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Enzyme catalysis in microgravity: steady-state kinetic analysis of the isocitrate lyase reaction

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Abstract

Two decades of research in microgravity have shown that certain biochemical processes can be altered by weightlessness. Approximately 10 years ago, our team, supported by the European Space Agency (ESA) and the Agenzia Spaziale Italiana, started the Effect of Microgravity on Enzyme Catalysis project to test the possibility that the microgravity effect observed at cellular level could be mediated by enzyme reactions. An experiment to study the cleavage reaction catalyzed by isocitrate lyase was flown on the sounding rocket MASER 7, and we found that the kinetic parameters were not altered by microgravity. During the 28th ESA parabolic flight campaign, we had the opportunity to replicate the MASER 7 experiment and to perform a complete steady-state analysis of the isocitrate lyase reaction. This study showed that both in microgravity and in standard g controls the enzyme reaction obeyed the same kinetic mechanism and none of the kinetic parameters, nor the equilibrium constant of the overall reaction were altered. Our results contrast with those of a similar experiment, which was performed during the same parabolic flight campaign, and showed that microgravity increased the affinity of lipoxygenase-1 for linoleic acid. The hypotheses suggested to explain this change effect of the latter were here tested by computer simulation, and appeared to be inconsistent with the experimental outcome.

Keywords: Microgravity; Parabolic flight; Enzyme catalysis; Isocitrate lyase

1. Introduction

Besides the great deal of data concerning plant, animal, and human physiology, there are reports indicating that weightlessness can also affect certain biochemical cell processes. Most of the observations are merely confined to a macroscopic level, and, in addition, investigators do not seem to have detected a consistent and uniform response to the microgravity stimulus [1,2]. Above all, nothing is known about the actual causes of the observed effects, and how the microgravity signal might be conveyed to the cell machinery.

It was to identify the physical–chemical link between microgravity and its potential cell sensors that approximately 10 years ago we started the Effect of Microgravity on Enzyme Catalysis (EMEC) project, with the financial support from

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the European Space Agency (ESA) and the Agenzia Spaziale Italiana (ASI). During the MASER 7 campaign (April 16–May 3 1996, Kiruna, Sweden), we showed that microgravity does not alter the kinetic parameters (saturation velocity and Michaelis constant) for the cleavage reaction of isocitrate lyase (EC 4.1.3.1) [3]. The EMEC experiment, although technically successful as a whole, left two questions pending: (i) the problem of the high variability affecting the velocity measurements, that have been already discussed elsewhere [3,4], and (ii) the unfinished state of the study, since the unique flight of the sounding rocket did not permit the collection of data about the reverse condensation reaction.

Recently, the 28th ESA parabolic flight campaign (May 15–26, 2000, Bordeaux, France) gave us the opportunity to replicate the MASER 7 experiment and also to perform a complete steady-state analysis of isocitrate lyase reaction in microgravity conditions. The results have been published in a short, preliminary form in Ref. [5]. In the present paper we describe the whole experiment in detail, also devoting some attention to the tricky procedure for data acquisition. Our kinetic analysis shows that neither the forward nor the reverse enzyme reactions are altered by microgravity. On the other hand, during the same campaign, another Italian team found that in microgravity the Michaelis constant of soybean lipoxygenase-1 for linoleic acid is decreased to one fourth of that of the ground control [6,7]. The hypotheses suggested to explain this effect are discussed here and tested by computer simulation.

2. Experimental

The experimental hardware was the EMEC module, a multichannel fiber-optics radiometer which allows 16 simultaneous measurements. The module optics, mechanics, and electronics are described in great detail in Refs. [3,4].

The microgravity conditions were realized by means of special parabolic-flight manoeuvres of an A300 Zero-G aircraft. The microgravity periods (20 s at $\sim 10^{-2}$ g) were alternated with hypergravity phases (1.8 g) of similar length, and longer standard conditions. Each daily flight session consisted of 30 parabolas, and the entire campaign included three sessions. The inside of the aircraft, which hosted the module and the crew devoted to its control, was pressurised at standard pressure; the temperature was approximately 22–23 °C and was continuously monitored and recorded by several sensors applied on the module both to the assay cells and the injection syringes.

