Effect of pH on the Inhibition of the Nicotinamide–Adenine Dinucleotide-Specific Isocitrate Dehydrogenase from Baker's Yeast by Anions

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1. The sensitivity of the NAD⁺-specific isocitrate dehydrogenase from baker's yeast towards inhibition by anions decreases with decrease in pH. The patterns of the pH-dependence of the enzymic activity can be explained by this effect. 2. In the presence of a high isocitrate concentration, citrate, unlike AMP, has no antagonizing effect on the inhibition of the enzyme by anions. In the presence of AMP, citrate inhibits the enzyme at high isocitrate concentration and activates at low isocitrate concentration. 3. The effects on the enzymic activity of the previous incubation of the enzyme were studied in relation to the substrate concentration, the chloride concentration and the presence of citrate and AMP.

The NAD⁺-specific isocitrate dehydrogenase [*threo*-D₃-isocitrate–NAD⁺ oxidoreductase (decarboxylating), EC 1.1.1.41] from baker's yeast (Hathaway & Atkinson, 1963; Atkinson, Hathaway & Smith, 1965) and *Neurospora crassa* (Sanwal, Zink & Stachow, 1964; Sanwal, Stachow & Cook, 1965; Sanwal & Cook, 1966) is activated by AMP or citrate at low isocitrate concentrations, and it has been suggested that these effects may play a role in metabolic regulation (Hathaway & Atkinson, 1963; Sanwal *et al.* 1964). The enzyme does not obey 'normal' kinetics, since plots of reaction rate against substrate concentration yield sigmoidal curves. The kinetic behaviour of the yeast enzyme has been explained by a model involving the existence on the enzyme of many interacting binding sites for the reactants (Atkinson *et al.* 1965).

Cennamo, Montecuccoli & Bonaretti (1965, 1967*a*) have shown that some simple anions inhibit the enzyme from baker's yeast. The inhibition is competitive with isocitrate, and the 'activating' effect of AMP appears to be due to the antagonizing of this inhibition. The occurrence of a random mechanism for the binding of the reactants to the enzyme protein was suggested as a possible explanation of the anomalous kinetic behaviour of the yeast enzyme (Cennamo *et al.* 1967*a*).

The present paper deals chiefly with the effects of pH on the inhibition by anions. The activation by citrate and the effects of preincubation of the enzyme under different conditions are also reported. A preliminary account of part of this work has been published (Cennamo, Montecuccoli, Bonaretti & Razzoli, 1967*b*).

MATERIALS AND METHODS

Enzyme purification. The preparation used was the 'Cy eluate', purified about 40-fold from baker's yeast essentially by the method of Kornberg & Prier (1951), as previously reported (Cennamo *et al.* 1967*a*).

Enzyme assay. The enzymic activity was determined at 22° by measuring the rate of NAD⁺ reduction at 366 mμ with an Eppendorf photometer, in 10 mm.-light-path cuvettes. The final volume of the reaction mixtures was 3 ml.; unless otherwise specified, the reaction was started by the addition of the enzyme. Measurements of pH were carried out in the reaction mixtures immediately after the determination of the activity.

Materials. Trisodium *threo*-D₃L₃-isocitrate (Vickery, 1962) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The isocitrate concentrations given always refer to a single isomer. NAD⁺ and AMP were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Magnesium acetate was used to supply Mg²⁺ ions.

Kinetic data. The difference between the extinction readings taken 90 sec. and 30 sec. after the addition of the enzyme was assumed to represent the initial rate. The results of the kinetic experiments are plotted by the double-reciprocal method of Lineweaver & Burk (1934), or by the logarithmic method proposed by Monod, Changeux & Jacob (1963). By the latter method, the values of $\log[v/(V_{\max} - v)]$ are plotted against $\log s$ (v being the initial velocity, $V_{\max}$ the maximum velocity and s the substrate concentration). The slope, n , of the straight line obtained by this plot corresponds to the apparent order of the reaction with respect to the substrate.

