

# Glycolysis activity in flight muscles of birds according to their physiological function. An experimental model in vitro to study aerobic and anaerobic glycolysis activity separately

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**Abstract** An experimental system in vitro is presented to assess the activity of the entire glycolysis in tissue extracts, which allows determining aerobic and anaerobic glycolysis activities separately. Glycolysis activity has been measured in pectoral and supracoracoideus muscles of the homing pigeon and the domestic fowl. These muscles support different aspects of flight in the two birds and are representative models of the two kinds of basic movements, endurance and sprint. The results obtained showed that in type I red fibers (pigeon pectoral), glucose produced a high glycolytic activity, while it was a poor substrate for type IIb white fibers (fowl pectoral and the two supracoracoideus). White fibers, however, attained its maximum glycolytic activity with phosphorylated glucose as substrate. These results demonstrated the validity of the experimental system as a method for assaying the two kinds of glycolytic activity in tissues, and supply new information about the biochemical and physiological features of these types of fibers.

**Keywords** Energy metabolism · Muscle metabolism · Glycolysis activity · Glycolysis assay · Bird muscle · Flight muscle

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## Introduction

The movements of the animals can be divided into two basic kinds of exercises: endurance—the continuous exercise for long periods of time—supported by type I muscle fibers and sprint—very quick and short activities—executed by type IIb muscle fibers. Usually, real movement entails a combination of both, specially in fast movements, and also by intermediate types of fibers; see reviews in [1–3]. The net ATP sources of them are aerobic metabolism, from glucose and fatty acids, for endurance, and anaerobic glycolysis for sprint.

The two kinds of glycolysis, aerobic and anaerobic, can be considered as two extreme models of metabolism, each one being defined by their substrate and end product [4]. Aerobic glycolysis is mainly fed by blood glucose, although muscle glycogen can also be used as a supplementary fuel, and the resulting pyruvate is metabolized via Krebs' cycle [5–7]. Anaerobic glycolysis is fed by phosphorylated glucose derived from muscle glycogen, which is converted into lactate; see reviews in [2, 6, 8–10]. These conclusions have been derived from biochemical assays of isolated enzymes [11, 12], histochemical analysis [13, 14], and physiological studies of global effects in vivo [1, 4, 15]. However, to date, there are no direct data on the activity of the entire glycolysis and its design in each type of fiber.

A major cause of this lack of information is that many studies have been carried out in mammals, where muscles are formed by a mixture of fibers. This fact has been discussed by Rome and colleagues who worked with fish and frog, where the fibers are separated in different muscles [16, 17]; this allowed them to obtain specific data on several aspects of the physiology of each type of fiber. Nevertheless, a full characterization of the metabolic pathways still remains unknown.

In this work we present a new approach that allows assessing the activity of the entire glycolysis as a full system, and the quantification of each kind of metabolism (aerobic or anaerobic) separately. This assay of the glycolytic activity is based on previous approaches described by us [18–20] that have been later applied by several authors to study glycolytic activity and its control [21–27]. Here we have measured the aerobic activity *in vitro*, as the response of the system to free glucose as substrate, and the anaerobic activity, as the rate increase over the former state after adding glucose 6-phosphate (glc 6-P), which represents the product released from the breakdown of muscle glycogen.

In order to obtain results specifically related to each type of fiber we have selected two species of birds highly specialized in a given kind of flight: the homing pigeon (*Columba livia*) as a long-distance bird, and the domestic fowl (*Gallus domesticus*) as a short-distance bird. In each bird, we have selected muscles that are highly specialized in a particular physiological role and that contain only one specific type of fiber: the pectoral (*Pectoralis profundus*) and the supracoracoideus (*Pectoralis superficialis*). The pectoral muscles of the homing pigeon and the fowl are quite different, and that of the homing pigeon is a full red muscle, typical case of endurance machinery which supports the regular sustained flight of the bird, with a 100% content of type I fibers [28, 29] and so its metabolism must be essentially aerobic. However, that of the fowl, specialized in short fast flights, only has type IIb fibers [30], and so its metabolism must be essentially anaerobic. On the other hand, the supracoracoideus muscles accomplish the most complicated kinematic phase of the wing beat cycle, supporting wing upstroke, a movement that requires great power [31, 32]. Therefore, this is a typical case where the force-time ratio must be high and its metabolism must be essentially anaerobic in all types of birds.