The goal of the experiment was to exploit the microgravity phase of the parabolic flights to perform a steady-state kinetic analysis of the isocitrate lyase reaction, to be compared with the results obtained on ground. For this purpose, the flight sessions were planned as follows:

Day 1: Analysis of the reverse (condensation) reaction at fixed glyoxylate concentrations (0.04, 0.06, 0.10, 0.20 mM) and varying succinate concentrations (range 0.30–2.04 mM).
Day 2: Analysis of the reverse (condensation) reaction at fixed succinate concentrations (0.3, 0.6, 1.19, 2.0 mM) and varying glyoxylate concentrations (range 0.04–0.20 mM).
Day 3: Analysis of the direct (cleavage) reaction at fixed succinate concentrations (none, 0.5, 1.0, 2.0 mM) and varying isocitrate concentrations (range 0.05–0.30 mM).

Each control experiment was carried out on the afternoon of the same days, on ground, inside the aircraft. The day-3 session, in addition to supplying data for the cleavage-reaction analysis, was necessary to establish the order of product release through succinate inhibition study. It should be remembered that we have studied the enzyme kinetic mechanism on several occasions [8–11].

2.1. Enzyme purification

Isocitrate lyase was purified from germinating *Pinus pinea* seeds as described in Ref. [12].

2.2. Enzyme assay

Isocitrate lyase catalyses the reversible cleavage of isocitrate into succinate and glyoxylate:

$$\text{isocitrate} \rightleftarrows \text{glyoxylate} + \text{succinate}$$

To measure the rate of isocitrate cleavage, the reaction was chemically coupled with phenylhydrazine which reacts with glyoxylate to form glyoxylate–phenylhydrazone. This compound has a peak in absorbance at 324 nm ($\varepsilon = 17$ $mM^{-1}\,cm^{-1}$) and can therefore be quantified spectrophotometrically [4]. The reaction mixture, 1.45 ml final volume, contained:

- 66 mM Hepes, pH 7.0
- 6 mM $MgCl_2$
- 4 mM phenylhydrazine–HCl
- approximately 2 μg isocitrate lyase
- suitable amount of *threo*-D_s-isocitrate and succinate

The condensation reaction was assayed by measuring isocitrate formation with isocitrate dehydrogenase ($NADP^+$) as the ancillary enzyme [10]. The reduction of the coenzyme to NADPH was followed at 324 nm. Since NADPH has a peak absorption at 340 nm ($\varepsilon = 6.2$ $mM^{-1}\,cm^{-1}$), the extinction coefficient at 324 nm was determined from standard NADPH solutions, and it was found to be 5.2 $mM^{-1}\,cm^{-1}$. The reaction mixture, 1.45 ml final volume, contained:

- 66 mM Hepes, pH 7.0
- 6 mM $MgCl_2$
- 0.5 mM $NADP^+$
- 0.2 U isocitrate dehydrogenase
- approximately 2 μg isocitrate lyase
- suitable amounts of succinate and glyoxylate

In both cases, reactions were started by injection of the enzyme solution (0.25 ml). Details about the three-step injection sequence are given in Ref. [3,4].

2.3. Data acquisition and processing

The light intensity emerging from the assay cells (transmittance) was measured by photodiodes and supplied to the monitoring and recording systems as electrical potential differences (V), at a sample rate of 2.5 readings s^{-1}. Absorbances were then calculated as the minus *log* of the relative transmittances. To obtain the relative transmittance, the output values were normalized to the average of the reading made a few seconds (50–60 data points) before the injection of the enzyme solution, i.e. when the transmittance could be considered 100%. Initial velocity values were finally determined from plots of absorbance vs. time.

A complete picture of the procedure for data acquisition is described in the first section of Section 3.

