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# RELATIONSHIP BETWEEN HYDROXYPYRUVATE AND THE PRODUCTION OF OXALATE IN VITRO

CARMEN LLUIS and JORGE BOZAL

*Department of Biochemistry, Faculty of Chemistry, University of Barcelona, Barcelona (Spain)*

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## SUMMARY

Chicken liver lactate dehydrogenase (L-lactate : $NAD^+$ oxidoreductase, EC 1.1.1.27) catalyses the reversible reduction reaction of hydroxypyruvate to L-glycerate. It also catalyses the oxidation reaction of the hydrated form of glyoxylate to oxalate and the reduction of the non-hydrated form to glycolate. At pH 8, these latter two reactions are coupled. The coupled system equilibrium is attained when the $NAD^+$/NADH ratio is greater than unity.

Hydroxypyruvate binds to the enzyme at the same site as the pyruvate. When there are substances with greater affinity to this site in the reaction medium and their concentration is very high, hydroxypyruvate binds to the enzyme at the L-lactate site. In vitro and with purified preparation of lactate dehydrogenase, hydroxypyruvate stimulates the production of oxalate from glyoxylate-hydrated form and from NAD; the effect is due to the fact that hydroxypyruvate prevents the binding of non-hydrated form of glyoxylate to the lactate dehydrogenase in the pyruvate binding site. At pH 8, the L-glycerate stimulates the production of glycolate from glyoxylate-non-hydrated form and NADH since hydroxypyruvate prevents the binding of glyoxylate-hydrated form to the enzyme.

## INTRODUCTION

The most significant metabolic precursors of oxalate, the final product of metabolism in mammals, are ascorbic acid and glyoxylate. The excess oxalate producing hyperoxaluria comes from the glyoxylate, the oxidation of which may be catalysed by xanthine oxidase, glycolic acid oxidase, or by lactate dehydrogenase. The part played by this last named enzyme in pathological metabolic states leading to the hyperproduction of oxalate from glyoxylate [1–4] has recently been brought to light.

The different forms of hyperoxaluria [5, 6] give rise to diseases differentiated by the metabolic pathway leading to the glyoxylate, precursor of the oxalate, and which are originated by vitamin or enzyme deficiencies. Hyperoxaluria is classified according to the nature of the products accompanying the oxalate in urine. Type II

primary hyperoxaluria or L-glyceric aciduria is due to a genetic deficiency of D-glycerate dehydrogenase, the absence of which causes an accumulation of hydroxypyruvate which, in turn, is reduced to L-glycerate by lactate dehydrogenase L-Glycerate does not accumulate in the urine of healthy individuals, but its level is high in those suffering from a lack of D-glycerate dehydrogenase [7] Therefore, although hydroxypyruvate is not a direct metabolic precursor of the oxalate, its conversion into L-glycerate is associated with the excess production of oxalate, characteristic of hyperoxaluria.

The relationship between the reduction of hydroxypyruvate, with the aid of lactate dehydrogenase, and the excess production of oxalate in vivo has not yet been clearly established. It has been postulated [8] that the $NAD^+$ produced in this reduction is reconverted to NADH in the oxidation of glyoxylate to oxalate, catalysed by this same enzyme. An attempt has been made in this paper to establish the conditions in which the reactions of reduction of the hydroxypyruvate and oxidation of the glyoxylate, catalysed by the purified preparations of lactate dehydrogenase, may be associated in vitro to give rise to the appearance of excess oxalate.

Only spectroscopic changes in NADH have been measured in the hydroxypyruvate action on the glyoxylate oxidation and these have not been quantitatively correlated with changes in concentrations of substrates or products. The observed initial rates of changes in NADH concentrations are the result of several competing reactions which will be mentioned and discussed in experimental part

## METHODS

Chicken liver lactate dehydrogenase (L-lactate · $NAD^+$ oxidoreductase, EC 1.1.1 27) crystallized according to Lluis et al [9] has been used The specific activity of the preparation is 275 units/mg protein (1 unit = 1 $\mu$mol converted NADH/min); 1 g of the purified preparation contains 200 mg of protein. Freshly prepared solutions of the following products in an appropriate buffer, after pH adjustment (pH-meter) are used as substrates or inhibitors· glyoxylic acid, lithium hydroxypyruvate, 50 % sodium DL-lactate, DL-glyceric acid and sodium oxamate (Merck), sodium pyruvate, NAD and NADH (Boehringer) and sodium oxalate (UCB). The buffer used is 50 mM sodium phosphate, pH 7 4 or pH 8 0

The rate of NADH oxidation at $\lambda = 340$ nm was followed to measure initial reaction rates, at $30 \pm 0$ 1 °C in a PYE UNICAM S-P 1700 recording spectrophotometer in 3-ml cells and 1 cm light path Addition of the enzyme initiated the reaction.

