Calculation of half-lives, τ_n, using eqn. (5) and $k = 2.5 \times 10^{-5}$ (min^{-1}) determined experimentally by GRIBNAU and TESSER[2]

n	τ_n (days) Consecutive order model[2]	Random order model
1	19.16	19.16
2	46.67	34.17
3	74.67	43.89
4	101.94	51.11
5	129.72	56.67
6	157.50	61.67

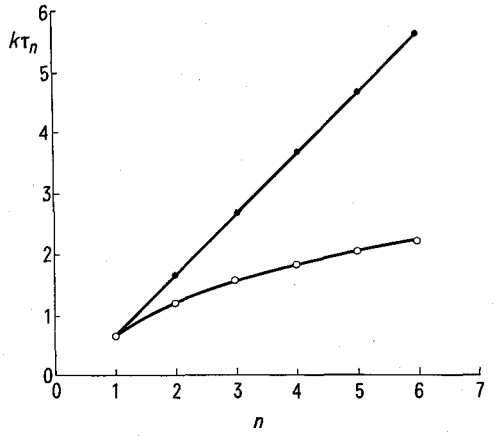

Fig. 2. Comparison of the $k\tau_n$ values computed from the consecutive order model[2] (●—●) and the random order model (○—○).

The time course of ligand release is computed from eqn. (4) (Figure 1). The shapes of these curves are qualitatively the same as in the GRIBNAU/TESSER model, although the analytical expression of the leakage-function is different (cf. eqn. (4)).

$k\tau_n$ values were computed from eqn. (5) using the iterative procedure of Newton and Raphson. They are compared with the values calculated by GRIBNAU and TESSER (Figure 2, Table). It is especially noteworthy that the increase of the $k\tau_n$ values with increasing n is not so steep as in the GRIBNAU/TESSER model. The interpretation of this somewhat surprizing result is that the stability gained by an additional point of attachment is partially offset by an increased probability of cleavage of a ligand-matrix bond. For $n = 1$, both models must yield the same $k\tau_1$. This condition is fulfilled by the randon model presented here (Figure 2).

Summary. A leakage function describing the hydrolytic release of ligand molecules covalently attached to insoluble supports by the CNBr method has been derived. Statistical factors were taken into account. The results of this random order model are compared with those of a consecutive order model proposed by GRIBNAU and TESSER.

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On the Role of Divalent Cations in the Reaction Mechanism of Malic Enzyme

NADP-linked malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) is known to require a divalent cation for activity[1]. Usually Mn^{2+} or Mg^{2+} are the better activators, but other cations, such as Co^{2+} and Ni^{2+}, are often able to replace Mn^{2+} or Mg^{2+}, at least to some extent[2].

We report here the results of some kinetic experiments on the activation of the NADP-linked malic enzyme partially purified from a marine Pseudomonad[3] by divalent cations.

The malic enzyme from the marine Pseudomonad was activated by several divalent cations. Mn^{2+}, Mg^{2+} and Co^{2+} were considerably more effective than Cd^{2+} and Ni^{2+}. When experiments with varying concentrations of divalent cation at fixed concentrations of the substrates L-malate (1 mM) and NADP (0.3 mM) were performed, the apparent V_{max} values obtained for the activation by Mn^{2+} and Mg^{2+} were similar, but the value for Co^{2+} was about half. The apparent K_a values were about 10^{-6} M, 2×10^{-6} M and 8×10^{-5} M for Co^{2+}, Mn^{2+} and Mg^{2+}, respectively.

The nature of the divalent cation used as activator affected the apparent kinetic constants for the substrates. Figure 1 shows the double reciprocal plots for the substrate L-malate obtained in the presence of 1 mM $MnCl_2$, $MgCl_2$ or $CoCl_2$. Substrate inhibition, previously reported for malic enzyme from other microorganisms[2], was clearly observed in the presence of Co^{2+} or Mn^{2+}, but not in the presence of Mg^{2+}. The apparent K_m values for L-malate obtained from the data of Figure 1 were 31,100 and 179 μM, in the presence of Co^{2+}, Mn^{2+} or Mg^{2+}, respectively. The apparent V_{max} value obtained in the presence of Co^{2+} was, however, considerably lower than those attained in the presence of Mn^{2+} or Mg^{2+} (Figure 1). The apparent K_m for NADP (not shown in the Figure) showed less variation with the nature of the divalent cation; under similar experimental conditions (1 mM L-malate) the values were 17, 24 and 22 μM, in the presence of Co^{2+}, Mn^{2+} or Mg^{2+}, respectively.