2.4. Kinetic analysis

To evaluate the effect of varying substrate and product concentrations on the initial velocity, double reciprocal plots were used as a preliminary diagnostic tool. The apparent kinetic constants (apparent V_{max} and K_m) were determined from these plots by using non-weighted nonlinear regression (a procedure which yields the same results as using weighted linear regression) [13]. Rough estimates of the kinetic parameters were obtained from replots (suitable apparent constant vs. fixed substrate or product concentration), and then they were refined applying nonlinear regression to fit the complete velocity equation to the whole set of initial velocity data. In our experience, such strict procedure can only provide unbiased interpretation of the inhibition patterns and reliable values of the kinetic constants [13].

All the statistics, nonlinear regression analysis, and computer simulation were performed using the program SYSTAT 5.1 (Systat Software Inc., Richmond, CA) running on a iMac (Apple Computer Inc.).

3. Results

3.1. Addendum to the data acquisition procedure

The simple data processing described under Materials and methods should have been sufficient, as it had been for the MASER 7 experiment [3], if we had not met with the unexpected problem of a heavy noise due to the instability of the Xenon lamp arc, which proved to be sensitive to gravity variations. An example of the extent of lamp noise is shown in Fig. 1a, where the time-course of one photodiode output is plotted for three consecutive parabolas: before, during, and at the end of an