The results reported here show that both types of fibers (type I and type IIb) have a highly specialized metabolism for aerobic and anaerobic glycolysis, respectively. In addition, they supply new information on the activity and regulation of glycolysis in each type of muscle fiber, which is consistent with other data obtained by different experimental approaches.

## Experimental model

The metabolic system studied is shown in Fig. 1. It is an extension of the simplified system presented in previous works of our group, which only included the first part of glycolysis (until the triose-phosphate), and the flux was measured as NADH variation [19, 20, 33]. This system demonstrated to be an useful tool to study several aspects

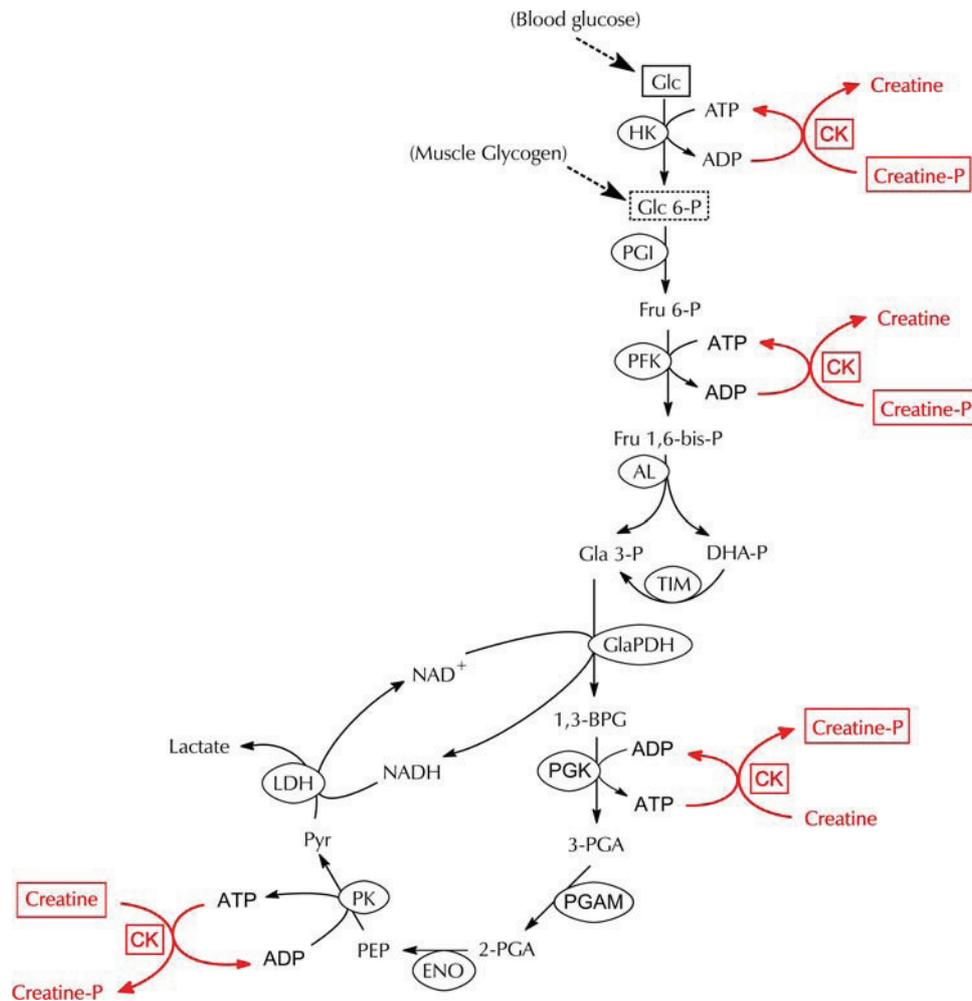
of glycolysis activity and its control [19, 21–25, 27, 33]. The novelty of the system we present here is that it allows measuring the activity of the entire glycolytic pathway.

The system contains the soluble fraction of the tissue extract, all coenzymes necessary for the conversion of glucose into lactate, and the full creatine system as ATP buffer, to guarantee the steady state. Since both aerobic (type I) and anaerobic (type IIb) fibers have high LDH activity [11], and our experimental system has no mitochondria, LDH works here as a full sink of pyruvate, driving all glycolytic flux to lactate. Then, the activity of the system is measured by deviating all flux to L-lactate, which is assayed with a specific electrode.

