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# Application of metabolic control analysis to the study of toxic effects of copper in muscle glycolysis

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**Abstract Experimental and model studies have been performed to characterise the effects of $Cu^{2+}$ on the activities of individual glycolytic enzymes and on the flux and internal metabolite concentrations of the upper part of glycolysis in mouse muscle extracts. $Cu^{2+}$ significantly inhibited the triosephosphate production from glucose with an $IC_{50}$ of about 6.0 μM. At a similar extension $Cu^{2+}$ inhibited hexokinase and phosphofructokinase, with an $IC_{50}$ of 6.2 μM and 6.4 μM respectively, whereas the effects on the activities of aldolase, phosphoglucose isomerase and the internal metabolite levels were not significant. Flux control coefficients and flux response coefficients were determined in the presence of copper concentrations between 0 and 10 μM. The same values of flux control coefficients for hexokinase and for phosphofructokinase (0.8 and 0.2 respectively) were found in absence and in presence of copper. At $Cu^{2+}$ equal to the flux $IC_{50}$, the response coefficient was −1. The elasticity coefficients for hexokinase and phosphofructokinase at $Cu^{2+}$ equal to the $IC_{50}$ were also −1. A mathematical model was used to analyze the effect of copper on glycolysis under different conditions using experimental kinetic parameters and rate equations for enzymatic reactions of the upper part of glycolysis.**


*Key words:* Glycolysis; Copper; Inhibition; Metabolic Control Analysis

## 1. Introduction

The increase in industrial activity in the last few decades has resulted in a considerable increase in the presence of heavy metals in the environment [1–4].

Pollutants such as heavy metals easily enter living organisms and accumulate in several tissues [5–7]. It is well described that several heavy metals have a high affinity for the free electron pairs of the S-H groups [5,8]. Therefore, heavy metals irreversibly inhibit several enzymes of the metabolism at the same time, e.g. by interfering with their S-H groups, and they affect most metabolic pathways simultaneously. Metabolic Control Analysis (MCA) has been used to quantify the effect at the systemic level of many simultaneous minor lesions at various enzymes.

Since its development by Kacser and Burns [9] and Heinrich and Rapoport [10] extensive theoretical and experimental work has been performed by MCA using different methods (for a review see [11]). One of the most important challenges of MCA is the possibility of a rational and quantitative evaluation of the relation between the kinetic properties of the enzyme and the degree of control that they exert on the systemic variables of interest in a metabolic pathway. In general the variables of interest analyzed have been the flux and intermediate metabolite levels ([12–14,11] and references therein).

MCA predicts that the control of the flux is not only located in one step but it tends to be shared between more than one enzyme in a pathway and MCA predicts that the distribution of control between the different steps of a pathway can change when external conditions or parameters are changed. A corollary is that MCA allows analysis of the case in which a single agent affects the cell at many steps in a metabolic pathway.

In this study we focused on the effect of copper on glycolysis in muscle which is straightforward with respect to bioenergetic processes. We restricted this study to the effects of copper because it appears to be a representative heavy metal in terms of its environmental impact and its effects are well described in the literature.

The aim of this study is to identify the sites of action of copper in the upper part of glycolysis and also to illustrate that MCA is a useful quantitative and predictive tool for the investigation of the action of a toxic agent on a metabolic pathway.

## 2. Materials and methods

### 2.1. Chemicals

ATP (sodium salt), NADH, glucose (Glc), glucose-6-P (G6P), fructose-6-P (F6P), fructose-1,6-bisphosphate (FBP), phosphocreatine (PC), hexokinase (HK), glucose-6-phosphate isomerase (GPI), phosphofructokinase (PFK), aldolase (ALD), triose-phosphate isomerase (TPI), α-glycerol-3-phosphate dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PDH), creatine kinase (CK), $CuCl_2$, trypsin inhibitor, HEPES and MOPS were purchased from Sigma Chemical. Bio-Rad protein assay was purchased from Bio-Rad Laboratories Gmbh. All other chemicals (analytical grade) were purchased from Panreac.