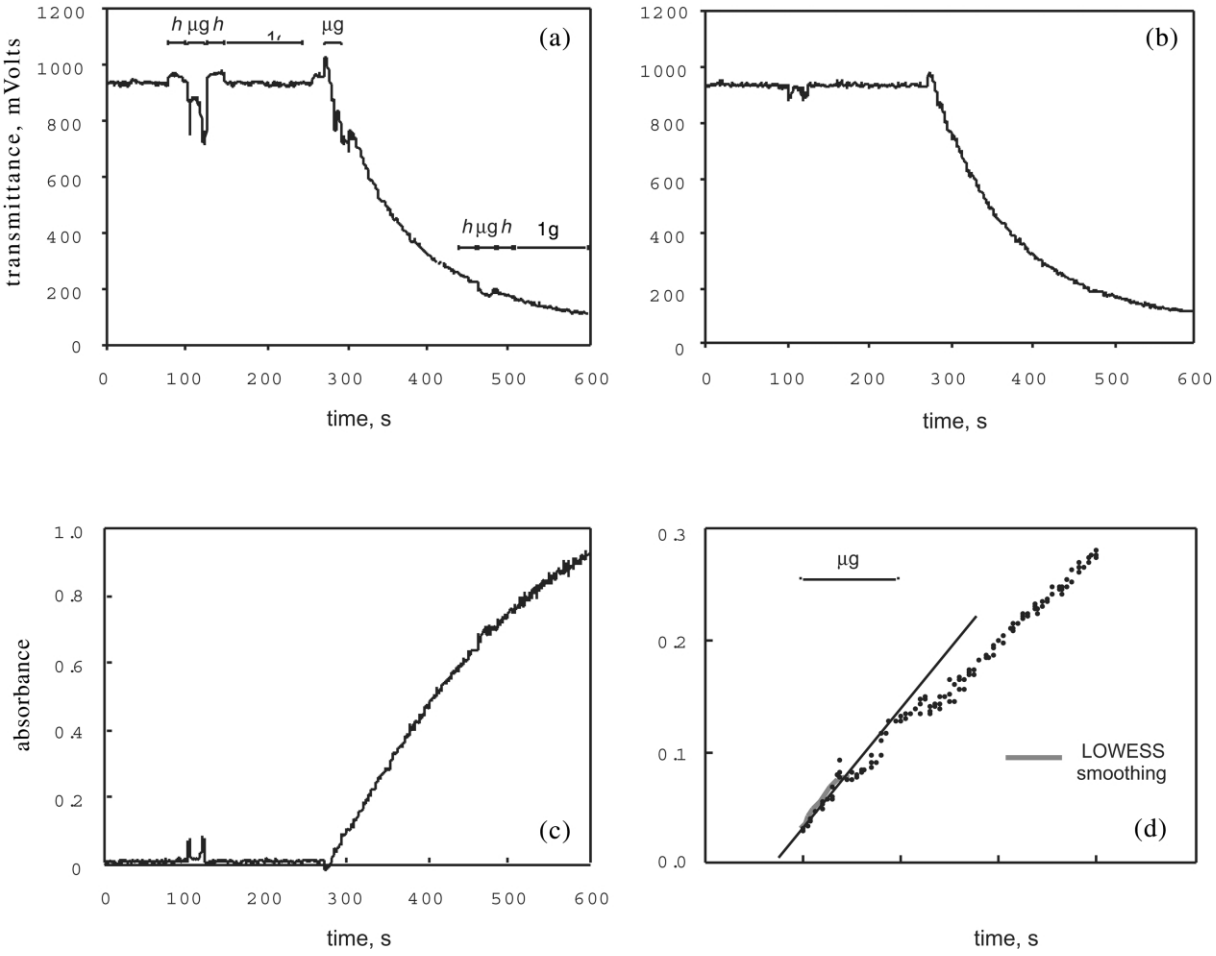


Fig. 1. The four-step procedure for calculating initial velocities. (a) Direct photodiode output; (b) after baseline correction; (c) after transformation to absorbance; (d) detail of the microgravity phase and application of the LOWESS (locally weighted least square) smoothing algorithm.

enzyme assay. Because fluctuations were approximately ±250 mV (corresponding to ±0.035 absorbance units), a correction to eliminate the noise was crucial to get correct estimates of the initial velocities. This was obtained by subtracting a relative baseline (Fig. 1b) calculated from the reference photodiode, and it worked satisfactorily in most cases. In the event of persisting fluctuations, we found that a further improvement was possible by applying the smoothing algorithm LOWESS (locally weighted least square), according to Cleveland [14]. Whether or not the LOWESS function was to be used, initial velocities were determined after transforming transmittance data to absorbance values, Fig. 2c,d, as described under Materials and methods.

During the injection sequence, the monitoring system of the EMEC module undergoes a 5–6 s programmed black-out (see the flat regions that precede the microgravity phases in Fig. 1a,b), to devolve all the electronics resources to the accuracy and precision of the injected volumes. To avoid loss of data and time cutting during the crucial phase of the experiment, we decided to start the injection procedure 6–7 s before the microgravity condition was reached. In this way

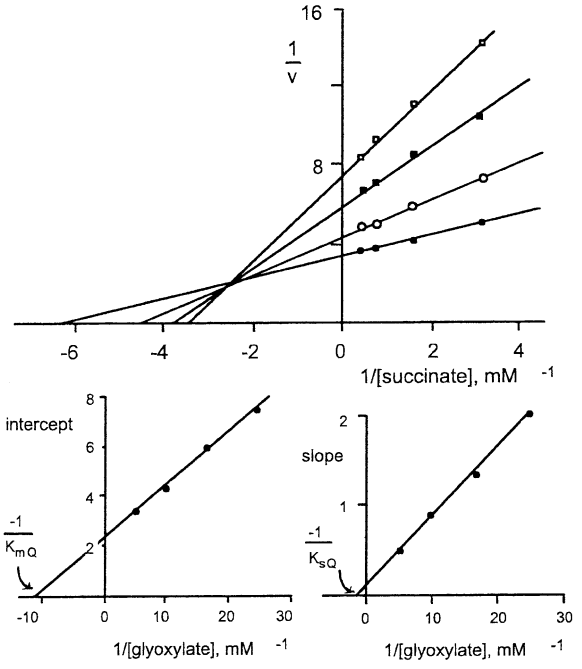


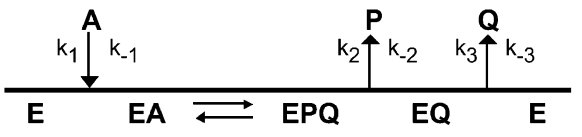
Fig. 2. Condensation reaction in microgravity. Effect of varying succinate concentration in the presence of different fixed glyoxylate concentrations (● 0.20, ○ 0.10, ■ 0.06, □ 0.04 mM). Below: $1/v$-axis intercept and slope replots vs. the reciprocal concentration of the fixed substrate.

we could have full 20 s enzyme kinetics in microgravity.

