The effects of bivalent cations on ribulose biphosphate carboxylase/oxygenase

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The half-saturation constants for binding of the bivalent cations (Mg^{2+} , Ni^{2+} , Co^{2+} , Fe^{2+} and Mn^{2+}) to ribulose biphosphate carboxylase/oxygenase from *Glycine max* and *Rhodospirillum rubrum* were measured. The values obtained were dependent on the enzyme and the cation present, but were the same for both oxygenase and carboxylase activities. Ribulose biphosphate rather than its cation complex was the true substrate. The kinetic parameters $V_{max.(CO_2)}$, $V_{max.(O_2)}$, $K_{m(CO_2)}$, $K_{m(O_2)}$, and $K_{i(O_2)}$ were determined for both enzymes and each cation activator. The evolutionary and mechanistic implications of these data are discussed.

Ribulose biphosphate carboxylase/oxygenase (EC 4.1.1.39) from all sources examined to date catalyses two separate reactions: the carboxylation of ribulose biphosphate by CO_2 and the oxygenation of ribulose biphosphate by O_2 . These reactions are competitive (Laing *et al.*, 1974). A bivalent cation has been shown to be essential for both reactions of the higher-plant enzyme (Weissbach *et al.*, 1956; Andrews *et al.*, 1973) and the *Rhodospirillum rubrum* enzyme (Akazawa *et al.*, 1970; Ryan *et al.*, 1974).

Christeller & Laing (1979) showed that the Michaelis constants for the two gaseous substrates are different in the presence of Mg^{2+} ions compared with Mn^{2+} ions. Wildner & Henkel (1978) and subsequently Robison *et al.* (1979) have attributed differential effects on maximum velocities to changes in the half-saturation constants for the different reactions. This discrepancy is accounted for, and the study of Michaelis constants and V_{max} effects is extended to cover the known range of bivalent cations which catalyse the reactions.

Materials and methods

Enzyme purification

Ribulose biphosphate carboxylase was isolated from soya-bean (*Glycine max* var. Amsoy) leaves as described by Laing & Christeller (1976). *Rhodospirillum rubrum* (N.C.I.B. 8255) was grown as described by Tabita & McFadden (1974) or cultured serially on 2.5% CO_2 in H_2 (Schloss *et al.*, 1979). *R. rubrum* carboxylase was purified as previously described (Christeller & Laing, 1978), as were

glycolate oxidase (EC 1.1.3.1) and phosphoglycolate phosphatase (EC 3.1.3.18) (Christeller & Laing, 1979). Ribulose biphosphate carboxylase was stored at 2°C as a suspension in a 360 g/litre $(NH_4)_2SO_4$ solution and discarded after 1 month.

Assay of activity

The assays to measure the half-saturation constants were performed in scintillation vials previously flushed with N_2 . The enzyme was incubated for 3–10 min under saturating bicarbonate and variable cation concentrations to allow complete activation before initiating the 30 s assay with ribulose biphosphate. No significant loss of CO_2 to the gas phase occurred under these conditions.

The assays and data analysis of carboxylase and oxygenase activities for determination of V_{max} , $K_{m(CO_2)}$, $K_{m(O_2)}$ and $K_{i(O_2)}$ were performed as described by Christeller & Laing (1979). Because Fe^{2+} is rapidly oxidized in solution by O_2 under assay conditions, it was not possible to obtain meaningful parameter values for oxygenase activity. Measurements of carboxylase activity with Fe^{2+} were performed in the presence of 2.5 mM-sodium dithionite as an added precaution against the presence of O_2 . Sodium dithionite at 2.5 mM had no effect on carboxylase activity. As described for oxygenase assays in the presence of Mn^{2+} (Christeller & Laing, 1979), it was also necessary to add excess EDTA to the assay after the addition of acid when Ni^{2+} or Co^{2+} was the activating cation. This prevented formation of a high background colour in the subsequent phosphoglycollate determination. Other assay details are given in the Figure legends.

Ribulose biphosphate was synthesized (Horecker *et al.*, 1958) and further purified (Laing & Christeller, 1976).

Results and discussion

Cation specificity of soya-bean ribulose biphosphate carboxylase

Weissbach *et al.* (1956) showed that Mg^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} and Ni^{2+} were all activators of ribulose biphosphate carboxylase from spinach. As these assays were performed in air, a reinvestigation of specificity was carried out under an N_2 atmosphere, because Christeller & Laing (1979) demonstrated extreme sensitivity of Mn^{2+} -catalysed carboxylase activity to oxygen concentration. No additional activators were found among those tested, i.e. Zn^{2+} , Ca^{2+} , Pd^{2+} , Cd^{2+} , Hg^{2+} , Ba^{2+} , Cu^{2+} and Y^{3+} . Subsequent studies were therefore confined to Mg^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} and Ni^{2+} .