### 2.2. Preparation of muscle extracts

Leg muscle of 8–16 week old C57BL/6 mice (IFFA Credo, Spain) was minced with scissors and 1 g muscle was homogenised in 2.5 ml standard buffer (50 mM Hepes buffer, pH 7.4, containing 100 mM KCl, 10 mM $NaH_2PO_4$ and 10 mM $MgCl_2$) by using a Potter-Elvehjem glass homogeniser at low speed (1000 rev/min). 1 mg of trypsin inhibitor/3 ml homogenate was added. The homogenate was centrifuged at $31\,000 \times g$ for 30 min. The resulting supernatant was filtered through a Sephadex G25 column (5.5×1.6 cm) to remove endogenous metabolites. All procedures were carried out at 4°C. The protein concentration of the extract was measured and, if necessary, the extract was diluted to adjust the protein concentration up to 12 mg/ml.

### 2.3. Determination of protein concentration

Protein concentrations were determined using the Bradford method (Bio-Rad Laboratories) [15].

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*2.4. Pre-incubation of muscle extract with $CuCl_2$*

The extract was incubated with $CuCl_2$ (0–10 μM) in standard buffer at 37°C for different times. The protein concentration in the incubation mixture was 1.2 mg/ml.

*2.5. Determination of the flux for the conversion of glucose to triosephosphates*

The steady state fluxes were measured in standard buffer at 37°C by coupling the reaction with excess TPI/GDH activities. The final concentrations in the cuvette were 2 mM NADH, 2 mM MgATP, 10 mM Glc, 20 mM PC, 3 U/ml CK, 7 U/ml TPI, and 2 U/ml GDH. NADH consumption was monitored at 385 nm ($\varepsilon_{NADH}^{385nm} = 0.75\ mM^{-1}\ cm^{-1}$) as described by Puigjaner et al. [16] using a Shimadzu-UV-2101-PC spectrophotometer with 1 cm light path cells.

The reaction was started by adding 100 μl reaction mixture to 900 μl of extract pre-incubated with the appropriate amount of $CuCl_2$ (0–10 μM) for 60 min.

*2.6. Determination of metabolite concentrations*

When the NADH consumption was constant, the consecutive reactions were stopped at different times by addition of ice-cold $HClO_4$ at a final concentration of 10% and neutralised to pH 7.0 with an appropriate amount of KOH/MOPS (6 M/0.6 M). After 10 min, the precipitate was removed by centrifugation for 10 min at $14\,000 \times g$. The supernatants were used for the enzymatic determination of G6P, F6P and FBP according to Bergmeyer [17].

*2.7. Modulation of steady state flux and metabolite concentration by external enzymes and determination of flux control coefficients*

For classical titration analysis the steady state flux and metabolite concentrations were measured in the presence of different amounts of the commercial enzyme (HK, GPI, PKF or ALD). In each case the appropriate amounts of commercial enzyme were added to the extract pre-incubated with $CuCl_2$ (0–10 μM) for 60 min at 37°C. The reaction was started by the addition of 100 μl of the reaction mixture containing the different amounts of commercial enzyme to 900 μl of the pre-incubated extract. Flux control coefficients ($C_{vi}^{J}$ = d ln (flux)/d ln (enzyme$_i$ activity)) in absence and presence of $Cu^{2+}$ with regard to HK, GPI, PFK and ALD were obtained using Small and Kacser's method for large changes in enzyme activities [18]. The flux control coefficient according to this method is computed as:

$$C_J^{vi} = \frac{1 - J_i/J_f}{({}^{vf}/_{vi} - 1)/({}^{vf}/_{vi})}$$

where $J_i$ and $J_f$ are the initial and final metabolic flux respectively, $v_i$ and $v_f$ are the initial and final enzyme activities and $C_{vi}^{J}$ is the flux control coefficient at the initial steady state. $J_i$ and $v_i$ were measured according the described above in basal conditions and $J_f$ and $v_f$ were measured after adding an excess of the *i*-enzyme. Typically 10–20-fold excess of the endogenous activities was used for titration. This method of calculating control coefficients was preferred to others (see [11] for a review) because the error associated with the enzyme activity added is negligible when large amounts of commercial enzyme are added. This is especially important since added commercial enzymes can also be partially inactivated by copper. We checked that this method gives similar results in the absence of copper compared to other classical titration methods in the absence of copper when 10, 20 and 50% of the endogenous activities were used for titration [16].

*2.8. Measurements of enzyme activities of the extract*

Activities of HK, GPI, PFK and ALD were measured in the standard buffer with 900 μl of pre-incubated extract mixture (the pre-incubated extract mixture contained the appropriate concentration of $CuCl_2$ and, also, when titration experiments were performed, the appropriate amounts of commercial enzymes) at 37°C. GPI and ALD activities were determined according to Bergmeyer's methods [17].PFK activity was measured according to the method of Brand and Söling [19]. HK activity was measured as described by Grossbard and Schimke [20].

*2.9. Determination of response coefficients of the flux*

Response coefficients of the flux ($R_{Cu}^{J}$=d ln (flux)/d ln [$Cu^{2+}$]) at different copper concentrations were obtained from the slope of the log (flux) versus log [$Cu^{2+}$] experimental curve for each copper concentration.

*2.10. Determination of elasticity coefficients with regard to $Cu^{2+}$*

Elasticity coefficients ($\varepsilon_{Cu}^{v}$ = d ln (enzyme activity)/d ln [$Cu^{2+}$]) at any copper concentration were calculated from the slope of the log (enzyme activity) versus log [$Cu^{2+}$] experimental curve for the desired copper concentration.

*2.11. Computer modelling of the pathway*

The model was constructed using an IBM-PC version of the simulation and the control package MIST [21]. The rate equations of HK, GPI, PFK and ALD used for the kinetic model are listed in Table 1. The equations are the same as those obtained by Puigjager et al. [16], in the absence of copper, except those for HK and PFK, which were modified to include the dependence of enzyme activity with regards to copper concentration, determined experimentally in this paper. To derive the appropriate rate equations of HK and PFK,

Table 1
Rate equations used in the kinetic model and kinetic parameters for HK, GPI, PFK, and ALD in the extract

| | | |
|---|---|---|
| HK | $v = \dfrac{V_{max}^{HK}[Glc]}{K_m^{HK} + [Glc]\left(1 + \dfrac{[G6P]}{K_I^{G6P}}\right)}$ | $K_m^{HK} = 0.4019$<br>$K_I^{G6P} = 0.111$<br>$V_{max}^{HK} = (63.0 - 4.68 * [Cu^{2+}])$ |
| GPI | $v = \dfrac{\left(\dfrac{V_{max}^{GPI_f}[G6P]}{K_m^{GPI_f}}\right) - \left(\dfrac{V_{max}^{GPI_b}[F6P]}{K_m^{GPI_b}}\right)}{1 + \dfrac{[G6P]}{K_m^{GPI_f}} + \dfrac{[F6P]}{K_m^{GPI_b}}}$ | $K_m^{GPI_f} = 0.48$<br>$K_m^{GPI_b} = 0.272$<br>$V_{max}^{GPI_f} = 12474$<br>$V_{max}^{GPI_b} = 18125$ |
| PFK | $v = \dfrac{V_{max}^{PFK}[F6P]^n}{(S_{0.5}^{PFK})^n + [F6P]^n}$ | $SPFK_{0.5} = 0.061$<br>$V_{max}^{PFK} = (434 - 33.33 * [Cu^{2+}])$<br>$n$=1.4744 |
| ALD | $v = \dfrac{V_{max}^{ALD}[FBP]}{K_m^{ALD} + [FBP]}$ | $K_m^{ALD} = 0.1297$<br>$V_{max}^{ALD} = 6000$ |