3.2. Kinetic analysis

A number of studies indicates that under steady-state assumptions, plant isocitrate lyase follows a sequentially ordered uni–bi/bi–uni kinetic mechanism in which succinate is released first from the ternary EPQ complex, and glyoxylate is the first substrate to enter the active site in the condensation reaction [8,9,15].

The complete reaction scheme, according to Cleland's notation, can be represented as follows:



where **E** is free enzyme, **A** isocitrate, **P** (succinate) the first product released, and **Q** (glyoxylate) the second product released.

Reverse reaction. The initial velocity equation of the reverse reaction can be written as:

$$v = \frac{V_r[\mathbf{P}][\mathbf{Q}]}{K_{s\mathbf{Q}}K_{m\mathbf{P}} + K_{m\mathbf{Q}}[\mathbf{P}] + K_{m\mathbf{P}}[\mathbf{Q}] + [\mathbf{P}][\mathbf{Q}]} \quad (1)$$

or

$$v = \frac{V_r[\mathbf{P}][\mathbf{Q}]}{K'_{i\mathbf{P}}K_{m\mathbf{Q}} + K_{m\mathbf{Q}}[\mathbf{P}] + K_{m\mathbf{P}}[\mathbf{Q}] + [\mathbf{P}][\mathbf{Q}]} \quad (2)$$

In fact $K_{s\mathbf{Q}}K_{m\mathbf{P}} = K'_{i\mathbf{P}}K_{m\mathbf{Q}}$.

In terms of rate constants, the content of the kinetic constants is as follows:

V_r (saturation velocity of the reverse reaction) $= k_{-1}[E_T]$

$$K_{s\mathbf{Q}} = \frac{k_3}{k_{-3}}$$

$$K_{m\mathbf{P}} = \frac{k_2 + k_{-1}}{k_{-2}}$$

$$K_{m\mathbf{Q}} = \frac{k_{-1}}{k_{-3}}$$

$$K'_{i\mathbf{P}} = \frac{k_3(k_2 + k_{-1})}{k_{-1}k_{-2}}$$

Eqs. (1) and (2) predict that the effect of either **P** or **Q** as the varied substrate will be the same, since the equations are 'symmetric' with respect to **P** and **Q**. More specifically, we expect to observe patterns which are typical of a 'mixed-type effect' (both apparent V_{max} and K_m vary), when either **P** or **Q** are varied, i.e. the families of double reciprocal plots ($1/v$ vs. $1/[\mathbf{P}]$ and $1/v$ vs. $1/[\mathbf{Q}]$) at any fixed concentration of the other substrate will intersect above or below the x-axis, depending on the relative values of $K_{m\mathbf{Q}}$ and $K_{s\mathbf{Q}}$.

Figs. 2 and 3 show the two families of double reciprocal plots obtained in microgravity, when succinate (Fig. 2) or glyoxylate (Fig. 3) concentrations are varied. The slope effect on both primary plots indicates that the mechanism is sequential: no irreversible step occurs before the second substrate binds to the enzyme. Rough