Interaction between cation and enzyme

Determination of the true half-saturation constant is complicated by two factors: the dual role in both activation and catalysis and the presence of chelating agents other than enzyme being present. Laing & Christeller (1976) showed that the correct expression for measurement of activity was:

$$n_e \propto \frac{E_t \cdot [CO_2][Me^{2+}]}{[CO_2][Me^{2+}] + K_c K_a} \quad (1)$$

where n_e , E_t and K_c are defined in the original reference, and Me^{2+} is the metal ion. Under conditions of assay where the activation state is not altered, i.e. preincubation and assay at the same cation concentration and assay times of 60 s or less, hyperbolic

kinetics are obtained and an observed binding constant can be calculated from the expression

$$n_e \propto \frac{E_t \cdot [Me^{2+}]}{[Me^{2+}] + K_a'} \quad (2)$$

Inspection of (1) and (2) shows that

$$K_a' = \frac{K_c \cdot K_a}{[CO_2]} \quad (3)$$

and the apparent binding constant (K_a) can be evaluated, since both K_c for the soya-bean (Laing & Christeller, 1976) and the *R. rubrum* (Christeller & Laing, 1978) enzyme and the CO_2 concentration in solution are known. Secondly, only the chelation to ribulose biphosphate needs to be considered, since protein concentration is negligible and cation-bicarbonate association constants are small (Sillen & Martell, 1964). The free cation concentration at half-maximum velocity (K_1) is calculated from the known total ribulose biphosphate and cation concentrations and an approximate apparent binding constant. The constant used was that obtained with fructose biphosphate as a substrate analogue. This appears to be justified, despite conformational differences between fructose biphosphate and ribulose biphosphate, as the values obtained (see Table 1) were of identical ranking and very similar to those of 2-phosphoglycerate (Sillen & Martell, 1964). Thus phosphate-phosphate distances appear to be of little importance, and use of fructose biphosphate values should not influence conclusions. The values for the observed binding constant (K_a'), the apparent binding constants (corrected for CO_2 activation) (K_a) and the half-saturation constant (corrected for chelation) (K_1) are shown in Table 1. The latter values closely follow the inverse order of stability

Table 1. Cation-binding constants for bivalent-cation activation of ribulose biphosphate carboxylase from soya-bean and *R. rubrum*

The data are the results of a typical single experiment obtained by fitting the individual data points for at least seven cation concentrations in triplicate. The enzyme assay and analysis are described in the Materials and methods section. The definitions of the three constants and the relationships between them are described in the text. Cation association constants used were: Mn^{2+} , $1883 M^{-1}$; Fe^{2+} , $1132 M^{-1}$; Co^{2+} , $1090 M^{-1}$; Ni^{2+} , $882 M^{-1}$; Mg^{2+} , $383 M^{-1}$ (J. T. Christeller, unpublished work).

Enzyme	Cation	Cation-binding constants (mM)		
		K_a'	K_a	K_1
Soya-bean	Mn^{2+}	0.018 ± 0.003	0.057 ± 0.010	0.022 ± 0.004
	Fe^{2+}	0.063 ± 0.010	0.158 ± 0.026	0.080 ± 0.013
	Co^{2+}	0.024 ± 0.003	0.075 ± 0.011	0.039 ± 0.006
	Ni^{2+}	0.181 ± 0.037	0.560 ± 0.12	0.350 ± 0.072
	Mg^{2+}	0.342 ± 0.027	1.065 ± 0.084	0.843 ± 0.067
<i>R. rubrum</i>	Mn^{2+}	0.060 ± 0.009	0.028 ± 0.004	0.014 ± 0.002
	Fe^{2+}	0.111 ± 0.020	0.208 ± 0.038	0.107 ± 0.020
	Mg^{2+}	0.482 ± 0.050	0.225 ± 0.023	0.186 ± 0.019

to the Irving–Williams series, suggesting that the bivalent cation probably binds through a hard ligand such as oxygen.