The inhibitions have been characterised by the double reciprocal plot of $v$ vs $[S]$ The binding site of two substrates, in the presence of the same enzyme, has been determined by Dixon's method [10] $K_{is}$ and $K_{ii}$ are inhibition constants calculated from the slope and intercept, respectively

## RESULTS

*Characterization of the hydroxypyruvate-lactate dehydrogenase-NADH system*

The enzyme's optimum pH in the hydroxypyruvate (24 mM)-lactate dehydrogenase (3 $\mu$g purified preparation/ml)-NADH (0.1 mM) system is 7 4 With the same concentrations of enzyme and NADH, the double reciprocal plot of the

initial reaction rates, obtained with variable concentrations of hydroxypyruvate (0.3–5 mM) is linear, $K_m = 0.5$ mM The hydroxypyruvate (10 mM or more) inhibits as a result of excess substrate. The double reciprocal plot of the initial reaction rates vs. the NADH concentrations (0 03–1 mM), obtained with a constant concentration of hydroxypyruvate (5 mM and 3 $\mu$g of purified enzyme preparation/ml) is linear; $K_m = 0.01$ mM. Substrate inhibition by NADH excess (0 1 mM or more) is also observed.

*Hydroxypyruvate binding site in lactate dehydrogenase*

In the hydroxypyruvate (0.3–5 mM)-lactate dehydrogenase (3 $\mu$g purified preparation/ml)-NADH (0.025–0 1 mM) system, the L-lactate (11.5 and 14 mM) is a non-competitive inhibitor with respect to the hydroxypyruvate ($K_{is} = 3.6$ mM; $K_{ii} = 2$ mM) when the NADH is at a saturating concentration (0 1 mM) and is an uncompetitive inhibitor (6.6 and 16 1 mM) with respect to NADH ($K_{ii} = 1.2$ mM) when the hydroxypyruvate is at a saturating concentration (5 mM). This suggest that the L-lactate and the hydroxypyruvate does not bind to the same enzyme site

The results obtained with non-equimolar mixtures of pyruvate and hydroxypyruvate, when the hydroxypyruvate concentration is higher or lower than the pyruvate concentration, are given in Table I When higher hydroxypyruvate concentrations are used, it is shown that the experimental values agree with the theoretical ones if it is assumed that both substrates compete for the same enzyme site. If the hydroxypyruvate concentration is lower than that of the pyruvate, there is no competition between the substrates. The phenomenon observed is in correlation with the $K_m$ values of both substrates: $K_{m(\text{hydroxypyruvate})} = 0.45$ mM, $K_{m(\text{pyruvate})} = 0\ 06$ mM. Non-equimolar mixtures of hydroxypyruvate and glyoxylate (Table II) show a similar behaviour. Therefore, it may be stated that when the hydroxypyruvate

TABLE I

NON-EQUIMOLECULAR MIXTURES OF PYRUVATE AND HYDROXYPYRUVATE

Theoretical rates for competing ($V_T^1$) or not competing ($V_T^2$) substrates for the same site, $V_{exp}$ = experimental rates, pH 7 4, [enzyme] = 6 6 $\mu$g of purified preparation/ml, [NADH] = 0 1 mM Pyruvate-lactate dehydrogenase-NADH system $K_{m(pyr)} = 0\ 066$ mM, $V_{(pyr)} = 12\ 5\ \ 10^{-2}\ \Delta A$/min Hydroxypyruvate-lactate dehydrogenase-NADH system $K_{m(\text{hydroxypyr})} = 0\ 5$ mM, $V_{(\text{hydroxypyr})} = 25\ 6\ \ 10^{-2}\ \Delta A$/min Results are expressed as $\Delta A/\text{min} \times 10^2$

| Pyruvate (mM) | Hydroxypyruvate (mM) | $V_T^1$ | $V_T^2$ | $V_{exp}$ |
|---|---|---|---|---|
| 0 01 | 0 1 | 5 5 | 6 3 | 5 2 |
| | 0 3 | 10.4 | 11.8 | 10 6 |
| | 0 5 | 13.4 | 15 1 | 12 8 |
| | 0 7 | 15 4 | 17 2 | 15 2 |
| 0 01 | 0 04 | 6 6 | 6 9 | 7 0 |
| | 0 06 | 8 0 | 8 3 | 9 1 |
| | 0 08 | 9 1 | 9 4 | 11 5 |
| 0 03 | 0 04 | 7 2 | 8 1 | 8 8 |
| | 0 06 | 8 5 | 9.5 | 10 4 |
| | 0 08 | 9 4 | 10 6 | 13 8 |