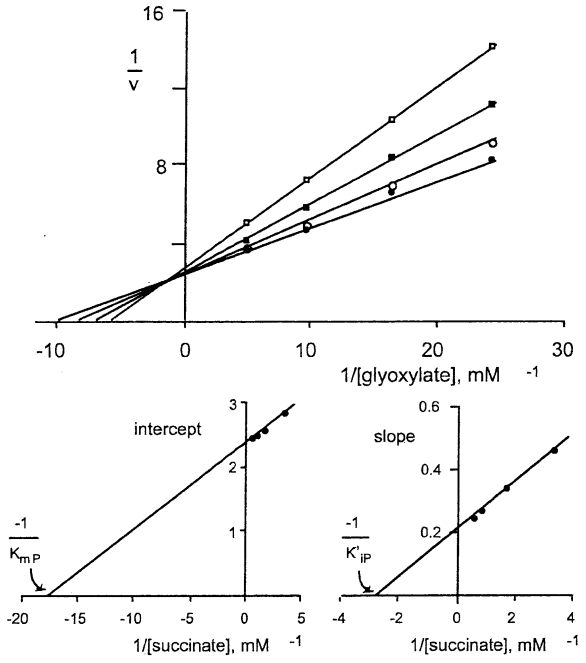


Fig. 3. Condensation reaction in microgravity. Effect of varying glyoxylate concentration in the presence of different fixed succinate concentrations (● 1.99, ○ 1.19, ■ 0.60, □ 0.30 mM). Below: $1/v$-axis intercept and slope replots vs. the reciprocal concentration of the fixed substrate.

estimates of the kinetic constants contained in Eqs. (1) and (2) were calculated from appropriate replots, shown in the same figures, below each primary plot. Refined values of these constants were determined from nonlinear fitting of the velocity equations to the complete set of experimental data, using the rough estimates as start points for the minimization algorithm. The values obtained in microgravity are compared in Table 1 with those obtained in ground controls.

This first approach, although consistent with a sequentially ordered bi–uni steady-state mechanism of the reverse reaction, does not enable the discrimination as regards which substrate is **P** and which is **Q**. Product inhibition studies can help to identify the order of product release. Indeed, the complete velocity equation predicts that in the absence of **Q**, **P** acts as a mixed-type inhibitor with respect to *A*, whereas, in the absence of **P**, **Q** acts as a competitive inhibitor. Of the two products, we had only the chance to study the effect of succinate, because of the limited number of flight sessions and the fact that the assay of enzyme activity for the cleavage reaction is based on the measurement of glyoxylate formation. Product inhibition analysis may even appear redundant, since the values of the kinetic constants determined in microgravity and in ground controls were almost identical (Table 1). However, this was a planned test, which, in addition, gave the opportunity to compare both the cleavage reaction parameters and the succinate inhibition constants. Fig. 4 shows the double reciprocal plots for the cleavage reaction in the presence of different concentrations of succinate, as performed in microgravity. The patterns, which are characteristic of mixed-type inhibition, confirms that succinate is the first product released. The kinetic parameters for the cleavage reaction and the succinate inhibition constants, as obtained in microgravity and in ground controls, are compared in Table 1.

4. Discussion

Our results indicate that isocitrate lyase reaction occurs in the same way both in microgravity and

Table 1
Kinetic parameters ± standard error for the condensation and cleavage reactions of *Pinus pinea* isocitrate lyase

	Standard g	Microgravity
Condensation reaction		
$V_r/[E_T]$ (s^{-1})	11.4 ± 0.3	11.7 ± 0.3
K_{sQ} (mM)	0.54 ± 0.17	0.60 ± 0.25
K_{mQ} (mM)	0.092 ± 0.004	0.088 ± 0.005
K_{mP} (mM)	0.063 ± 0.017	0.053 ± 0.020
K'_{iP} (mM)	0.37 ± 0.03	0.36 ± 0.04
Cleavage reaction		
$V_f/[E_T]$ (s^{-1})	17.5 ± 0.2	17.4 ± 0.2
K_{mA} (mM)	0.039 ± 0.002	0.043 ± 0.002
K_{sA} (mM)	0.14 ± 0.02	0.15 ± 0.01
K_{iP} (mM)	1.2 ± 0.2	1.3 ± 0.1
K_{eq} (M^{-1})	830 ± 200	910 ± 230

V_r and V_f are saturation velocities of the reverse and forward reactions, respectively. K_m indicates Michaelis constants for the subscript ligand; K_s indicates dissociation constants ($K_{sA} = k_{-1}/k_1$); $K_{iP} = (k_2 + k_3)/k_{-2}$; $[E_T]$ is total enzyme concentration. K_{eq}, the equilibrium constant of the glyoxylate + succinate = isocitrate reaction, was calculated from the Haldane equations: $K_{eq} = \frac{V_r K_{mA}}{V_f K_{sQ} K_{mP}} = \frac{V_r K_{sA}}{V_f K_{iP} K_{mQ}}$

