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Redistribution of the flux-control coefficients in mitochondrial oxidative phosphorylations in the course of brain edema

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This work describes the control exerted by dicarboxylate carrier and succinate dehydrogenase activities on the oxidative phosphorylations in rabbit brain mitochondria as an edema develops. Vasogenic edema leads to an uncompetitive inhibition of succinate dehydrogenase activity and to a large decrease of oxidative phosphorylations linked to succinate utilisation. Naftidrofuryl treatment in vivo restores both a high succinate dehydrogenase activity and a normal respiratory rate. In order to quantify the control of oxidative phosphorylations by the succinate dehydrogenase step, we applied the control analysis (Kacser, H. and Burns, J.A. (1973) in Rate Control of Biological Processes (Davies, D.D., ed.), pp. 65–104, Cambridge University Press, London; Heinrich, R. and Rapoport, T.A. (1974) Eur. J. Biochem. 42, 89–95). By using two inhibitors, one (phenylsuccinate) acting only on the dicarboxylate carrier and another (malonate) acting on both the dicarboxylate carrier and the succinate dehydrogenase, a method was developed to calculate the control coefficients of these two steps. The main result is that in mitochondria isolated from normal tissue succinate dehydrogenase exerted no control, but in the course of edema this enzymatic step became a controlling one: a transition from zero to a high control coefficient (0.5) was observed from the onset of intracellular edema for the threshold value of water/dry-weight tissue of 4.6.

Introduction

One of the best-known experimental models of brain insult leading to edema is the cryogenic lesion initially described by Klatzo [1]. In this model, which is relevant to the understanding of a number of clinical situations in neurosurgery, the main cellular disorder seems to be disturbances in lipid-protein organization (see Ref. 2 for a review); this affects several membrane-bound enzymes. Thus, cytochromes, ATPase complex and succinate dehydrogenase, which are involved in mitochondrial oxidative phosphorylations, decrease to differing degrees as vasogenic edema increase [3,4]. The consequence of such events is a large modification in the regulatory mechanisms and the functioning of this metabolic pathway. Therefore, in order to identify the key enzymes involved in the pathophysiological development of vasogenic brain edema, we applied the concept introduced by Kacser and Burns [5] and Heinrich

Abbreviations: DCIP, 2,6-dichlorophenol indophenol; naftidrofuryl, 3-(1-naphtyl)-2-tetrahydrofurylpropionic acid-diethylester-hydrogenoxalate; PMS, phenazine methosulfate.

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and Rapoport [6]; this led to a quantitative evaluation of the various enzymatic steps controlling metabolic flux. A description of the control distribution modifications during the course of edema must be a useful approach for understanding the initial functional alterations leading to irreversible damage. Moreover, it may be expected that drug-induced changes in the rate of metabolic processes are brought about by changes at one or more controlling steps in these processes. Identification of the rate-controlling steps in a metabolic pathway and knowledge of the particular enzymatic effects of drugs constitute useful tools for choosing the adequate treatment.

As an illustration, we quantified the participation of succinate dehydrogenase activity in the impairment of oxidative phosphorylations during the course of edema. The flux control coefficient of succinate dehydrogenase increases as edema increases, thus indicating that the modification of this activity plays an important role in respiratory rate control. This feature is almost completely responsible for the decrease of oxidative phosphorylations; indeed, treatment by a drug (naftidrofuryl) acting only in vivo at the succinate dehydrogenase level [7] is able to re-establish normal efficiency of oxidative phosphorylations.

Materials and Methods

Induction of edema. Rabbits (Fauve de Bourgogne bride) weighing about 2.2 kg were anaesthetized with ketamine chlorhydrate intravenous injections (30–40 mg per kg). According to the general procedure described by Klatzo [1], a freezing lesion was produced in four distinct sites of the right hemisphere by application of liquid nitrogen cryode (diameter, 4 mm) for 35 s onto the intact bone.

