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Quantitative analysis of anaerobic metabolism in resting anoxic muscle by ^{31}P and ^{1}H MRS

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Abstract

^{31}P and ^{1}H MRS high resolution measurements at 4.7 T were carried out in isolated frog (*Rana esculenta*) gastrocnemius muscle during anoxia to assess, using reference compounds, the concentration of high energy phosphates ($\sim P$ = phosphocreatine (PC) plus adenosinetriphosphate (ATP)), inorganic phosphate (P_i), phosphomonoesters (PME) and lactate (La). Two sets of measurements were performed, with (**p**) and without (**up**) muscle IAA poisoning and the time course of the metabolite concentration changes was described. The rate of phosphocreatine hydrolysis during the first phase of anaerobiosis, when no lactate is accumulated in either case, appears to be greater in **p** than in **up** preparations. This finding can be explained with the sizeable accumulation of phosphomonoesters (PME) in the former. The efficiency of anaerobic glycolysis, i.e. the $\sim P/La$ ratio, recalculated taking into account also PME changes, was found to be 1.48 ± 0.28, a value higher than that obtained by previous chemical measurements and close to the maximum stoichiometric $\sim P/La$ value. Hence, the in vivo substrate of glycolysis, in the resting anoxic frog gastrocnemius, appears to be almost exclusively glycogen. © 1997 Elsevier Science B.V.

Keywords: High energy phosphate; NMR spectroscopy, ^{31}P; NMR spectroscopy, ^{1}H; Anaerobic metabolism; Gastrocnemius muscle; (*Rana esculenta*)

1. Introduction

Nuclear magnetic resonance spectroscopy (MRS) has been widely used for non-invasive studies of metabolism in several biological systems [1–5]. For this purpose, a combined analysis of two complementary nuclei such as ^{31}P and ^{1}H has also been adopted [4,6–8].

In the present work, the time course of anaerobic metabolism in the resting in anoxic conditions isolated frog gastrocnemius muscle was investigated by means of joint ^{31}P and ^{1}H MRS. This approach is based on simultaneous measurements of high energy phosphate ($\sim P$ = phosphocreatine (PC) and ATP), inorganic phosphate (P_i), phosphomonoester (PME) levels, intracellular pH (pH_i) and lactate concentration ([La]). As is well known, lactate is the end product of anaerobic glycolysis, the main energy yielding process in living cells in the absence of oxygen, the primary one being the hydrolysis of high energy phosphates, mainly PC. Thus, changes in

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lactate as well as in phosphate metabolites concentration allow to establish the muscle energy cost under physiological and/or pathological conditions. Serial biochemical assays quantitatively estimate lactate and ~ P in the tissues as a function of variables such as the level of metabolism and changes in physical (e.g. temperature) and chemical (e.g. pH and anoxia) parameters. However, all require disruption of the cells. Thus, time-dependent changes in metabolite concentrations within given cell populations cannot be obtained by these procedures. Besides, samples cannot be taken at regular time intervals from the same preparation for the assessment of metabolic profiles, which implies large experimental variability. The application of MRS to intact tissues offers the possibility of monitoring time-dependent changes of intracellular metabolites whose time-course can therefore be determined.

This paper reports a quantitative assessment of muscle anaerobic metabolism based on the kinetics of the most significant metabolic intermediates monitored over several hours.

Quantitation of ^{31}P and ^{1}H spectra was obtained adding known amounts of reference compounds to the muscle preparation. Tissue metabolite concentrations were then calculated in terms of micromoles per gram of wet weight. The reliability of the quantitative procedure for lactate analysis was also tested by comparing the calculated MRS concentrations with those obtained by a standard enzymatic method. Certain limitations affecting the adopted quantitative method will be discussed in the text (see Section 4).

The main results of this study are:

1. The assessment of the effects of IAA poisoning on the accumulation of various phosphate compounds, particularly PME.
2. The direct assessment of the efficiency of anaerobic glycolysis in vivo, i.e. of the ~ P/La ratio.
3. The identification of the substrates (glycogen and/or glucose) contributing energy to the system.

2. Materials and methods

2.1. MRS apparatus and measuring procedure

MRS high resolution measurements were carried out on a 4.7 T Bruker AM WB spectrometer equipped with a double tuneable probehead at the ^{31}P-(81 MHz) and ^{1}H-(200 MHz) MRS resonance frequencies and a gradient drive unit utilized for microimaging experiments. Since, in this configuration the instrument was not equipped with a field-frequency stabilization (lock channel), at the beginning of the experiment the field frequency was set on the H_2O frequency. All experiments were carried out using a 15 mm insert and introducing the sample in a non-spinning 10 mm tube. ^{31}P and ^{1}H one-dimensional spectra were recorded. ^{31}P spectra (spectral width 5000 Hz, 4096 data points) were acquired by applying an excitation hard pulse of $45° = 15$ μs and a recycle time of 7 s, enough to prevent partial saturation effects (see Section 2.2). Each spectrum was the average of 64 or 128 FID's, thus requiring a total time of about 8 or 15 min, respectively. The PC peak was used as chemical shift reference ($\delta = 0.00$ ppm). A line broadening of 10 Hz was applied before Fourier transformation. ^{1}H spectra (spectral width 2200 Hz, 8192 data points) were acquired at regular intervals for monitoring lactate accumulation. The lactate methyl resonance was resolved from the overlapping lipid multiplet and the water signal was suppressed by adopting a zero and double quantum filter sequence, the coherences being selected by gradient pulses. The sequence, $90°$-τ-$90°$-t_1-11-τ-Acquire, proposed by Trimble [9], was described in detail previously [10]. The $90°$ hard pulse was 13.8 μs, the evolution and refocussing time $\tau = (2J)^{-1}$ 68 ms (lactate coupling constant, $J = 7.34$ Hz), the multiple and zero quantum evolution period, t_1, 6 ms. The binomial 11 pulse, tailored with the maximum on the lactate methine and the null on the methyl resonance was used to selectively excite the CH resonance, transferring coherence from the methine to the methyl. Double and zero quantum coherences were selected by applying two gradients (2.54 G cm^{-1}) during the t_1 period around a $180°$ binomial pulse selective on the CH signal. The refocussing gradient (5 G cm^{-1}) was applied during the J refocussing period, τ. No phase cycling was adopted. The lactate methyl chemical shift (1.33 ppm) was referred to sodium-3-trimethylsilylpropionate (TSP) used as internal reference. For each spectrum 128 FID's were acquired, with a repetition time of 1 s for a total acquisition time of about 10 min. A line broadening of 3 Hz was applied before Fourier transformation.