Eqn. (3) predicts a very large effect of CO₂ concentration on the observed binding constant. Thus only when both carboxylase and oxygenase activities are assayed from a single reaction mixture is a direct comparison of their cation-saturation response possible. An experiment using soya-bean enzyme and Mg²⁺ is shown in Fig. 1. The observed constants for carboxylase are $K_a' = 1.06 \pm 0.07$ mM,

$V_{max}' = 0.539 \pm 0.011 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, and for oxygenase $K_a' = 1.00 \pm 0.15$ mM, $V_{max}' = 0.111 \pm 0.004 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. These values, allowing for [CO₂] and competitive inhibition by alternative substrates, correspond to K_a values of 0.98 mM and 0.93 mM and V_{max} values of 1.24 and 0.265 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for carboxylase and oxygenase activities respectively.

The bivalent cation appears to be required for two roles, satisfied by binding of a single cation per large subunit (Wishnick *et al.*, 1970; Mizziorko & Mildvan, 1974). Firstly, Mizziorko & Mildvan (1974) found that carboxyribitol biphosphate abolished the interaction between Mn²⁺ and HCO₃⁻ on the enzyme, suggesting a direct involvement of the cation at the active site. This direct role is also implicated by the data of Christeller & Laing (1979), where different cations altered the K_m values of the gaseous substrates. Secondly, O'Leary *et al.* (1979) showed that the cation has a role in stabilizing the CO₂ at the activating site. The mechanism responsible for this is not known, but because the activating CO₂ is bound before cation binding (Lorimer *et al.*, 1976) it is unlikely that the equilibrium constant (K_c) is affected by the nature of the cation present.

The data of Fig. 1 show that the half-saturation constant for Mg²⁺ is independent of the activity being measured. The observed K_a' does, however, depend strongly on the CO₂ concentration, and this largely accounts for reports (Wildner & Henkel, 1978; Robison *et al.*, 1979) of a different response to cation concentration for carboxylase compared with oxygenase activity. For example, for carboxylase Robison *et al.* (1979) calculated K_a' values of 0.15 mM for carboxylase and 4 mM for oxygenase for Mg²⁺ and the *R. rubrum* enzyme. Recalculation of their data gives K_a values of 0.175 mM for carboxylase and 0.138 mM for oxygenase activity in better agreement with Christeller & Laing (1978) (0.089 mM) and Table 1 (0.225 mM).

Thus it appears that although the cation may affect the relative affinities of O₂ and CO₂ for the active site (Christeller & Laing, 1979), the reverse situation does not occur. This also is consistent with evidence (Laing & Christeller, 1976) that the cation does not need to rebind with each catalytic cycle.

Fig. 1. Double-reciprocal plot of Mg²⁺ concentration against soya-bean ribulose biphosphate carboxylase and oxygenase activities

The reaction was carried out at 25°C in serum-capped 25 ml flasks equilibrated with 100% O₂ in the gas phase. The reaction mixture, containing 30 mM-Tris/HCl, pH 8.2, MgCl₂ as indicated, 6.75 mM-NaH¹⁴CO₃ (0.44 mCi/mmol) and 0.298 mg of enzyme in 0.97 ml, was incubated for 10 min. The assay was initiated with 30 μl of 10 mM-ribulose biphosphate and stopped after 30 s with 100 μl of 1 M-HCl. A sample (100 μl) was removed for radioactivity determination and the remainder analysed for phosphoglycollate. The data points (○, carboxylase; □, oxygenase) represent the means of three determinations and the lines are those fitted to the individual untransformed data.

Table 2. Effect of Mg²⁺ concentration on the apparent K_m for ribulose biphosphate and maximum velocity for soya-bean ribulose biphosphate carboxylase

The data were obtained from the experiment described in Fig. 2. Concentrations of free and complexed ribulose biphosphate (Rbu-P₂) were calculated by using a value for the dissociation constant of 2 mM for Mg²⁺–ribulose biphosphate.

	Total Mg ²⁺ added ...	10 mM	20 mM	30 mM
K_m for Rbu-P ₂ ⁴⁻ (μM)		10.5 ± 0.8	8.4 ± 0.8	9.2 ± 1.0
K_m for Mg-Rbu-P ₂ ²⁻ (μM)		53.0 ± 3.9	84.3 ± 8.0	139.0 ± 16.0
K_m for total Rbu-P ₂ (μM)		63.6 ± 4.6	92.7 ± 8.8	149.0 ± 17.0
V_{max} (μmol · min ⁻¹ · mg ⁻¹)		1.13 ± 0.02	1.10 ± 0.02	1.07 ± 0.03

Interaction between Mg^{2+} , ribulose biphosphate ($Rbu-P_2$) and enzyme

When different saturating concentrations of Mg^{2+} were present during the assay, the apparent K_m (total $Rbu-P_2$) increased with increasing Mg^{2+} concentration (Table 2). When the uncomplexed ribulose biphosphate concentration (predominantly $Rbu-P_2^{4-}$) was calculated, the K_m was independent of Mg^{2+} concentration (Fig. 2), and the K_m for ($Mg-Rbu-P_2$) also changed greatly (Table 2). The V_{max} was unaffected by Mg^{2+} , suggesting that the complex is not inhibitory to the carboxylase reaction.