Quantification of edema. Since edema may be defined as an increase in tissue water content, the water/dry-weight ratio yielded the most accurate and straightforward quantitative assessment. The water content of the samples was measured by drying to a constant weight at 120 °C. The water/dry-weight ratio was given by

$$r = \frac{\text{wet tissue weight} - \text{dry tissue weight}}{\text{dry tissue weight}}$$

Condition of naftidrofuryl injections. Intravenous injections of naftidrofuryl (0.5 mg of naftidrofuryl in 2.5 ml of physiological serum per kg of rabbit) were performed 2 h before sacrifice (injection speed: 27 ml/h). The animals were killed by cardiac arrest at various times (from 6 h to 24 h) after cold injury. Chemical assays were carried out on normal or on edematous right hemispheres. The superficial necrotic areas produced by cryode applications were always removed before mitochondrial isolation.

Isolation of mitochondria. The right cerebral hemispheres were removed into an ice-cold isolation medium (0.25 M sucrose/10 mM Tris-HCl/0.5 mM EDTA. pH 7.4). Each hemisphere was rapidly cut out, chopped finely and washed three times. Mitochondria were isolated according to Clark and Nicklas [8]. Protein concentration was estimated by the biuret method using bovine serum albumin as standard.

The cytochrome $a + a_3$ content of isolated mitochondria was measured at room temperature with a Perkin-Elmer 356 spectrophotometer; the wavelength pair and extinction coefficient used to calculate cytochrome $a + a_3$ concentration from dual-wavelength measurements were:

$$\Sigma_{\text{redox}}^{605-630\,\text{nm}} = 24 \text{ (Ref. 8)}$$

No significant difference in organelle yield from normal and edematous hemispheres was observed when the marker used was cytochrome $a + a_3$.

Respiration studies. Mitochondrial oxygen consumption was measured at 30 °C in a 3 ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Gilson) in the following basal medium: 0.2 M sucrose, 20 mM Tris-HCl, 10 mM Tris-phosphate, 15 mM KCl, 0.3 mM EDTA, bovine serum albumin 0.3% (w/v) (pH 7.2).

Succinate dehydrogenase (EC 1.3.99.1) assays. Mitochondria (1 mg protein) were incubated at 30 °C for 10 min in the following medium: 0.2 M sucrose, 20 mM Tris-HCl, 10 mM Tris-phosphate, 15 mM KCl, 0.3 mM EDTA, bovin serum albumin 0.3% (w/v), 3.75 mM KCN, 0.8 μM rotenone at pH 7.2 containing 3 mg Triton X-100 per mg protein. Then 0.225 mM DCIP and various concentrations of succinate were added. The

reaction was started by addition of 1.6 mM PMS. DCIP reduction was monitored at 600 nm on a SP8-100 spectrophotometer Philips ($\epsilon_{600\,nm}$ = 21000 $M^{-1} \cdot cm^{-1}$ for DCIP).

Results

Impairment of oxidative phosphorylations and succinate dehydrogenase activity

In order to study overall oxidative phosphorylations, the respiration of mitochondria isolated from cerebral hemispheres excised at different times after the cold injury was measured (Fig. 1A). The respiratory rate observed in the presence of phosphate plus ADP (State 3) decreased largely as the water/dry-weight ratio (r) increased.

Naftidrofuryl (3-(1-naphtyl)-2-tetrahydrofurylpropionic acid-diethylester-hydrogenoxalate) is a spasmolytic vasodilating agent used for the treatment of cerebral and peripheral ischemia [9]. Intravenous injections of this drug performed, as indicated in the Materials and Methods section, 2 h before sacrifice, completely restored a normal respiratory rate of mitochondria isolated from edematous hemispheres (Fig. 1B). A previous study has shown that the consequence of drug administration is, at the mitochondrial level, an increase of succinate dehydrogenase activity. However, other enzymatic activities implicated in oxidative phosphorylations are not affected [7].

The decrease of the respiration rate in State 3 observed in mitochondria isolated from cerebral hemispheres excised at different times after the cold injury (Fig. 1A) is a direct consequence of a loss of the maximal oxidative phosphorylations activity. In effects, the ADP/O ratio does not change during edema [3] and the respiratory control indice decreases as the respiratory rate in State 3 as an edema develops (compare Fig. 2 to Fig. 1A). Moreover, it can be noted that drug administration re-establishes a normal respiratory indice value (not shown).

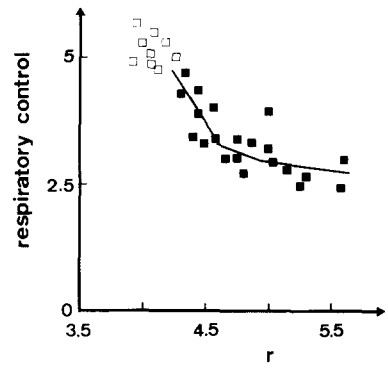

Fig. 2. Change in respiratory control during the course of edema Experimental conditions were as in Fig. 1A.