The areas under the phophates and lactate peaks were calculated using a numerical integration routine on the Bruker spectrometer. No T_1 correction factor was applied to calculate concentrations.

2.2. Model samples

The validity of the quantitation of phosphate metabolites was tested on a model solution containing 15 mM PC, 5 mM ATP, 10 mM P_i and 10 mM 1P-fructose in 1.5 ml Ringer. Fully relaxed ^{31}P spectra (90° hard pulse: 50 μs; relaxation delay: 60 s) were acquired. Comparing the peak areas with those measured in spectra acquired with the acquisition parameters adopted for the experiments on muscles (45° hard pulse, recycle time 7 s), no correction factor for preventing saturation effects appeared to be required. Moreover, the concentrations calculated using 1P-fructose as standard reference (see Section 2.4) corresponded to the actual ones with an error, similar for each metabolite, of about ±2%.

The influence on lactate methine and methyl chemical shifts of physico-chemical variables such as temperature, pH, ^2H_2O percent and ionic strength have been described in a previous paper [11]. Temperature variations in the 10–30°C range, pH changes from 6 to 8, and the ionic strength of the Ringer had no effect on lactate chemical shifts in model samples containing lactate 10 mM in Ringer.

Lactate standard samples at known concentrations (1, 1.5, 2, 5, 10, 15, 20, 25, 30 and 35 mM in Ringer) were prepared and the ^1H zero and double quantum filter spectra acquired as described in the Section 2. The lactate methyl areas resulted linearly related to the concentrations ($Y_{area} = 3.44[La] + 3.09$; $r^2 = 0.99$). In addition, the calculated concentrations corresponded to the actual ones (±1.6%) over the studied range.

The experiments on the phosphates and lactate model samples were repeated four times with a precision in the peak areas of ±3%.

Lactate longitudinal (T_1) and transversal (T_2) relaxation times calculated on the lactate 30 mM sample by the inversion-recovery and the spin-echo sequences, respectively resulted as follows:

$T_{1CH_3} = 1.38 \pm 0.06$ s; $T_{2CH_3} = 1.04 \pm 0.07$ s.

From fully relaxed spectra a T_1 correction factor of 1.2 was calculated for the lactate methyl area. This correction factor was not used in the calculation of [La] since the latter referred to the same peak resonance.

2.3. Muscle preparation

Frogs (*R. esculenta*) were kept at room temperature in tap water and supplied with mealworms until the onset of the experiments. These were carried out during the spring season on five pairs of gastrocnemii (wet weight 0.48 ± 0.04 g). Each muscle was dissected out and perifused with Ringer (NaCl 75 mM, $NaHCO_3$ 25 mM, KCl 5.1 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1.6 mM) at 25°C, pH 7.7, equilibrated with 2% CO_2 in O_2 to allow restoration of the basal metabolite levels prior to the onset of the experiment. The muscle preparations were then carefully introduced under nitrogen atmosphere into a 10 mm NMR tube and soaked in 1 ml of $^2H_2O/H_2O$ (4:1, v/v) deoxygenated Ringer containing 5 mM TSP. The proportion of ^2H_2O in Ringer was the greatest compatible with a normal metabolite kinetics, particularly for lactate [12]. Two sets of MRS measurements were performed at 25°C. One set was carried out on five preparations (**up**) in order to study lactic metabolism. ^{31}P and ^1H spectra were recorded alternatively at 30 min intervals until the lactate methyl area reached a plateau (about 20 h from the onset of anaerobiosis). The second set of measurements was carried out on the contralateral muscles (**p**) aimed at assessing alactic metabolism (~ P hydrolysis). For this purpose, glycolysis was inhibited by adding 1 mM monoiodoacetic acid (IAA) to the Ringer contained in the tube. ^{31}P spectra were recorded at intervals of 15 min as long as the β peak of ATP was detectable (about 7.5 h), ^1H spectra were recorded at the beginning and at the end of the experiment.

2.4. Quantitative calibration

In order to calculate absolute phosphates and [La], known amounts (10 mM) of both 1P-fructose and lactate were added as references to the perifusion solution at the end of the experiment. The quantitation method was a simplification of that widely reported in a previous paper [11]. For each experiment

a 10 mm flat bottom NMR tube was used and the weight of the dissected muscle chosen so that the muscle plus the perifusion volume resulted less or equal to 1.5 ml. Thus, the sample (that is, the muscle plus the perifusion medium), introduced in the magnet, was totally within the sensitivity volume of the radio frequency coils. By this procedure, the concentrations of the metabolites could be referred to the whole muscle volume. From ^{31}P spectra, by comparing the signal area to that of 1P-fructose, the micromoles of muscle phosphate metabolites were calculated and whence the concentrations of the muscle wet weight could be obtained. Preliminary experiments, carried out adding 1P-fructose from the beginning of the experiment, showed no changes of the linewidth or of the area of 1P-fructose peak, as well as of the linewidth of muscle metabolites during the transient.

In any case, 1P-fructose was then added at the end of the experiment, in order to avoid any possible interference with muscle metabolism. [La] were calculated by the same procedure, in the latter case the assessment of La accounted for both muscle and perifusate. No change in the lactate methyl chemical shift was observed during the transient, showing the absence of paramagnetic effects on the resonance.

2.5. Enzyme assays

The kinetics of lactate production, calculated from the MRS approach described above, was compared to that determined by an enzymatic procedure in a previous paper (submitted). The enzymatic experiments were carried out on 54 frog gastrocnemii (weight 0.40 ± 0.09 g). Each muscle, prepared as described in the Section 2.3 was soaked in 1 ml of Ringer, put into a 10 mm MRS tube and placed in a thermostated bath at 20°C. At increasing incubation times, related to the onset of the MRS experiments, one or two muscles were frozen by pliers precooled in liquid nitrogen and then homogenized and analyzed for lactate by a standard enzymatic assay [13]. [La] was determined taking into account both the fraction contained in the frozen muscles and that diffused into the incubation solution. MRS experiments were carried out on two frog gastrocnemii at 20°C as described in the Section 2.4.