The data confirm that Mg^{2+} and ribulose biphosphate bind separately to the enzyme. It has been shown, by a range of techniques, that the metal ion can bind to the enzyme in the presence of CO_2 and in the absence of ribulose biphosphate to activate the higher-plant enzyme (Lorimer *et al.*, 1976; Laing & Christeller, 1976; Wildner, 1976; Vater *et al.*, 1977; Grebanier *et al.*, 1978; O'Leary *et al.*, 1979), and ribulose biphosphate binds to the inactive enzyme (Laing & Christeller, 1976; Vater *et al.*, 1977). The assignment of these binding sites to the catalytic site is unproven. The separate binding of ribulose biphosphate and cation is consistent with evidence (Laing & Christeller, 1976) that the cation does not need to rebind with each catalytic cycle. The failure of $Mg-Rbu-P_2$ to bind at

the active site does not rule out a direct interaction between these species on the enzyme, but suggests that the nature of any interaction is different from that in solution.

Kinetic constants for soya-bean and *Rhodospirillum* enzyme

The values for the maximum velocities, Michaelis and inhibition constants for both carboxylase and oxygenase activities are shown in Table 3. The values obtained generally agree well with previous estimates where these are available (Akazawa *et al.*, 1978). The failure of Ni^{2+} to promote catalysis of either activity of the *R. rubrum* enzyme, and of Co^{2+} to promote carboxylase activity, confirms the results of Robison *et al.* (1979).

The ribulose biphosphate carboxylase/oxygenase enzymes from higher plants and from *R. rubrum* are quite distinct in structure (Kawashima & Wildman, 1970; McFadden, 1973) and in the environment under which they operate and have evolved. Since *R. rubrum* is an anaerobe and its enzyme has a simpler structure, it seems likely that this protein more closely resembles any common ancestral enzyme (McFadden & Tabita, 1974), and that the complexity of the higher-plant enzyme may be due to the stress imposed on plant growth in an atmosphere of changing O_2 and CO_2 concentrations (Badger, 1980).

Laing *et al.* (1974) showed that, since $K_{m(CO_2)} = K_{I(CO_2)}$ and $K_{m(O_2)} = K_{I(O_2)}$ for ribulose biphosphate carboxylase/oxygenase, the ratio of the two activities can be expressed in terms of the four kinetic parameters and two environmental variables:

$$\frac{v_{(O_2)}}{v_{(CO_2)}} = \frac{V_{max.(O_2)}}{V_{max.(CO_2)}} \cdot \frac{K_{m(CO_2)}}{K_{m(O_2)}} \cdot \frac{[O_2]}{[CO_2]}$$

It is probable that a decrease in this ratio would be a useful evolutionary strategy in air, provided that absolute values of $v_{m(CO_2)}$ in air were not decreased equally. By rewriting the net photosynthesis equation of Laing *et al.* (1974) as $P_N = v_{(CO_2)} \{1 - [t \cdot v_{(O_2)}/v_{(CO_2)}]\}$ and using their value of t (the proportion of glycolate released in photorespiration) = 0.25, the data in Table 3 illustrates the extent to which the physiological combination of higher-plant enzyme and Mg^{2+} has fulfilled this expectation. Assuming values in the chloroplast stroma for $[CO_2]$ of $11 \mu M$ and for $[O_2]$ of 21%, a ratio of $v_{(O_2)}/v_{(CO_2)} = 0.98$ is calculated. All other combinations of enzyme and cation give a ratio greater than 4, indicating that a net CO_2 -fixation rate could not be supported. Since a change in the ratio can be caused by changes in any or all parameters, it is of interest to note what changes have occurred when comparing the two enzymes, and to what extent the parameters may be interrelated.