$V_{max}$ and $K_m$ are expressed in nmol/min mg protein and mM respectively.

we take into account that copper is an irreversible inhibitor and combines with the enzyme in a stoichiometric manner: $E+nCu^{2+} \rightarrow ECu_n^{2+}$ (inactive). Consequently, the $V_{max}$ of the enzyme depends on the copper concentration according to the following equation: $V_{max} = k_{cat}(E_{TOTAL} - ECu_n^{2+}) = k_{cat}(E_{TOTAL} - Cu^{2+}/n)$. Calculation of the flux control coefficients and response coefficients in the presence of $CuCl_2$ were computed by numerical differentiation.

## 3. Results and discussion

### 3.1. Inhibition of glycolytic enzymes by $Cu^{2+}$

To establish the conditions of the experiments, the time dependency of the inhibition of HK, PGI, PFK and ALD by $Cu^{2+}$ was examined. Mouse muscle extract at a protein concentration of 1.2 mg/ml was pre-incubated with different $Cu^{2+}$ concentrations (0–10 μM) for 2 h at 37°C. Every 20 min the activities of HK, GPI, PFK and ALD were measured. In the range of $Cu^{2+}$ concentrations tested, the activities of HK and PFK decreased until they reached a limit value after 60 min. GPI and ALD activities remained constant during this interval of time for all the $Cu^{2+}$ concentrations tested. The decrease in the activity of the enzymes due to inactivation by temperature after 60 min was negligible. On the basis of these results a pre-incubation time of 60 min was chosen to ensure that the reaction of the enzymes with copper was completed.

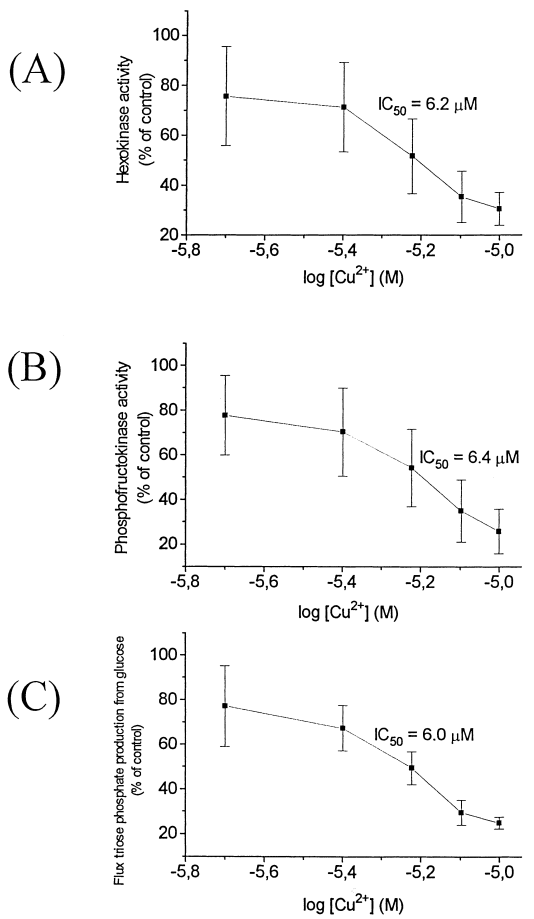


Fig. 1. Effect of $Cu^{2+}$ on mouse muscle extract hexokinase activity (A), phsophofructokinase activity (B) and triosephosphate production steady state flux from glucose (C). All values were mean ± S.D. of three separate determinations. Extracts were pre-incubated with copper for 60 min. For other details see Section 2. Control values of steady state hexokinase and phosphofructokinase activities and triosephosphate production flux were respectively: 57.7, 212.7 and 47.5 nmol/min mg protein.