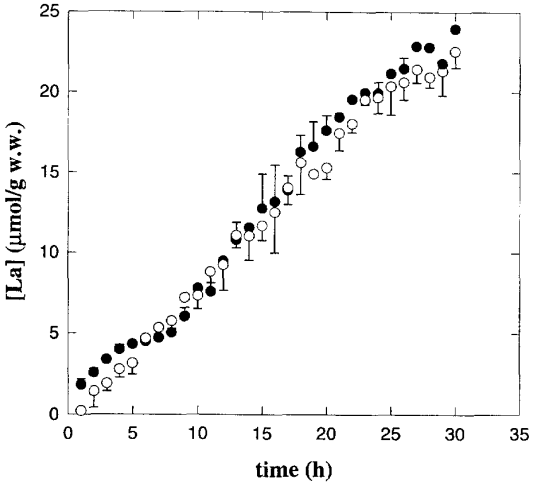


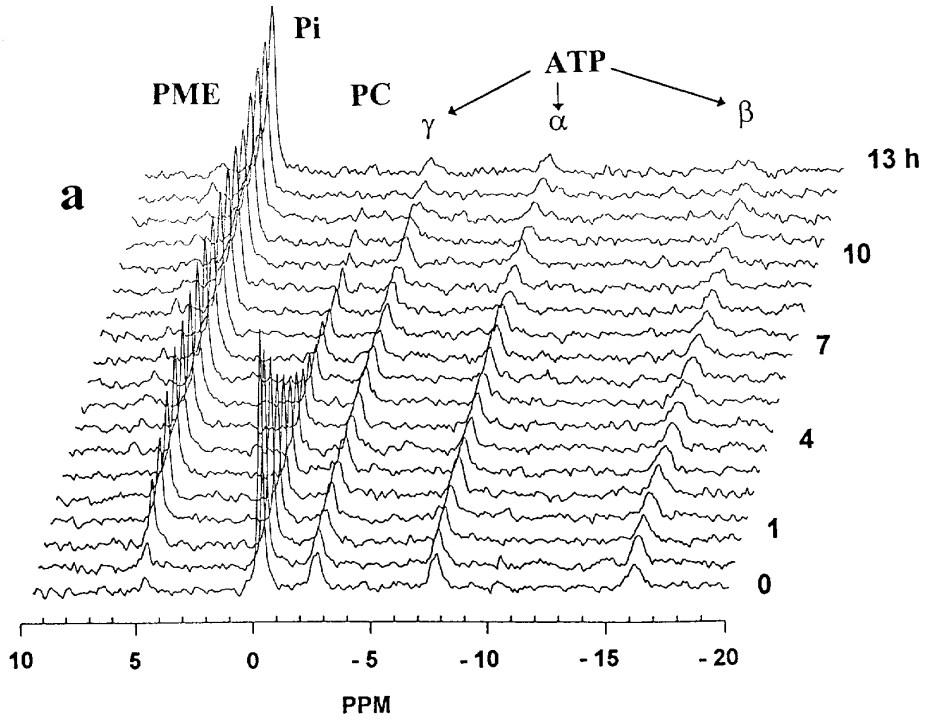
Fig. 1. Lactate concentrations versus time calculated from MRS (●) and enzymatic (○) experiments at 20°C. Each point represents the mean value and the bar the standard deviation. MRS mean kinetics was performed on two muscles. Each enzymatic value is the mean of one or two muscle samples. To perform a kinetics over than 30 muscles were used.

Fig. 1 reports the experimental mean values and the standard deviations of [La] obtained by MRS (filled circles) and enzymatic determinations (open circles). From statistical F test [14] the MRS and the enzymatic computed concentrations did not result significantly different ($P > 0.10$).

3. Results

The ^{31}P and ^{1}H spectra recorded as a function of time in an unpoisoned anoxic gastrocnemius are