RESULTS

Effect of pH on the inhibition by anions. Fig. 1 shows the effect of pH on the inhibition by increasing

Fig. 1. Effect of NaCl on the activity of the NAD⁺-specific isocitrate dehydrogenase from yeast, at different pH values. The reaction mixtures contained, in a final volume of 3 ml.: 1 mM-isocitrate, 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.04 ml. of 'C_γ eluate' (see the text) and imidazole-HCl or tris-HCl buffer (final concn. of chloride 27 mM). Imidazole buffers: ○, pH 6.3; ■, pH 6.8. Tris buffers: ●, pH 7.6; □, pH 8.3.

concentrations of sodium chloride. Imidazole-hydrochloric acid buffers were used at pH 6.3 and 6.8, and tris-hydrochloric acid buffers at pH 7.6 and 8.3. The buffers contained a constant amount of chloride anion, corresponding to 27 mM final concentration in the reaction mixtures, in the absence of added sodium chloride. The isocitrate concentration used (1 mM) was almost saturating except at pH 8.3. The results in Fig. 1 show that the sensitivity of the enzyme towards the inhibition by chloride decreases with pH; at pH 8.3, the chloride concentration contributed by the buffer appears to be already inhibitory. In consequence of this behaviour, whereas the enzymic activity decreases with pH when the chloride concentration is minimal, at higher chloride concentrations the lowering of pH leads to an increase in activity. Fig. 1 also shows that, at the lowest pH values,

Fig. 2. Plots of enzymic activity against isocitrate concentration at pH 6.3 (imidazole-HCl buffer, ○) and pH 7.6 (tris-HCl buffer, ●), in the absence of AMP (a) or in the presence of 1.67 mM-AMP (b). The reaction mixtures contained, in a final volume of 3 ml.: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.04 ml. of 'C_γ eluate' (see the text) and imidazole-HCl or tris-HCl buffer (final concn. of chloride 27 mM).

Fig. 3. Effect of pH on the enzymic activity in the presence of 3 mM-isocitrate (a) or 0.33 mM-isocitrate (b). The reaction mixtures contained, in a final volume of 3 ml.: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.04 ml. of 'C_γ eluate' (see the text) and imidazole-HCl (○, □, △, +) or tris-HCl (●, ■, ▲) buffer (final concn. of chloride 27 mM). (a) Curve A: no addition; curve B: 0.13 M-NaCl; curve C: 0.13 M-NaCl and 0.5 mM-AMP. (b) Curve A: no addition; curve B: 0.5 mM-AMP; curve C: 0.13 M-NaCl; curve D: 0.13 M-NaCl and 0.5 mM-AMP.

sodium chloride exhibits an activating effect when its concentration is increased before reaching the inhibitory value; such an effect had previously been observed at pH 7.6 and explained by an unspecific influence of the ionic strength (Cennamo *et al.* 1967a).

In Fig. 2(a), plots of activity against the concentration of isocitrate are shown. The experiments were carried out at pH 6.3 and 7.6 in the presence respectively of imidazole and tris buffers providing a fixed chloride concentration (27 mM). The maximal activity at pH 6.3 is about one-quarter of that at pH 7.6; however, at pH 6.3 the maximum is reached at a concentration of isocitrate (about 0.1 mM) at which no activity is observed at pH 7.6. As shown in Fig. 2(b), the same effect is also observed in the presence of AMP; in this case, the curves are displaced towards lower concentrations of isocitrate. At pH 6.3, in the presence of 1.67 mM-AMP, maximum activity is obtained at about 0.01 mM-isocitrate. These results can be explained by the influence of pH on the inhibition by anions.

In Fig. 3, plots of the enzymic activity against pH are shown. The experiments were carried out at two isocitrate concentrations and under different conditions, in the presence of imidazole-hydrochloric acid and tris-hydrochloric acid buffers containing a constant (27 mM) chloride concentration. In the experiments of Fig. 3(a) 3 mM-isocitrate was present. A comparison between curve A (no addition) and curve B (with 0.13 M-sodium chloride) shows that the inhibition is evident only at pH values above 7.3 and approaches completion above pH 8. Therefore the presence of sodium chloride decreases the pH optimum of the enzymic activity from about 8 to 7.2, producing a pH-activity curve very similar to that obtained at low (0.33 mM) isocitrate concentration in the absence of sodium chloride (curve A in Fig. 3b). In both cases the

