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STUDIES ON GLYCOLYSIS *IN VITRO*: ROLE OF GLUCOSE PHOSPHORYLATION AND PHOSPHOFRUCTOKINASE ACTIVITY ON TOTAL VELOCITY

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Abstract—1. An *in vitro* glycolysis system has been developed to study the regulation of glycolysis on kinetic structure basis, in order to determine the extent of regulatory effects on the whole system of individual enzymes according to their kinetic data, in rat liver and muscle.

2. Hexokinase or glucose-6-phosphate addition to the system with glucose as substrate increases lactate production rate by 2.5 in liver and by 10 in muscle, which suggest glucose phosphorylation step is a limiting step in this system.

3. Fructose 2,6-bisphosphate addition to the system increases lactate production rate in liver only when glucose is the substrate, but not with glucose-6-phosphate as substrate.

4. There is a linear relationship between glycolytic activity, as lactate produced per min and protein quantity, which suggests that this system can also be used to assay glycolytic activity in tissue extracts. Specific glycolytic activity found, as μmol of L-lactate produced per min, per protein mg was 0.1 for muscle and 0.01 for liver.

INTRODUCTION

The study of glycolysis regulation has been centered mainly on the kinetics of its individual enzymes and their regulating effects. In fact, glycolysis regulation has been assumed mainly to PFK and pyruvate kinase activities, giving special emphasis to ATP inhibition on PFK, this being the primary physiological effect on *in vivo* glycolysis regulation (Passonneau and Lowry, 1964; Goldhammer and Paradies, 1979). However, in skeletal muscle this effect is unclear since, although ATP inhibits muscle PFK (Uyeda, 1979; Foe and Kemp, 1982) ATP concentration determines the extent of the regulating mechanism of the contractile system in this tissue, because affinity of troponin for calcium depends on the saturation degree of myosin ATPase by ATP (Weber and Bremel, 1971; Bremel *et al.*, 1973; Marston *et al.*, 1980). This in turn suggests a constant ATP concentration maintenance by the adenylate kinase and phosphocreatine systems in this tissue in order to ensure calcium regulation of the contractile system. Moreover, the discovery of F26P as a strong activator of PFK (Van Schaftingen *et al.*, 1980; Uyeda *et al.*, 1981; Hers and Van Schaftingen, 1982) and the confirmation that its concentration in liver cells varies according to glycolytic activity controlled by hormones (Richards *et al.*, 1981; Richards and Uyeda, 1982) suggest that under some conditions glycolysis activity can be independent of ATP inhibition. Therefore, the elaboration of a simple model explaining glycolysis regulation based on individual effects observed in every enzyme is very difficult.

Furthermore, another point to consider is the entirety of the structural data of glycolytic enzymes, including not only the eleven for metabolic conversion of carbon skeleton of glucose into lactate, but also others such as those involved in biphosphoglycerate and F26P metabolism and those which directly relate products and substrates in this patway, such as ATPase, adenylate kinase and AMP deaminase, and gluconeogenic enzymes. Activity and kinetic data of all these enzymes, as well as intermediary metabolite concentrations, may be likewise considered in order to find a plausible model to explain glycolysis regulation in each organ or tissue. Although synthetic models based on PFK inhibition by ATP account for several physiological aspects, such as the Pasteur effect (Passonneau and Lowry, 1962; Krebs, 1972; Sols, 1976), the complexity of this system and the lack of many of these data necessarily mean that these models are incomplete (Boiteux and Hess, 1981). In the same way that the assumption to *in vivo* conditions of any effects found *in vitro* to system behaviour is commonly challenged, the contribution of any phenomenon found in an isolated system to the activity of a whole process of which they form a portion should also be taken with reserve. This is by no means an attempt to detract from the importance of kinetic data found in each of the glycolytic enzymes, but rather to determine the extent of their effect on the whole system where there are other known or unknown factors. One way to answer these questions is to study glycolysis with the complete system without removing or purifying enzymes in order to keep the structural ratio of their crude tissue extracts. Several authors have used similar

Abbreviations: cAMP, 3′,5′-cyclic AMP; F6P, fructose-6-phosphate; F26P, fructose 2,6-bisphosphate; G6P, glucose-6-phosphate; HK, hexokinase; PFK, phosphofructokinase.

experimental approach: Scopes (1973, 1974a,b) and Eagle and Scopes (1981) have shown many results in reconstituted glycolytic systems from rabbit muscle after enzyme isolation; Hess (1973) and Hess and Boiteux (1973) have described oscillations in NADH concentration with crude yeast extracts; Bañuelos and Gancedo (1978) have described a system to study total glycolysis in yeast *in situ*, among others. On the other hand, glycolytic activity could be a good metabolic parameter to study some physiological or pathological states in several tissues, such as transformed cells (Bustamante *et al.*, 1981). Our aim in this work is the study of glycolysis *in vitro* to analyze within the complete system the extent of effects on individual enzymes and to find a useful method for assaying glycolytic activity in tissue extracts. Our results show that glucose phosphorylation is the limiting step in rat liver and muscle glycolysis *in vitro* and that PFK activity regulates glycolysis velocity only when unphosphorylated glucose is the substrate. On the other hand, these results show that this *in vitro* system can be used to assay glycolytic activity in tissue extracts, giving a good linearity in both lactate an glycerol phosphate production rates, the two being similar in the first minutes of the assay.