The experiments start with the addition of glucose as substrate. The system then reaches a steady state (*State A*), where L-lactate is produced at a constant rate, which gives us the aerobic glycolytic activity of the tissue. Then, glc 6-P is added to trigger the anaerobic glycolysis. This enhances the activity of the system and the production of lactate to reach a second steady state (*State B*), where the slope of lactate production is higher than in *State A*. The difference between the two slopes gives us the activity of the system strictly due to anaerobic glycolysis. This effect has been extensively demonstrated in the simpler experimental system of the upper glycolysis that we presented previously [19, 26, 27, 33], and the purpose of this is to study the global effect in the whole pathway.

In the samples we have analyzed, LDH activity is in large excess over the other glycolytic enzymes [11], so it was not necessary to add more LDH to guarantee the full conversion of pyruvate to lactate, although this could be done for other tissues. In fact, control assays in which more LDH was added to the samples did not show more glycolytic activity in any case. On the other hand, since the extract contains the original mixture of cytosolic enzymes, the flux assayed from glucose or glc 6-P to lactate gives us the total glycolytic activity at steady state, independently of the fact that other pathways may also be working, as they actually do in the cell. First, leak fluxes from glucose or glc 6-P are negligible because of the high equilibrium constant of the whole conversion of glucose or glc 6-P to lactate [34], and because no other coenzymes (e.g., NADP<sup>+</sup>) are added to the incubation medium. Spurious fluxes to other products (e.g., toward glycerol-P) can occur as they take place in the cell, so they do not interfere in the assay.

The incubation medium was designed to simulate the conditions in the cell and to allow the maintenance of the steady state in our system. Berger et al. [35] determined that in skeletal muscle under contraction, the intracellular glucose concentration increased to 1.1–1.6 mM in all



**Fig. 1** Metabolic system that converts glucose or glc 6-P into L-lactate by the glycolysis pathway. The reaction mixture contains the muscle extract plus ATP, ADP,  $\text{NAD}^+$ , and NADH, as the required coenzymes, and creatine, creatine-phosphate and creatine kinase as ATP buffer system. Glucose and glc 6-P are added sequentially as substrates to reach *State A* and *State B*, respectively, and lactate is assayed with the L-lactate electrode. Abbreviations: *enzymes*: AL fructose-bisphosphate aldolase (EC 4.1.2.13); CK creatine kinase (EC 2.7.3.2); ENO enolase (EC 4.2.1.11); GlcPDH glyceraldehyde 3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12); HK

hexokinase (EC 2.7.1.1); LDH L-lactate dehydrogenase (EC 1.1.1.27); PFK phosphofructokinase (EC 2.7.1.11); PGI phosphoglucose isomerase (EC 5.3.1.9); PGAM phosphoglycerate mutase (EC 5.4.2.1); PGK phosphoglycerate kinase (EC 2.7.2.3); PK pyruvate kinase (EC 2.7.1.40); TIM triose-phosphate isomerase (EC 5.3.1.1). *Metabolites*: 1,3-BPG 1,3-bisphosphoglycerate; Fru 1,6-bis-P fructose 1,6-bisphosphate; DHA-P dihydroxyacetone-phosphate; Fru 6-P fructose 6-phosphate; Gla 3-P glyceraldehyde 3-phosphate; Lact L-lactate; 2-PGA 2-phosphoglycerate; 3-PGA 3-phosphoglycerate; PEP phosphoenolpyruvate; Pyr pyruvate

cases, independently of several physiological conditions, as fed or starved situation, diabetes or insulin administration. This concentration of glucose virtually saturates muscle hexokinase (94%), since its  $K_M$  for glucose is 0.1 mM. In our experiments, the starting glucose concentration was 7.5 mM (98.7% hexokinase saturation). At the end of the assays, the maximum production of lactate recorded was 5 mM; this represents an expenditure of 2.5 mM glucose and a final concentration of 5 mM glucose, with a negligible change in hexokinase saturation (98%). Thus, the steady state of the system is guaranteed, which is also

demonstrated by the constant rate of glucose-induced lactate production recorded in the first part of the experiments.

On the other hand, Aragón et al. [36] demonstrated that glc 6-P concentration during anaerobic glycolysis in muscle under isometric contractions raised from its basal value of 0.33 mM up to 7.3 mM, fed by glycogen depletion. Therefore, we used glc 6-P at that concentration as a typical representative metabolite of glycogen breakdown for anaerobic glycolysis. Coenzymes concentrations were also as they occur in vivo [34]; all experiments were carried out at 40°C, which is the corporal temperature of both birds.