addition of AMP produces pH-activity curves (curve C in Fig. 3a and curve B in Fig. 3b) similar to that obtained at high isocitrate concentration in the absence of sodium chloride. These results explain the observations by Hathaway & Atkinson (1963) that the pH optimum of the NAD⁺-specific isocitrate dehydrogenase from baker's yeast depends on the substrate concentration, and that an increase of isocitrate concentration or an addition of AMP leads to an increase of pH optimum. According to the present results, this behaviour is due to the influence of pH on the sensitivity of the enzyme to the inhibition by anions. The dependence of the enzymic activity on pH can be modified by the addition of salt independently of the substrate concentration. As shown in Fig. 3(b), in the presence of 0.33 mM-isocitrate, the addition of sodium chloride produces a further decrease of the pH optimum below 6.5, and AMP brings about an opposite effect in this case also (curves C and D).

It is noteworthy that, if the enzymic activity is modified by various other factors apart from the isocitrate and anion concentrations, such as the concentration of NAD⁺ or Mg²⁺, no comparable effect on the pH optimum is observed. As shown in Fig. 4(a), the activity is strongly affected when the NAD⁺ concentration is decreased from 0.33 mM to 0.17 mM in the presence of 3 mM-isocitrate (curve A), or when it is increased to 1 mM in the presence of 0.33 mM-isocitrate (curve B); however, there is no remarkable modification of the pH optimum in comparison with the curves obtained in the presence of 0.33 mM-NAD⁺ at the corresponding isocitrate concentrations (curve A in Fig. 3a and curve A in Fig. 3b respectively). Essentially the same result is obtained when the concentration of Mg²⁺ is lowered from 3.3 mM to 0.17 mM, at the two concentrations of isocitrate used (Fig. 4b). The decrease in activity above pH 8 in the presence of

Fig. 4. Effect of pH on the enzymic activity under different conditions. The reaction mixtures contained, in a final volume of 3 ml.: 0.04 ml. of 'Cy eluate' (see the text) and imidazole-HCl (○, □) or tris-HCl (●, ■) buffer (final concn. of chloride 27 mM). (a) Curve A: 3 mM-isocitrate, 0.17 mM-NAD⁺ and 3.3 mM-Mg²⁺; curve B: 0.33 mM-isocitrate, 1.0 mM-NAD⁺ and 3.3 mM-Mg²⁺. (b) Curve A: 3 mM-isocitrate, 0.17 mM-Mg²⁺ and 0.33 mM-NAD⁺; curve B: 0.33 mM-isocitrate, 0.17 mM-Mg²⁺ and 0.33 mM-NAD⁺.

3mm-isocitrate (curve *A*) is probably due to a decrease in the concentration of free Mg^{2+} by the formation of complexes with tris base (O'Sullivan & Perrin, 1964), which was present in increasing concentrations at increasing pH values.

Fig. 5 shows the kinetics with respect to isocitrate at pH 6.2 in the presence of 0.33mm- Mg^{2+} . The

Fig. 5. Logarithmic plot of initial rate against isocitrate concentration. The reaction mixtures contained in a final volume of 3ml.: 0.33mm-NAD⁺, 0.33mm- Mg^{2+} , 0.1ml. of 'C_γ eluate' (see the text) and 33mm-potassium phosphate buffer, pH 6.2. O, No addition; ●, 1.67mm-AMP. Reaction rates are expressed as $\Delta E_{366}/\text{min.}$, and isocitrate concentrations as molarities.

results are plotted by the logarithmic method; the apparent order of the reaction with respect to isocitrate is 2.5 in the absence of AMP and 1.3 in the presence of 1.67mm-AMP. This behaviour is similar to that observed at pH 7.6 (Cennamo *et al.* 1967a).

Effects of citrate. In Table 1, the effects of AMP and citrate on the inhibition of the enzyme by chloride at pH 6.2 and pH 7.6 are compared. The results show that AMP is able to counteract the inhibition at any substrate concentration. On the other hand, citrate, when present at a concentration (3mm) that is sufficient to enhance the enzymic activity at low substrate concentration in the absence or in the presence of added sodium chloride, had no effect on the inhibition induced by sodium chloride in the presence of 3mm-isocitrate. Considering that the inhibition by anions is kinetically competitive with isocitrate, the effect of citrate appears to be similar to that of a comparable concentration of isocitrate and is therefore evident when the substrate concentration is sufficiently low.