Fig. 2. Stacked plots of ^{31}P and ^{1}H MRS spectra at 200 MHz from a resting frog gastrocnemius perifused with Ringer at 25°C. The changes of the phosphate metabolites and lactate in anoxic conditions are shown by the sequence of the spectra. The numbers on the right refer to the hours elapsed from the onset of anoxia. (a) ^{31}P spectra acquired by SI = 4 K; SW = 5000 Hz; 45° = 15 μs; NS = 128. (b) Expansions from ^{1}H spectra acquired using a zero and double quantum filter sequence (SI = 8 K; SW = 2200 Hz, 90° = 12.3 μs; NS = 128) described in the text, showing the lactate methyl resonance at 1.33 ppm. In the shown experiment the basal lactate signal is not visible.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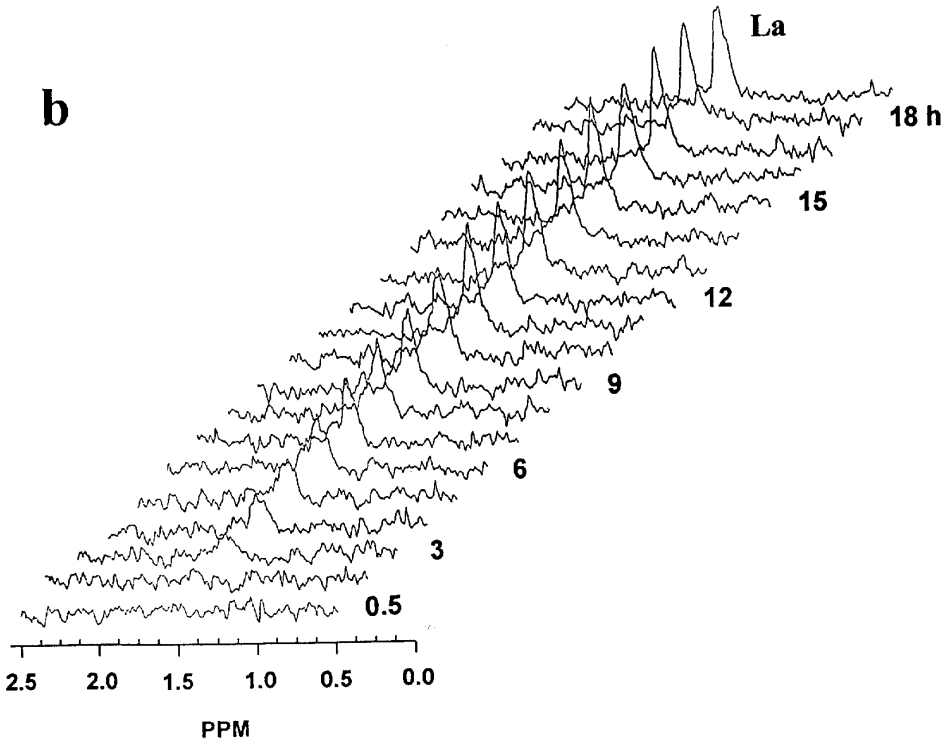
- 20
PPM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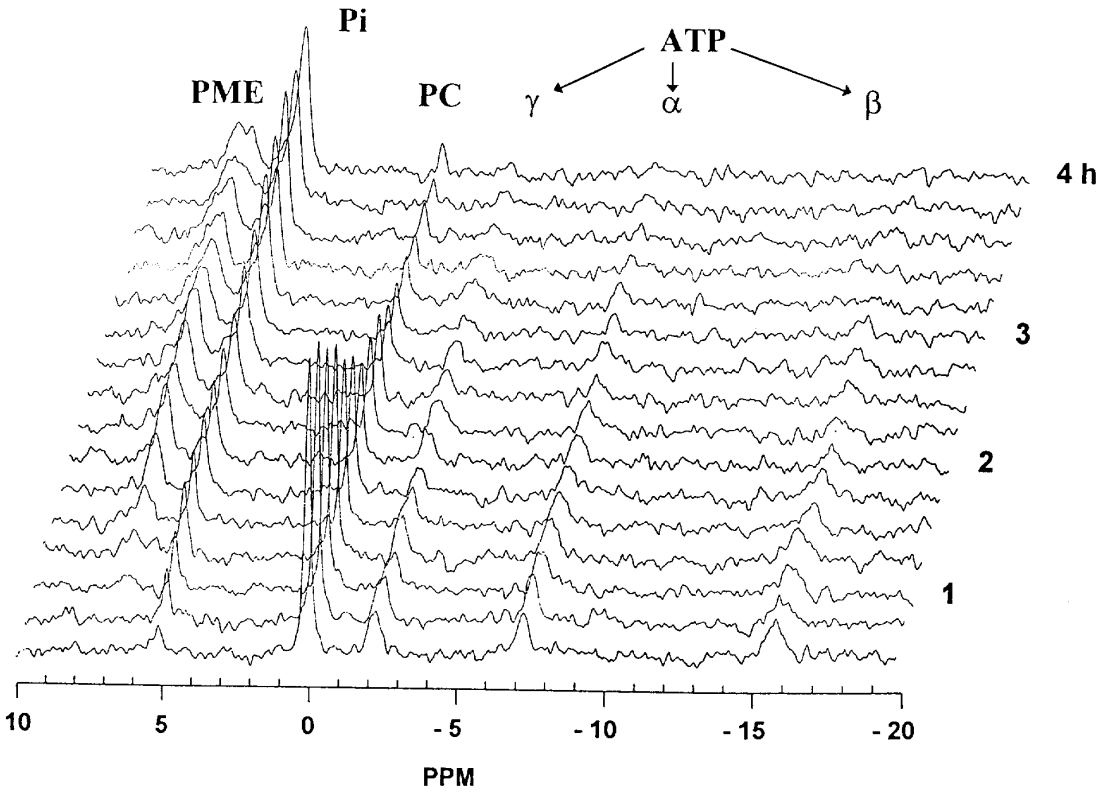

Fig. 3. Stacked plot of the ^{31}P spectra from an IAA poisoned muscle (**p**). The acquisition parameters were the same as in Fig. 2 (NS = 64).

shown in Fig. 2a and b. Fig. 3 is the stacked plot of the ^{31}P spectra recorded in the poisoned contralateral preparation.

As indicated in the figures, the main resonances in the ^{31}P spectra, from left to right, are PME, P_i, PC and the γ, α and β groups of ATP. The α and γ ADP phosphate resonances overlap the α and γ ATP groups, the β ATP peak being the only one available for a quantitative determination. As may be seen from both figures, the intensity of γ and β peaks decreases with time to the same extent, thus ruling out the accumulation of a detectable amount of ADP, the latter metabolite being transformed into AMP and IMP, which are not distinguishable from PME. PME resonance includes mostly hexose monophosphates, fructose 1,6-diphosphate, 3-phosphoglyceraldehyde, 3-phosphoglyceric acid and other triose phosphates. All these compounds have resonances close to each other therefore appearing as a single broad peak. Thus, a careful quantitation of each metabolite appears to be beyond reach. Similar to PME, the basal P_i is often not easily detectable in the resting muscle. During anoxia, both P_i and PME are readily accumulating. Most of the oxygen stored in the muscle is

Fig. 4. Concentration versus time plots of the phosphate compounds and lactate in unpoisoned (**up**) and IAA poisoned (**p**) preparations. All experimental data (**up**: filled symbols; **p**: open symbols) are shown. Symbols: (a) phosphocreatine (∇); (b) ATP (□); (c) inorganic phosphate (△); (d) phosphomonoesters (◇); (e) lactate (○); (f) intracellular pH (hexagon). Concentrations are expressed in μmol g^{-1} of muscle wet weight. Time zero corresponds to the beginning of anaerobiosis (which was set arbitrarily when the PC peak starts decreasing sharply) and not to the onset of MRS recording.

