Studies with a Reconstituted Muscle Glycolytic System

THE RATE AND EXTENT OF CREATINE PHOSPHORYLATION BY ANAEROBIC GLYCOLYSIS

By ROBERT K. SCOPES*

Agricultural Research Council, Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

(Received 20 November 1972)

A mixture of purified muscle glycolytic enzymes was reconstituted and the mixture shown to behave in a fashion analogous to that occurring *in vivo*. Glycolysis leads to ATP production in muscle and results in the phosphorylation of creatine. The extent of this phosphorylation by anaerobic glycolysis was shown to depend to a small extent on the relative proportions of available P_i and creatine initially, but more importantly on the first step in glycolysis, in this case the enzyme phosphorylase. With less than 0.1 % of the phosphorylase in the *a* form, only about one-third of the creatine was phosphorylated in 30 min, whereas with 4 % or more of phosphorylase *a*, 90 % of the creatine was phosphorylated within this time. Inclusion of an adenosine triphosphatase decreased the steady-state concentration of phosphocreatine in the system. Calculations of the theoretical concentrations of ADP and AMP showed that phosphorylase *b* was almost inactive even in the presence of $9\ \mu\text{M}$ -AMP, because of ATP inhibition. With phosphorylase *a* present, glycolysis was able to continue at least until the calculated concentration of MgADP^- was only $7\ \mu\text{M}$, and AMP in the sub- μM olar range. The relation of these values to measured concentrations of nucleotides and to phosphorylase *a* percentages in intact muscle is discussed.

Fast-acting skeletal muscles have a high capacity for anaerobic glycolysis, enabling ATP to be synthesized for a short period at many times the rate via oxidative phosphorylation. The substrate for these rapid bursts of glycolysis is mainly glycogen and the products are lactate and ATP. The splitting of ATP that occurs simultaneously, which indeed is the stimulus for the glycolytic burst, is the main source of the acid that is produced in parallel with lactate. The ATP/ADP ratio is buffered against sudden changes by the enzyme creatine kinase, with phosphocreatine providing the initial source for ADP rephosphorylation, before glycolysis is activated. During recovery from activity and at rest depleted supplies of phosphocreatine in the muscle are restored by further glycolysis and oxidative phosphorylation until a steady state is reached when a substantial proportion of the creatine in the muscle is phosphorylated. In frogs this proportion may be as high as 90 % (Maréchal & Beckers-Bleukx, 1966; Gilbert *et al.*, 1971), though values for mammals are lower (see, e.g., Bendall, 1960; Hohorst *et al.*, 1962). With large animals it is virtually impossible to obtain any sample of muscle that has not been stimulated by the sampling technique resulting in a loss of phosphocreatine before extraction. The highest value observed

in this laboratory, in both biopsy and immediate post-mortem samples, was 65 % of creatine phosphorylated in a pig that had been completely immobilized by injection with MgSO_4 (Lister *et al.*, 1969). This is the same value as that reported by Hohorst *et al.* (1962) for rats. Nevertheless, there was sufficient phosphate present, in the form of P_i and hexose monophosphates, to have allowed theoretical values of 90 % creatine phosphorylation to be reached. It is of interest to know whether such a high value of creatine phosphorylation could ever exist in mammals in the resting state (at 37°C) as it does in frogs (at $0\text{--}3^\circ\text{C}$).

As an extension of previous work on muscle glycolysis (Newbold & Lee, 1965; Newbold & Scopes, 1967, 1971*a,b*; Scopes & Newbold, 1968), simplified systems consisting of dialysed muscle extracts were studied; these extracts could be cleared of all ATPase† by centrifugation. As a result, the effect of adding back different quantities and types of ATPase could be studied. Analysis of the progress of glycolysis was carried out enzymically by adapting the analyses to automation and eventually by using fluorimetry (Scopes, 1972*a*). Since nearly all the glycolytic enzymes were required for these analyses and were mostly already produced in the laboratory it was decided to take the final step in simplification, and

* Present address: Department of Biochemistry, La Trobe University, Bundoora, Vic. 3083, Australia.

† Abbreviation: ATPase, adenosine triphosphatase.

reconstitute the glycolytic sequence by putting together the purified enzymes in the relevant proportions. This necessitated developing one or two new procedures for isolating certain enzymes from muscle (e.g. phosphoglycerate kinase, EC 2.7.2.3; Scopes, 1969), to avoid having to use yeast enzymes. All the major enzymes used were crystalline or crystallizable preparations with specific activities close to the maximum activity reported for each enzyme. The final experimental mixture had a total protein concentration of 40 mg/ml, about the same amount as the enzymes' concentration in muscle (in mg/g), and several orders of magnitude greater than the concentrations at which the individual enzymes are usually studied.