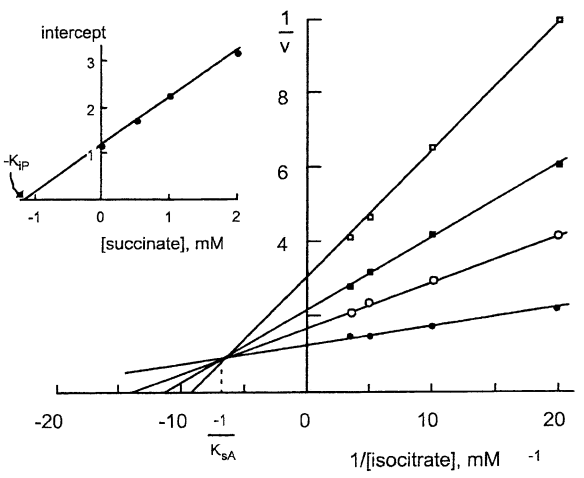


Fig. 4. Cleavage reaction in microgravity. Effect of varying isocitrate concentration in the presence of different fixed succinate concentrations (● none, ○ 0.5, ■ 1.0, □ 2.0 mM). *Inset:* $1/v$-axis intercept replot vs. the concentration of the fixed substrate.

in standard conditions. The comparison of the kinetic parameters, as determined in microgravity and at 1 g, does not show significant differences for either the cleavage or the condensation reactions; neither is the equilibrium constant altered in microgravity (Table 1). Moreover, the analysis of the cleavage reaction is consistent with the results previously obtained during the MASER 7 campaign [3].

The present findings do not agree with those obtained during the same parabolic flight campaign by another team. The latter found that in microgravity, the Michaelis constant of soybean lipoxygenase-1 for linoleic acid is decreased to one fourth of that of the ground control [6,7]. The authors described this effect as apparent, attributing it primarily to the unequal substrate dispersion (with the result that the formation of the enzyme–substrate complex is hampered) which probably forms when the assay mixture is in a gravitational field, contrary to uniform distribution favored by the weightlessness [7,16]. In short, the 'ideal' K_m is the one measured in microgravity.

Based on this supposition, we studied an assay model by computer simulation and we found that such an explanation was inconsistent with the experimental results. In this model, we assumed that, at standard g, starting from homogeneous distribution—as a consequence of the stirring produced by the injection whirl—the substrate tends to migrate toward the top of the assay cell, through subsequent degrees of narrower normal distributions, as schematized in Fig. 5. We also assumed that the enzyme concentration remains uniform.

For each different substrate distribution, we simulated velocity data with the same concentrations of linoleic acid used in the lipoxygenase experiment (15, 30, 60, 120 μM), the same V_{max} value (23 μM min^{-1}), and various 'ideal' K_m values; these velocity data were then used to calculate the apparent kinetic constants. To this end, it should be noted that both the 'ground' and 'flight' K_m values reported in the cited paper (10.5 and 2.6 μM) are quite probably misprints [7], and the actual values must in reality be tenfold higher (105 and 26 μM, respectively), as one promptly realizes by observing the patterns of the velocity profiles (see Fig. 1c in Ref. [7]). To fix the correct

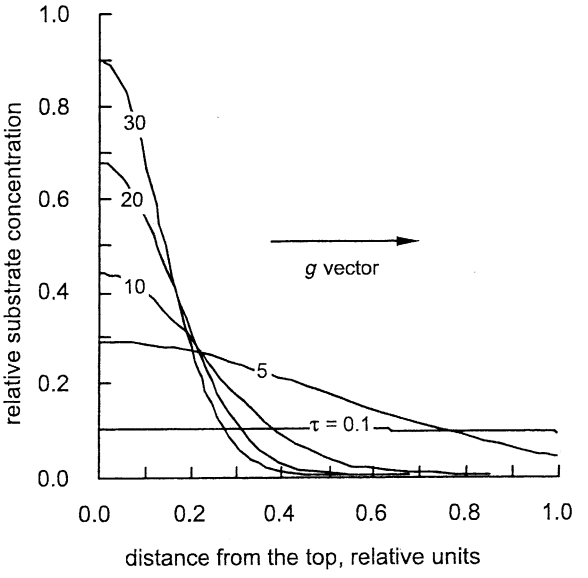


Fig. 5. A model of the relative distribution of linoleic acid within the assay cell, in standard g, at different times after mixing the solution. Data were obtained by integrating in subsequent 0.1 intervals the probability function of the normal distribution: $\phi_{(x)} = e^{-\tau x^2}$ τ value, indicated for each distribution curve, can be regarded as a relative time unit.