## Materials and methods

### Reagents

Enzymes, coenzymes, metabolites, protease inhibitors, and HEPES were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were from Panreac (Barcelona, Spain). *Buffer R* (the regular buffer used for kinetic experiments and as a general solvent for all other reagents) contained 50 mM-HEPES, 100 mM-KCl, 10 mM-MgCl<sub>2</sub>, and 10 mM-sodium phosphate [HNa<sub>2</sub>PO<sub>3</sub>/H<sub>2</sub>NaPO<sub>3</sub> about (2/1)], adjusted at pH 7.4 with the appropriate ratio of phosphates. *Buffer H* (used for tissue homogenization) was prepared by adding 100 µl of the cocktail of protease inhibitors and 30 mg of Sigma Trypsin inhibitor type II (Ovomucoid from chicken egg white) to 100 ml of Buffer R. Buffer H was maintained in a bath of ice-cold water, and always used at 2–4°C. The cocktail of protease inhibitors contained a mixture of 7 mg-PMSF, 7 mg-E-64, 50 mg-pepstatin A, 15 mg-benzamidine, and 8 mg-1, 10-phenantroline per ml. Amounts of 1–2 ml were prepared, and kept frozen in 100 µl vials at –20°C.

### Animals and tissues

Eight-two-year-old specimens of homing pigeons (*Columba livia*) were supplied by a professional pigeon trainer; they had been maintained under a controlled diet and a training schedule that consists of flying hundreds of kilometers every week. Eight-two-year-old specimens of domestic fowls (*Gallus domesticus*) were supplied from a farm, where they lived free-ranged under a controlled diet and allowed to fly at will. All animals were, thus, well trained according to their natural flight specialty, which allowed them to develop their regular physiological behavior and their specific energy metabolism. The procedures followed were in accordance with institutional guidelines. Animals were treated in full accordance with the Spanish and European Union legislation on ethical rules; and appropriate measures were taken to minimize pain or discomfort.

### Tissue extracts

Birds were killed by cervical dislocation, breast feathers were removed and a fine longitudinal cut in the skin along the breastbone line was made with a lancet, allowing an easy access to the breast muscle packages. Pectoral and supracoracoideus muscles were dissected by separating them with a lancet or scissors. These muscles are arranged in two packages on both sides of the breastbone. Pectorals are large muscular packages located just below the skin, covering the full thoracic cavity, including the ribs. Supracoracoideus are smaller and located below the pectorals,

just above the ribs. They are easy to separate and dissect because of their different color (the pectoral is darker due to its greater amount of myoglobin), and because each package is separated by the aponeuroses. For the experiments, only the most internal part of each muscle was taken, since there nearly all fibers are specific of one type or the other [29]. All these operations were carried out in the cold room at 4°C, and the muscles were kept clean during the whole procedure by washing them abundantly with ice-cold saline solution (0.85% w/v NaCl in distilled water).

Immediately after the dissection, about 10 g of the tissues were washed with cold saline solution, finely chopped with a meat grinder and subsequently homogenized with a tissue micro-homogenizer (Biospec, Bartlesville, Okla, USA) at 1 g of tissue per 4 ml of Buffer H, at 25,000 rpm, by five intervals of 1 min, and then in a Potter-Elvehjem with a Teflon pestle driven by a motor at 5,000 rpm, for three periods of 2 min. The vessel containing the sample was kept in ice-cold water. Homogenates were centrifuged at 15,000 rpm, in a Sorvall RC2-B centrifuge with a SS-34 rotor for 1 h at 2–4°C. The supernatants (a mean of 4.6 ml) were taken as tissue soluble extracts that contained all cytosolic enzymes. The tissue extracts were kept in ice-cold water until taken for kinetic experiments and protein assays. Protein was quantified by Bradford's method [37], usually giving values between 15 and 20 mg/ml. ATP was assayed by the hexokinase method as previously described [38].