TABLE II

NON-EQUIMOLECULAR MIXTURES OF GLYOXYLATE AND HYDROXYPYRUVATE

Theoretical rates for competing ($V_T^1$) or not competing ($V_T^2$) substrates for the same site, $V_{exp}$ = experimental rates, pH 7 4, [enzyme] = 6 6 $\mu$g of purified preparation/ml, [NADH] = 0.1 mM Glyoxylate-lactate dehydrogenase-NADH system $K_{m(glyox)} = 3$ mM, $V_{(glyox)} = 5\ 1\ 10^{-2}\ \Delta A$/min Hydroxypyruvate-lactate dehydrogenase-NADH system $K_{m(hydroxypyr)} = 0\ 7$ mM, $V_{(hydroxypyr)} = 7\ 7\ 10^{-2}\ \Delta A$/min Results are expressed as $\Delta A/\text{min} \times 10^2$

| Hydroxypyruvate (mM) | Glyoxylate (mM) | $V_T^1$ | $V_T^2$ | $V_{exp}$ |
|---|---|---|---|---|
| 0 015 | 0 66 | 1 | 1 1 | 2 4 |
| | 1 33 | 1 6 | 1 8 | 2 5 |
| | 2 6 | 2 4 | 2 6 | 3 1 |
| | 20 0 | 4 4 | 4 6 | 4 9 |
| 0 3 | 0 66 | 2 6 | 3 1 | 2 8 |
| | 1.33 | 2 9 | 3 7 | 2 6 |
| | 2 6 | 3 3 | 4 5 | 3 9 |
| | 20 0 | 4 6 | 6 6 | 4 8 |

concentration is low and the pyruvate or glyoxylate concentrations are moderately high, the hydroxypyruvate is displaced from the characteristic pyruvate binding site and must bind itself to another site on the enzyme molecule, probably the L-lactate binding site.

Oxamate is a competitive inhibitor of lactate dehydrogenase with respect to pyruvate. Its effect on the hydroxypyruvate-lactate dehydrogenase (6 6 $\mu$g purified preparation/ml)-NADH (0.1 M) may be illustrative as to the site where the hydroxypyruvate binds. The inhibition caused by the oxamate (0 3 and 0.5 mM) is purely non-competitive if the hydroxypyruvate varies from 0.005 to 0.1 mM, or is mixed non-competitive if the concentrations of both are similar ([oxamate] = 3–5 mM; [hydroxypyruvate] = 1–10 mM). The inhibition by oxamate (0.05, 0.1 and 0.2 mM) is competitive if the hydroxypyruvate concentration varies 1 to 10 mM. The nature of the inhibition by oxamate changes gradually and may be related to the displacement of the hydroxypyruvate from its binding site when there is a high concentration of substrates with greater affinity.

*Oxido-reduction of glyoxylate at pH 8*

At pH 8, the glyoxylate may be oxidized (with NAD) or reduced (with NADH) by lactate dehydrogenase. The reaction rates in the glyoxylate-lactate dehydrogenase-NAD system at pH 8, show two straight lines of differing slopes (Fig 1). If the reaction times are very short, the slope is steeper than if the times are extended. This phenomenon is independent of the glyoxylate concentration (0.8–4 mM). At pH 8, the glyoxylate (hydrated form)-lactate dehydrogenase-$NAD^+$ produces NADH, this latter reduces the anhydrous form of the glyoxylate (glyoxylate (anhydrous form)-lactate dehydrogenase-NADH system) to glycolate. Therefore, there must be a $NAD^+$/NADH ratio at which an equilibrium is attained between the oxidation and reduction of the glyoxylate; that is, there may be an initial mixture of $NAD^+$, NADH, and glyoxylate with which no absorbance increments or decrements are