Fig. 3 shows the mechanism of succinate dehydrogenase inhibition by comparison of the double reciprocal plots of this activity measured in mitochondria isolated from normal and edematous brain. The two kinetic parameters V_m and K_m

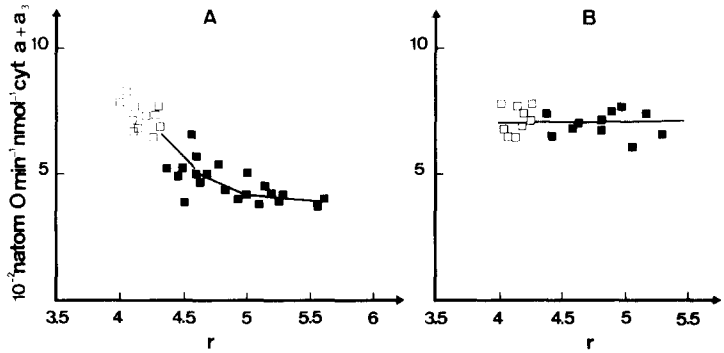

Fig. 1. Change in respiratory rate during the course of edema: effect of naftidrofuryl. Mitochondria (1 mg protein/ml) were incubated at 30 ° C in the basal medium (see Materials and Methods section) with 3 mM succinate and 0.8 μM rotenone. The State 3 respiration was obtained after addition of 0.13 mM ADP. Each point corresponds to the mean of three measurements carried out on a particular mitochondrial preparation. Mitochondria isolated from: (A) normal tissue (□); edematous tissue (■); and (B) normal tissue after naftidrofuryl treatment (□); edematous tissue after naftidrofuryl treatment (■).

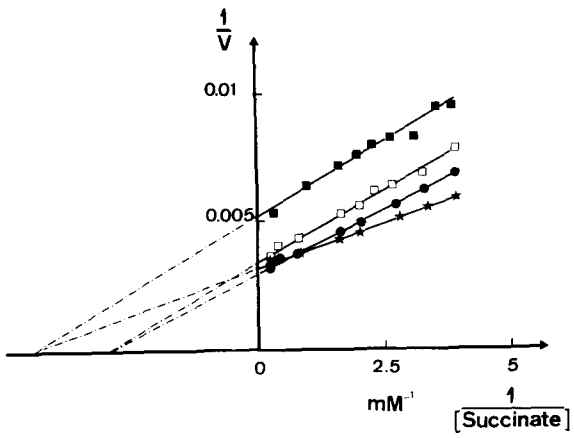

Fig. 3. Effects of edema and naftidrofuryl treatment on succinate dehydrogenase activity. Mitochondria (1 mg protein) were incubated 10 min at 30 °C in the basal medium (see the Materials and Methods section and the legend to Fig. 1) containing 3 mg Triton X-100 per mg protein. Then 0.225 mM DCIP and different concentrations of succinate were added. The reaction was started by addition of 1.6 mM PMS. The rate of succinate dehydrogenase activity is given as nmol DCIP reduced per min per nmol cytochrome $a + a_3$. Mitochondria are isolated from: normal tissue ($r = 3.9$) (□), edematous tissue ($r = 4.5$) (■), normal tissue after naftidrofuryl treatment ($r = 3.9$) (●), and edematous tissue after naftidrofuryl treatment ($r = 4.5$) (★).

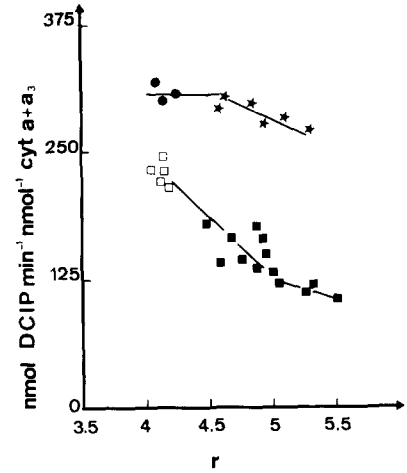

Fig. 4. Change in succinate dehydrogenase activity during the course of edema: effect of naftidrofuryl treatment. The experimental conditions were as in legend to Fig. 3, except that succinate concentration was 3 mM. Mitochondria are isolated from: normal tissue (□), edematous tissue (■), normal tissue after naftidrofuryl treatment (●) and edematous tissue after naftidrofuryl treatment (★).