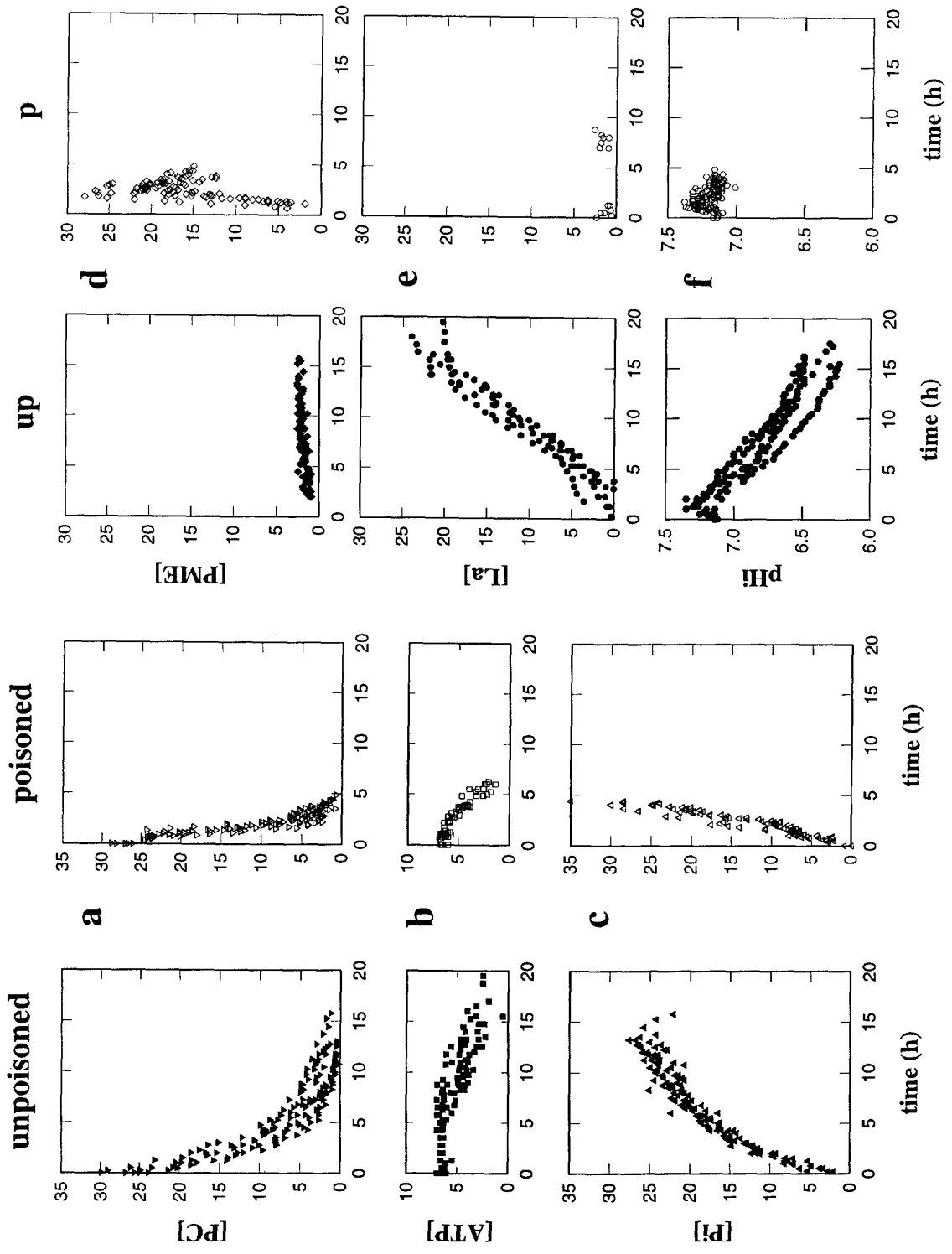
unpoisoned
poisoned
up
p
a
b
c
d
e
f
[PC]
[ATP]
[Pi]
[PME]
[La]
pHi
time (h)

Table 1
Basal concentrations of phosphocreatine (PC), ATP and lactate (La) as determined by ^{31}P and ^{1}H MRS for unpoisoned (**up**) and poisoned (**p**) muscles

	PC (μmol g^{-1})	ATP (μmol g^{-1})	La (μmol g^{-1})
up	26.26[a]	6.46	1.02
	±2.28	±0.36	±1.68
p	27.38	6.32	1.18
	±1.11	±0.33	±0.67

[a]The average concentration levels (±S.D.) are referred to 1 g of muscle wet weight.

consumed during the preparation of the experimental set-up; anyway, as may be seen in Figs. 2 and 3, the onset of the investigated kinetics was set arbitrarily (time = zero) when the PC peak starts decreasing sharply, which coincides with the onset of full muscle anaerobiosis.

The mean values of phosphate metabolites and lactate (the latter not always clearly visible) at the beginning of the experiments appear for both **up** and **p** muscles in Table 1. The time-dependent concentration values of the phosphate compounds, lactate and intracellular pH for all muscles in **up** (filled symbols) and **p** (open symbols) conditions are shown in Fig. 4. The exhaustion of the PC and ATP stores in **up** muscles occurs in a time 2.5 times longer than for the **p** preparations. The experimental data were fitted by the Marquardt–Levenberg algorithm adopted by the Sigma Plot software (Jandel). The PC level ([PC]) in both **up** and **p** preparations decreases according to a single-exponential function of the type:

$$[PC]_t = [PC]_0 \exp(-k_{PC} \cdot t) \quad (1)$$

with different time courses.

up: PC splitting rate (k_{PC}) = (0.26 ± 0.06) h^{-1}; $r^2 = 0.94 \pm 0.01$ (exhaustion time = 20 h),

p: PC splitting rate (k_{PC}) = (0.87 ± 0.19) h^{-1}; $r^2 = 0.94 \pm 0.01$ (exhaustion time = 7.5 h).

As appears from panel b, ATP concentration ([ATP]) keeps rather constant for both **up** and **p** muscles until [PC] falls below ~ 15% of its initial level, then progressively decreases according to an approximately linear function:

$$[ATP]_t = [ATP]_0 - k_{ATP} \cdot t \quad (2)$$

where $k_{ATP} = (0.44 \pm 0.12)$ h^{-1} for **up**, $r^2 = 0.89 \pm 0.15$; and $k_{ATP} = (1.20 \pm 0.09)$ h^{-1} for **p**, $r^2 = 0.94 \pm 0.11$, respectively.

