

Viewpoint:

Carbon isotope effect predictions for enzymes involved in the primary carbon metabolism of plant leaves

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Abstract. Carbon isotope effects of enzymes involved in primary carbon metabolism are key parameters in our understanding of plant metabolism. Nevertheless, some of them are poorly known because of the lack of *in vitro* experimental data on purified enzymes. Some studies have focused on theoretical predictions of isotope effects. Here we show how quantum chemical calculations can be adapted for calculation of isotope effects for the Rubisco-catalysed carboxylation and oxygenation reactions and the citrate synthase reaction. The intrinsic isotope effect of the carboxylation by Rubisco appears to be much smaller than previously thought, being close to the overall isotope effect of the reaction that is, between 25 and 30 per mil. The same applies to the enzyme citrate synthase, that catalyses the first step of the Krebs cycle, with an isotope effect of around 23 per mil. Combined with the isotope effects of equilibrium reactions calculated with β -factors, the Krebs cycle then has an overall isotope effect that depletes organic acids in ^{13}C .

Keywords: carbon isotopes, carbon metabolism, carboxylation, citrate synthase, enzymes, oxygenation, Rubisco.

Introduction

Disentangling the metabolic network in plant cells is a major challenge in plant biology. The use of stable carbon isotopes as tracers (^{13}C labelling) is a powerful means by which to reach this aim and is now currently achieved with nuclear magnetic resonance (NMR, for a review see Bligny and Douce 2001). But ^{13}C natural-abundance studies with isotope ratio mass spectrometry have also been used successfully for more than 20 years (O'Leary 1981): the isotope signature in CO_2 and organic compounds, as well as the distribution of isotopes among molecules of an organic compound are routinely analysed to study enzymatic reactions (O'Leary 1980). It is now well established that photosynthetic CO_2 fixation discriminates against the heavy ^{13}C isotope so that C_3 plants are on average 20‰ depleted in ^{13}C compared with atmospheric CO_2 (Troughton 1971). This effect has been extensively studied with gas-exchange measurements, and also modelled, pointing to the main fractionating steps of CO_2 diffusion and enzymatic CO_2

fixation by Rubisco (Farquhar *et al.* 1982). Respiration of leaves has also been studied and it has been demonstrated that the CO_2 produced in the dark after leaf illumination is ^{13}C -enriched by up to 6‰ compared with leaf sucrose (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001). Although the $^{13}\text{C}/^{12}\text{C}$ fractionation by Rubisco has been repeatedly studied *in vitro* (Roeske and O'Leary 1984; Roeske and O'Leary 1985; Guy *et al.* 1987), the enzymatic fractionations that can occur during the Calvin cycle after CO_2 fixation by Rubisco, as well as during sucrose breakdown by the respiratory pathway, are poorly known. Some of them have been measured *in vitro* on animal or yeast enzymes, but the extent to which those isotope effects are similar in their plant counterparts is unknown. However, the isotope effects of these reactions can give important clues to metabolic fluxes if the isotope distribution in molecules is known, as has already been analysed in reactions involving carbon isotopes of organic acids in fruit cells (Schmidt 2003) and in the yeast glycolytic pathway with D/H isotopes

Abbreviations used: DHAP, dihydroxyacetone-phosphate; E4P, erythrose-4-phosphate; FBP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; PGA, phosphoglyceric acid; R5P, ribose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; X5P, xylulose-5-phosphate.

(Zhang and Martin 1995). In the case of fruit cells, the carbon isotope distribution in organic acids, using the isotope effect of the pyruvate dehydrogenase enzyme, revealed that this reaction was only half complete (50% of the reactants were consumed, the rest going to other reactions, e.g. fermentation or phosphoenolpyruvate carboxylation) (Schmidt 2003). Thus, isotope effects are critical parameters for investigation of metabolism. Theoretical calculations of heavy atom isotope effects can help in this effort. The theory of kinetic isotope effects was developed some time ago (Bigeleisen 1949; Melander 1960), but until recently the lack of transition state geometry and structure of biological reactions created a major barrier to applying this theory to enzymatic reactions. More recently, geometric data on crystallised enzymes as well as progress in computational quantum chemistry have improved, and there is an expectation that some isotopic data may be obtained regarding a few key metabolic reactions. More general papers also dealing with non-biological reactions are available (Paneth 1995) and a comparison of the isotope effect predicted via quantum chemistry with the measured one during the enzymatic reaction of aspartate trans-carbamylase has been published (Pawlak *et al.* 1998). The purpose of the present paper, then, is to present the current status of the modelling of isotope effects on reactions involved in plant primary carbon metabolism, and to compare such models with experimental data where available.

A key feature of the isotope fractionation by a particular enzyme is the fractionation associated with just the carbon-bond-making or -breaking step. This is called the intrinsic isotope effect. The overall fractionation is then a modification of the intrinsic effect depending on the mechanism of the overall reaction, e.g. internal back reactions. A key message of the present paper will be that the intrinsic isotope effect of the carboxylation by Rubisco is very likely much smaller than previously thought (Roeske and O'Leary 1984), being close to the overall isotope effect of the reaction. The intrinsic isotope effect is also close to the overall isotope effect in the enzyme citrate synthase, that catalyses the first step of the Krebs cycle, where we calculate an isotope effect of around 20 per mil. Combined with the other steps, the Krebs cycle has an overall isotope effect that depletes the organic acids in ^{13}C .

Theory

Transition-state theory for kinetic isotope effects

Under the assumptions of transition-state theory, the rate constant k of an elemental reaction depends on the energetic properties of the transition state (that is, the intermediate molecule species) compared with that of the reactants. The kinetic isotope effect can be calculated with the ratio of the rate constants of the two isotopic species considered

(denoted as 1 and 2 here), according to the following equation (Melander 1960):

$$\frac{k_1}{k_2} = \left(\frac{M_1^\neq}{M_2^\neq} \cdot \frac{M_2^R}{M_1^R} \right)^{3/2} \times \left(\frac{\mu_1^\neq}{\mu_2^\neq} \cdot \frac{\mu_2^R}{\mu_1^R} \right)^{1/2} \times \prod_i^{3N-6} \frac{\sinh(u_{i1}^R/2)}{\sinh(u_{i2}^R/2)} \cdot \prod_i^{3N^\neq-7} \frac{\sinh(u_{i2}^\neq/2)}{\sinh(u_{i1}^\neq/2)} \times \frac{\kappa_1}{\kappa_2}, \quad (1)$$

where M are the molecular masses, μ are the moments of inertia component (usually made of three parts standing for each space component), κ the transmission coefficient (fraction of molecules that passes the energy barrier in the forwards direction). The superscripts R and \neq are related to the reactants and the transition state, respectively. u is given by:

$$u = hv/k_B T, \quad (2)$$

where v is the stretching frequency, h the Planck's constant, k_B the Boltzmann's constant, and T the absolute temperature. \sinh is the hyperbolic sine and is defined as: $\sinh(x) = 0.5 \times (e^x - e^{-x})$.