Citrate can also be an inhibitor of the enzyme (Hathaway & Atkinson, 1963). The experiments in Fig. 6 were carried out at pH 7.6. The inhibition by citrate is particularly evident in the presence of AMP (Fig. 6b), whereas in the absence of AMP the activating effect of citrate prevails (Fig. 6a). In the presence of AMP, citrate inhibits the enzyme at high substrate concentrations and activates it at low substrate concentrations. When the results of curve *B* in Fig. 6(b) are plotted by the logarithmic method, the order of the reaction with respect to isocitrate is calculated to be approx. 1.2.

Table 1. *Effects of citrate and AMP on the inhibition of the enzyme by sodium chloride*

All reaction mixtures contained, in a final volume of 3ml.: 0.33mm-NAD⁺, 3.3mm- Mg^{2+} , and tris-HCl buffer (final concn. of chloride 27mm), pH 7.6, or 33mm-potassium phosphate buffer, pH 6.2; 0.04ml. of 'C_γ eluate' (see the text) was used at pH 7.6, and 0.10ml. at pH 6.2.

pH	Additions	$10^3 \times$ Enzyme activity ($\Delta E_{366}/\text{min.}$) in the presence of		
		—	Citrate (3mm)	AMP (0.17mm)
7.6	Isocitrate (3mm)	36	36	35
	Isocitrate (3mm) + NaCl (0.10M)	19	18	35
	Isocitrate (3mm) + NaCl (0.20M)	2	1	33
	Isocitrate (0.33mm)	23	33	35
	Isocitrate (0.33mm) + NaCl (0.01M)	8	26	37
	Isocitrate (0.33mm) + NaCl (0.07M)	2	6	33
	Isocitrate (0.17mm)	7	27	33
6.2	Isocitrate (3mm)	30	32	28
	Isocitrate (3mm) + NaCl (0.33M)	15	15	25
	Isocitrate (3mm) + NaCl (0.40M)	5	4	24
	Isocitrate (0.33mm)	24	31	26
	Isocitrate (0.33mm) + NaCl (0.17M)	6	22	28
	Isocitrate (0.33mm) + NaCl (0.20M)	2	9	27
	Isocitrate (0.017mm)	3	16	24

Fig. 6. Double-reciprocal plots of initial rate against isocitrate concentration: effect of citrate in the absence of AMP (a) or in the presence of AMP (b). The reaction mixtures contained, in a final volume of 3 ml.: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.04 ml. of 'C₇ eluate' (see the text) and tris-HCl buffer, pH 7.6 (final concn. of chloride 27 mM). (a) Curve A: no addition; curve B: 3 mM-citrate. (b) Curve A: 1.67 mM-AMP; curve B: 1.67 mM-AMP and 3 mM-citrate. Reaction rates are expressed as $10 \times \Delta E_{366}/\text{min}$.

Effects of preincubation of the enzyme. In the experiments reported in Fig. 7, the enzyme was incubated for various times up to 10 min. under different experimental conditions before the beginning of the reaction. In mixtures that were not incubated the reaction was started by addition of the enzyme. In the incubated mixtures, NAD⁺ and Mg²⁺ were present during incubation unless otherwise specified, and the reaction was started with isocitrate.

When a high (3 mM) isocitrate concentration is used for assay, the enzymic activity is only slightly decreased by preincubation of the enzyme in the absence of isocitrate (curve A). On the other hand, a marked decrease of the activity is observed when isocitrate is added after 5–10 min. of incubation at a low (0.33 mM) final concentration (curve H).

However, the decrease of the enzymic activity by preincubation is also obtained at high (3 mM) isocitrate concentration in the presence of sodium chloride; a decrease is observed not only at an inhibitory concentration of sodium chloride (0.13 M; curve G) but also at a final concentration (0.05 M) that is insufficient to inhibit the enzyme in the non-incubated mixture (curve B). Curves similar to curve B are obtained when the three compounds taking part in the enzymic reaction (isocitrate, NAD⁺ and Mg²⁺) are added together after the incubation, or when they are present during the incubation either individually or in all possible combinations of two; e.g., in the experiment of curve C, isocitrate and Mg²⁺ were present during

the incubation with sodium chloride (in this case, the reaction was started with NAD⁺). The decrease of activity induced by incubation with sodium chloride is also observed if citrate is present, together with NAD⁺ and Mg²⁺, during the incubation (curve D). AMP, however, prevents the decrease of the activity when present during the preincubation or even when added together with isocitrate at the end of the incubation period (curves E and F).