were modified, with the result that the V_m/K_m ratio remained approximately constant; this indicated an uncompetitive inhibition of this membrane-bound enzyme. Drug administration induced an increase of V_m, K_m remaining constant in both normal and edematous tissue mitochondria (Fig. 3). This resulted in a better efficiency of succinate dehydrogenase activity in mitochondria isolated from normal and edematous brains after treatment than in controls, where succinate was at an almost saturating concentration (Fig. 4).

Control on oxidative phosphorylations exerted by dicarboxylate carrier and succinate dehydrogenase activities

The fact that an increase of succinate dehydrogenase activity induced by drug administration is able to re-establish a normal efficiency of oxidative phosphorylations (see Figs. 1 and 4) indicates that this enzyme catalyzes an important step for respiratory-activity control.

In a metabolic pathway, the flux control coefficient C_i of an enzymatic step is defined as:

$$C_i = \left.\frac{\partial \ln J}{\partial \ln v_i}\right|_{\text{at steady state}} \quad (1)$$

where J is the whole flux and v_i is the rate of the isolated step in the same conditions as in the pathway. In our work, the control coefficients were determined using specific inhibitors, according to the equation:

$$C_i = \left.\frac{\frac{\partial \ln J}{\partial I}(I=0)}{\frac{\partial \ln v_i}{\partial I}(I=0)}\right|_{\text{at steady state}} \quad (2)$$

where I is the concentration of the specific inhibitor of the step i.

However, no specific inhibitor of succinate dehydrogenase alone is available and succinate externally added as respiratory substrate is involved in two successive steps: transport by dicarboxylate carrier and succinate dehydrogenase activity. To overcome these difficulties, we used two inhibitors, one (phenylsuccinate) acting only on the dicarboxylate carrier, since it is not trans-

ported into mitochondria [10] and the other (malonate) acting on both steps.

Since phenylsuccinate is a competitive inhibitor, the flux control coefficient of dicarboxylate carrier is given by [11]:

$$C_i = \frac{-K_i(1+S/K_m)}{J}\frac{dJ}{dI} \quad (3)$$

Thus, the flux control coefficient of the dicarboxylate carrier was calculated using a K_i for phenylsuccinate of 0.7 mM and a K_m for succinate of 1.2 mM [12]; it was assumed here that these kinetic parameters did not vary during the course of edema. As shown in Fig. 5, this flux control coefficient slightly decreased when edema increased, both with and without the injection of naftidrofuryl.

Malonate is a competitive inhibitor [13] transported into mitochondria by the dicarboxylate carrier and acting on succinate dehydrogenase.

The whole flux is a function of the rate of dicarboxylate carrier v_1 and of succinate dehydrogenase v_2:

$$J = J(v_1, v_2)$$

where v_1 and v_2 are dependent on the malonate concentration.

Thus, we can write:

$$\frac{\partial \ln J}{\partial [mal]} = \frac{\partial \ln J}{\partial \ln v_1}\frac{\partial \ln v_1}{\partial [mal]} + \frac{\partial \ln J}{\partial \ln v_2}\frac{\partial \ln v_2}{\partial [mal]}$$

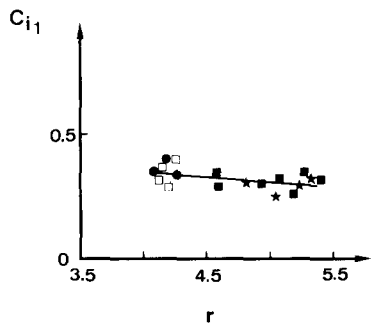

Fig. 5. Evolution of control coefficient of dicarboxylate carrier Flux-control coefficient was calculated from phenylsuccinate inhibition curves. Experimental conditions were as in legend to Fig. 1. Mitochondria isolated from: normal tissue (□), edematous tissue (■), normal tissue after naftidrofuryl treatment (●) and edematous tissue after naftidrofuryl treatment (★).

and for (mal) = 0, we obtain, according to Eqn. 1:

$$\frac{\partial \ln J}{\partial [mal]}(mal=0) = C_1\frac{\partial \ln v_1}{\partial [mal]}(mal=0) + C_2\frac{\partial \ln v_2}{\partial [mal]}(mal=0) \quad (4)$$

where C_1 and C_2 are the flux-control coefficients of the two involved steps.