The component κ includes tunneling effects (that is, the possibility that a small mass particle passes through the energy barrier as a consequence of the Heisenberg uncertainty principle) as well as recrossing of the energetic barrier. Generally, it is thought that the tunneling effect is negligible at ordinary temperatures with heavy atoms like ^{13}C so that the isotope effect associated with κ is considered to be of minor importance (Van Hook 1971; Saunders 1986).

If v^\neq denotes the imaginary frequencies, the imaginary frequencies of the isotopic species 1 and 2 are usually linked by the relationship:

$$\frac{v_1^\neq}{v_2^\neq} = \sqrt{\frac{m_2}{m_1}}, \quad (3)$$

where m is the reduced mass of the molar masses w and w' of the atoms linked by the bond j (that is, $1/m = 1/w + 1/w'$). If the heavy isotopic species is the number 2, $m_2 > m_1$ so that the heavy isotopomer has a lower imaginary frequency. For example, for a C–C bond formation, the reduced mass is calculated by $1/m_1 = 1/12 + 1/12$ for the light isotopomer and $1/m_2 = 1/12 + 1/13$ for the heavy isotopomer. The ratio is then 1.040 and the imaginary frequency for the light isotope is ~ 1.0198 times that of the heavy isotope. With $u = hv^\neq/k_B T$ (eqn 2) where v^\neq is the imaginary frequency, and if $u_{j2}^R = u_{j1}^R$ (for example, when the bond j is formed during the reaction and does not exist in the reactants),

that in the appendix of Farquhar (1979). Accordingly, the subscripts of the rate constants k are the same here as in the appendix of Farquhar (1979).

The rate constant k_7 is likely very low as the carboxylation step is thought to be irreversible (Andrews and Lorimer 1987; Cleland *et al.* 1998). Nevertheless, we assume here that the backward reaction can occur and be isotopically sensitive (for a reason that will become apparent below). The step with the rate constant k_8 is quite complex and includes intramolecular re-arrangements, hydration and cleavage of the C₆ molecule. This cleavage step is not here accompanied by a backward reaction. This is because there is now experimental evidence that the reaction is fully committed to the formation of 3-phosphoglycerate so that the C–C cleavage of the C₆ molecule is irreversible (Andrews and Lorimer 1987). The isotope effect is then the following (see Appendix):

$$\alpha = \alpha_i \times \frac{{}^{13}k_7 + k_8}{{}^{12}k_7 + k_8}, \quad (5)$$

where α_i ($= {}^{12}k_6 / {}^{13}k_6$) is the intrinsic isotope effect, that is, the isotope effect of the carboxylation step. If k_7 is very close to zero (i.e. if the decarboxylation is unlikely) compared with k_8 or if the decarboxylation is not accompanied by an isotope effect, then the isotope effect is very close to the intrinsic isotope effect. Although this model may be oversimplified, it suggests that the isotope effect measured *in vitro* should be nearly equal to the isotope effect of the carboxylation step itself. Thus, very likely, the intrinsic isotope effect of spinach Rubisco was greatly over-estimated by Roeske and O'Leary (1984).

The isotope effect of the carboxylation step itself (that is, α_i) can now be investigated with quantum chemistry methods at the *ab initio* (not semi-empirical) level with molecular models that are close to the natural reaction. With a given molecular model, these methods calculate the electronic structure, the geometry and so the energy of the transition state. This also allows calculation of the stretching frequencies of the bonds and, using the energy level of the reactants, gives the activation energy of the reaction considered. The geometric data from the crystallised enzyme with its substrate can be used to add geometric constraints or include more atoms (of the catalytic site) involved in the reaction in the model.

We take advantage of such a study, done with 2,3,4-trihydroxy-2-pentene as a model for RuBP-enediol (Fig. 1), and for which the corresponding transition structure has been investigated (Tapia *et al.* 1995). The imaginary frequency value of the transition-state formed during CO₂ attack on the C-2 of 2,3,4-trihydroxy-2-pentene is $390i$ (Tapia *et al.* 1995) and with the ratio $v_{12}^\ddagger / v_{13}^\ddagger = 1.0198$ (as described in the Theory section), we have an imaginary frequency of $382i$ for the heavy isotope. This means that u_{j12}^\ddagger is 1.8834 (eqn 2)

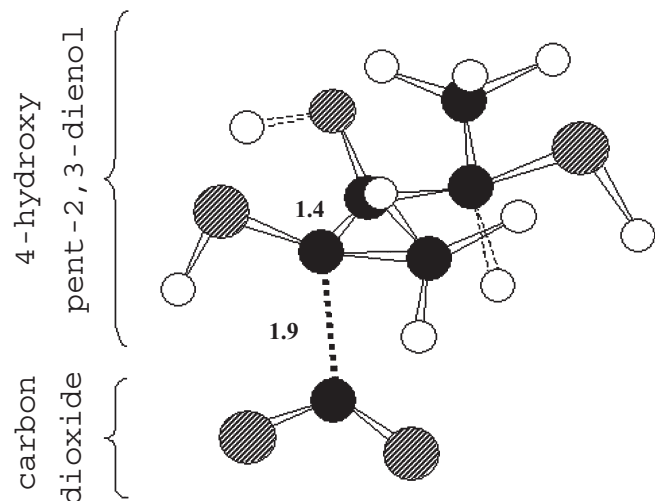


Fig. 1. Molecular model for the transition state structure of RuBP carboxylation by Rubisco. This figure is a tentative summary of the geometry of the transition state structures that have been published by Tapia *et al.* (1995), Moliner *et al.* (1999), Andrés *et al.* (1999) and Mauser *et al.* (2001). Approximate distances are given in Å. Carbon, oxygen, and hydrogen atoms are represented by black, hatched, and white discs, respectively. The thick dotted line indicates the C–C bond that is made during the reaction. The C atom in CO₂ is δ^+ -polarised and carries out an electrophilic attack on the C-2 of the dienol. The dienol can give, in a reversible way, the enolate associated species (i.e. with C–O[−]), which is why the bond between the O and the H atom is dashed here.