L-malate is known to be able to form complexes with divalent cations; the stability constant for the L-malate-Mn complex is greater than that for the L-malate-Mg complex[4]. The stability of the L-malate-Co complex might be expected to be of the same order or greater than that for L-malate-Mn, considering the usual order of effectiveness of the divalent cations to form complexes with organic ligands[4]. Two main roles for the divalent cation in the reaction mechanism of malic enzyme seem possible, both involving the ability of L-malate to form complexes with divalent cations. First, the L-malate-Me complex might be the true substrate of the reaction, as in the case of the $MeATP^{2-}$ complex for the kinases[5]; second, free cation might bind to the enzyme, and then act as a link between

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the active site and L-malate. The latter possibility is implicit in the reaction mechanism proposed by HSU et al.[6]. Experiments performed with the malic enzyme from *Mycobacterium*[2] suggested that the first mechanism mentioned was not followed, and favored the direct binding of cation to the enzyme. Furthermore, PARVIN et al.[2] suggested that the substrate inhibition by excess L-malate, also observed in the case of the enzyme from *Pseudomonas* (Figure 1), was due to complexation by excess substrate of the free cation required as activator. The crystalline enzyme from pigeon liver binds Mn^{2+} in the absence of other ligands[6]; the enzyme from *Pseudomonas* seems to be able to bind Mg^{2+} independently of the presence of the other reactants, since we have found partial protection of the enzyme against thermal inactivation at 70 °C by 1.3 m*M* $MgCl_2$ in the absence of other ligands (MASSARINI and CAZZULO, unpublished experiments).

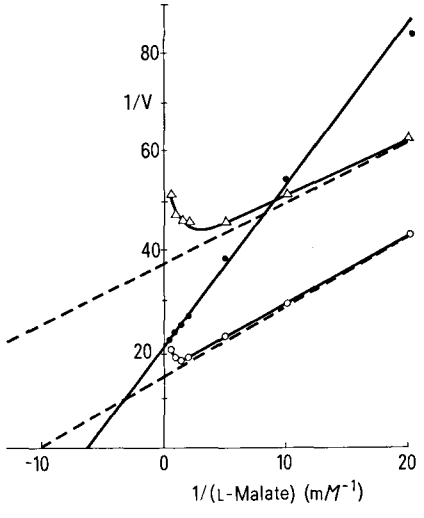

Fig. 1. Effect of the nature of the divalent cation activator on the saturation curve for L-malate. The reaction mixtures contained (in μmoles) in a final volume of 1 ml: *Tris*-HCl buffer (pH 7.6), 100; NH_4Cl, 20; NADP, 0.3; $MnCl_2$ (○), $MgCl_2$ (●) or $CoCl_2$ (△), 1; 0.4 μg of protein, and L-malate as stated (in reciprocal scale) on the abcissa. The reaction was followed as the increase in absorbance at 340 nm in a UNICAM SP 1800 B recording spectrophotometer at 30 °C. 150-fold purified malic enzyme[3] was used; further purification was prevented by the instability of the highly purified enzyme. The enzyme preparations were exhaustively dialyzed before use against 50 m*M* *Tris*-HCl buffer (pH 7.6) containing 0.1 m*M* EDTA. When EDTA was omitted, the enzyme presented, in the absence of added divalent cation, an activity equal to about 50% of the activity attained in the presence of 2 m*M* $MnCl_2$. The apparent K_m values for L-malate in the presence of Co^{2+} or Mn^{2+} were obtained from the asymptotes (dotted lines), according to CLELAND[7].