The activities reported in Fig. 7 were initial activities, measured by the increase of the extinction at 366 mμ between 30 sec. and 90 sec. after the reaction was started. In Fig. 8, the time-course of the reaction, followed by the increase in the extinction at 366 mμ, is reported for two reaction mixtures previously incubated for 5 min. in the absence or in the presence of 0.05 M-sodium chloride. The reaction was started by the addition of 3 mM-isocitrate. In the mixture previously incubated without sodium chloride the activity decreased with time as usual, whereas in the mixture incubated with sodium chloride the activity increased at first so that after about 3 min. the reaction velocity was about the same in both mixtures. It appears therefore that the decrease in the enzymic activity that is slowly induced by incubation with sodium chloride, and can be demonstrated by measuring the initial velocity, is also slowly reversed during the reaction in the presence of a suitable substrate concentration. On the other hand, the restoration

Fig. 7. Effect of preincubation on the enzymic activity, under different conditions. Samples (0.04 ml.) of 'C_γ eluate' (see the text) were incubated at 22° for the indicated times in a volume near to 3 ml., in the presence of tris-HCl buffer, pH 7.6 (final concn. of chloride 27 mM), and the following additions: curves A and H: 0.33 mM-NAD⁺ and 3.3 mM-Mg²⁺; curves B and F: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺ and 0.05 M-NaCl; curve C: 3 mM-isocitrate, 3.3 mM-Mg²⁺ and 0.05 M-NaCl; curve D: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.05 M-NaCl and 3 mM-citrate; curve E: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺, 0.05 M-NaCl and 0.33 mM-AMP; curve G: 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺ and 0.13 M-NaCl. The reaction was started with the following additions (final volume, 3 ml.): curves A, B, D, E and G: 3 mM-isocitrate; curve C: 0.33 mM-NAD⁺; curve F: 3 mM-isocitrate and 0.33 mM-AMP; curve H: 0.33 mM-isocitrate. Curves E and F coincide. In the non-incubated mixtures (zero incubation time) the reaction was started with enzyme.

of the initial activity induced by AMP, after incubation with sodium chloride, appears to be rapid (curve F in Fig. 7).

The decrease in the activity at a low isocitrate concentration induced by preincubation of the enzyme was previously noticed (Cennamo *et al.* 1967a) and the possibility was considered that the effect was due to inactivation of the enzyme by Mg²⁺. According to the present results, it appears likely that in this case the low chloride concentration provided by the buffer is sufficient to induce a remarkable decrease in the activity during preincubation.

DISCUSSION

Hathaway & Atkinson (1963) showed that the AMP-induced activation of the NAD⁺-specific isocitrate dehydrogenase from baker's yeast, reported by Kornberg & Pricer (1951), can be

Fig. 8. Increase of the E_{366} during the enzymic reaction, which was started after previous incubation of the enzyme in the absence of NaCl (curve A) or in the presence of 0.05 M-NaCl (curve B). Both mixtures contained, in a volume near to 3 ml.: 0.04 ml. of 'C_γ eluate' (see the text), 0.33 mM-NAD⁺, 3.3 mM-Mg²⁺ and tris-HCl buffer, pH 7.6 (final concn. of chloride 27 mM). After 5 min. at 22°, the reaction was started by the addition of 3 mM-isocitrate (final volume 3 ml.).

observed at low isocitrate concentrations only. According to previous work from our Laboratories (Cennamo *et al.* 1967a), the effect of AMP appears to be due to antagonizing of the inhibition of the enzyme caused by some simple anions. Since this inhibition is competitive with isocitrate, the occurrence of the AMP effect only at low isocitrate concentrations is understandable. The present work indicates that some aspects of the pH-dependence of the enzymic activity can be similarly explained by the inhibition of the enzyme by anions, since the sensitivity of the enzyme to this inhibition appears to decrease at low pH values. This behaviour explains the dependence of the pH optimum on the isocitrate concentration and the higher affinity for isocitrate at low pH values, reported by Hathaway & Atkinson (1963), and accounts for the opposite effects on the enzymic activity observed by varying the pH under different experimental conditions. The experiments of Figs. 1 and 2 indicate that a decrease in pH may lead to a decrease in the enzymic activity at high isocitrate concentration and low anion concentration, whereas the opposite effect is observed under the reverse conditions.