and u_{j13}^\ddagger is 1.8468 at $T=298$ K (eqn 3). Using eqn (4), we have:

$$\frac{k_{12}}{k_{13}} = \frac{\sinh(u_{j12}^\ddagger / 2)}{\sinh(u_{j13}^\ddagger / 2)} = 1.02537.$$

This calculated isotopic effect value of around 1.0254 is very different from the assumed intrinsic isotope effect of 1.040 (Roeske and O'Leary 1984); by contrast it is near the overall observed isotope effect (1.029), supporting our prediction using eqn (5) (Table 1). Nevertheless, there are several computational studies with different geometries that can be a source of variation in the estimate of the isotope effect: the distance C₂(RuBP)–C₃(RuBP) varies from 1.38 (Mauser *et al.* 2001) to 1.41 Å (that is, 0.138–0.141 nm) (Moliner *et al.* 1999) and the distance C(CO₂)–C₂(RuBP) varies from 1.84 (Moliner *et al.* 1999) to 1.94 Å (Andrés *et al.* 1999). Although these distance variations are quite large, they would lead to minor variation in the isotope effect [assuming that the C(CO₂)–C₂(RuBP) bond is similar to a classical oscillator of energy $h\nu$, these distance variations would lead to a variation of the calculated isotope effect of less than 1%].

A similar procedure can be followed with oxygen isotopes in O₂ using the same molecular model (2,3,4-trihydroxy-2-pentene). $v_{16}^\ddagger / v_{18}^\ddagger = 1.0247$ and so using the imaginary

Table 1. Comparison of measured fractionation with theoretical kinetic fractionations obtained through computer quantum chemistry methods at 25°C

Fractionations (in per mil) are calculated with $\Delta (\text{‰}) = 1000 \times (\alpha - 1)$. *, Fractionations during Rubisco-catalysed carboxylation that are given with respect to dissolved CO₂

Reaction	Model	Fractionation (‰)	Reference
Theoretical fractionations		Calculated in this paper	To obtain v in eqn (4)
O ₂ fixation by Rubisco	3,4-dihydroxypentaneone	20.8	Tapia <i>et al.</i> (1995)
CO ₂ fixation by Rubisco	3,4-dihydroxypentaneone	18.3–20.8	Oliva <i>et al.</i> (1999)
Citrate synthase	Addition of HSCOCH ₂ ⁻ to acetone	25.3	Tapia <i>et al.</i> (1995)
Measured fractionations		23.0–27.1	Mulholland and Richards (1998)
O ₂ fixation by Rubisco	Spinach enzyme pH 8.5	20.7	Guy <i>et al.</i> (1987)
CO ₂ fixation by Rubisco	Spinach enzyme pH 8	29.0	Roeske and O'Leary (1984)*
	Spinach enzyme pH 8.5	29.4	Guy <i>et al.</i> (1987)*
Citrate synthase	–	20.0	Schmidt (2003)

frequency of 635i (Tapia *et al.* 1995) gives a frequency of 619i for ¹⁸O. Thus $u_{j16}^{\#}$ is 3.0645 and $u_{j18}^{\#}$ is 2.9906, giving an oxygen isotope effect of 1.0415 using eqn (4). Equation (4) corresponds to the isotope effect occurring when the oxygen atom that makes a link with the carbon atom of RuBP is considered separately from the other one. The second oxygen atom of O₂ that does not participate in the reaction is nevertheless taken into account in classical isotope effect measurements as the isotope ratio of molecular oxygen includes both oxygen atoms. As a consequence, a symmetry number of 2 should be added (see the Appendix for the supporting theory) and the predicted isotope effect is $\sim 1 + 0.0415/2 = 1.0207$. It is striking that the isotope effect measured on the spinach enzyme is the same (Guy *et al.* 1987; Table 1).

The theoretical considerations (eqn 5) suggest that the isotope effect is not influenced by the rate of enolisation k_9 , and that there should be no impact of deuteration of RuBP in C-3 (²H-3-RuBP). However, from observations, deuteration does change the carbon isotope effect to 1.021 (Roeske and O'Leary 1984). This 8 per mil reduction below that observed with normal RuBP (1.029), is less than the 15 per mil predicted by Roeske and O'Leary (1984). The latter value was obtained using the deuterium isotope effect to calculate the value of k_9 with ²H-RuBP. The origin of the deuteration effect is unclear. The hydrogen atom in C-3 has been proposed to be removed by Lys-201 of the enzyme and transferred to the oxygen atom in C-2 (Cleland *et al.* 1998, but see King *et al.* 1998). If so, deuteration would modify the electronic structure of the C-2 atom and then the energetic properties of the transition structure. This would in turn influence the isotope effect during the CO₂ attack.

Taken together the isotopic data do not agree with a Michaelis-like mechanism with CO₂ reversibly binding first, but are in accordance with RuBP binding first, followed by a concerted mechanism, with more or less simultaneous CO₂

attack on the C-2 carbon atom and an –OH bond formation on the C-3 carbon atom (Cleland *et al.* 1998).

What is the origin of the variation in the Rubisco isotope effect?

Equation (5) indicates that the carbon isotope effect of Rubisco does not depend on the other steps of the reaction (except if a decarboxylation isotope effect occurs), nor on RuBP or oxygen concentrations. That is, the isotope effect during the Rubisco-catalysed carboxylation depends on neither the RuBP binding rate nor the oxygenation of RuBP (see the Appendix for the supporting theory). Indeed, the carbon isotope discrimination during photosynthesis follows a linear relationship with respect to p_i/p_a , the ratio of internal to atmospheric CO₂ partial pressure (Farquhar *et al.* 1982): $\Delta = a + (b - a) \cdot p_i/p_a$, where b is the isotope fractionation of the Rubisco-catalysed carboxylation. The slope of this relationship is constant and does not depend on CO₂, strongly suggesting that the isotope effect of Rubisco is not sensitive to the CO₂/O₂ partitioning.