We studied the order of addition of Mg^{2+} and the substrates of the reaction in the kinetic experiments shown in Figures 2 and 3. When L-malate was the variable substrate, at different fixed levels of $MgCl_2$ (Figure 2A), the equilibrium ordered initial velocity pattern[7] was obtained, i.e.: saturation with L-malate appeared to eliminate the requirement for the divalent cation, since the same V_{max} was obtained irrespective of the $MgCl_2$ concentration. When the results were plotted as a function of the reciprocal $MgCl_2$ concentration (Figure 2B), the lines intersected to the left of the ordinate; the secondary plot of the slopes as a function of the reciprocal L-malate concentration (Figure 2C) was a straight line through the origin. This demonstrates that the addition of Mg^{2+} and L-malate to the malic enzyme from *Pseudomonas* is ordered; the cation must be the first to bind, and cannot leave the active site once L-malate is also bound[7]. This strongly suggests that the cation acts as a link between L-malate and the active site. Similar experiments were not performed with Mn^{2+} or Co^{2+}, because of the problems arizing from the low EDTA concentration (0.5–1.0 μ*M*) necessarily present (see legend to Figure 1), which interfered with the very low Mn^{2+} or Co^{2+} concentrations able to elicit activation.

When the concentrations of NADP and $MgCl_2$ were varied in the presence of a fixed concentration of L-malate, the double reciprocal plots for NADP (Figure 3A) or for $MgCl_2$ (Figure 3B) intersected to the left of the ordinate, and the secondary plot of slopes as a function of the reciprocal NADP concentration (Figure 3C) had a finite intersection, as is the normal case[7]. These results suggest that the addition of Mg^{2+} and NADP to the malic enzyme from *Pseudomonas* did not follow an obligatory order, being probably random. This is in good agreement with the demonstration by HSU and LARDY[8] that the pigeon liver enzyme binds NADP or NADPH in the absence of other ligands. L-malate, on the other hand, can

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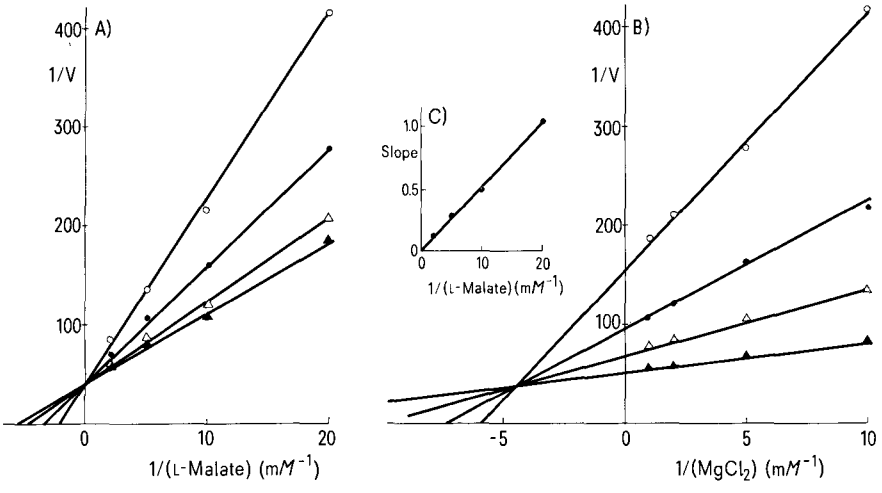

Fig. 2. Effect of the variation of the concentrations of L-malate and $MgCl_2$ on the reaction catalyzed by malic enzyme. The experimental conditions were similar to those described in the legend to Figure 1, except for the concentrations of L-malate and $MgCl_2$. A) effect of variable concentrations of L-malate, in the presence of 0.1 (○), 0.2 (●), 0.5 (△) or 1.0 (▲) m*M* $MgCl_2$. B) effect of variable concentrations of $MgCl_2$, in the presence of 0.05 (○), 0.1 (●), 0.2 (△) or 0.5 (▲) m*M* L-malate. C), secondary plot of the slopes from the lines in Figure B, as a function of the reciprocal concentration of L-malate.