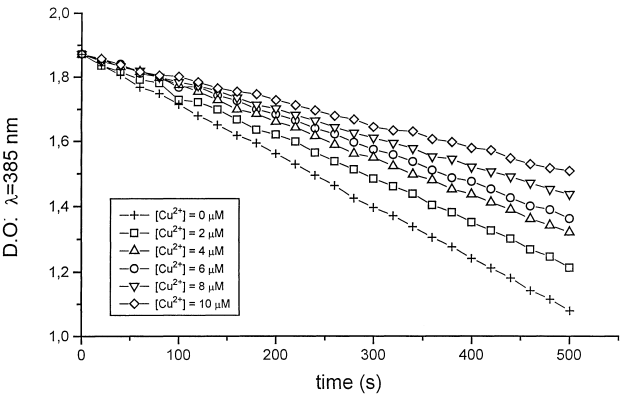


Fig. 2. Time courses of the NADH consumption in muscle extract in the absence and presence of $Cu^{2+}$. The reaction of triosephosphate formation from glucose was coupled with excess TPI/GDH. Extracts were pre-incubated with copper during 60 min. For other details see Section 2.

$Cu^{2+}$ inhibited hexokinase activity in a concentration-dependent manner (Fig. 1A). At 2 μM and 10 μM $Cu^{2+}$, this inhibition was about 25% and 70% respectively. The $IC_{50}$ for $Cu^{2+}$ was 6.2 μM (Fig. 1A). $Cu^{2+}$ also inhibited phosphofructokinase activity to the same extent as hexokinase. The $IC_{50}$ for $Cu^{2+}$ was 6.4 μM (Fig. 1B).

### 3.2. Effects of $Cu^{2+}$ on steady state flux of the triosephosphate production and on steady state intermediate metabolite concentrations

The extract was pre-incubated with $Cu^{2+}$ (0–10 μM) in standard buffer at 37°C for 60 min. Fig. 2 shows typical time courses of the triosephosphate production flux by muscle extract from glucose. The basal rate (47.5 nmol/mg protein/min), which was constant for several minutes, was signifi-

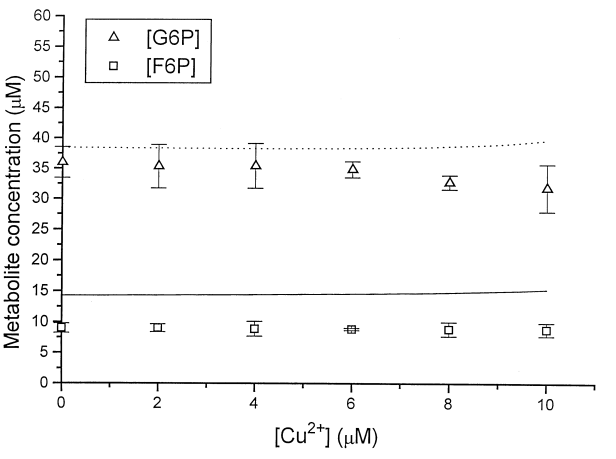


Fig. 3. Dependence of the steady state glucose-6-phosphate (△) and fructose-6-phosphate (□) concentrations on copper concentrations. The metabolite concentrations were determined when the flux of triosephosphate production from glucose by the extract proceeds at a constant rate. The extract was pre-incubated with copper for 60 min. For other details see Section 2. The glucose-6-phosphate and the fructose-6-phosphate concentrations predicted with the mathematical model constructed are indicated by a dotted line and a solid line respectively.

cantly decreased by the addition of $Cu^{2+}$. The $IC_{50}$ of $Cu^{2+}$ of the triosephosphate production from glucose was 6.0 μM (Fig. 1C). The concentrations of G6P and F6P determined at 250 and 500 s in the extract pre-incubated without $Cu^{2+}$ were constant (36.0 ± 2.6 μM and 9.0 ± 0.8 μM), suggesting that glucose conversion proceeds at steady state rate. The steady state basal concentrations of G6P and F6P were not significantly altered when the extract was pre-incubated with different concentrations of $Cu^{2+}$ (Fig. 3).

