.8 \times 10^{-3}$ M for ATP, ADP and AMP respectively.

The variation of the degree of inhibition by ATP with pH is shown in Fig. 2. In agreement with earlier reports (6, 19), the optimum pH was found to be near 9.0. However, ATP inhibition was very little in evidence at this pH (4.5 %). This result may in part explain why this phenomenon has not previously been observed. The degree of inhibition increases at lower pH values and is maximal near pH 7.5.

The relationship between reaction velocity at pH 7.4 and $NAD^+$ concentration is non-Michaelian (Fig. 3A). Plots of $\log \frac{v}{V_{max.}-v}$ against log [$NAD^+$] [Hill

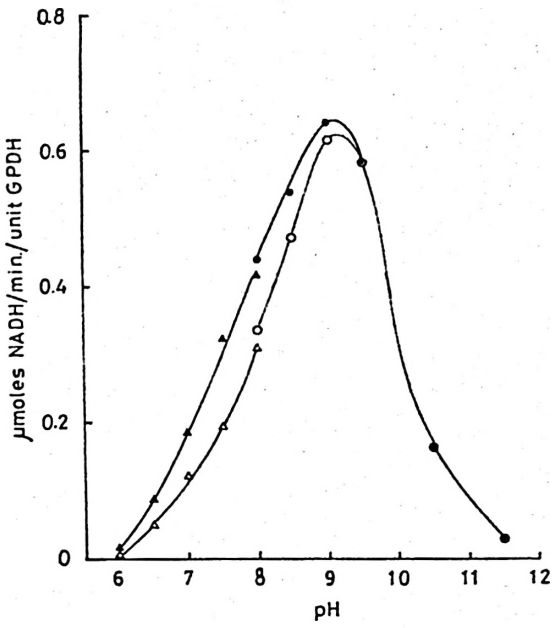


FIG. 2. Variation of the degree of inhibition of GPDH by ATP (1 mM) with pH. Assays were as described in the text, with $2 \times 10^{-4}$ M NAD and $4.5 \times 10^{-4}$ M G3P. Closed symbols represent rates in absence of ATP; open symbols those in the presence of 1 mM ATP.
△, ▲ Tris; ○, ● veronal

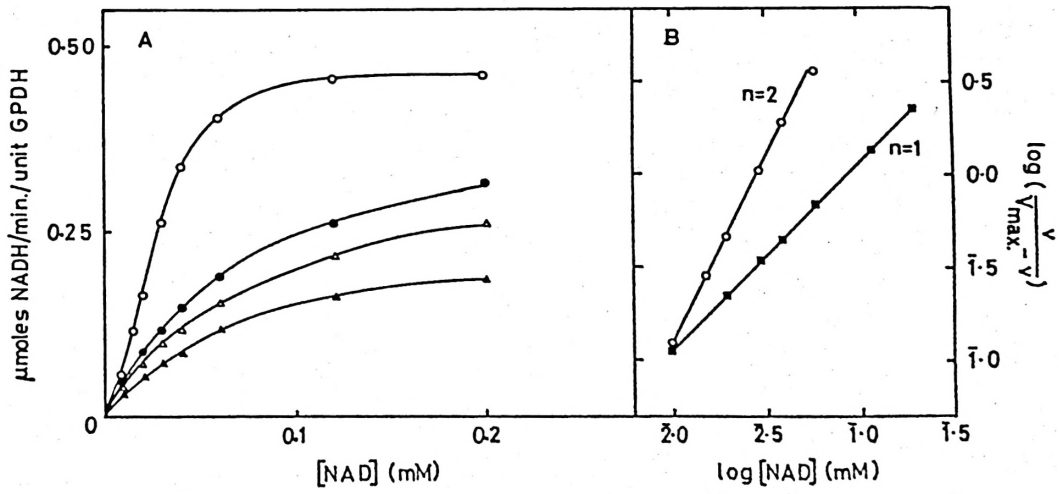


FIG. 3. A) Relationship between reaction velocity at pH 7.4 and $NAD^+$ concentration. Assay conditions were as described in the text. The G3P concentration was $4.5 \times 10^{-4}$ M.
○ No ATP; ● 0.4 mM ATP; △ 1 mM ATP; ▲ 4 mM ATP.
B) Hill plot of the same data. ○ No ATP; ■ represents values with 0.4, 1 and 4 mM ATP.