It is possible that NAD⁺-specific isocitrate dehydrogenases from other sources behave like the yeast enzyme. Cox & Davies (1967) have shown that the enzyme from pea mitochondria is also subject to anion inhibition. The occurrence of this inhibition might explain the results of Klingenberg, Goebell & Wenske (1965), who have extensively studied the influence of pH on NAD⁺-specific isocitrate dehydrogenase from animal tissues; in this case, also, the pH-dependence of the enzymic activity is influenced by the isocitrate concentration. Sanwal *et al.* (1965) and Sanwal & Cook (1966), working with the enzyme from *Neurospora crassa*, observed that 'normal' kinetics with respect to isocitrate are obtained in the presence of AMP at pH 6.5 but not at pH 7.5, and suggested that an allosteric site binding isocitrate is inactive in the presence of AMP at the lower pH. This behaviour could, however, also be due to the lower sensitivity of the enzyme towards anion inhibition at the lower pH value. With the yeast enzyme, the present and the previous results (Cennamo *et al.* 1967a) indicate that AMP has a nearly-normalizing effect on the kinetics at pH 6.2, as well as at pH 7.6.

The peculiar behaviour of the NAD⁺-specific isocitrate dehydrogenase has been interpreted by different models (Atkinson *et al.* 1965; Sanwal & Cook, 1966; Klingenberg *et al.* 1965), all of which assume the existence on the enzyme protein of several interacting binding sites at least for some of the compounds participating to the reaction. However, other explanations appear to be possible. Cennamo *et al.* (1967a) suggested that the occurrence of alternative reaction pathways could explain the kinetic behaviour of the yeast enzyme. A theoretical appraisal of such a mechanism was presented by Ferdinand (1966), and was discussed in relation to the kinetics of some regulatory enzymes, particularly phosphofructokinase. Further, effects such as those observed with regulatory enzymes seem possible according to a kinetic model (Rabin, 1967), related to the induced-fit hypothesis of Koshland (1964), which postulates the existence of many conformations of the active site of the enzyme protein.

On the other hand, the enzyme-incubation experiments presented here raise some doubt on the correspondence of the initial-rate measurements to true steady-state conditions, and therefore on the validity of kinetic studies with this enzyme, particularly at low substrate concentrations and in the presence of relatively high anion concentrations.

Hathaway & Atkinson (1963) proposed that the activation of the NAD⁺-specific isocitrate dehydrogenase by AMP might regulate the tri-carboxylic acid cycle in response to metabolic demand for ATP. In this respect, the inhibition of

the enzyme by anions could be considered as a 'background sensitization' providing the basis for the modulating effect of AMP. Bernofsky & Utter (1966) have shown that the AMP effect is also demonstrable with yeast mitochondria. Therefore it appears probable that the effects observed *in vitro* reflect the conditions existing *in vivo*; it has been reported that the internal pH of the yeast cell is about 6 (Eddy, 1958) and (Fig. 2) at this pH the maximum velocity of the reaction is lower than at pH values near 8, but is reached at lower concentrations of isocitrate. On the other hand, changes in the intracellular pH could induce wide modifications in the enzymic activity. Another regulatory effect might be exhibited *in vivo* by citrate, which is essentially an activator of the reaction at low substrate concentration; a role of 'precursor activation' was attributed by Sanwal, Zink & Stachow (1963) to this effect with the enzyme from *Neurospora crassa*. The kinetics of the reaction are also influenced *in vitro* by the nature of the metal ion activator; it has been observed (Cennamo, Montecuccoli & Bonaretti, 1966) that 'normal' kinetics are obtained at pH 7.6, in the presence of AMP, when Zn²⁺ is used instead of Mg²⁺. Synergic effects between different metal ions have also been shown (Cennamo *et al.* 1965); these effects have been observed at low substrate concentrations, and could play a role *in vivo*.

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