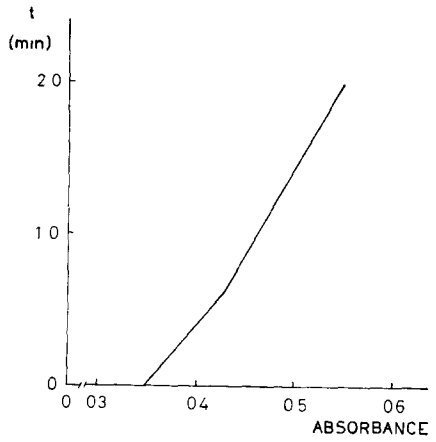



Fig 1. Initial rates from glyoxylate-lactate dehydrogenase-$NAD^+$ system [glyoxylate], 4 mM, [NAD], 3 3 mM, 50 mM sodium phosphate buffer, pH 8, [enzyme], 6 6 $\mu$g of purified preparation/ml.

observed at $\lambda = 340$ nm. The results obtained with different concentrations of glyoxylate (2–7.8 mM) show that the $NAD^+$/NADH ratio is always greater than unity (33–66), this seems to suggest that, at this pH, the reduction reaction is more favourable than the oxidation reaction. It may be assumed, therefore, that the NADH formed by the glyoxylate (hydrated form)-lactate dehydrogenase-$NAD^+$ system may, in turn, be active in the reduction of the glyoxylate (anhydrous form) to glycolate.

*Action of hydroxypyruvate in the oxidation of glyoxylate*

In the presence of $NAD^+$ and enzyme, the hydroxypyruvate undergoes no change and is a competitive inhibitor (0.0003–0.001 mM; $K_{is} = 2\ \mu$M) with respect to the glyoxylate (0.15–1 mM) in the glyoxylate-lactate dehydrogenase (6.6 $\mu$g purified preparation/ml)-NAD (3.3 mM) system.

When studying the effect of the hydroxypyruvate on the glyoxylate-lactate dehydrogenase-$NAD^+$ system at pH 8, we have taken into consideration all the reactions that may take place between the substrates initially present, which are the hydrated and non-hydrated forms of the glyoxylate the $NAD^+$ and hydroxypyruvate and the products which appear in the reaction medium. The initial reaction is as follows:

$$(OH)_2CH\text{-}COOH + NAD \xrightarrow{E} \text{oxalate} + NADH + H^+$$

When the NADH appears, the following reactions may take place:

$$HOC\text{-}COOH + NADH + H^+ \xrightarrow{E} \text{glycolate} + NAD^+$$

$$\text{Hydroxypyruvate} + NADH + H^+ \rightleftharpoons \text{L-glycerate} + NAD^+$$

The initial reaction rates obtained in vitro with mixtures of glyoxylate, $NAD^+$, hydroxypyruvate and lactate dehydrogenase at pH 8 are given in Table III. The velocity plots, obtained by the spectrophotometric method, show two straight lines with a genuine inflection point between them, similar to what is observed in the

TABLE III

ACTION OF HYDROXYPYRUVATE IN THE GLYOXYLATE-LACTATE DEHYDROGENASE-NAD SYSTEM, pH 8

[Enzyme] = 3 $\mu$g of purified preparation/ml, $[NAD]^+$ = 3 3 mM, [glyoxylate] = 4 mM $V$ and $V'$ initial rates calculated from the first and second slope of velocity recording plots

| Hydroxypyruvate (mM) | $\Delta A$/min $\times 10^2$ | | Activation or inhibition (%) | | Effect of hydroxypyruvate on the velocity plots |
|---|---|---|---|---|---|
| | $V$ | $V'$ | 1st slope | 2nd slope | |
| 0 | 6 5 | 3 7 | – | – | |
| 0 0005 | 6 7 | 3 7 | 4 | 0 | |
| 0 0008 | 7 7 | 3 8 | 19 | 4 | Increments |
| 0 001 | 8 9 | 4 3 | 37 | 16 | |
| 0 005 | 7 0 | 4 3 | 8 | 10 | |
| 0 01 to 0 1 | 6 5 | 3 7 | – | – | – |
| 0 5 | 2 8 | 1 8 | 57 | 51 | |
| 1 | 1 9 | 1 2 | 70 | 67 | Decrements |
| 3 | 0.8 | 0 4 | 87 | 89 | |