The fall of [PC] is accompanied by an increase in P_i concentration ([P_i]). As appears from panel c, the P_i accumulation trend in **up** is different from that in **p**. In fact, for the **up** muscles the time course of P_i build-up can be described by a power function such as:

$$[P_i]_t = [P_i]_0 + t^{k_{P_i}} \quad (3)$$

where $k_{P_i} = (0.45 \pm 0.08)$ h^{-1}; $r^2 = 0.94 \pm 0.01$.

By contrast, in the **p** condition the process is better described by a linear relationship:

$$[P_i]_t = [P_i]_0 + k_{P_i} \cdot t \quad (4)$$

where $k_{P_i} = (6.54 \pm 0.07)$ h^{-1}; $r^2 = 0.94 \pm 0.01$.

From panel d it may be shown that in **up** muscles, after an initial rise, the PME level ([PME]) keeps constant at ~ 2 μmol g^{-1}. By contrast, in **p** muscles, during the first three hours of anoxia, PME accumulate at a constant rate (8.40 ± 0.82 μmol g^{-1} h^{-1}), the concentration reaching a maximum (25–30 μmol g^{-1}), after which [PME] tends to decrease progressively until exhaustion (~ 10–15 μmol g^{-1}).

[La] (Fig. 4, panel e) for **up** muscles remains at a level not significantly different from basal up to about the 4th hour from the onset of anaerobiosis, then increases linearly reaching a plateau at ~ 23 μmol g^{-1}. The rate of lactate accumulation from the 4th hour to the 15th hour is 1.62 ± 0.22 μmol g^{-1} h^{-1}. In **p** muscles, the average [La] at the end of the experiment is practically the same as that at the beginning ($P > 0.05$), thus showing that the IAA concentration used for the study was adequate for preventing anaerobic glycolysis.

^{31}P MRS is known to be also a reliable method of determining the intracellular pH in intact muscles, based on the chemical shift of the P_i resonance from a titration curve obtained in appropriate conditions [1]. Panel f shows the pH_i kinetics in both **up** and **p** conditions. From the extrapolated value of the P_i chemical shift at zero time, the average pH_i value for both **up** and **p** muscles was calculated to be 7.15 ± 0.03. Before anaerobic glycolysis becomes noticeable (**up**), as well as in the absence of lactic acid accumulation (**p**), a net alkalinization is detectable. The further acidification down to pH_i levels of ~ 6.35, observed in **up** muscles, is attributable to lactic acid accumulation. The process is described by an exponential function:

$$pH_i = 5.76 + 1.59 \exp(-k \cdot t) \quad (5)$$

where $k = (0.058 \pm 0.01)\ h^{-1}$; $r^2 = 0.98 \pm 0.01$.

The time course of the slight acidification observed in **p** preparations, cannot be established.

4. Discussion

4.1. Quantitative calibration

Different methods are reported in the literature to achieve an absolute quantitation of MRS spectra. Many studies have been reported in terms of ratios of one metabolite peak area to another used as standard [1,15]. It is assumed that the change in the ratio between a given peak and the reference one reflects the change in concentration of the former metabolite. However, the assumption is made that the standard compound is not changing during the experiment.

The use of an endogenous reference such as tissue water [16] or total creatine [17,18] to quantitate proton spectra as well as [ATP] [8,19,20] to quantitate ^{31}P spectra has been also adopted. In this way, both metabolite and reference are referred to the same sample volume and the spectra are obtained under identical conditions. However, any change in the actual concentration of the reference, owing to intrasubject variability or relaxation time variations will not be accounted for.

In the present study known amounts of reference compounds were added to the perifusion solution to quantitate both phosphate metabolites and lactate. The procedure assured that the whole muscle and the perifusion medium were completely within the MRS coils so that the concentration values were referred to the whole volume. Calibration studies showed that the areas were linearly related and consistent with the actual. Moreover, the MRS [La] were well in agreement with the enzymatic data.

However, the MRS quantitation is intrinsically limited by the fact that the environment of the intracellular and perifusate metabolites is not the same, mainly owing to differences in susceptivity. Moreover, in order to quantitate a kinetic trend, changes in peak areas must reflect changes in concentration, so that other parameters such as relaxation time variations must not interfere with the measurements. As reported by the literature, ^{31}P NMR relaxation time changes do not affect the quantitative determination of phosphate metabolites (PC, P_i, ATP) during brain ischemia [21]. Parenthetically it is reported that variations in O_2 supply, such as shifts from normoxia to hypoxia, transient ischemia as well as inhomogeneous distribution of deoxy- and oxy-hemoglobin could generate bulk susceptibility gradients, altering T_2^* [8,22].

The investigated muscle preparations were in anoxic conditions, since at the very beginning of the experiment all O_2 stores (i.e. free O_2, oxyhemoglobin and oxymyoglobin) were likely to be depleted. Indeed, neither the linewidth nor the area of the phosphate reference changed during the transient, nor the linewidth of the investigated phosphate metabolites, confirming that no factors affecting T_2^* were acting. Finally, as recently reported on isolated frog gastrocnemius muscle [23], changes in lactate methyl chemical shift, reflecting the susceptibility effect of myoglobin, were not observed in the present experiment.

4.2. Metabolite kinetics

As may be seen from Table 1, at the beginning of the experiment, [ATP], [PC] and [La] are essentially the same in both **up** and **p** preparations ($P > 0.05$). This is a pre-requisite for the comparison of the kinetics of the investigated metabolites in both experimental conditions.

In anaerobiosis, PC (Fig. 4a) decreases as a single exponential in both **up** and **p** preparations according to an accepted model [4]. In the **up** preparations, the rate of PC hydrolysis is the result of two separate processes characterized by different time courses. Indeed, [ATP] is kept constant by the creatine kinase (CK) reaction. [PC] measured during this phase is fitted by a linear function with rate constant $k_{PC} = (-4.60 \pm 0.35)\ \mu mol\ g^{-1}\ h^{-1}$; $r^2 = 0.96 \pm 0.01$. After about 4 h from the onset of anaerobiosis, the energy supplied to the muscle by glycolysis increases substantially, with La accumulation. For the **p** preparations, the PC hydrolysis during the first 2.5 h, that is before the drop of [ATP], can be interpolated by a linear function, with a rate constant, $k_{PC} = (-12.88 \pm 1.32)\ \mu mol\ g^{-1}\ h^{-1}$; $r^2 = 0.96 \pm 0.02$, that is much greater than for **up** muscles. Thus, based on $\Delta PC/\Delta t$, the energy cost of **p** vs. **up** preparations, before the activation of anaerobic glycolysis, would

appear significantly greater. This observation is supported by the finding of a greater accumulation of PME in **p** vs. **up** muscles, a reaction requiring energy consumption.