Fig. 2. Effect of Mg^{2+} concentration on the apparent Michaelis constant for ribulose biphosphate of soya-bean ribulose biphosphate carboxylase

The enzyme was initially activated in 20 mM- $NaHCO_3$, 20 mM- $MgCl_2$ and 50 mM-Tris/HCl, pH 8.2, at $25^\circ C$ for 10 min. Activity was assayed by addition of $10 \mu l$ of enzyme ($15 \mu g$) to 20 mM- $NaH^{14}CO_3$ (0.67 mCi/mmol), ribulose biphosphate and Mg^{2+} as indicated, and 50 mM-Tris/HCl, pH 8.2, in 0.5 ml. Reaction was stopped after 30 s at $25^\circ C$ with 0.1 ml of 1 M-HCl. The data points represent the means of three values and the line represents the fit to the untransformed individual values. Ribulose biphosphate consumption was 10% or less. ●, 10 mM- $MgCl_2$; ○, 20 mM- $MgCl_2$; □, 30 mM- $MgCl_2$.

Table 3. Kinetic constants for soya-bean and *R. rubrum* ribulose biphosphate carboxylase/oxygenase with different cations

The values are the means $\pm$ S.E.M. for two to four separate determinations of the parameters. Storage, enzyme assays and data analysis are described in the Materials and methods section.

Parameter	Cation	Mn ²⁺	Fe ²⁺	Co ²⁺	Ni ²⁺	Mg ²⁺
$V_{\max.(\text{CO}_2)}$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)						
Soya-bean		0.30 $\pm$ 0.01	0.19 $\pm$ 0.05	0.18 $\pm$ 0.04	0.70 $\pm$ 0.17	1.65 $\pm$ 0.19
<i>R. rubrum</i>		1.43 $\pm$ 0.54	0.59 $\pm$ 0.08	0.00	0.00	3.44 $\pm$ 0.65
$V_{\max.(\text{O}_2)}$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)						
Soya-bean		0.29 $\pm$ 0.01	—	0.69 $\pm$ 0.11	0.15 $\pm$ 0.02	0.65 $\pm$ 0.06
<i>R. rubrum</i>		0.53 $\pm$ 0.02	—	0.53 $\pm$ 0.07	0.00	0.89 $\pm$ 0.04
$K_{\text{m}(\text{CO}_2)}$ (mM-HCO ₃ ⁻)						
Soya-bean		1.38 $\pm$ 0.38	1.17 $\pm$ 0.01	3.13 $\pm$ 0.63	3.96 $\pm$ 0.40	2.52 $\pm$ 0.57
<i>R. rubrum</i>		5.3 $\pm$ 2.8	4.1 $\pm$ 1.5	—	—	15.8 $\pm$ 2.4
$K_{\text{m}(\text{O}_2)}$ (% O ₂)						
Soya-bean		1.70 $\pm$ 0.21	—	15.2 $\pm$ 3.8	4.79 $\pm$ 3.37	35.4 $\pm$ 6.5
<i>R. rubrum</i>		11.5 $\pm$ 4.8	—	6.0 $\pm$ 3.8	—	24.2 $\pm$ 9.4
$K_{\text{I}(\text{O}_2)}$ (% O ₂)						
Soya-bean		1.00 $\pm$ 0.11	—	13.8 $\pm$ 3.4	4.10 $\pm$ 0.40	33.5 $\pm$ 3.0
<i>R. rubrum</i>		8.3 $\pm$ 3.1	—	—	—	24.1 $\pm$ 1.3

Table 4. Correlations between the kinetic parameters of ribulose biphosphate carboxylase/oxygenase

The parameter values from Table 3 were used to obtain the correlations. Correlations are non-significant (ns) or significant at the levels of: 5%, *, 1%, **, and 0.1%, ***, Correlations indicated by † were obtained with the data pair including a K_{m} value for Mg²⁺—soya-bean enzyme omitted.

Parameters	No. of observations	Correlation	Significance
$V_{\max.(\text{CO}_2)}$, $K_{\text{m}(\text{CO}_2)}$	8	0.900	**
$V_{\max.(\text{CO}_2)}$, $K_{\text{m}(\text{CO}_2)}$ †	7	0.979	***
$V_{\max.(\text{O}_2)}$, $K_{\text{m}(\text{O}_2)}$	7	0.692	ns
$V_{\max.(\text{O}_2)}$, $K_{\text{m}(\text{O}_2)}$ †	6	0.886	*
$V_{\max.(\text{CO}_2)}$, $V_{\max.(\text{O}_2)}$	7	0.613	ns
$K_{\text{m}(\text{CO}_2)}$, $K_{\text{m}(\text{O}_2)}$	6	0.332	ns
$K_{\text{m}(\text{CO}_2)}$, $K_{\text{m}(\text{O}_2)}$ †	5	0.910	*