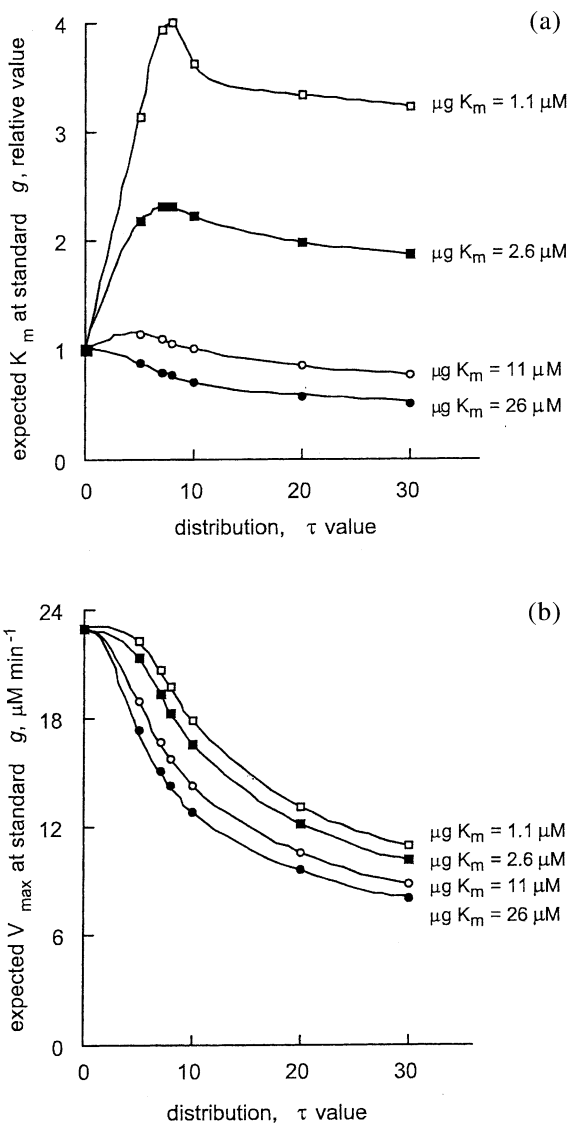


Fig. 6. Expected values for the apparent kinetic constants (a, K_m; b, V_{max}) of an enzyme that obeys Michaelis–Menten kinetics, as a function of varying substrate distribution in solution (Fig. 5 for the definition of τ value). The model assumes that when the substrate has a uniform distribution, $V_{max} = 23$ μM min^{-1} and K_m values are those termed μg K_m in the figures.

values of the Michaelis constants, although in no way does this impair the outcome of the lipoxygenase experiment, it is essential to support the subsequent reasoning. Indeed, as shown in Fig. 6a, the apparent K_m value determined according to the standard-g model, is higher than the 'ideal' one only in the case in which the latter value is less than 11 μM; in other words, on condition that the substrate concentrations used in the assays are all beyond the 'ideal' K_m value or close to saturation. Note that a fourfold increase in the apparent K_m value can be observed, for a given distribution ($\tau = 8$), provided that the 'ideal' K_m is 1.1 μM. In addition, the apparent V_{max} values are decreased on all occasions (more and more markedly as the substrate concentration moves away from uniform distribution), independently of the 'ideal' K_m values (Fig. 6b).

In conclusion, we think that the contrasting results of the two enzyme-catalysis experiments should be an incitement to continue this kind of research in microgravity, since more reliable and robust hypotheses are required to explain the reasons for the differences detected.

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