The isotope effect is nevertheless sensitive to any backward reaction (decarboxylation) rate (k_7 , see eqn 3). The decarboxylation of the β -ketoacid (the product of carboxylation) has been observed, but mainly with non-activated (decarbonylated) Rubisco. The decarboxylation reaction (possibly) proceeds via the reverse step of carboxylation, followed by the β -elimination of the phosphate group (in C-1) (Pierce *et al.* 1986). Little CO₂ production has been detected with the activated (carbonylated) enzyme fed with the 6-carbon product of carboxylation (β -ketoacid): Pierce *et al.* (1986) found that the partitioning ratio decarboxylation/(hydration + decarboxylation) is around 0.05 in *Synechococcus* and 0.07 in *Rhodospirillum*, and can be much more if metals other than Mg²⁺ are linked to the enzyme. Consequently, it is generally thought that the decarboxylation is negligible in the physiological conditions; some uncertain features of the active site

may promote hydration and C₂–C₃ cleavage so that the β-ketoacid is fully committed in the forward direction (Mauser *et al.* 2001). Nevertheless, we assume here that even though the rate is very low, decarboxylation may occur. This assumption has already been made by Roeske and O’Leary (1985) for the *Rhodospirillum rubrum* enzyme to explain the low isotope effect measured in this organism (α around 1.017 in that study). In such a case, the CO₂ that is back-released will have an impact on the isotope effect if the decarboxylation fractionates between isotopes. Indeed, decarboxylations generally fractionate against ¹³C (O’Leary 1980), so that ¹²k₇ > ¹³k₇. The isotope effect of eqn (5) can be re-written as follows (see the Appendix):

$$\alpha = \alpha_i(1 - \varepsilon_7) + \left(\alpha_i \varepsilon_7 \times \frac{k_8}{k_6 C} \times \frac{k_9 + k_{10}}{k_9 + k_8} \right) \cdot \frac{C}{K_C}, \quad (6)$$

where ε₇ is defined by ¹²k₇/¹³k₇ – 1 (that is, ε₇ is the fractionation associated with k₇) and K_C is the apparent Michaelis constant for CO₂. Note that we include C in this equation to ensure that terms within the brackets are dimensionless. Even though the mechanism of the reaction is not assumed to involve a Michaelis CO₂–enzyme complex, the form of the kinetics of the overall reaction is unchanged: the velocity v of the reaction can still be written as v = V_{max}C/[K_C + C] where K_C is an apparent Michaelis constant (Farquhar 1979).

The carboxylation step itself (rate k₆) is rate limiting (Cleland *et al.* 1998) so that the ratio k₈/k₆C is high, and presumably the third ratio between the brackets may be of order unity, assuming the k are of the same order of magnitude. More importantly, eqn (6) emphasises the dependence of the isotope effect upon K_C: for a given CO₂ concentration, a high K_C value decreases the isotope effect following a hyperbolic relationship.

A tentative plot of measured α against K_C for different Rubiscos can be drawn in order to see the trend apparent in eqn (6). Such a plot is shown in Fig. 2 where the points were obtained with Rubiscos from various organisms. As would be expected if, say, k₇ were to vary, there is a decreasing dependence of α on K_C so that the estimated value for (α_i – ε₇) is around 1.0204 and the value of

$$\frac{k_8}{k_6} \times \frac{k_9 + k_{10}}{k_9 + k_8} \cdot \varepsilon_7$$

is around 0.1 μmol L⁻¹ (Fig. 2). Note that the isotope effect does not diverge to infinity when K_C tends towards zero in Fig. 2, because the maximum value obtained for k₇ → 0 is α = α_i as given by eqn (6) [and eqn (A5b) of the Appendix]. A simple interpretation of the relationship of Fig. 2 would be that α_i is around 1.030 and ε₇ is nearly 10%. However, the intrinsic isotope effect α_i might also vary (decrease) from low K_C to high K_C Rubiscos. Whatever

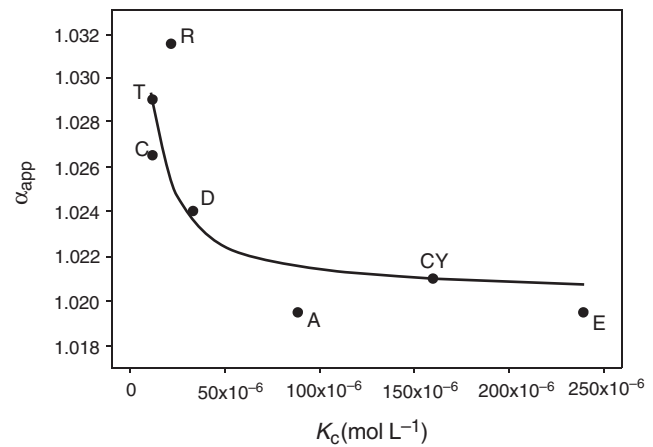


Fig. 2. Representation of the carbon isotope effect of Rubisco-catalysed carboxylation as a function of the Michaelis constant for CO₂ for various organisms. A, α-proteobacteria (*Rhodospirillum*), C, Chlorophyceae, CY, cyanobacteria, D, Diatoms, E, endosymbionts of *Riftia*, R, Rhodophyceae, T, C₃ Tracheophytæ. Data are from the review by Raven (1996) and the paper by Robinson *et al.* (2003) for *Riftia*. Note that the isotope effect associated with the points labeled C, D, and R are inferred by Raven (1996) from the carbon isotope composition of organisms grown in high CO₂. The solid line is a hyperbolic regression $y = y_0 + a \times x$ ($R^2=0.607$). The regression is significant ($F=7.72$, $P < 0.05$) and gives $y_0 = 1.02037$ and $a = 1.0772 \cdot 10^{-7}$.

the significance of the relationship in Fig. 2, it is clear that the intrinsic isotope effect (α_i) is less than the value 1.040, as assumed by Roeske and O’Leary (1984), and is much closer to the apparent isotope effect (α). This conclusion is supported by the calculations that we made with the frequency results of quantum chemistry computations and that give an isotope effect of around 1.025. Parenthetically, this result does not mean that Rubisco fractionation, denoted b or b₃ in papers related to gas exchange or water-use efficiency (Farquhar *et al.* 1982; Farquhar and Richards 1984), is now less than previously thought. The value for b is not being challenged here, but rather its chemical significance and its linkage with other Rubisco properties that are key to its function.