Then from Eqn. 4 we obtain:

$$C_2 = \frac{\dfrac{\partial \ln J}{\partial [mal]}}{\dfrac{\partial \ln v_2}{\partial [mal]}} - C_1\frac{\dfrac{\partial \ln v_1}{\partial [mal]}}{\dfrac{\partial \ln v_2}{\partial [mal]}} \quad (5)$$

In order to calculate C_2 we have to estimate the different terms involved in Eqn. 5. C_1 is known for the phenylsuccinate inhibition curves. Since, after injection of naftidrofuryl, succinate dehydrogenase activity from normal or edematous tissue was higher than that observed in controls (see Fig. 4), the fact that no supplementary increase of respiratory activity under these conditions was detected shows that this enzymatic step exerted no control on oxidative phosphorylations. Then, after naftidrofuryl injection, C_2 is nil and in this conditions, d ln v_1/d [mal] can be calculated from the peculiar expression of Eqn. 4 in this case:

$$\frac{\partial \ln v_1}{\partial [mal]} = \frac{1}{C_1}\frac{\partial \ln J}{\partial [mal]}\Big|_{\text{at steady state + naftidrofuryl}} \quad (6)$$

As naftidrofuryl injection does not affect the kinetic properties of the dicarboxylate carrier, this value of d ln v_1/d [mal] is also the one in the absence of naftidrofuryl. d ln v_2/d [mal] can be obtained for each particular condition from the titration curves of succinate dehydrogenase with malonate. One example of such titration curves obtained with mitochondria isolated from normal or edematous tissue is given in Fig. 6. Finally, d ln J/d [mal] is calculated from the malonate inhibition curve of the respiratory flux (Fig. 6).

Putting all these values together in Eqn. 5 allows the determination of the flux-control coefficient C_2 of the succinate dehydrogenase step on respiratory flux. Fig. 7 shows the evolution of the flux-control coefficient of succinate dehydrogenase on respiratory activity as a function of the

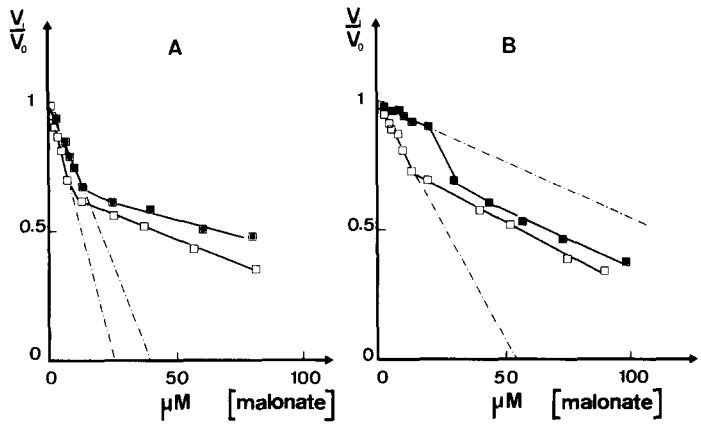

Fig. 6. Inhibition by malonate of oxygen-consumption flux and succinate dehydrogenase activity. Experimental conditions were as described in legends of Figs. 1 and 3. Succinate concentration was 2 mM. Mitochondria were isolated from edematous tissue ($r = 5.52$) (A); from normal tissue ($r = 4.26$) (B). ■, oxygen-consumption flux; □, succinate-dehydrogenase activity.

course of edema. Up to an r value of 4.6, this coefficient was nil: but between 4.6 and 5, it increased rapidly to a value of 0.5. Afterwards, the flux-control coefficient of succinate dehydrogenase remained constant at this high value.