MATERIALS AND METHODS

Chemicals

Porcine heart lactate dehydrogenase type XVIII, yeast HK type C-300, rabbit muscle PFK type III, rabbit muscle aldolase type I, yeast G6P dehydrogenase type XI, rabbit muscle glycerophosphate dehydrogenase type I, ATP. ADP, cAMP, IMP, NAD^+, $NADP^+$, NADH, fructose-1,6-biphosphate, F26P, glucose, G6P, β-alanine, caproic acid, creatine phosphate, levulinic acid, L-lactic acid and 2,3-biphosphoglycerate were obtained from Sigma Chem. Co. (St Louis, Missouri, U.S.A.); all other reagents were analytical reagent grade, purchased from E. Merck (Darmstadt, F.R.G.) or Koch–Light (Coinbrook, Bucks, U.K.). In all isotachophoresis assays, doubly distilled and deionized water was used to prepare all solutions including electrolytes, spacers and nucleotide standards, which were used within 72 hr after preparation.

Biological samples

Male and female Wistar albino rats (180–220 g) fed on a standard laboratory diet, were used in all experiments. Liver and skeletal muscle (biceps femoris) were obtained under ether anesthesia, cooled, chopped and homogenized at 1 g/3 ml in 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mM NaCl and 2.5 mM $MgCl_2$, by using a Potter–Elvejeim homogenizer with Teflon pestle, in an ice-cold bath. The homogenates were clarified twice by centrifugation at 27,000 *g* in a Sorvall RC-5B centrifugue (I. Sorvall Inc., Newtown, Connecticut, U.S.A.) at 3–4°C for 20 and 10 min, respectively. The resulting surneageants were used immediately for kinetic glycolysis experiments, and their protein concentrations were determinated by the method of Lowry *et al.* (1951).

Kinetic experiments

Liver and muscle samples (soluble fractions) were incubated with the appropriate substrates, effectors and recycling products, according to the experiment for developing *in vitro* glycolysis, in a shaker bath at 30°C during 70–80 min, taking 1 ml aliquots at convenient times (usually every 5–10 min) for assay of necleotides, substrates and products of the reaction. Bacterial growth was avoided by addition of 1 mg/ml penicillin and 1 mg/ml streptomycin to incubation mixture. However, in repeated experiments without antibiotic addition no differences were observed. Agar plates seeded with the incubation mixture without addition of antibiotics give 1000 colonies at 80 min at 30°C, which is not sufficient for detectable bacterial activity. Chicken egg white trypsin inhibitor (Sigma, type II) was always added to all incubation mixtures at 0.5 mg/ml. Control experiments to assure anaerobic metabolic conditions, as correspond to mitochondria free samples were developed, in the same conditions, in a Clark Oxygen electrode (Rank Brothers, Cambridge, England) without observing oxygen depletion.

Extensive series of experiments were carried out to study the more appropriate concentrations of substrates, effectors and recycling products for development of *in vitro* glycolysis. The incubation mixture for a typical experiment to assay glycolytic activity was: 7 mM G6P, 15 mM ADP, 1 mM ATP, 7 mM NAD^+, 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate. In several experiments, in order to study the glucose phosphorylation step, 7, 20 and 100 mM glucose was used as the substrate instead of G6P. Likewise, several experiments were carried out without cAMP, fructose-1,6-biphosphate or 2,3-biphosphoglycerate. ATP concentration was also modified between 0.3–1.5 mM. Tissue samples appropriately diluted in 0.1 M potassium phosphate buffer, pH 7.4 (0.18 M K^+), 5 mM NaCl and 2.5 mM $MgCl_2$ to obtain 3.5–7.0 protein mg/ml for liver and 0.5–1.0 protein mg/ml for muscle in the incubation mixture were added to incubation medium according to the experiment. All reagents used in the experiments including those described above and added enzymes, were dissolved in the same buffer. The total volume of the incubation mixture was 7–10 ml according to the necessary number of aliquots. Reactions were started by adding 0.1 and 0.05 ml of tissue extract for liver and muscle, respectively, per ml of whole mixture. Calibration curves to study the linear relationship between glycolytic activity and protein concentration in the assay medium were obtained by using the incubation mixture described above, with 7 mM G6P as substrate, and diluting the sample in a range between 1.25–6.8 and 0.12–1.35 protein mg/ml for liver and muscle, respectively. In several experiments, as is shown below, certain reagents were added to incubation mixtures at 30 or 40 min from starting time, to study the behaviour of the system in these new conditions. These additions were always made in 0.1 ml. In all cases controls were also developed without glucose or G6P to determine basal interference in L-lactate and glycerol phosphate production and possible glycogen use. Glycolytic activity is expressed in μmol of L-lactate produced per minute in our conditions from glucose or G6P, according to the experiment. Specific glycolytic activity was calculated as μmol of L-lactate or glycerol phosphate produced per minute, per protein mg.