plots (10)] show $n$ values of 2.0 (Fig. 3B). In the presence of ATP, however, rate response curves for $NAD^+$ have the usual hyperbolic form predicted by the Michaelis-Menten relationship (Fig. 3A); Hill $n$ values are decreased to unity (Fig. 3B).

Lineweaver-Burk reciprocal plots of these data (not shown) show that increasing ATP concentrations serve to decrease the $V_{max.}$ whilst having no effect on the $K_m$ for $NAD^+$ ($2.7 \times 10^{-5}$ M).

## Discussion

STANCEL and DEAL (17) have suggested that the point of action of ATP on yeast GPDH is located at the $NAD^+$ binding site. The results presented above indicated that ATP inhibits GPDH non-competitively with respect to $NAD^+$. Although there are obvious structural similarities between these two nucleotides, the possibility remains that ATP binds at the

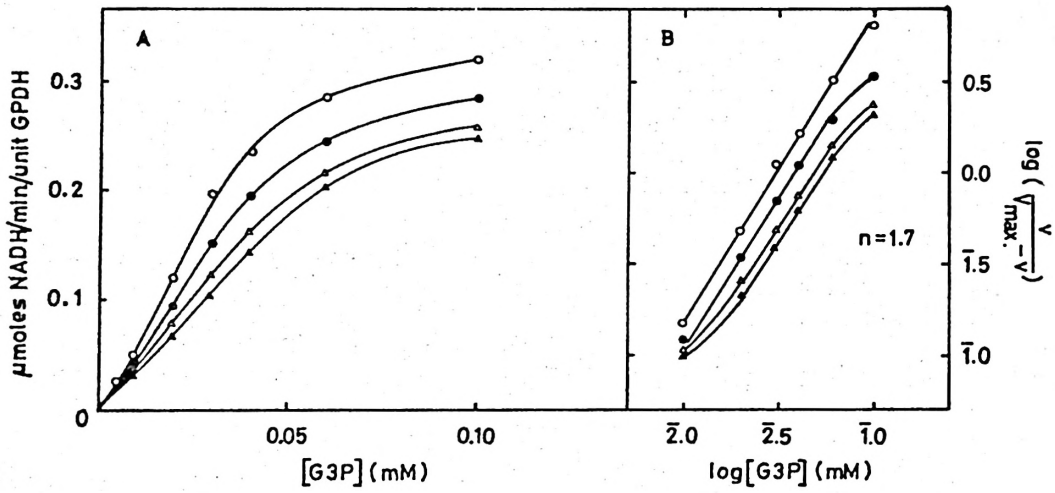


FIG. 4. A) Plot of reaction velocity against increasing G3P concentration. Assay conditions were as described in the text. The $NAD^+$ concentration was $2 \times 10^{-4}$ M.
B) Hill plot of the same data. ○ No ATP; ● 0.4 mM ATP; △ 1 mM ATP; ▲ 4 mM ATP.

Plots of reaction velocity against increasing G3P concentrations are sigmoid (Fig. 4A). ATP inhibits GPDH with respect to G3P, but, in distinction from the effect with $NAD^+$, does not alter the degree of sigmoidicity of the rate response curves. $V_{max.}$ for G3P is unchanged by increasing ATP. These results are seen more clearly in Fig. 4B. Hill $n$ values are approx. 1.7. Increasing ATP concentrations (up to $4 \times 10^{-3}$ M) increase the $S_{(0.5)}$ (2) for G3P from $3.2 \times 10^{-5}$ M to $5.4 \times 10^{-5}$ M.

phosphate site on the enzyme. This would be consistent with the observed inhibitory patterns with respect to G3P and $NAD^+$ if interaction occurs between the respective G3P and phosphate binding sites present.