[ATP] (Fig. 4b) keeps constant for both **up** and **p** muscles as far as [PC] reaches ~ 15% of its initial value. From this level onward, the muscle begins to utilize ATP, as the energy generated by the PC hydrolysis becomes insufficient to cope with an apparently constant energy demand.

With regard to the P_i kinetics (Fig. 4c), there are differences between **up** and **p** muscles. For the former, P_i increases according to a power function, reaching a plateau at a molar level corresponding roughly to the basal value of PC prior to the beginning of the experiment. At variance with the above results, the rate of P_i accumulation in the **p** preparations is constant and no plateau is reached. These differences may be accounted for by the simultaneous time-dependent changes of [PME] (see below).

In order to verify the stoichiometric correspondence between the decrease of PC and the increase of P_i (i.e. of equilibrium between the CK reaction and ATP hydrolysis), the P_i versus PC time-dependent changes for both **up** (●) and **p** (○) preparations are compared in Fig. 5 using (Eq. (3) or Eq. (4)) vs. Eq. (1). A stoichiometric equivalence between ΔP_i and ΔPC has been reported previously by several authors [24–27]. At variance with the conclusions of the above mentioned papers, the present data appear to follow variable trends around the identity line. Indeed, for **up** metabolism, in the early phase of anoxia, the produced P_i appears to exceed the hydrolyzed PC. Subsequently, at a time roughly corresponding to the glycolysis enhanced, the rate of increase of P_i starts being lower than expected, finally increasing again toward muscle exhaustion. Activation of glycolysis could be due to the initial increase of P_i, since high P_i is known to enhance the activity of phosphofructokinase [28]. Later, when the flow rate of glycolysis becomes constant, the rise of P_i becomes somewhat lower than the drop of PC, likely because part of P_i is taken up by two reactions, i.e. glycogen phosphorylation and conversion from 3-phosphoglyceraldehyde to 1,3-diphosphoglycerate. Although the level of glycolytic intermediates becomes likely stable after a few hours, the amount of P_i taken up by these intermediates will be utilized for ADP phosphorylation, a reaction associated to lactate accumulation. When approaching exhaustion, neither the creatine kinase reaction nor glycolysis can supply enough energy. At this stage the muscle takes up energy from ATP hydrolysis, reaction accompanied by a stoichiometric increase of P_i. In **p** muscles which rely only on the CK reaction, P_i keeps constantly below the identity line: this is likely the consequence of the progressive accumulation of PME which requires a further transfer of P_i.

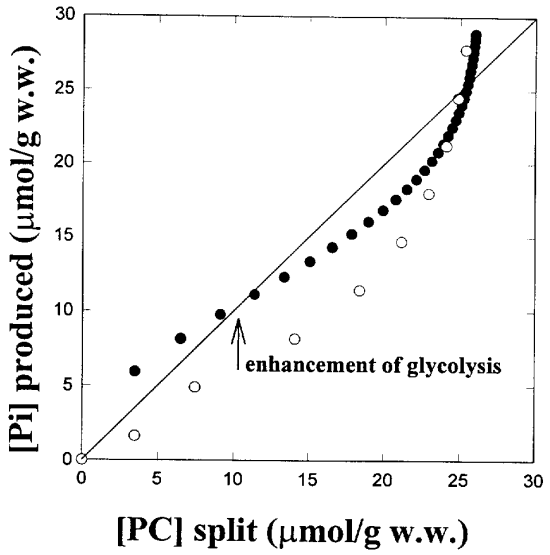

Fig. 5. Plot of split phosphocreatine vs. inorganic phosphate built up in **up** (filled circles) and **p** (open circles) conditions. The identity line PC: $P_i = 1$ (continuous) is also shown.

[La] in the **up** muscles (Fig. 4e) does not change significantly from the control value during the first hours from the onset of anaerobiosis. Thereafter, La increases at a constant rate up to a maximum level, reached when glycolysis is likely to be inhibited by the acidification of the cytoplasm or, alternatively, by the exhaustion of the substrate. The delay in the enhancement of the glycolysis, as shown by La accumulation, is also reflected by the level of PME in the **up** muscles (Fig. 4d), whose level during the first hours of the experiment keeps lower the detectable threshold. Later, PME tends to increase slightly keeping constant during the phase of La accumulation, indicating that the velocity of the flux along the glycolytic chain is somewhat constant. By contrast,

PME accumulation rate of **p** muscles increases rapidly as a consequence of the blockade of glycolysis. Incidentally, the accumulation of glycolytic intermediates is a typical finding in patients affected by muscle metabolic disorders resulting in impaired glycolysis (phosphofructokinase, phosphoglycerate mutase, phosphoglycerate kinase deficiencies) [29–31]. Prior to the exhaustion of ~ P, PME tend to decrease. Since the utilization of such intermediates as substrates of glycolysis appears unlikely, they might have become invisible to ^{31}P MRS as a consequence of a dephosphorylation. The latter change could explain the increase in the rate of P_i accumulation observed during the last phase of the **p** experiment (see Fig. 4c). Alternatively, the P_i increase could be ascribed to ATP hydrolysis: in the latter case an identical change of P_i in **up** and **p** muscles should be observed, wich is in contrast with the present experimental data.

The pH_i initial level (Fig. 4f), the same in both **up** and **p** preparations, was consistent with the data reported in the literature [32,33], as well as the observed alkalinization [2] and the final pH_i value, reached by the **up** muscles [4]. The slight acidification observed in **p** preparations, not attributable to lactate accumulation, is due to ATP hydrolysis, occurring when muscle is approaching exhaustion (see Fig. 4b).

From the present data, relationships between metabolic variables were calculated resulting in agreement with those reported in the review by Kemp and Radda [4]. Indeed, lactate was related to PC concentrations by a similar curvilinear function. The same was true for pH_i versus PC, whereas pH_i was linearly related to lactate ($r^2 = 0.93$). For calculations the authors used as ~ P/La ratio the maximum stoichiometric value of 1.5, assuming that the substrate of glycolysis is exclusively glycogen. In the present study, the ~ P/La ratio value was calculated.