Substrate and product assays

Aliquots from incubation medium, obtained in the course of the kinetic experiments (1 ml) were deproteinized by addition of 1 ml of 1 M $HClO_4$, neutralized after 10 min with 0.1 ml of 5 M K_2CO_3 (to pH 6–7) and centrifuged at 3500 *g* for 10 min to remove the prepcipitated proteins. 0.1 ml of this extract was used for each enzymatic end-point assay of glucose, G6P, L-lactate and glycerol phosphate according to Bergmeyer (1974) by a continuous recording UV absorbance variation of NAD(P)H to end point, measured at 340 nm with a programmable Hitachi 100–80A Spectrophotometer (Hitachi Ltd, Tokyo, Japan) at 25°C. Nucleotides were assayed by analytical isotachophoresis as previously described (Pérez *et al.*, 1982a), performed on a LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden) with a polytetrafluorethylene capillary column (230 × 0.5 mm) at 20°C using 5 mM HCl corrected to pH 3.89 ± 0.03 (critical value) with β-alanine as leading electrolyte, and 5 mM caproic acid, pH 3.94 as termining

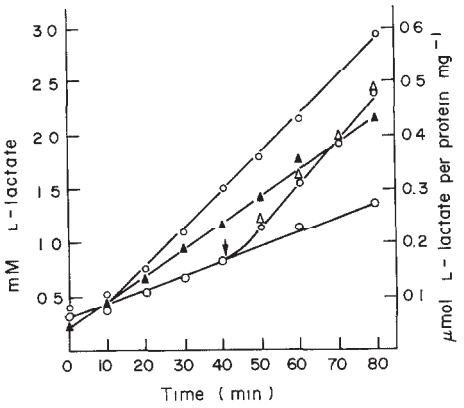

Fig. 1. L-Lactate production rate in rat liver glycolysis system *in vitro*. Soluble fractions were prepared from rat liver as described under "Materials and Methods" and incubated during 80 min with 7 mM glucose (○) and 7 mM G6P (●, ▲) as the initial substrate, with 15 mM ADP, 1 mM ATP and 7 mM NAD^+ in 0.1 M potassium phosphate buffer, pH 7.4, having added at starting time nothing (▲), and 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate (○, ●). Reactions were developed at 30°C taking 1 ml aliquots every 10 min to assay L-lactate. In separate experiments with 7 mM glucose as substrate (○), 7 mM G6P (●) or 2.5 U/ml HK (△) were added to the incubation mixture (arrow).

electrolyte. 0.1% (v/v) Triton X-100 (Sigma) was added to the leading electrolyte to reduce electroendosmosis. Isotachophoretic analysis of all nucleotides (ATP, ADP, AMP, IMP-cAMP, NAD^+ and NADH) in each sample was achieved in 20 min at 50 μA initial constant current which was reduced to 25 μA when the voltage reached 4.5 kV. Running zones were detected by UV signal at 254 nm, registered with a LKB 2066 recorder and their peak areas calculated with a Hewlett–Packard 3390A Integrator (Hewlett–Packard, Amstelveen, The Netherlands). For achieving nucleotide peaks separation, 2 μl of spacer solution (0.7 mM creatine phosphate, 0.5 mM malonic acid, 0.5 mM G6P, 0.3 mM levulinic acid and 0.1 mM lactic acid) were injected in the column with 2 μl of diluted sample. It was not necessary to add phosphate as spacer since it is present in the sample in sufficient concentration. It was not possible to resolve cAMP from IMP peak in our assays, and in our results these two nucleotides are mixed.

RESULTS

Since we are interested in the study of glycolytic activity, our *in vitro* system has been designed in maximal velocity conditions (V_{max}), at high glucose or G6P, inorganic phosphate, NAD^+ and ADP concentrations. This is suitable to find the limiting step in the reaction sequence, as well as to study the regulation effects on activity of enzymes which control glycolysis flux. In fact, our system is not in psysiological conditions for first substrate concentrations, but it allows us to analyze the kinetic structure of the system concerning activity of individual enzymes and the relationship between this activity and that of total glycolysis.