glyoxylate-lactate dehydrogenase-$NAD^+$ system in the absence of inhibitor (Fig. 1) The presence of low concentrations of hydroxypyruvate increases the reaction rates In the presence of high hydroxypyruvate concentration, a drop in the reaction rates is observed. Spectrophotometric absorbance increments are observed in all cases due to the production of NADH When considering the ensemble of possible reactions, it may be assumed that at the start of the reaction the NADH concentration will be infinitely low and no reaction with the hydroxypyruvate, the concentration of which is also low, will be observed. On the other hand, the hydroxypyruvate will bind to the enzyme in its preferred site, to which non-hydrated form of the glyoxylate also binds and this, in view of its lesser affinity, may be displaced by the hydroxypyruvate. This fact will imply that when the hydrated form of the glyoxylate is reduced (preferred reaction) the equilibrium between both forms of the glyoxylate is displaced towards the latter form, which is the genuine substrate of the glyoxylate (hydrated form)-lactate dehydrogenase-NAD system and this would produce an apparent activation of the system. When the hydroxypyruvate concentrations in the medium are high, they may react with the NADH, which would manifest itself as a reduction of the rate values.

The same effects, although less notable, also appear when the second straight line of the velocity plots is analysed. In fact, after the early part of the reaction has elapsed, the NADH concentration in the medium is higher and may reduce the hydroxypyruvate Independently of the increments or decrements observed in the reaction rates when measuring the NADH absorbance variation, this effect is an increase in the production of oxalate.

*Effect of* L-*glycerate on the glyoxylate-lactate dehydrogenase-NADH system*

In the glyoxylate (1.2–10 mM)-lactate dehydrogenase (6 6 $\mu$g purified preparation/ml)-NADH (0 1 mM) system (pH 7 4), the L-glycerate (4 1 and 8 3 mM) is a non-competitive inhibitor ($K_{11} = K_{1s} = 2.6$ mM) with respect to the glyoxylate

TABLE IV

ACTION OF L-GLYCERATE IN THE GLYOXYLATE-LACTATE DEHYDROGENASE-NADH SYSTEM, pH 8

[Enzyme], 3 $\mu$g of purified preparation/ml, [NADH], 0 1 mM, [Glyoxylate], 2 mM

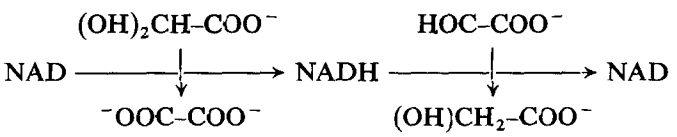
| L-Glycerate (mM) | $V$ ($\Delta A$/min $\times 10^2$) | Activation or inhibition (%) | Effect of L-glycerate on the velocity plots |
|---|---|---|---|
| 0 | 5 4 | – | |
| 0 0041 | 6 6 | 22 | Increments |
| 0 0083 | 6 | 11 | |
| 0 041 | 5 8 | 7 | |
| 4 1 | 4 8 | 7 4 | |
| 8 3 | 3 1 | 43 | Decrements |

The presence of L-glycerate in the above system, in vitro at pH 8, produces variable effects depending on its concentration (Table IV). This shows that the lower concentrations of L-glycerate produce increases in the glyoxylate reduction rate, whilst the higher concentrations produce a slowing down. The effects described are similar to those observed in the glyoxylate oxidation system in presence of variable concentrations of hydroxypyruvate. The increased rate observed in the presence of the lower concentrations of L-glycerate may be interpreted, if it is accepted that this binds to the same enzyme site as to which the hydrated form of glyoxylate, whose binding it prevents, whereby the reduction of the non-hydrated form is facilitated. All of this brings about a reduction in the NADH concentration, as shown by the absorbance readings. The inhibition observed in the presence of the higher concentrations of L-glycerate is only apparent, since this will be oxidized by the NAD coming from the glyoxylate-lactate dehydrogenase-NADH system.

## DISCUSSION

The results described indicate that the hydroxypyruvate binds to the same enzyme site as the pyruvate (as happens with the inhibition produced by the L-lactate and with the non-equimolecular mixtures of pyruvate-hydroxypyruvate or glyoxylate-hydroxypyruvate if hydroxypyruvate concentrations are high) Hydroxypyruvate is displaced from the same enzyme site as the pyruvate, probably to the same enzyme site as L-lactate, when in the reaction medium are present pyruvate or glyoxylate concentrations higher than hydroxypyruvate concentrations.