When the two enzymes are compared in pairs with the cation as a common factor, certain features are apparent. In all cases but one when the $V_{\max.(\text{CO}_2)}$ or $V_{\max.(\text{O}_2)}$ increases, then the corresponding $K_{\text{m}(\text{CO}_2)}$ or $K_{\text{m}(\text{O}_2)}$ also increases. Similarly, in all cases but one, when one $V_{\max.}$ increases the other increases and when one K_{m} increases so does the other. Inspection of parameter values suggests that with Mg²⁺ the higher-plant $K_{\text{m}(\text{CO}_2)}$ and $K_{\text{m}(\text{O}_2)}$ values are the exceptional values, since the ratio of K_{m} values is anomalous, whereas the ratio of $V_{\max.}$ values is unexceptional. This perhaps explains why the $V_{\max.(\text{CO}_2)}$ has decreased in the higher-plant enzyme with the apparent ensuing disadvantage. The concomitant and greater decrease in $K_{\text{m}(\text{CO}_2)}$ not only gives an increased net velocity at atmospheric CO₂ concentrations, but also provides a competitive advantage over O₂. This competitive advantage is amplified by the increased $K_{\text{m}(\text{O}_2)}$.

When the parameter values are assessed together, the results (Table 4) generally support the conjecture that the parameters are closely linked. The correla-

tions imply firstly that $k_{\text{cat.}}$ is a major component of the rate constants determining the K_{m} for carboxylase and perhaps oxygenase activities. Secondly, the correlations are consistent with the two activities having the same rate-limiting step. Because both cation and gaseous substrate affect $V_{\max.}$, this step involves comparable forms of the quaternary complex. Similar conclusions have been reached by Badger & Collatz (1977) from the temperature-dependence of these kinetic parameters.

Other workers find a similar situation. Kung & Marsho (1976) and Rhodes *et al.* (1980) found no alteration in the $V_{\max.(\text{CO}_2)}/V_{\max.(\text{O}_2)}$ ratio within experimental limits for several mutants, cultivars, species and nuclear substitution lines of tobacco ribulose biphosphate carboxylase. Hall *et al.* (1978) attributed small variations in $V_{\max.}$ ratios to endogenous factors. Examples of changes in one parameter without alteration of another, however, have been described. Nelson & Surzycki (1976a) described a mutant of *Chlamydomonas* with a decreased $V_{\max.(\text{CO}_2)}$ in Hepes [4-(2-hydroxyethyl)-

Table 5. *Kinetic constants for ryegrass ribulose biphosphate carboxylase*

Six plants of each cultivar were pooled and 20g each of leaf tissue ground in 80ml of 50mM-Tris/HCl/10mM-MgCl₂/10mM-NaHCO₃/0.25mM-EDTA/5mM-mercaptoethanol, pH8.0 at 0°C. Homogenates were filtered and centrifuged (10000g/10min). The supernatants were made up with buffer to 100ml and 22.6g of (NH₄)₂SO₄ was added. After centrifugation 6.7g of (NH₄)₂SO₄ was added to 70ml of the supernatant. The precipitate was resuspended in 5ml of the above buffer and passed through a Sephadex G-25 column (30cm x 1cm) equilibrated with the same buffer. The desalted protein solutions obtained were used directly. The cultivars were grown together in a glasshouse and the enzymes purified simultaneously.

Cultivar	Grasslands no.	Ploidy	$K_m(\text{HCO}_3^-)$ (mM)	$V_{\max}(\text{CO}_2)$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Reveille	AT 62	4n	4.11 ± 0.29	2.57 ± 0.07
Tetilla	BT 540	4n	3.39 ± 0.21	1.71 ± 0.07
Perma	A 3765	2n	3.84 ± 0.19	2.17 ± 0.07
Gremie	A 3316	2n	3.59 ± 0.24	1.84 ± 0.08

1-piperazine-ethanesulphonic acid] buffer but not in Tris, but the $K_m(\text{CO}_2)$ was unchanged. Subsequent data, however (Nelson & Surzycki, 1976b), suggest that the mutation may be to the activation process. Garrett (1978) showed a change in $K_m(\text{CO}_2)$ while the $V_{\max}$ remained unaltered in diploid and tetraploid ryegrasses (*Lolium*). W. A. Laing & J. T. Christeller (Table 5) were unable to show any significant differences between the cultivars.

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