Anaerobic glycolysis, as L-lactate production *in vitro* from glucose and from G6P, is shown in Figs 1 and 2 for rat liver and muscle, respectively. A good linearity in lactate production rate is obtained in these experiments, as corresponds to a constant lactate production rate during the whole of the experiment, allowing us to calculate glycolytic activity in these conditions. In experiments with unphosphorylated glucose used as substrate, further addition to the incubation medium of 7 mM G6P or 2.5 U/ml HK, in separate experiments, enhances the lactate production rate by 2.5 in liver and by 10 in muscle, ATP concentration being used between 0.3 and 1 mM, with the same results, also giving a good linearity for lactate. Thus, the glucose phosphorylation step is a specially interesting point in this system, since when we add the phosphorylating enzyme or its reaction product to this system the lactate rate is significatively increased, suggesting therefore, that the enzymatic glucose phosphorylation step is limiting; in fact, the addition of this enzyme gives the same result as the addition of all glycolytic enzymes. According to these results, the glucose phosphorylation step is more critical in muscle than in liver. Hexokinase is the glycolytic enzyme with least activity in liver and muscle (Scrutton and Utter, 1968); we have also assayed this effect at high glucose concentration (0.1 M) as initial substrate, as well as high ATP concentration (5 mM), obtaining in all cases the same results by addition of G6P or HK, resolving, therefore, the possible function of glucokinase and the saturation degree of both phosphorylating enzymes by ATP. Because aldolase activity is also small in liver, we also have tested its addition (2.5 and 5.0 U/ml) to the liver glycolysis assay in experiments like those shown in Fig. 1 without noting changes in the lactate production rate. Results about PFK addition are shown below, in Fig. 5. We have assayed HK activity in our enzymatic extract before and after centrifugation of

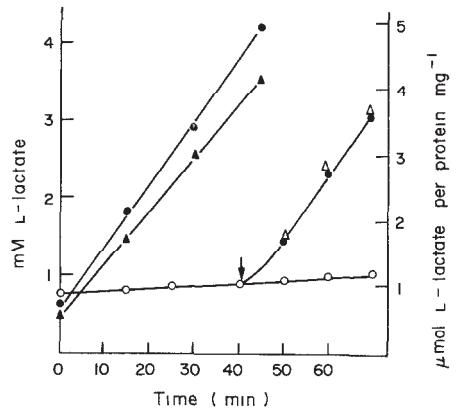

Fig. 2. L-Lactate production rate in rat muscle glycolysis system *in vitro*. Soluble fractions were prepared from rat muscle as described in "Materials and Methods" and incubated during 70 min with 7 mM glucose (○) and 7 mM G6P (●, ▲) as the initial substrate, with 15 mM ADP, 1 mM ATP and 7 mM NAD^+ in 0.1 M potassium phosphate buffer, pH 7.4, having added at starting time nothing (▲), and 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate (○, ●). Reactions were developed at 30°C taking 1 ml aliquots every 10 min to assay L-lactate. In separate experiments with 7 mM glucose as substrate (○), 7 mM G6P (●) or 2.5 U/ml HK (△) were added to the incubation mixture (arrow).

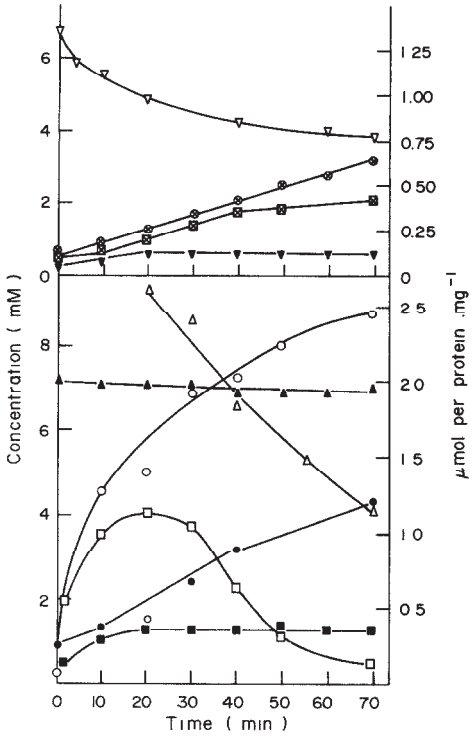

Fig. 3. Substrate, product and nucleotide concentration during *in vitro* glycolysis development of rat liver, with G6P as the substrate. Soluble fraction was prepared from rat liver as described under "Materials and Methods" and incubated during 70 min with 7 mM G6P as the substrate, 15 mM ADP, 0.3 mM ATP, 7 mM NAD^+, 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were developed at 30°C taking 1 ml aliquots every 10 min to assay glucose (▼), G6P (▽), L-lactate (⊗) and glycerol phosphate (⊠) by enzymatic end-point method, and ATP (○), ADP (△), AMP (□), IMP (●), NAD^+ (▲) and NADH (■) by isotachophoresis. cAMP was not resolved from IMP as mentioned in the text.

homogenate and we have not found differences in its activity, which discards the possible loss of HK by binding it to removed mitochondrial fraction.