### Hexokinase assay

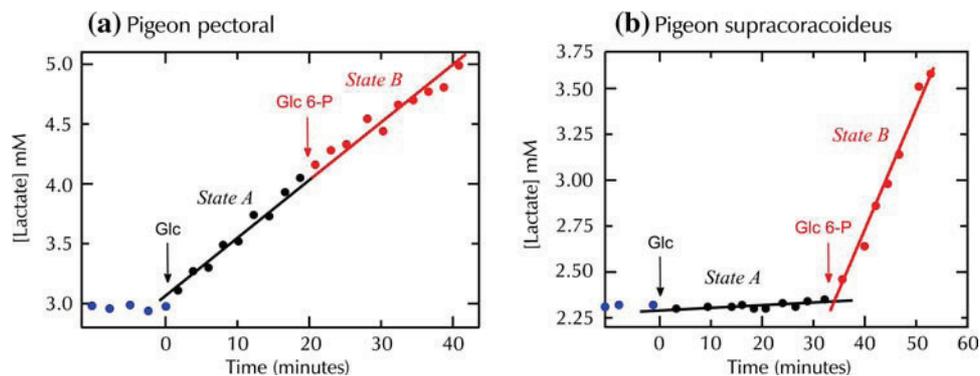
Hexokinase was assayed by a method modified from that described by Bergmeyer et al. [39]. The reaction mixtures (1 ml) contained 3 mM-ATP, 0.35 mM-NADH, 5 U-pyruvate kinase, and 10 U-lactate dehydrogenase, all these reagents dissolved in Buffer R. The volume of tissue extract used in the assay depended on the activity of each sample, and was usually 0.3–1.5 µl for the fowl, and 5–15 µl for the pigeon, according to their activity, completing the volume to 1 ml with Buffer R. Experiments were carried out in a double beam Varian Cary-1 Bio spectrophotometer equipped with a holder for 6 + 6 cuvettes, and with a Peltier-effect system for the temperature control, which was adjusted at 40°C. Reactions were monitored by means of a continuous recording at 340 nm of the NADH decay spent in the lactate dehydrogenase reaction. Hexokinase activity is given as nmoles of NADH consumed per minute (mU), per mg of protein.

### Glycolytic activity

The reaction mixture contained 3 mM-ATP, 0.5 mM-ADP, 1 mM-NAD<sup>+</sup>, 0.5 mM-NADH, 20 mM-creatine, 1 mM-creatine-P, 25 U-creatine kinase, and the muscle extract

(150–550  $\mu\text{l}$  for the fowl, and 750–1800  $\mu\text{l}$  for the pigeon, according to their activity), in 5 ml of Buffer R. The reactions were carried out in 25 ml Erlenmeyer flasks placed in a shaking water bath at 40°C. The kinetic progress of the reaction was monitored by assaying L-lactate in 25  $\mu\text{l}$  aliquots taken from the reaction medium at fixed times with a *YSI Sport 1500* lactate analyzer (YSI Inc., Yellow Springs, Ohio, USA). This lactate electrode is a portable instrument designed for the clinical biochemical control of sport training in situ, which allows to measure L-lactate concentration in very small volumes (25  $\mu\text{l}$ ) of blood [40]. Its high sensitivity (0.01 mM), wide range of linear response (from 0.01 to 10 mM), the accuracy of the measurements and the short time needed to determine a lactate concentration (90 s) makes this instrument an ideal device for the purpose of these experiments. The output signal of the analyzer was connected to a computer, and data was processed with an in-house software specifically built for this purpose.

The reaction mixtures were first cleansed of intermediates by having the system running for 10–15 min until a clear horizontal baseline with no lactate formation was observed. The reaction was then triggered by the addition of 37.5  $\mu\text{l}$  of 1 M-glucose to give a final concentration of 7.5 mM in the incubation medium. Once the system had been in the resulting *steady state A* long enough to allow an accurate calculation of the lactate production slope, 10  $\mu\text{l}$  of 600 mM glc 6-P was added to give 6 mM in the incubation mixture, and the slope of the resulting *steady state B* was calculated again. Control experiments were made to check the activity of the systems directly with glc 6-P as substrate, without previous addition of free glucose, obtaining in all cases the same results as for State B. ATP concentration and pH were assayed after each experiment to check the performance of the buffer systems, giving similar values as at the beginning.



**Fig. 2** Progress curves of the entire glycolysis activity (the pathway shown in Fig. 1) with extracts from pigeon muscles (pectoral, 1.80 ml; supracoracoideus, 1.75 ml). The reaction mixtures (5 ml) were carried out in 25 ml Erlenmeyer flasks, containing 3 mM-ATP, 0.5 mM-ADP, 1 mM-NAD<sup>+</sup>, 0.5 mM-NADH, 20 mM-creatine, 1 mM-creatine-P,