Kinetic properties of enzymes such as Rubisco vary genetically, and cause variation in the isotopic fractionation. Conversely, isotope fractionation could be used for the assessment of relative limitations by biochemical and diffusional components of CO₂ fixation by plant leaves (e.g. C₃, C₄ pathways). The interpretation of the latter also needs an understanding of the Rubisco isotope effect, particularly if comparisons are made across divergent phyla and so, further studies giving insights on the ¹²C/¹³C fractionation during the carboxylation reaction by Rubisco and the evolution of such a fractionation from one taxonomic group to another are now necessary.

Other enzymes of the Calvin cycle

The Calvin cycle is made up of alternative transketolisations and aldolisations, catalysed by transketolase and

(trans)aldolase, respectively. Aldolase catalyses the following reactions: dihydroxyacetone-phosphate + 3-phosphoglyceraldehyde → fructose-1,6-bisphosphate and dihydroxyacetone-phosphate + erythrose-4-phosphate → sedoheptulose-1,7-bisphosphate. Transketolase catalyses the following reactions: 3-phosphoglyceraldehyde + fructose-6-phosphate → erythrose-4-phosphate + xylulose-5-phosphate and 3-phosphoglyceraldehyde + sedoheptulose-7-phosphate → ribose-5-phosphate + xylulose-5-phosphate. Because these reactions break and make C–C bonds, it is likely that they are fractionating steps. The isotope composition of carbohydrates that are produced by the cycle is influenced by their isotope effects. Aldolase catalyses the reaction dihydroxyacetone phosphate + 3-phosphoglyceraldehyde → fructose-1,6-bisphosphate (FBP) that is a key reaction in the chloroplast as well as in the cytosol, because it converts triose phosphates to hexoses. The isotope effect of aldolase has been studied *in vitro* on the rabbit enzyme by Gleixner and Schmidt (1997) and they found both kinetic and thermodynamic isotope effects in the C-3 and C-4 positions during fructose production (Table 2). The predominance of one type of isotope effect over the other depends on the kinetic status of the reaction (that is, far from equilibrium or at full equilibrium). Mathematically, the general expression of the isotope effect as a function of time t is given by (see the Appendix):

$$\alpha = \frac{R_{\text{triose}}}{R_{\text{FBP}}} = \frac{k^{12}}{k^{13}} \cdot \frac{1 - e^{-(k^{12}+k_{-1}^{12})t}}{1 - e^{-(k^{13}+k_{-1}^{13})t}} \cdot \frac{(k^{13}e^{-(k^{13}+k_{-1}^{13})t} + k_{-1}^{13})}{(k^{12}e^{-(k^{12}+k_{-1}^{12})t} + k_{-1}^{12})}$$

where k is the rate constant of the forward reaction (FBP production) and k_{-1} that of the backward reaction. The superscripts 12 and 13 stand for the light and heavy isotopomers, respectively. When $t \rightarrow 0$, α tends towards $k^{12}/k^{13} = \alpha_{\text{kinetic}}$ and when $t \rightarrow +\infty$, it tends towards:

$$\frac{k^{12} k_{-1}^{13}}{k^{13} k_{-1}^{12}} = \frac{K^{12}}{K^{13}} = \alpha_{\text{equilibrium}}$$

Thus, one may expect that the enzyme will show an intermediate isotope effect in the case of continuously displaced equilibrium (that is, the equilibrium is never reached because the continuous removal of products by a subsequent reaction of the Calvin cycle ‘pulls’ the forward reaction of the equilibrium).

It has been experimentally shown that the carbon isotope distribution in the glucose molecule is not uniform, with the C-3 and C-4 positions being ^{13}C -enriched (Rossmann *et al.* 1991). This is in accordance with the equilibrium isotope effects of 0.997 and 0.996 found *in vitro* for the aldolase reaction during FBP production, thus favouring the ^{13}C isotope. However, it remains difficult to imagine that the aldolase reaction reaches full equilibrium with the Calvin cycle running in an illuminated leaf. This raises the question of the *in vivo* isotope effect of this particular reaction and the possible other fractionations that can occur and contribute to the pattern of carbon isotope composition in glucose observed by Rossmann *et al.* (1991).

A metabolic model that takes into account the main reactions of the Calvin cycle that break or make C–C bonds has been used by Tcherkez *et al.* (2004) in order to ‘reverse-calculate’ the isotope effects that are at the origin of the isotope distribution in glucose. With a typical photorespiration rate v_o/v_c of 0.5 (where v_c is the rate of

Table 2. Theoretical fractionations obtained from ‘reverse modelling’ or equilibrium β -factor calculations for both aldolase and transketolase, and measured fractionations in aldolase
Fractionations (in per mil) are calculated with $\Delta = 1000 \times (\alpha - 1)$

Reaction	Model	Fractionation (‰)	Reference for calculations
Theoretical fractionations			
(Trans)aldolase	‘Reverse’ modelling	+2.6 in C-3 –7.8 in C-4	Tcherkez <i>et al.</i> (2004)
	β -factors	–18.2 in C-3 –7.3 in C-4	Galimov (1985)
Transketolase	‘Reverse modelling’	+4.7 in C-1 0.0 in C-2	Tcherkez <i>et al.</i> (2004)
	β -factors	+8.0 in C-1 0.0 in C-2	Galimov (1985)
Measured fractionations			
Aldolase (FBP production)	Rabbit enzyme <i>in vitro</i>		
	Equilibrium	–3.6 in C-3 –4.9 in C-4	Gleixner and Schmidt (1997)
	Kinetic	+12.3 in C-3 –8.7 in C-4	

carboxylation and v_o the rate of oxygenation of RuBP by Rubisco), they found predicted isotope effects of aldolase around 1.003 and 0.992 in the C-3 and C-4 positions (Table 2). Those values are intermediate between equilibrium and kinetic *in vitro* isotope effects, indicating that the aldolase reaction may not reach its equilibrium in the cell. It was also the case for the transketolase reaction, with an isotope effect around 1.005 in the same conditions (Table 2). Similarly, it suggests that the transketolase reaction is not at full equilibrium in the chloroplast but rather at a slightly displaced equilibrium. These data are in agreement with the fact that the so-called 'equilibrium steps' of the Calvin cycle such as aldolase and transketolase reactions may act as metabolic regulators of the carbon flow through the cycle, a feature that would not be observed for full equilibrium reactions (for a review, see Fridlyand and Scheibe 1999).