The effect of fructose 1,6-bisphosphate, cAMP and 2,3-bisphosphoglycerate, as regulating or recycling glycolytic products, was also studied. Fructose 1,6-bisphosphate was added as activator of pyruvate kinase in order to eliminate its inhibition by ATP, at 0.2 mM, according to results previously described (Pérez *et al.*, 1982b). All three products, at used concentrations, enhance glycolytic activity by 45% in liver and by 20% in muscle. These results are also expressed in Figs 1 and 2. This effect makes these reagents suitable for inclusion in the medium to assay glycolytic activity, as well as in further experiments in order to obtain highest values of glycolysis velocity necessary to have the system in maximal velocity (V_{max}) conditions for the study of kinetic structure of the glycolytic system, at saturating concentrations of all substrates (G6P, ATP, ADP, NAD, P_i, etc.) of the glycolysis system. A good linearity for lactate was obtained, however, in all cases. Individual effects of each product (results not shown) give fructose-1,6-biphosphate as the larger activator and cAMP as the smaller, in liver and muscle. Higher concentrations of these activiators are unnecessary. The effect of F26P addition is shown below in Fig. 5.

Figures 3 and 4 show the changes in the concentration of glycolysis substrates and products for liver and muscle, respectively, using 7 mM G6P as substrate and 0.3 mM ATP at starting time. In these conditions there is no notable variation in glucose concentration, a considerable G6P descent being observed, which is consistent with isomerization to F6P, near equillibrium, and with the increase of lactate and glycerol phosphate. In both tissues studied glycerol phosphate production occurs at the same rate as lactate in the first 40 min, lactate being the only assayed product which shows a constant linearity during the whole assay time until reaching a concentration of 4–6 mM, unaltered by the loss of

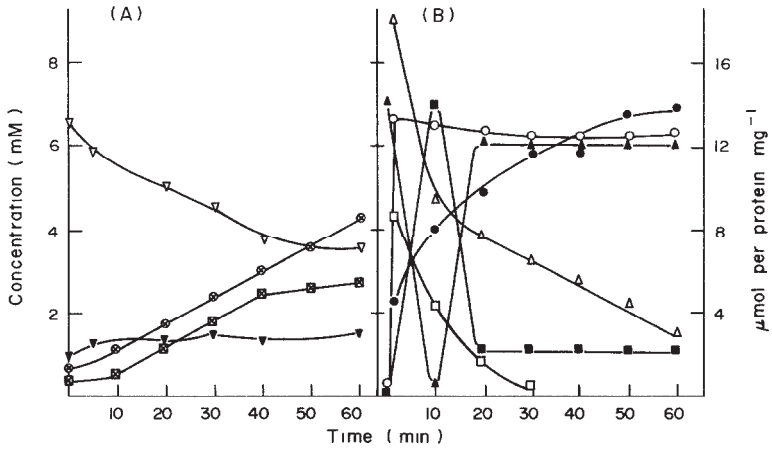

Fig. 4. Substrate, product and nucleotide concentrations during *in vitro* glycolysis development of rat muscle, with G6P as the substrate. Soluble fraction was prepared from rat muscle as described under "Materials and Methods" and incubated during 60 min with 7 mM G6P as the substrate, 15 mM ADP, 7 mM Nad^+, 0,3 mM ATP, 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate in 0.1 M potassium phosphate buffer, pH 7.4. Reaction was developed at 30°C taking 1 ml aliquots every 10 min to assay glucose (▼), G6P (▽), L-lactate (⊗) and glycerol phosphate (⊠) by enzymatic end-point method, and ATP (○), ADP (△), AMP (□), IMP (●), NAD^+ (▲) and NADH (■) by isotachophoresis. cAMP was not resolved from IMP as mentioned in the text.