4.3. ~ P / La ratio calculation

MRS makes it possible to assess continuously the absolute concentration changes of the metabolites turnover during anoxia in both **up** and **p** conditions. From these data the efficiency of anaerobic glycolysis could be calculated as the number of moles of high energy phosphates (~ P) resynthetized by 1 mole of La produced. Observing that in both **up** and **p** conditions ADP does not accumulate to a significant extent (see the height of the γ and β ATP peaks of Figs. 2 and 3), ADP must be transformed into AMP and eventually into IMP. Thus, the total pool of high energy phosphates (~ P) available for muscle metabolism is given by the sum of PC plus the concentration of ATP (2 ~ P).

The efficiency values of anaerobic glycolysis previously reported in the literature [34,35] were based on the hypothesis that the difference between the rate of decay of ~ P as a function of time in the IAA-poisoned ($\Delta \sim P_p/\Delta t$) and that of the unpoisoned preparations ($\Delta \sim P_{up}/\Delta t$) was totally compensated for by the energy released by anaerobic glycolysis ($\Delta La/\Delta t$). Thus, the equation adopted for the calculation of the ~ P/La ratio was the following:

$$\Delta \sim P_p - \Delta \sim P_{up}/\Delta La = \sim P/La. \quad (6)$$

However, the use of this equation implies that in **up** and **p** conditions the ~ P kinetics are identical. The calculated ~ P/La ratio was found to be, on the average, ≅ 1.3 [34]. The ~ P/La value calculated from Eq. (6) using the data obtained in the present study exceeds 1.5, i.e. the maximal stoichiometric value starting from glycogen. This is because the above made assumption is erroneous. Indeed, the rate of phosphocreatine hydrolysis in the **up** preparation during the early phase of the experiment, when lactate is not produced, is lower than that for the **p** muscle, as if the energy consumption of latter was higher. As is well known, when glycolysis is inhibited by IAA (**p**), the reaction fructose 6-phosphate → fructose 1,6-diphosphate which requires energy transfer, is not compensated for by ATP resynthesis. Thus, the energy balance between lactic and alactic metabolism may be established only in the absence of accumulation of glycolytic intermediates (fructose 1,6-diphosphate and 3-phosphoglyceraldehyde), or taking into account the actual concentration of each of these metabolites. Since, the latter cannot be sorted out from the experimental PME peaks, a simplified procedure to calculate the ~ P/La ratio was adopted. Based on the hypothesis that resting metabolism in the **up** preparation keeps constant and on the finding that in the earlier part of the experiment lactate accumulation is absent or negligible, the energy being

yielded only by the PC hydrolysis as discussed above (see Section 4.2), a straight line was drawn extrapolating the PC experimental data vs. time (Fig. 6 dashed line). This function describes the theoretical ~ P splitting rate ($\Delta P_t/\Delta t$) after the onset of anoxia in the absence of anaerobic glycolysis. This line would coincide with the ($\Delta \sim P_p$) vs. time function in the absence of PME accumulation. The function ($\Delta \sim P_t - \Delta \sim P_{up}$), that is the difference between the theoretical function ($\Delta \sim P_t$) and the line drawn through the experimental points ($\Delta \sim P_{up}$) obtained in the **up** condition, allows to calculate the energy equivalent of the accumulated lactate. Thus the correct ~ P/La ratio can be calculated by the following modified equation:

$$(\Delta \sim P_t - \Delta \sim P_{up})/\Delta La \quad (7)$$

The calculated average value (from the 4th to 15th hour from the onset of anaerobiosis) of the ~ P/La ratio is 1.48 ± 0.28. This figure is close to the maximum stoichiometric ~ P/La value. Thus the substrate, in the resting anoxic frog muscle, appears to be almost exclusively glycogen.

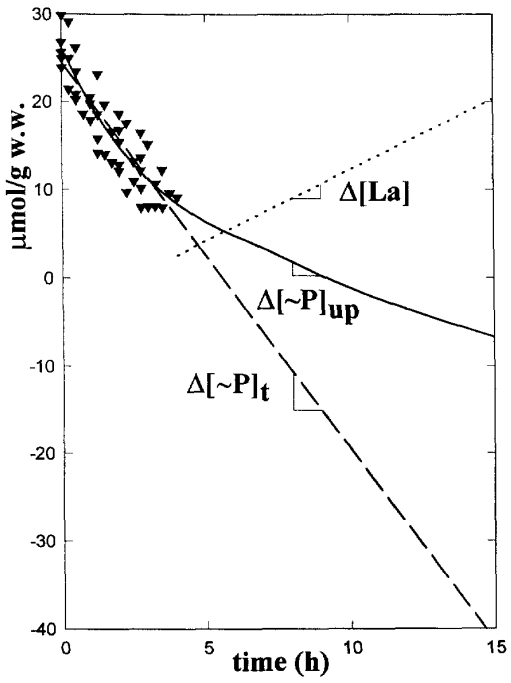

Fig. 6. Simplified ~ P/La ratio calculation procedure. The dashed straight line ($\Delta \sim P_t$), interpolates the [PC] (▼) data obtained in the **up** condition during the first four hours of anaerobiosis when lactate is absent or negligible; the solid line ($\Delta \sim P_{up}$) interpolates the actual ~ P decay in the **up** condition; the pointed line shows the time course of lactate accumulation.

Another possible source of error affecting the values of ~ P/La ratio reported in the literature derives from the use of enzymatic methods for the assessment of both ~ P and La. Freeze clamping, as is well known, is often characterized by a sizeable and unpredictable underestimate of ~ P from PC in-between experiments. This would obviously affect the measurement of the correct ~ P/La value.

5. Conclusions

Combined ^{31}P and ^{1}H MRS allowed to gain a dynamic insight into the muscle anaerobic metabolic pathways. The assessment of the kinetics of the most relevant metabolites by the use of adequate standards, made it possible to describe the energetics of the muscle in anoxia and to calculate the efficiency of anaerobic glycolysis in vivo.

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