Respiratory pathway

The respiratory pathway has been extensively studied regarding carbon isotope composition of respired CO_2 and of carbohydrates that can feed respiration (Ghashghaie *et al.* 2003). It has been repeatedly observed that the CO_2 dark-respired by leaves is ^{13}C -enriched compared with sucrose, the probable respiratory substrate. This has been related to the ^{13}C -enrichment in the C-3 and C-4 positions in the glucose molecule that is decarboxylated by the pyruvate dehydrogenase (PDH) reaction (Ghashghaie *et al.* 2001), and has been called 'fragmentation fractionation' (Tcherkez *et al.* 2004). The predominance of the PDH decarboxylation over the decarboxylations of the Krebs cycle in the dark may then be responsible for the ^{13}C -enrichment in CO_2 . Accordingly, it has been found that the substrate shift from carbohydrates to fatty acids, which are decarboxylated only by the Krebs cycle, is accompanied by a ^{13}C -depletion in the respired CO_2 (Tcherkez *et al.* 2003). However, the fact that CO_2 inherits directly the ^{13}C -abundance of the corresponding C atoms of glucose is surprising: it is as if there were no isotope effects throughout the respiratory pathway. Yet, the (yeast) PDH irreversible reaction has a kinetic isotope effect of around 1.023 during CO_2 production (Melzer and Schmidt 1987). Assuming that a similar isotope effect occurs in the plant enzyme, it would suggest that the PDH reaction is almost complete in the dark (i.e. all the pyruvate molecules in the mitochondria enter the PDH reaction) so that it cannot fractionate between isotopomers.

The enzymes of the Krebs cycle itself may also fractionate between carbon isotopes. It is assumed (i), that most of the reactions of the cycle are at equilibrium, with free enthalpy values close to zero or slightly positive (Siedow and Day 2000) and so equilibrium isotope effects can occur during these reactions, and (ii), that the decarboxylations (α -ketoglutarate dehydrogenase and isocitrate dehydrogenase) and the citrate synthase reaction are irreversible, with very negative values of free enthalpy

($\Delta_r G$, negative values indicate that the chemical potential of the reactant is more than that of the product), so that a kinetic isotope effect may occur.

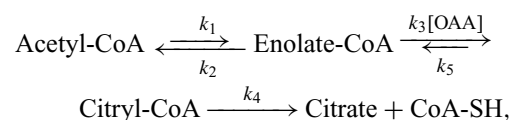
Irreversible reactions

The isotope effect of the NADP-dependent isocitrate dehydrogenase (the mitochondrial one is NAD-dependent) has been found to be 0.996 *in vitro* thus favouring the ^{13}C isotope for CO_2 production (O'Leary 1971). The isotope effect of the α -ketoglutarate dehydrogenase is not known but this enzyme probably has a similar isotope effect to the PDH reaction that is, around 1.023 (Melzer and Schmidt 1987), as the mechanism of the reaction is very similar. Moreover, it is probable that this reaction is incomplete in some plant cells because some of the α -ketoglutarate molecules are directed to nitrogen assimilation (glutamate synthesis), thus allowing the enzyme to fractionate. The citrate synthase reaction has not been investigated directly, but a very similar α -ketoacid-acetyl-CoA condensation reaction (ketomethionin-acetyl-CoA aldolase) has an isotope effect favouring ^{12}C (Butzenlechner *et al.* 1996), an estimate of which is 1.020 (Schmidt 2003).

Citrate synthase has been studied by computational quantum chemistry both with semi-empirical and *ab initio* methods (Mulholland and Richards 1998). The condensation reaction of citrate synthase was modelled by acetone (CH_3COCH_3), representing the carbonyl group of oxaloacetate, and HSCOCH_2^- to represent the enolate form of acetyl-CoA (Fig. 3). The natural reaction starts with the deprotonation of acetyl-CoA, followed by enolisation. This favours a nucleophilic attack of the C-3 of acetyl-CoA on the C-2 of oxaloacetate. The imaginary frequency for enolate addition found by Mulholland and Richards (1998) is around $400i$, and we use here this value to calculate the kinetic isotope effect during the reaction using eqn (4). The ratio $v_{12}^\ddagger / v_{13}^\ddagger$ is equal to 1.0198 (as described in the Theory section) and we have an imaginary frequency of $392i$ for the heavy isotope. This means that u_{j12}^\ddagger is 1.9304 and u_{j13}^\ddagger is 1.8929. Using eqn (4), we have:

$$\frac{k_{12}}{k_{13}} = \frac{\sinh(u_{j12}^\ddagger / 2)}{\sinh(u_{j13}^\ddagger / 2)} = 1.0256,$$

that is, an (intrinsic) isotope effect around 1.025 (Table 1). As for Rubisco, one may compare this value with the isotope effect actually measured. The relationship between the overall isotope effect of the reaction and the intrinsic isotope effect can be obtained assuming the following mechanism:



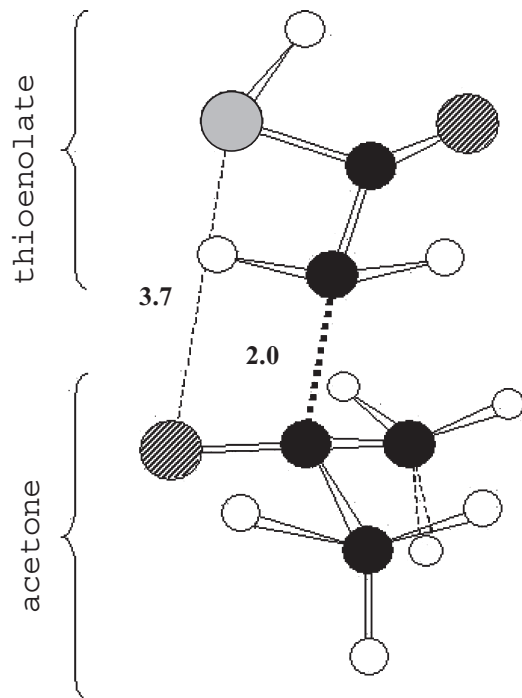


Fig. 3. Molecular model of the transition state structure of the condensation of acetyl-CoA and oxalo-acetate catalysed by citrate synthase. Approximate distances are given in Å. Carbon, oxygen, sulfur and hydrogen atoms are represented by black, hatched, grey and white discs, respectively. The thick dotted line indicates the C–C bond that is made during the reaction: the C-2 carbon atom of the thioenolate carries out a nucleophilic attack on the C-2 of acetone. The thin dotted line stands for the S–O distance.