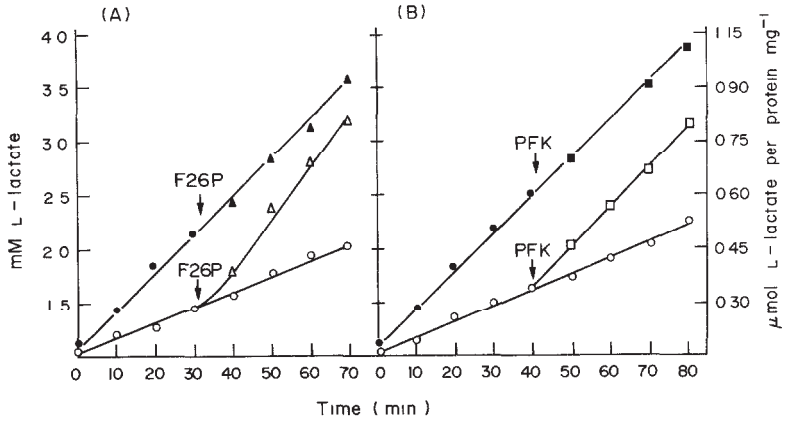

Fig. 5. Effect of fructose-2,6-biphosphate and PFK additions on lactate production rate in rat liver glycolycis system *in vitro*. Soluble fractions were prepared from rat liver as described under "Materials and Methods" and incubated during 80 min with 20 mM glucose (○) and 7 mM G6P (●) as substrate, with 15 mM ADP, 1 mM ATP, 7 mM NAD^+, 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were developed at 30°C taking 1 ml aliquots every 10 min to assay L-lactate. (A) 10 μM F26P addition to the system, indicated by arrows, when G6P was the substrate does not alter the lactate rate (▲), while the same addition when unphosphorylated glucose was the substrate increases the lactate rate (△). (B) 5 U/ml PFK were added giving the same effect of F26P addition. PFK addition, indicated by arrows give enhanced rate only with glucose as the substrate (□).

glycerol phosphate linearity. This glycerol phosphate production at velocity similar to lactate in both tissues is certainly a point of interest. We have, in fact, in our system the conversion of G6P in lactate and glycerol phosphate in both tissues, which means glycolysis is not the only glucose–lactate conversion, since only half of the carbon source is converted to lactate. Linearity in lactate production rate is maintained, however, during all experiment time (70–80 min) but linearity in glycerol phosphate production rate is suddenly lost at 40 min giving a new constant velocity of 25% in liver and 18% in muscle from their initial velocities. Changes in ATP, ADP and AMP concentrations during the experiments are very different in the two tissues studied, specially for ATP. In liver, there is a continuous ATP increase, consistent with the glycolysis process, whereas in muscle ATP remains at a constant concentration of 6 mM after the first 10 min. The important ADP descent in both tissues in the first minutes can be attributed to adenylate kinase, whose activity is shown by changes in the concentration of AMP, not added in the incubation mixture. IMP as catabolic product from adenine nucleotides, not resolved from cAMP in our isotachophoretic assays, also shows an increase during glycolysis development, in agreement with the high AMP deaminase activity in these tissues. In muscle there is no regular ATP increase, and the ADP descent is compensated by IMP increase, almost double that in liver. This constant ATP concentration, typical in muscle, is in agreement with the metabolic conditions of this tissue, as discussed below, and also agrees with data from Scopes (1973). NAD^+/NADH ratio is another point of interest. In liver, after the first minutes, where there is a little variation from its initial value, this ratio is constant remaining in a steady state during the whole experiment. In muscle, there is also a similar steady state after the first 20 min, during which an important NADH increase and a consistent NAD^+ descent occurs, from initial concentrations, to overtake the steady state at a later time in the experiment.

PFK, or its great activator F26P, addition to the liver glycolytic system gives the results shown in Fig. 5. Both F26P and PFK addition enhance lactate production rate, only when unphosphorylated glucose is the substrate used in the experiment. In muscle, however, we have not found this activating effect with F26P. In liver, when the substrate is G6P we do not find this activation effect on lactate production rate by F26P or PFK addition, its velocity being of the same order as that from glucose after the addition of the activator, shown in the same Fig. 5. These results show that glycolysis rate from glucose depends on the glucose phosphorylation step and PFK, glycolysis velocity from glucose with F26P added from starting time being like that shown in Fig. 5 after the addition of this activator. However, subsequent addition to this last system of 7 mM G6P also enhances the lactate production rate, again by nearly 2.5 showing PFK-activated glycolysis dependence on the glucose phosphorylation step (Fig. 6). Table 1 summarizes these results.

These results show that with this system one can assay the glycolysis activity in tissue extracts assaying L-lactate every 5–10 min with appropriately diluted samples. In our experiments with rat liver and muscle, we have assayed the glycolytic activity, expressed as μmol of lactate produced per min from G6P, in the range from 0.5 to 5.0 protein mg in 4 ml of the assay mixture for muscle and in the range 5 to 30 protein mg in the same volume of assay mixture for liver. In all cases we have obtained a good linearity in the glycolytic activity and protein quantity relationship, with specific glycolytic activities as μmol of L-lactate min^{-1} protein mg^{-1}, of 0.01 for liver and 0.1 for muscle. (Fig. 7).