Discussion

It is now well established in mitochondria from various origins that control of oxidative phosphorylations takes place over many steps, and that the distribution of control as measured by flux-control coefficients varies according to different steady states [11,14–17]. Since, stimulation of succinate dehydrogenase activity by naftidrofuryl injection does not induce an increase of respiratory rate in mitochondria from normal brain (see Figs. 1 and 4), this step cannot be considered as a control under these conditions, as generally reported. However, in the course of edema, the decrease of oxidative phosphorylations supported by succinate oxidation seems largely linked to succinate dehydrogenase alteration. Indeed, naftidrofuryl, acting only at the succinate dehydrogenase level, is able to restore a normal respiratory rate in edema brain mitochondria (Fig. 1). The theoretical and experimental approaches, called control analysis (see Ref. 18 for a recent review), can lead to a quantitative description of such an evolution in an intact metabolizing system. In our work, there were two main difficulties for the application of the inhibitor method. The first one is a usual one: indeed, in theory, for the determination of the flux-control coefficient of an enzymatic step within a metabolic pathway, v_i (see Eq. 2) has to be measured under the same conditions. However, this is often difficult and sometime impossible to realise. For measuring the succinate dehydrogenase activity alone, it is necessary to by-pass the dicarboxylate carrier step and thus to add a low concentration of Triton for permeabilizing the mitochondria to substrate and inhibitor. It is assumed here that the kinetic properties of succinate dehydrogenase are not significantly modified by this treatment. The second difficulty is the lack of specific inhibitor of suc-

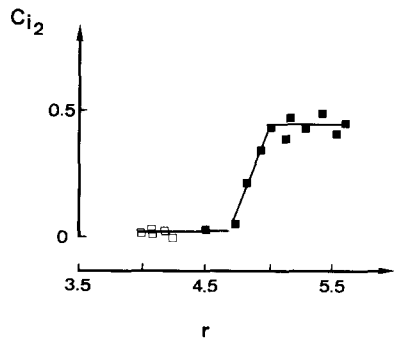

Fig. 7. Control coefficient of succinate dehydrogenase as a function of edema development. Values were calculated from the experiments like those presented in Fig. 6. Mitochondria are isolated from: normal tissue (□) or edematous tissue (■).

cinate dehydrogenase. But by using two inhibitors, one (phenylsuccinate) acting only on the dicarboxylate carrier and the other (malonate) acting on both steps, it is possible to evaluate the different flux-control coefficients. It must be underlined that the threshold transition between no control and a high succinate dehydrogenase control coefficient occurs for a water/dry-weight tissue ratio of 4.6 when the activity of this enzymatic step is strongly reduced (compare Figs. 1 and 7). One of the predictions of the theory is that the sum of all the control coefficients of a given flux is equal to 1. When the edema is extensive (r above 4.8) the sum of the two control-flux coefficients calculated in this work ($C_1 + C_2$) is about 0.8; this indicates that most of the control is confined to these two steps.

It should be noted that the flux-control coefficient of the dicarboxylate carrier have no relevance to the in vivo situation, since only endogenously formed succinate is utilized by mitochondria in situ. Moreover, concerning the control exerted by the succinate dehydrogenase it could be noted that the ATP production linked to pyruvate or pyruvate + malate oxidations in isolated mitochondria is largely controlled by succinate dehydrogenase activity during edema [4]. However, alternative transmembranal metabolic pathway (i.e., pyruvate cycle) by-passing this step could operate and play an energetic role in isolated mitochondria [4]. Thus, it is difficult to evaluate the actual consequence of the decrease of the succinate dehydrogenase activity on the control of oxidative phosphorylations in vivo.

Anyway, even if the decrease of respiratory activity could be related to the alteration of the succinate dehydrogenase velocity, the course of edema alone is not a direct consequence of oxidative phosphorylations impairment, as previously proposed [4]: naftidrofuryl treatment, which completely restores oxidative phosphorylations, does not prevent the edema from evolving, as measured by brain water increase (Fig. 1). This agrees with the previous conclusions that the trapping of Na^+ within the cells which leads to intracellular uptake of water during vasogenic edema is caused by impairment of Na^+-K^+-ATPase and not by a deficit in the overall energy production [3,4].

One of the aims of this work is to show that the application of control theory is a useful approach for understanding the initial functional alterations leading to the pathologic process. Indeed, it may be expected that drug-induced changes in the rate of a particular metabolic process are brought about by changes at one or more controlling steps in this process. Identification of the rate-controlling steps in a metabolic pathway and knowledge of the enzymatic effects of drugs constitute one of the useful tools for choosing adequate treatment in certain pathologies.

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