The oxidation of the glyoxylate, catalyzed by lactate dehydrogenase, has been studied at pH 8. Under these conditions, glyoxylate would also be reduced to glycolate by the same enzyme. The reaction velocity plots obtained and the value of the $NAD^+/NADH$ ratio, when an equilibrium is obtained in the coupled system, show that the reduction reaction takes place in preference to the oxidation reaction and they suggest that the glyoxylate conversions are adapted to the following scheme:



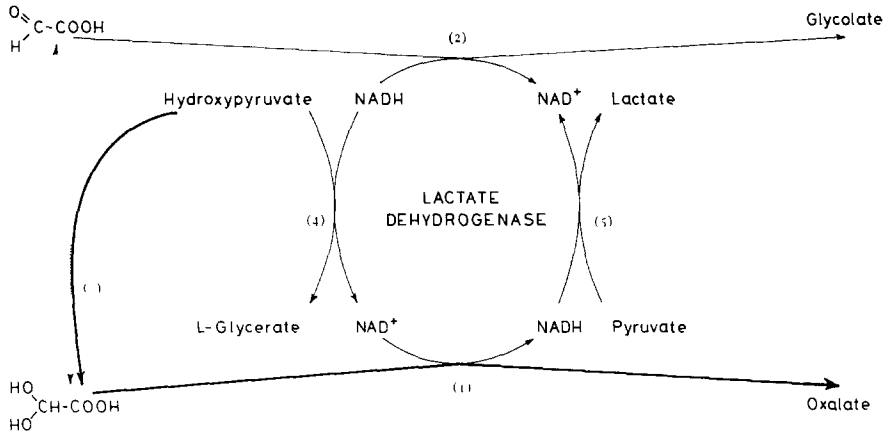



Fig 2 Relationships between several reactions catalyzed by lactate dehydrogenase

The opposite effects induced by hydroxypyruvate on the glyoxylate-lactate dehydrogenase-$NAD^+$ system, at pH 8, may be explained by reference to Fig. 2 The hydrated form of the glyoxylate, which is converted into oxalate at the same enzyme site as the L-lactate (step 1) and the non-hydrated form, which is converted into glycolate at the enzyme site to which the pyruvate binds (step 2) are in the reaction medium. The hydroxypyruvate, having a greater affinity for the same enzyme site than glyoxylate (non-hydrated form), prevents its binding. For this reason the equilibrium between the two forms of glyoxylate is displaced towards the hydrated form (step 3), which one is enzymatically oxidized (step 1) and an activating effect is observed when the increase in NADH concentration is read in the spectrophotometer. The activating effect produced by small hydroxypyruvate concentrations is only perceptible when the reaction begins; initially steps 2 and 4, will not take place because there is not NADH in the reaction medium. After the few first seconds of the reaction, when a sufficient NADH concentration has been reached, the processes 2 and 4 will consume it and the activation observed will be smaller. High concentrations of hydroxypyruvate should prevent the binding of the non-hydrated form of the glyoxylate to the enzyme and would facilitate, therefore, the conversion of the hydrated form into oxalate; nevertheless, hydroxypyruvate at high concentrations causes an inhibition if the spectrophotometric readings are taken into consideration. This does not mean that the production of oxalate is not stimulated under these conditions, but that, since the hydroxypyruvate concentration is high, step 4 takes place at a very appreciable level and NADH is consumed leading to the above observed apparent inhibition The hypotheses formulated are analogous to those which could be deduced from the results obtained with the study of the action of the L-glycerate in the glyoxylate-lactate dehydrogenase-NADH system.

The in vitro experiments we have discussed seem to suggest that higher concentrations of hydroxypyruvate inhibit binding of the non-hydrated form of glyoxylate to the enzyme and thereby promote the oxidation of the hydrated form of glyoxylate and thus increase oxalate production.

REFERENCES

1 Romano, M. and Cerra, M (1969) Biochim Biophys Acta 177, 421–426
2 Warren, W A (1970) J Biol Chem 245, 1675–1681
3 Banner, M R and Rosalki, S B (1977) Nature 213, 726–727
4 Duncan, R J S and Tipton, K F (1969) Eur J Biochem 11, 58–61
5 Smith, L-L H and Williams, H E (1974) in Hereditable Disorders of Aminoacids Metabolism Nyham, W L , ed ), pp 343–358, John Wiley and Sons, London
6 Williams, H E and Smith, L-L H (1968) Am J Med 45, 715–735
7 Williams, H E and Smith, L-L H (1968) New Engl J Med 278, 233–239
8 Williams, H E and Smith, L-L H (1971) Science 171, 390–391
9 Lluis, C , Gubert, S and Bozal, J (1975) Rev Esp Fisiol 31, 223–234
10 Dixon, M and Webb, E C (1964) Enzymes, 2nd edn , pp 84–86, Longmans Green, London