Similar sets of experiments carried out with muscle extract at different dilutions showed that the steady state rates ($J$) were proportional to the amount of extract pre-incubated either in absence or in presence of copper and no alterations in the steady state concentrations of G6P and F6P were detected at different dilutions (data not shown). These findings provide evidence that the summation theorem ($\Sigma C^J_{Ei} = 1$) for flux control coefficients is fulfilled even for the extract that was pre-incubated with copper.

The control coefficients determined using Small and Kacser's method for large changes in enzyme activities [18] were 0.8 and 0.2 for HK and PFK irrespective of whether the extract was pre-incubated in absence or in presence of $Cu^{2+}$ (2–10 μM). Control coefficients of GPI and ALD were negligible in basal conditions and in the range of copper concentrations tested. These data indicate that HK and PFK, the two enzymes that control the upper part of glycolysis in muscle extract, were inhibited to the same extent by copper and that GPI and ALD were practically not affected. The finding that the control coefficient distribution among the enzymes is the same in the presence and in absence of $Cu^{2+}$ is consistent with the similar $IC_{50}$ values found for HK and PFK and with the fact that metabolite concentrations were unchanged.

### 3.3. Determination of the response coefficient of $Cu^{2+}$ on the triosephosphate production flux

Fig. 4 shows the flux response coefficient at different copper concentrations obtained from the slope of the log (flux) versus log [$Cu^{2+}$] experimental curve (data in Fig. 1C) at each copper concentration. The response coefficient values were always negative and increased in absolute value with the concentration of $Cu^{2+}$.

At a $Cu^{2+}$ concentration of 6.0 μM, that gave 50% inhibition of the triosephosphate production flux from glucose, the response coefficient was −1.

The flux response coefficient at the $IC_{50}$ can also be computed from the multisite response equation that relates the kinetic properties of the enzymes (elasticities) with the systemic properties of the pathway (control and response coefficient), since elasticities and flux control coefficients were measured at the same copper concentration (see [11] for a review).

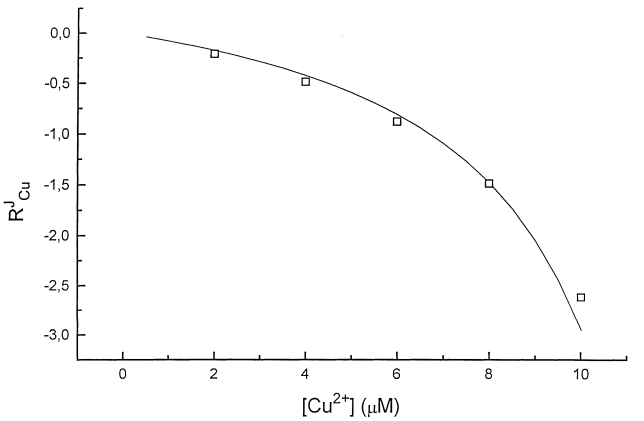


Fig. 4. Response coefficients of copper on triosephosphate production flux versus the copper concentrations. The solid line represents the flux response coefficient values calculated from the kinetic model constructed. The experimental values (□) were calculated from the data presented in figure 1C (as the slope of the log (flux) versus log [$Cu^{2+}$] experimental curve at each copper concentration).

From the data displayed in Fig. 1A,B, we can also compute the elasticity coefficients of hexokinase and phosphofructokinase with regards to copper as the slope of the log (enzyme activity) versus log [$Cu^{2+}$] experimental curve at the $IC_{50}$ obtained for the triosephosphate production flux. The elasticity values obtained for both enzymes were −1.