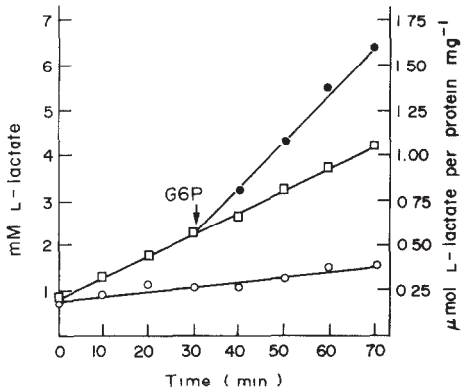

Fig. 6. Effect of glucose-6-phosphate on F26P activated rat liver glycolysis *in vitro*. Soluble fractions were prepared from rat liver as described under "Materials and Methods" and incubated during 80 min with 20 mM glucose as the substrate, with 15 mM ADP, 7 mM NAD^+, 1 mM ATP, 0.3 mM cAMP, 0.2 mM fructose-1,6-biphosphate and 0.5 mM 2,3-biphosphoglycerate in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were developed at 30°C taking 1 ml aliquots every 10 min to assay L-lactate. 10 μM F26P was also added at starting time (□) and 7 mM G6P was added at 30 min (arrow) on F26P activated system (□), giving an increase in its lactate production rate (●).

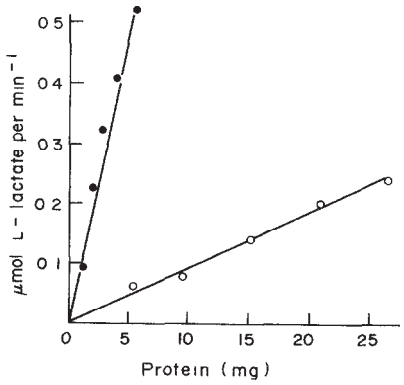

Fig. 7. Glycolytic activity and protein quantity relationship. Soluble fractions of rat liver (○) and muscle (●) were prepared as described under "Materials and Methods", diluted to obtain the appropriate protein concentration range in the incubation mixture and incubated at 30°C during 40 min with 7 mM G6P, 15 mM ADP, 1 mM ATP, 7 mM NAD^+, 0.3 mM cAMP, 0.2 mM fructose 1,6 biphosphate and 0.5 mM 2,3 biphosphoglycerate in 0.1 M potassium phosphate buffer, pH 7.4. Total volume of the incubation mixtures were 4 ml and 1 ml aliquots were taken every 5 min to assay L-lactate by enzymatic end-point method after $HClO_4$ protein precipitation. Slopes of curves allow to calculate specific glycolysis activity as μmoles of L-lactate produced per min, per protein mg (0.1 for muscle and 0.01 for liver).

DISCUSSION

The increase of lactate production rate, glucose being the initial substrate, by adding G6P or HK to liver and muscle *in vitro* glycolysis suggests that the glucose phosphorylation stage could be the limiting step in these systems, as corresponds to the zero order kinetic step in the reaction sequence, whose activity depends only on enzyme activity. However, in liver, using glucose as the substrate, there is also a rate increase when PFK or F26P is added, showing that PFK could also be a limiting step. When G6P is the substrate the system does not depend on PFK activity, since addition of this enzyme or its activator F26P does not alter the whole rate, which lead us to decide on the HK role as the limiting step in this *in vitro* system. In fact, once the HK step is surpassed, the system no longer depends on PFK. Furthermore, results of inverted experiments agree with this conclusion: with F26P-activated PFK the system continues to be dependent of HK, since when glucose is the substrate with F26P added at starting time, further addition of G6P again enhances the lactate production rate by 2.1. We therefore think that these results show glucose phosphorylation as the limiting step in this *in vitro* glycolysis from glucose. Concerning the *in vivo* situation, this result can show the discord between the glucose phosphorylation step activity and glycolysis activity from G6P, suggesting that these two tissues studied, liver and muscle, are adapted to carry out glycolysis mainly using phosphorylated glucose coming from glycogen.

In liver, PFK role in regulation of glycolysis is different according to the substrate used, glucose or G6P. When glucose is the substrate the extent of ATP

Table 1. L-Lactate production rate in rat liver *in vitro* glycolysis and effect of additions of glycolysis activators

Substrate	Rate × 10^3	Added product	Rate × 10^3	Activation factor
20 mM glucose	3.8	7 mM G6P	9.5	2.5
20 mM glucose	3.8	2.5 U/ml HK	9.5	2.5
20 mM glucose	3.8	10 μM F26P	12.1	3.2
20 mM glucose	3.8	5 U/ml PFK	9.1	2.4
7 mM G6P	9.5	10 μM F26P	9.5	1.0
7 mM G6P	9.5	5 U/ml PFK	9.5	1.0
20 mM glucose plus 10 μM F26P	12.1	7 mM G6P	25.5	2.1