## Results and discussion

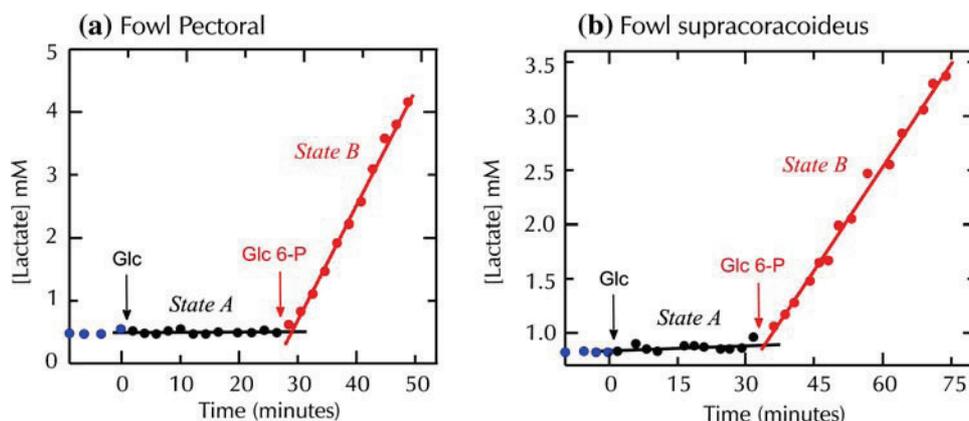
Figures 2 and 3 show the progress curves of lactate production obtained in typical kinetic experiments with pigeon and fowl muscles extracts, respectively, where glucose and glc 6-P were added as substrates in a sequential way as described above. Mean quantitative results are shown in Table 1.

The experimental model, discussed above, allows us to interpret the results as the ability of each tissue to use glucose or glc 6-P depending on their capacity to perform aerobic or anaerobic glycolysis, respectively. Results showed that all muscles assayed have a highly specialized metabolism. Red fibers of pigeon pectoral (Fig. 2a) reached a maximal glycolysis activity with glucose as substrate, but no further increase of activity was achieved with the addition of glc 6-P. Conversely, white fibers of pigeon supracoracoideus (Fig. 2b) and fowl pectoral and supracoracoideus (Fig. 3a, b) showed to have a very low glycolysis activity with glucose, but a high activity with glc 6-P.

The pigeon pectoral muscle is a typical aerobic tissue, as expected for a bird highly trained for long-distance flights, while the fowl pectoral muscle is clearly anaerobic, as corresponds to a bird highly specialized in short fast flights. The supracoracoideus muscle is very anaerobic in both species, which is consistent with their physiological function of supporting the wing upstroke. The fowl pectoral exhibited the highest anaerobic activity, even higher than in the fowl supracoracoideus, which is indeed also very high (3.7 times more than in the pigeon). It might be expected that the supracoracoideus were more anaerobic than the pectoral in all species of birds. However, since the flight of the fowl is very short and quick, based on continuous fast wing upstrokes, it is like the action of taking off but maintained for a longer time, and so the metabolism to support this must have a very high anaerobic activity.

and 25 U of creatine kinase. The progress of each reaction was monitored by taking samples of 25  $\mu\text{l}$  at controlled times to assay L-lactate. The first slope (*State A*) is the glycolytic activity with glucose as substrate, and the second one (*State B*) is the activity after glc 6-P addition. Mean values of the experiments are shown in Table 1

**Fig. 3** Progress curves of the entire glycolysis activity (the pathway shown in Fig. 1) with extracts from fowl muscles (pectoral, 150  $\mu$ l; supracoracoideus, 550  $\mu$ l). Description of experiments is as explained in Fig. 2. Mean values of the experiments are shown in Table 1



**Table 1** Results of the activity of the entire glycolysis and hexokinase obtained from pigeon and domestic fowl

Bird and muscle	Activity (mU/mg prot) substrate			% Activity substrate		Hexokinase (mU/mg prot)
	Glc (State A)	Glc 6-P (State B)	Glc – Glc 6-P (State A – State B)	Glc (State A)	Glc 6-P (State B)	
Pigeon pectoral	7.7 $\pm$ 0.75	7.7 $\pm$ 0.50	0 $\pm$ 0.33	100	0	56 $\pm$ 4.5
Pigeon supracoracoideus	0.2 $\pm$ 0.02	10.4 $\pm$ 1.02	10.2 $\pm$ 1.22	1.9	98.1	8.65 $\pm$ 0.87
Fowl pectoral	0.7 $\pm$ 0.06	304 $\pm$ 12.6	303 $\pm$ 15.4	0.2	99.7	6.70 $\pm$ 0.95
Fowl supracoracoideus	0.9 $\pm$ 0.08	37.9 $\pm$ 2.5	37 $\pm$ 2.7	2.4	97.6	7.00 $\pm$ 0.82