It should be noted that in the case of hexokinase the elasticity coefficient was measured at the same fixed concentration of glucose as that at which flux was measured. Accordingly, at the steady state conditions corresponding to a copper concentration equal to the $IC_{50}$, this elasticity value can be directly related with the response coefficient by means of the multisite response equation. The substrate concentration at which elasticity is computed for phosphofructokinase was different from the steady state concentration of substrate obtained when we measured the flux at a copper concentration equal to the $IC_{50}$. However, taking into account that copper is an irreversible inhibitor and thus only affects the apparent $V_{max}$ of the enzyme, the elasticity coefficient with regard to copper does not depend on the substrate concentration. The elasticity value obtained for phosphofructokinase can also be directly related with the response coefficient by means of the multisite response equation.

If we substitute these elasticity coefficient values together with the obtained flux control coefficients in the presence of copper at the $IC_{50}$ in the above formula we obtain the same value as that obtained experimentally (Fig. 4).

Table 2
Comparison between triosephosphate production flux determined experimentally and calculated from the model at different copper concentrations

| [$CuCl_2$] (μM) | Experimental flux Activity (nmol/min mg protein) | Calculated flux Activity (nmol/min mg protein) |
|---|---|---|
| 0 | 47.55 ± 4.09 | 45.40 |
| 2 | 36.63 ± 5.47 | 38.71 |
| 4 | 31.95 ± 2.07 | 31.96 |
| 6 | 21.58 ± 1.65 | 25.20 |
| 8 | 14.08 ± 1.45 | 18.41 |
| 10 | 11.86 ± 0.23 | 11.59 |

It should be noted that this equation can be used to compute a response coefficient with regard to copper from elasticity and flux control coefficients or to compute an elasticity coefficient or a flux control coefficient in presence of copper from the response coefficient measured experimentally. In this paper we measured experimentally the control and response coefficients and the elasticities and we use the multisite response equation to validate the experimental values obtained. In addition, from this equation it has also been demonstrated that the elasticity of phosphofructokinase with regard to copper has a value of $-1$ at the $IC_{50}$, independent of the substrate concentrations, which is in accordance with the fact that the enzyme is irreversibly inhibited by copper.

It should be noted that the response coefficient with regard to copper could be defined at any copper concentration different from zero. In order to have a quantitative index to compare the copper effect with the effect of other heavy metals or contaminants on the systemic properties of a metabolic pathway, we propose that the response coefficient should be computed at a concentration of copper that corresponds to the $IC_{50}$.

### 3.4. Modelling of the effect of $Cu^{2+}$

A mathematical model to analyze the effect of copper on glycolysis under different conditions using experimental kinetic parameters and rate equations for enzymatic reactions of the upper part of glycolysis was constructed. This model was based on our previous model constructed to analyze the control of the upper part of glycolysis in absence of copper [16]. In this paper the model was redefined to include the kinetic equations of HK and PFK, the dependence of Vmax with regard to copper, experimentally characterised hereby. Moreover, the model predicts that the steady state flux will be zero at a concentration of copper around 13 μM. This prediction of the model has been experimentally corroborated (data not shown). The rate equations used and the kinetic parameters determined experimentally using the same amount of muscle extract as for the flux measurements appear in Table 1. Using these data the steady state flux rate as well as the concentrations of G6P and F6P were computed at different concentrations of copper (0–10 μM) with results in good agreement with the experimental data (Fig. 3, Table 2).

The flux control coefficients for HK and PFK and the elasticity coefficients with regard to copper were also computed from the model and were in good agreement with the experimental values obtained. The flux response coefficient with regard to copper $R^{J}_{Cu}$ computed using the model were also in good agreement with the experimental values measured (see Fig. 4).

We conclude that the model constructed supports the control coefficients experimentally obtained and validate the consistence of all the data obtained. In addition, it will be easy to incorporate in the future, to the model, experimental data on the effect of other heavy metals or pollutants on glycolysis. This model will be a very valuable tool for continuous and on line simulation of the effects of substances that affect many enzymes simultaneously. Disposition of a realistic model of the synergistic effects of copper or/and other pollutants on glycolysis could in the future lead to the design of the best diagnostic conditions and to predict the possible toxic effect of simultaneously low concentrations of different pollutants.

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