Kinetic experiments were carried out with soluble fractions of rat liver, as described in "materials and methods", from indicated substrates, and velocity was measured by assaying L-lactate every 10 minutes in the incubation mixture. At 30–40 min of reaction time the shown reagents were added. Activation factors were calculated as the ratio between the rate after activator addition and that before it. Lactate production rate values are expressed as μmoles of L-lactate produced per minute, per protein mg. These results are the mean values of five experiments, giving a coefficient of variation $\sigma/\bar{X} \times 100$ of 2.7.

inhibition on total glycolysis can be seen, since PFK activation by F26P increases the lactate production rate, as does PFK addition. However, neither F26P nor PFK addition increases the lactate production rate in glycolysis from G6P, which means that PFK is not inhibited, the system being independent of PFK activity variation, no longer depending on ATP inhibition. In fact, in the first minutes of the reaction ATP concentration reaches 1–2 mM which is enough to inhibit PFK (Uyeda *et al.*, 1981; Van Schaftingen and Hers, 1981; Van Schaftingen *et al.*, 1981), and in the course of reaction ATP reaches values higher than 7 mM without changes in the initial lactate rate, this not being activated for PFK activation. According to these results glycolysis is regulated by PFK activity only when the substrate is glucose, but this does not occur on glycolysis from G6P. A possible explanation of this fact is the F6P concentration which should be high when G6P is the substrate and small when it is glucose. Moreover it is possible the synthesis of F26P in our *in vitro* system, more favoured at high G6P and F6P levels. In muscle, ATP remains constant after the first minutes. This fact is in agreement with the constant ATP concentration in this tissue, necessary for the regulation of the contractile system by troponin (Weber and Bremel, 1971; Marston *et al.*, 1980; Ebashi, 1974). In this tissue, the adenylate kinase and AMP deaminase account for it.

The glycerol phosphate production rate is another point of interest in this system; it is of the same extent as lactate in liver and muscle in the first 40 min suggesting that glycolysis is the conversion of G6P both in lactate and glycerol phosphate, which advises the assay of glycerol phosphate as well as of lactate in the glycolytic activity assays.

Finally it is necessary to stand out one fact, in light of the results of experiments of the addition of G6P, HK, F26P and PFK to this *in vitro* system, it can be observed that inversion of the order in the addition of these reagents to the incubation mixture in kinetic studies does not lead us to the same result. Thus, as can be seen in Table 1, addition of G6P to the system with glucose as the substrate increases the rate by 2.5 activation factor and addition of F26P to the system with G6P as substrate does not produce any increase in rate. On the other hand, addition of F26P to the system with glucose as substrate increases the lactate rate by a 3.2 activation factor, and addition of G6P to the system with glucose as substrate and with F26P at starting time again increases the lactate rate by 2.1 activation factor, giving a total activation factor in two steps of $3.2 \times 2.1 = 6.7$. However, this value is not obtained by inverting the order of reagent addition. We think that on the basis of the known ligand–enzyme interactions, these results are difficult to explain, there possibly existing other phenomena, such as enzyme–enzyme interactions. Further experiments to this end could answer this question and find an explanation with a plausible model for glycolysis regulation.

SUMMARY

An *in vitro* glycolysis system has been developed to study the regulation of glycolysis in order to determine the extent of regulatory effects on the whole system of individual enzymes according to their kinetic data. The experiments were carried out by incubation of soluble fractions from both rat liver and muscle with ADP, phosphate and other appropriate reagents, assaying the lactate production rate from other glucose or glucose-6-phosphate as the substrate. All nucleotides, as well as other products, were also assayed during the course of reaction. Hexokinase or glucose-6-phosphate addition to the system with glucose as substrate increases lactate production rate by 2.5 in liver and by 10 in muscle, which could indicate a close dependence in these two tissues of phosphorylated glucose use, such as that from glycogen. Fructose 2,6-bisphosphate addition to the liver system increases lactate production rate 3 fold approximately, only with unphosphorylated glucose as substrate but not with glucose-6-phosphate as substrate. Moreover, from glucose as substrate, adding fructose 2,6-bisphosphate from starting time, further glucose-6-phosphate addition again enhances the activity giving the highest observed velocity, activated by 6.7 with respect to a simple system using only glucose as substrate. These results seem to determine the extent of the individual kinetic effects on the whole system observed in phosphofructokinase regulation and suggest that this experimental approach could be suitable for studying glycolysis regulation. We have observed a good linearity in the glycolytic activity and protein quantity relationship in both, liver and muscle, obtaining values of specific glycolytic activity, as μmol of L-lactate produced per min, per protein mg, of 0.1 for muscle and 0.01 for liver, from glucose-6-phosphate. These results suggest that this system can also be used to assay glycolytic activity in tissue extracts.

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