Glycolysis activity is given as nmoles of L-lactate produced per minute (mU), per mg of protein. Values were obtained from eight specimens of each bird. Data are expressed as mean value  $\pm$  standard deviation. Each muscle sample was assayed six times in different experiments. Different analyses of the same sample gave a mean coefficient of variation ( $V = (s/\bar{x}) \cdot 100$ ) of 5.7

Results presented in Table 1 show that in all muscles hexokinase activity is high enough to account for the glucose-dependent glycolytic fluxes (State A). In red fibers, hexokinase can account for the full glycolytic flux, since it was not enhanced by a further addition of glc 6-P. On the contrary, hexokinase in white fibers cannot account for the high glycolytic activity when glc 6-P is used as substrate. This means that phosphorylated glucose derived from glycogen must be the physiological substrate in white fibers. The critical role of glucose phosphorylation step controlling glycolysis demonstrated here (which determines the substrate for each kind of glycolysis) was reported in our earlier works on metabolic control analysis [19, 27] and confirmed afterward by other authors [21, 23, 25] using the same and other experimental approaches.

Our results agree with other reports on the design of the two kinds of glycolysis according to the specific substrate [8–10]. Vock et al. [7] reported that in mammalian red muscle, under a highly intensive exercise, both muscle glycogen and blood glucose are processed aerobically, and that red fibers are also able to perform anaerobic glycolysis from muscular glycogen. This means that under a high stimulation of glycolysis, some fraction of pyruvate can be driven to lactate, making possible a supplementary ATP

source. This result is not in disagreement with ours, because red fibers have a high activity of lactate dehydrogenase [11], and we have seen that they can also process glc 6-P as substrate. On the other hand, differences between other results and ours are most likely due to the fact that assays have been carried out in mammals, which have mixed fiber muscles. For this reason we have drawn special attention to this feature and have selected two highly specialized bird muscles, each one particularly rich in one type of fiber. Nevertheless, it must be taken into consideration the remark made by Newsholme and Start [6], that anaerobic glycolysis in red fibers will be only important when the demand for energy is greater than the oxygen availability, which agrees with the results showed by Vock et al. [7].

The role of hexokinase in the two types of muscles indicates that it is integrated in the glycolytic pathway in red but not in white fibers. This fact can be explained by the occurrence of a channelling in white fibers where hexokinase could be associated to the glycogen synthesis pathway. This agrees with several reports describing the occurrence of two different pools of glc 6-P in muscle with a channelling for glycogen synthesis [41, 42].

Data of individual enzyme activities reported by Crabtree and Newsholme [11] also agree with our results, as

they had shown that in white fibers (fowl pectoral) hexokinase is about 100-fold lower than phosphofructokinase, while in red fibers (pigeon pectoral) it is only eight times lower. In addition, those data also agree with the coupling of the white fibers glycolysis with glycogen depletion, as activity of phosphorylase is there at equal levels to other glycolytic enzymes, while it is much lower in red fibers [11]. The high ratio between phosphorylase and hexokinase (about 100 times) in the fowl pectoral muscle clearly indicates that their glycolysis must be coupled with glycogen depletion, as well as that glucose plays there a poor role as substrate. All these results corroborate the metabolic specialization of this tissue, in close concordance with its physiological function. Thus, in white fibers, free glucose (taken from blood under physiological conditions) would go directly only to replenish glycogen, whose further depletion would be the main source for anaerobic glycolysis. This behavior is not surprising because the supply of glucose from blood would be a poor substrate for anaerobic glycolysis, as this pathway spends much more substrate than it can be supplied by the blood. In effect, comparing the ATP fluxes between the pigeon pectoral (the most aerobic) and the fowl pectoral (the most anaerobic), our results (Table 2) show that anaerobic glycolysis yields an ATP flux about three times higher than aerobic glycolysis, spending 38 times more glucose, which obviously cannot be supplied by the blood.