in which [OAA] denotes the oxalo-acetate concentration. This mechanism is very similar to that of Rubisco, OAA and Acetyl-CoA playing the role of CO₂ and RuBP, respectively. The isotope effect is:

$$\alpha = \alpha_i \times \frac{{}^{13}k_5 + k_4}{{}^{12}k_5 + k_4},$$

where α_i ($= {}^{12}k_3 / {}^{13}k_3$) the intrinsic isotope effect. Because the condensation reaction is essentially irreversible ($k_5 = 0$) (Shepherd and Garland 1969; Johansson *et al.* 1973; Mulholland and Richards 1998), we have the simple relationship $\alpha = \alpha_i$. Again, experimental data and theoretical data agree quite well: the overall isotope effect of the reaction (~ 1.020 , Schmidt 2003) is very close to the predicted isotope effect of the condensation step itself (~ 1.025).

Equilibrium reactions

The reactions of the Krebs cycle that are supposed near equilibrium can fractionate if there are thermodynamic (equilibrium) isotope effects. These isotope effects are not known but they can be tentatively calculated by the β -factors method (Galimov 1985). The simple ratio of the β -factor of the reactant over that of the product gives an

estimate of the isotope effect (that does not depend on the transition state). Figure 4 gives the equilibrium isotope effects obtained with this method. Clearly, although most of them favour the ¹³C isotope, these reactions do not fractionate a lot, with a value for discrimination of around -4% . As a consequence, they should not modify the isotope ratio in the organic molecules to a great extent.

Taken as a whole, the theoretical data on isotope effects occurring during the Krebs cycle reveal that this cycle is a source of ¹³C-depletion in organic acids that are intermediates in the cycle, as well as in respired CO₂. That is why it is likely that the ¹³C-enrichment of the dark-respired CO₂ found by Ghashghaie *et al.* (2001, 2003) is related to a large diversion of the acetyl-CoA molecules towards pathways other than the Krebs cycle; otherwise the isotope effects of this cycle would deplete the respired CO₂ in ¹³C.

Even if this provides a convincing framework for the ¹³C abundance in CO₂, some aspects are still unclear regarding primary carbon metabolism. Such is the case for the ¹³C abundance in amino-acids: for example, (i) glutamate does not have the same isotope composition as glutamine, although glutamine and glutamate are interconverted via a single reaction (Jamin *et al.* 1997); (ii) alanine, which directly comes from amination of pyruvate, is strongly ¹³C-enriched compared with other metabolites like glucose (Abelson and Hoering 1961), although pyruvate inherits one half of the glucose molecule through the glycolytic pathway. It is probable that parallel metabolic pathways or carbon sources contribute to production of amino acids, so that their carbon isotope composition does not follow a general rule. Multiplicity of carbon sources has indeed been shown with labelling methods during starch and sucrose synthesis in illuminated leaves (Nogués *et al.* 2004). Experimental data are now needed to see whether isotopic discrepancies in organic compounds such as amino acids have a similar explanation.

Concluding remarks

The theoretical predictions of isotope effects of reactions have progressed in the last few years, in part thanks to the improvement of quantum chemistry methods (Paneth 1995). Here we have used the data obtained by these methods to calculate the (intrinsic) isotope effects of three reactions involved in the primary metabolism of plants, namely, Rubisco, PDH and citrate synthase. In the case of the carboxylation and oxygenation of RuBP by Rubisco, we find isotope effect values that are in reasonable accordance with the experimental data. Other satisfying comparisons have already been made on the enzyme aspartate trans-carbamylase (Pawlak *et al.* 1998). Nevertheless, one should be aware that geometric parameters such as the conformation of the reactants or the transition state can change significantly the theoretical isotope effect

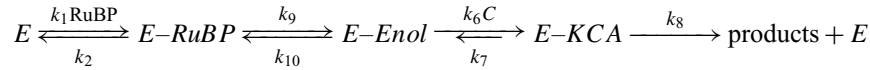
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Appendix: classical calculations of isotopic effects*Isotope effect of Rubisco*

The relationship between the isotope effect and the rate constants of the steps of the reaction can be simply obtained using the general expression of the rate of the reaction. The mechanism is:



where C and $E\text{-KCA}$ denote CO_2 and the six-carbon intermediate, respectively. The rate of the overall reaction with $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ is calculated considering that the two isotopomers compete for carboxylation. A similar system has already been studied by Farquhar (1979) with CO_2 and oxygen (competition between carboxylation and oxygenation). If RuBP is not limiting, the rate is:

$$v = \frac{V_c C / K_C}{1 + C / K_C + O / K_O}, \quad (\text{A1})$$

where O is the oxygen concentration, and K_C and K_O are the apparent Michaelis constants for CO_2 and O_2 . V_c is the maximum velocity of the carboxylase. Similarly, with two ^{12}C and ^{13}C -isotopomers of CO_2 , we have, neglecting oxygenation:

$$v^{12} = \frac{{}^{12}V_c {}^{12}C / {}^{12}K_C}{1 + {}^{12}C / {}^{12}K_C + {}^{13}C / {}^{13}K_C}, \quad (\text{A2})$$

and the symmetrical expression for $^{13}\text{CO}_2$. The ratio of the velocities is then:

$$\frac{v^{12}}{v^{13}} = \frac{{}^{12}V_c}{{}^{13}V_c} \times \frac{{}^{13}K_C}{{}^{12}K_C} \times \frac{{}^{12}C}{{}^{13}C}. \quad (\text{A3})$$

Dividing each side by $^{12}\text{C} / ^{13}\text{C}$, we have the overall isotope effect of the reaction:

$$\alpha = \frac{{}^{12}V_c}{{}^{13}V_c} \times \frac{{}^{13}K_C}{{}^{12}K_C}. \quad (\text{A4})$$