It seems probable that ADP and AMP bind to the same site as ATP, albeit more weakly. This is in contrast to the postulate that the terminal phosphate in ATP is of primary importance (17).

The alteration in apparent kinetic order (deduced from Hill $n$ values) seen in

experiments with ATP at low concentrations of $NAD^+$ may be correlated with the reported dissociation of the rabbit muscle enzyme by ATP (5).

At the present time it is difficult to assess the physiological significance of the inhibition by ATP. This problem is due in great part to the technical difficulties arising from the unfavourable equilibrium of GPDH at physiological pH's. These prevent the collection of relevant kinetic data with regard to phosphate binding under these conditions.

From an enzymological standpoint it is likely that important information regarding the structure, subunit interactions and reaction mechanism of rabbit muscle GPDH may be obtained from further ultracentrifugal and rapid reaction studies employing ATP and its analogues as specific inhibitors.

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## Summary

The inhibitory effects of adenine nucleotides (ATP, ADP and AMP) on rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase (GPDH) have been investigated. Concentrations for 50 % inhibition at $2 \times 10^{-4}$ M $NAD^+$ and $4.5 \times 10^{-4}$ M glyceraldehyde 3-phosphate (G3P) were determined as $1.5 \times 10^{-3}$ M; $4.6 \times 10^{-3}$ M and $6.8 \times 10^{-3}$ M for ATP, ADP and AMP respectively. The degree of inhibition, which is very small at pH 9, is maximal at approx. pH 7.5. The relationship between reaction velocity at pH 7.4 and $NAD^+$ concentration is sigmoid ($n = 2$) but becomes hyperbolic ($n = 1$) in the presence of ATP. Increasing ATP concentrations decrease the $V_{max.}$ whilst having no effect on the $K_m$ for NAD ($2.7 \times 10^{-5}$ M), suggesting non-competitive inhibition with respect to $NAD^+$. Plots of reaction velocity against increasing G3P concentrations are sigmoid ($n = 1.7$). ATP inhibits GPDH with respect to G3P without altering the apparent order of reaction. In this case, $V_{max.}$ is unchanged and the $S_{(0.5)}$ for G3P is increased from $3.2 \times 10^{-5}$ M to $5.4 \times 10^{-5}$ M. The metabolic and enzymological significance of the data is discussed.

## Resumen

Se estudió el efecto inhibidor del ATP, ADP y AMP sobre la D-gliceraldehido 3-fosfato-deshidrogenasa (GPDH) de músculo de conejo. Las concentraciones para alcanzar un 50 % de inhibición con ATP, ADP y AMP fueron, respectivamente, $1{,}5 \times 10^{-3}$ M; $4{,}6 \times 10^{-3}$ M y $6{,}8 \times 10^{-3}$ M, a concentraciones de $2 \times 10^{-4}$ M NAD y $4{,}5 \times 10^{-1}$ M de gliceraldehido 3-fosfato (G3P). El grado de inhibición es muy bajo a pH 9 y máximo a un pH cercano a 7,5. La relación entre la velocidad de reacción a pH 7,4 y la concentración de NAD es sigmoidea ($n = 2$) pero se hace hiperbólica ($n = 1$) en presencia de ATP. Las concentraciones crecientes de ATP hacen disminuir la $V_{máx.}$ pero no afectan la $K_m$ para NAD ($2{,}7 \times 10^{-5}$ M), sugiriendo una inhibición no competitiva con respecto al NAD. Las graficaciones de velocidad de reacción con respecto a concentraciones crecientes de G3P son sigmoideas ($n = 1{,}7$). El ATP inhibe a la GPDH con respecto al G3P sin alterar el orden aparente de la reacción. En este caso $V_{máx.}$ no se altera y $S_{(0.5)}$ para G3P aumenta de $3{,}2 \times 10^{-5}$ M a $5{,}4 \times 10^{-5}$ M. Se discute el significado metabólico y enzimático de los resultados obtenidos.

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