Since our system was carried out with concentrations of substrates and coenzymes in the range of intracellular concentrations *in vivo*, it reproduces the kinetic features of the real system, so from the data shown in Table 1 we can estimate the fluxes in the intact cell *in vivo*. Our results shown in Table 2 have been derived taking into account the dilution of the tissue, and considering that the cytosolic space is about 0.54 ml per gram of tissue [43]. Although there are no available data of glycolytic fluxes *in vivo* from these muscles to contrast our results, a comparison with some reported data from human muscles *in vivo* can be an

approximation to evaluate the soundness of this approach. Lactate fluxes in human muscles during the first seconds of highly intensive exercise (at highly anaerobic metabolism) have been reported by several authors giving values between 146 and 51 mM lactate/min [44], which is not far from our result of 44 mM/min for the most anaerobic system of our experiments (fowl pectoral). Fluxes of ATP production in human muscles under aerobic conditions have been determined [45] giving values between 24 and 63 mM/min, which are also similar to the value we have found in the pigeon pectoral (as the most aerobic tissue of our experiments), 20 mM/min. Finally, it is interesting to notice the increase of ATP production flux in the transition from aerobic to anaerobic metabolism, as a rapid and massive ATP source. Activation of ATP production flux in that transition has been shown to be by about 2.94 [44]. This value can be compared in our experiments with the ratio of ATP fluxes between fowl pectoral (the most anaerobic) and the pigeon pectoral (the most aerobic), see Table 2. This gives in our results a ratio of  $66/20 = 3.3$ . Thus, although some particular values obtained in this study can be lower than in the original systems *in vivo*, the ratio between them is very similar to the *in vivo* value. Nevertheless, it must be taken into account that we are comparing data of muscles from different sources.

We would like to remark the convenience of approaches like the one presented here, concerning the kinetic behavior of entire metabolic pathways that allow obtaining systemic data to study the metabolism of complex systems. The biochemical studies of individual enzymes, despite yielding valuable information, do not allow to fully comprehending the global performance of a complex system. On the other hand, physiological macroscopic studies *in vivo* provide important information on global processes, but do not allow deriving the details of functioning of each metabolic pathway and the specific role of individual steps controlling them. We have presented previously a work in which the pentose-phosphate pathway was studied by a similar

**Table 2** Possible fluxes of glycolysis and ATP production *in vivo* derived from the data shown in Table 1, calculated according to the dilution of the soluble fraction of the tissues, as explained in the text

	Fluxes 'in vivo' with respect to each substrate or product <sup>a</sup>					
	Glucose or glc 6-P		Pyruvate or lactate		ATP <sup>b</sup>	
	nmol/(min mg prot)	mM/min	nmol/(min mg prot)	mM/min	nmol/(min mg prot)	mM/min
Pigeon pect	3.85	0.56	7.7	1.11	138	20
Pigeon supr	5.2	0.75	10.4	1.5	15.6	2.26
Fowl pect	152	22	304	44	456	66
Fowl supr	19	2.74	38	5.49	57	8.23

<sup>a</sup> Concentrations in the cytosolic space, which is about 0.54 ml per gram of tissue [43]

<sup>b</sup> ATP fluxes were calculated assuming that all flux from glucose would be processed in the cell by the aerobic way, yielding 36 ATP's per glucose, and all flux from glc 6-P would be processed by the anaerobic way, yielding three ATP per glc 6-P

approach as herein, showing for the first time global data [46]. These results agreed with the behavior that had been inferred from isolated enzyme activities, but gave direct quantitative values on the whole system (e.g., the high increase of activity of the oxidative phase of the pentose-phosphate pathway in lactating mammary gland for fatty acid synthesis). The experimental system we present here to study kinetics of the entire glycolysis pathway follows this idea, completes previous works [19, 33] and will allow more comprehensive studies. It is our opinion that several features of muscle metabolism should be studied further in order to understand the details of muscle metabolism, which is a subject of major interest, and that this approach may be helpful in this task.

The reliability of the experimental model presented in this work is based upon having used muscles with a pure composition of fibers. The same approach can nevertheless be applied to more complex muscles, such as those of mammals, which have a mixture of fibers. The results obtained there would expectedly show the global aerobic/anaerobic capability and could permit a better understanding of their metabolism and physiology. Besides, this system can be developed by several means to study the effect of other substrates or variables, such as pH and temperature, which has been reported to have important influence on muscle energy metabolism, particularly in ectothermic animals [22, 47]. Likewise, the experimental system can be extended by adding more auxiliary enzymes, substrates, and coenzymes. For example, coupling between glycolysis and pentose-phosphate cycle could be studied by a combination of the systems describe here and elsewhere [46]. In addition, this approach can be used to complete studies on metabolic control analysis, since as far as we know, all experimental studies on this subject have been made in the more simplified glycolytic system described by us, which only included the first steps from glucose to the triose-phosphate [19, 20].

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