The values of K_C and V_c are (derived from the appendix of Farquhar 1979):

$$K_C = \frac{k_7 + k_8}{k_6} \times \frac{k_9 + k_{10}}{k_9 + k_8} \quad (\text{A5a})$$

and

$$V_c = \frac{k_8 k_9}{k_8 + k_9} [E]_0, \quad (\text{A5b})$$

where $[E]_0$ is the total enzyme site concentration. Substituting the V_c and K_C values into eqn (A4) gives:

$$\alpha = \alpha_i \times \frac{{}^{13}k_7 + k_8}{{}^{12}k_7 + k_8}, \quad (\text{A6})$$

where α_i ($= {}^{12}k_6 / {}^{13}k_6$) is the ‘intrinsic’ isotope effect, that is, the isotope effect of the carboxylation step. k_8 is assumed to be similar with both isotopes because the corresponding step (hydration and cleavage of the C_6 intermediate) does not involve the carbon atom inherited from CO_2 . This result is not modified when RuBP is limiting or when the oxygenation of RuBP is taken into account. If RuBP is limiting, the velocity is (Farquhar 1979):

$$v' = v \times \frac{A}{A + K_a'}, \quad (\text{A7})$$

where K_a' is the apparent Michaelis constant for RuBP and A is the concentration of RuBP. It is the same for ^{13}C and ^{12}C so that it cancels out in eqn (A3). When oxygenation is added, the denominator of v in eqn (A2) has the additional term O / K_O , and it has no effect on the ratio of eqn (A4).

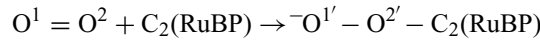
Equation (A6) can be re-written with the relationship $^{12}k_7 = (1 + \varepsilon_7)^{13}k_7$ (ε_7 is the discrimination associated with decarboxylation) and substituting k_7 using eqn (A5) as follows:

$$\alpha = \alpha_i (1 - \varepsilon_7) + \left(\alpha_i \varepsilon_7 \times \frac{k_8}{k_6 C} \times \frac{k_9 + k_{10}}{k_9 + k_8} \right) \cdot \frac{C}{K_C}, \quad (\text{A8})$$

where C is the (overall) carbon dioxide concentration. The concentration C is here added in both the numerator and the denominator in order to recall that $k_6 C$ has the same dimension as k_8 , and K_C has the dimension of a concentration.

Calculation of the isotope effect during Rubisco's reaction with oxygen

The oxygen isotope effect that occurs when the $\text{O}(\text{O}_2)\text{-C}_2(\text{RuBP})$ is formed, as given by eqn (4) is not equal to the isotope effect that would be measured using the overall isotope ratio in O_2 compared with that of the oxygen that is fixed to the C-2 of RuBP. This is because of the symmetry of the O_2 molecule. The reaction is described as follows, where for clarity, the oxygen atoms are labelled with numbers:



The fractionation given by eqn (4) is:

$$\Delta = \frac{\delta(2) - \delta(2')}{1 + \delta(2')} \approx \delta(2) - \delta(2')$$

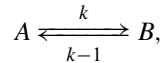
while the isotope fractionation that would be measured is as follows:

$$\Delta' = \frac{\frac{\delta(1) + \delta(2)}{2} - \frac{\delta(1') + \delta(2')}{2}}{1 + \frac{\delta(1') + \delta(2')}{2}} \approx \frac{\delta(1) - \delta(1')}{2} + \frac{\delta(2) - \delta(2')}{2}.$$

But the oxygen atom number 1 is not involved by the bond considered and so is not subjected to any isotope effect. So we have $\delta(1) = \delta(1')$ and the simple relationship: $\Delta' = \Delta / 2$. As the isotope effect is given by $\alpha = \Delta - 1$, we have $\alpha' = 1 + (\alpha - 1) / 2$.

Isotope effect of a reversible reaction

We assume here the following reversible reaction, with the reactants A and the product B :



where the rate constants of the forward and backward reactions are k and k_{-1} , respectively. For the ^{12}C and ^{13}C isotopes, we have: k^{12} , k^{13} and k_{-1}^{12} and k_{-1}^{13} . Mass balance equations are such that:

$$A + B = A_0, \quad (\text{A9})$$

which is true for each isotope: $A^{12} + B^{12} = A_0^{12}$ and similarly for ^{13}C . With a first order mechanism, we have the following differential equation:

$$\frac{dA^{12}}{dt} = -k^{12}A^{12} + k_{-1}^{12}B^{12} = -(k^{12} + k_{-1}^{12})A^{12} + k_{-1}^{12}A_0^{12}. \quad (\text{A10})$$

The same applies to ^{13}C :

$$\frac{dA^{13}}{dt} = -k^{13}A^{13} + k_{-1}^{13}B^{13} = -(k^{13} + k_{-1}^{13})A^{13} + k_{-1}^{13}A_0^{13}. \quad (\text{A11})$$

The solutions of (A10) and (A11) are exponential functions. For ^{12}C , we have:

$$A^{12} = z \cdot e^{-(k^{12} + k_{-1}^{12})t} + w,$$

where z and w are obtained at $t = 0$ so that:

$$A^{12} = \frac{A_0^{12} k^{12}}{k^{12} + k_{-1}^{12}} e^{-(k^{12} + k_{-1}^{12})t} + \frac{A_0^{12} k_{-1}^{12}}{k^{12} + k_{-1}^{12}}, \quad (\text{A12})$$

and the same for A^{13} . Combining Eqns (A9) and (A12), we have:

$$B^{12} = \frac{A_0^{12} k^{12}}{k^{12} + k_{-1}^{12}} \cdot \left(1 - e^{-(k^{12} + k_{-1}^{12})t}\right), \quad (\text{A13})$$

and similarly for B^{13} . The isotope effect is defined by the isotope ratio of the reactants divided by that of the products, that is, with eqns (A12) and (A13):

$$\frac{R_A}{R_B} = \frac{k^{12}}{k^{13}} \cdot \frac{1 - e^{-(k^{12} + k_{-1}^{12})t}}{1 - e^{-(k^{13} + k_{-1}^{13})t}} \cdot \frac{\left(k^{13} e^{-(k^{13} + k_{-1}^{13})t} + k_{-1}^{13}\right)}{\left(k^{12} e^{-(k^{12} + k_{-1}^{12})t} + k_{-1}^{12}\right)}.$$

When $t \rightarrow 0$, R_A / R_B tends towards k^{12} / k^{13} and when $t \rightarrow +\infty$, it tends towards:

$$\frac{k^{12} k_{-1}^{13}}{k^{13} k_{-1}^{12}} = \frac{K^{12}}{K^{13}},$$

as the exponential term tends to zero.