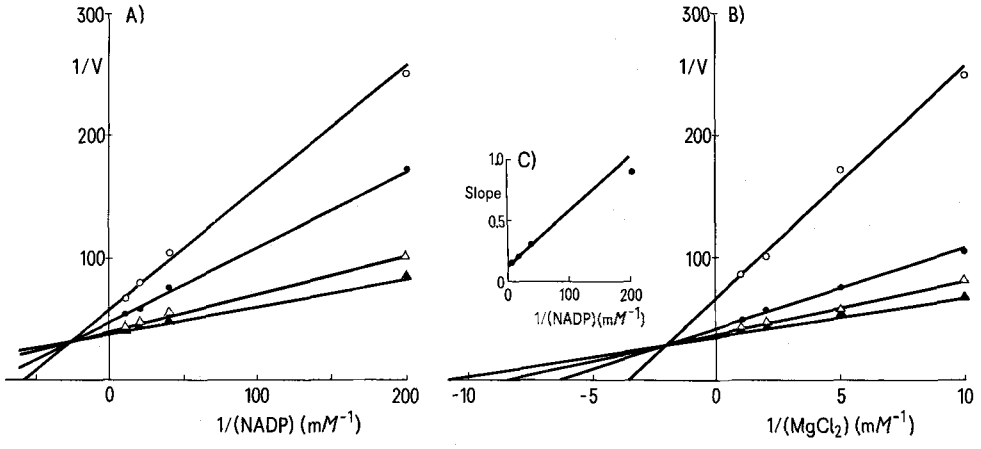

Fig. 3. Effect of the variation of the concentration of NADP and $MgCl_2$ on the velocity of the reaction catalyzed by malic enzyme. The experimental conditions were similar to those described in the legend to Figure 1, with 1 m*M* L-malate and the concentrations of NADP and $MgCl_2$ stated below. A) effect of variable concentrations of NADP, in the presence of 0.1 (○), 0.2 (●), 0.5 (△) or 1.0 (▲) m*M* $MgCl_2$ B) effect of variable concentrations of $MgCl_2$ in the presence of 0.005 (○), 0.025 (●), 0.05 (△) or 0.1 (▲) m*M* NADP. C) secondary plot of the slopes from the lines in Figure B as a function of the reciprocal concentration of NADP.

bind only after NADP is bound to the enzyme, to form the quaternary complex which undergoes reaction[6].

Assuming that the obligatory order of addition of L-malate after NADP also holds for the enzyme from *Pseudomonas*, we can propose that the ternary complex NADP-enzyme-Mg^{2+} reacts with L-malate through the metal ion, which acts as a link between L-malate and the active site. The different effectiveness of Co^{2+}, Mn^{2+} and Mg^{2+} as activators, and the different apparent K_m values for L-malate obtained in the presence of the same activators, could be explained on the basis of a decreasing ability of Co^{2+}, Mn^{2+} and Mg^{2+} to form the quaternary complex according to the Irving-Williams series[4] for the formation of complexes between divalent cations and organic ligands.

Summary. The kinetic order of addition of Mg^{2+} and L-malate to malic enzyme has been determined. Mg^{2+} is the first to bind, and probably acts as a link between the substrate and the active site.

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20 May 1975.

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[9] This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina, and the Consejo de Investigaciones de la Universidad Nacional de Rosario. JJC and EM are members of the Carrera del Investigador Científico of the former and the latter institutions, respectively.

Conserved Message of *o*-Diphenolase in Wheat Embryos (*Triticum aestivum*)

Our preliminary observations[1] indicated that the stimulation of *o*-diphenolase activity in wheat embryos, excized from germinating grains, is strongly inhibited by cycloheximide but not by actinomycin D. The occurrence of stable messengers capable of supporting general protein synthesis is reported in the ungerminated wheat embryos[2,3]. Other workers, however, observed the early synthesis of mRNA in wheat embryos which is not converted into stable message[4,5]. At present, information is not available about the nature of specific enzyme proteins whose translation is supported by conserved or newly formed messages in wheat embryos. In the present report, we provide evidence for the existence of a long-lived stable messenger of *o*-diphenolase in excized wheat embryos which supports the de novo synthesis of *o*-diphenolase enzyme under conditions of inhibited RNA synthesis.

Materials and methods. Embryos were excized from wheat grains (*Triticum aestivum*, var. Shera) presoaked for 10 h at 4 °C and sterilized with 0.02% mercuric chloride. The excized embryos were germinated in the dark at 25 °C on nutrient medium[6,7] containing 50 µg/ml of chloramphenicol. 40 embryos were homogenized in 0.05 *M* phosphate buffer (pH 6.6) and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant (crude extract) was employed for measuring *o*-diphenolase activity by following the procedure of WONG et al.[8]. One unit of enzyme activity is defined as the amount of enzyme which brings about a change in absorbance of

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