The present paper describes the reconstituted glycolytic system and metabolism during the development of the 'resting state' in which creatine is phosphorylated. The final percentage of creatine phosphorylation, which through the equilibrium of the creatine kinase reaction is related to the ATP/ADP ratio, has been determined in a variety of circumstances.

Materials and Methods

Glycolytic intermediates, nucleotides and co-factors were purchased from Boehringer und Soehne, Mannheim, Germany, and from Sigma Chemical Co., St. Louis, Mo., U.S.A. Some preparations were also supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Oyster glycogen was purchased from Sigma Chemical Co.; carnosine, serum albumin (Cohn fraction V), mercaptoethanol and dithiothreitol were from Koch-Light. Other chemicals were A.R. grade.

Rabbit and pig muscle were obtained and extracted as described previously (Scopes, 1969). Some enzymes were prepared from ox and horse muscle by using the same extraction procedure with muscle up to 24 h *post mortem*. 'Active Dried Yeast' was obtained from Distillers Co. (Yeast) Ltd., Morden, Surrey, U.K. Column chromatography was carried out in polymethyl methacrylate columns supplied by Wright Scientific Co. Ltd., Kenley, Croydon, Surrey, U.K., with microgranular cellulose ion-exchangers (Whatman CM 52 and DE 52), or Sephadex gels for gel filtration. All Sephadex columns were run in 0.1 M-NaCl-20 mM-Tris-0.2 mM-EDTA, pH 7.8, unless otherwise indicated.

Enzyme activities were measured spectrophotometrically, by using the necessary coupling enzymes to form or oxidize NADH or NADPH. In most cases the mixtures contained 30 mM buffer (β -glycerophosphate, imidazole or Tris), pH 6.5-8.5, depending on the optimum pH of the enzyme concerned, 50 mM-KCl, 3 mM-MgSO₄, 0.1 mM-EDTA and 0.2 mg of serum albumin/ml. Of particular importance was

the measurement of phosphorylase activity. Phosphorylase *a* was measured in a solution containing 20 mM-potassium phosphate, pH 7.2, 2 mM-MgSO₄, 1 mg of glycogen/ml, 10 μ M-glucose 1,6-diphosphate, 0.1 mM-NADP, and 1 unit each of phosphoglucomutase and glucose 6-phosphate dehydrogenase/ml. Phosphorylase *a+b* was measured in the same solution containing 0.5 mM-AMP. All activities were measured at or multiplied by a suitable factor to convert them into the activities at 30°C and expressed as μ mol of substrate utilized per min. Specific activities are expressed as μ mol of substrate transformed/min per mg of protein at 30°C.

Protein was determined at 205 nm by using an extinction coefficient of $E_{205}^{1\%} = 32$ (Goldfarb *et al.*, 1951), at 280 nm if the extinction coefficient was known, or by the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Enzymes were prepared from muscle extracts from rabbit or pig. The specific activities of the purified enzymes for the particular direction of reaction are given, and the methods of purification were as follows. Phosphorylase *b* (EC 2.4.1.1; sp. activity 25, glycogen $\rightarrow$ glucose 1-phosphate) was purified by the method of Fischer & Krebs (1958) with the modifications of DeLange *et al.* (1968). Phosphorylase *a* (EC 2.4.1.1; sp. activity 25, glycogen $\rightarrow$ glucose 1-phosphate) was prepared from phosphorylase *b* by using phosphorylase *b* kinase (DeLange *et al.*, 1968). Amylo-1,6-glucosidase (EC 3.2.1.33; sp. activity 1-2, phosphorylase limit dextrin $\rightarrow$ glucose) Brown & Brown (1966); this preparation also contained the necessary oligoglucan 1,4-1,4-transferase (EC 2.4.1.24), which is reported to be part of the same enzyme protein (Nelson *et al.*, 1968). Phosphofructokinase (EC 2.7.1.11; sp. activity 150-170, fructose 6-phosphate $\rightarrow$ fructose 1,6-diphosphate) Ling *et al.* (1965). Aldolase (EC 4.1.2.7; sp. activity 14-20, fructose 1,6-diphosphate $\rightarrow$ triose phosphate), Czok & Bücher (1960); also see below. Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8; sp. activity 250-300, dihydroxyacetone phosphate $\rightarrow$ glycerol 3-phosphate) Beisenherz *et al.* (1955). Triose phosphate isomerase (EC 5.3.1.1; sp. activity 6000, glyceraldehyde 3-phosphate $\rightarrow$ dihydroxyacetone phosphate) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12; sp. activity 80-150, glyceraldehyde 3-phosphate $\rightarrow$ 1,3-diphosphoglycerate) were purified as by-products of the preparation of phosphoglycerate kinase. Phosphoglycerate kinase (EC 2.7.2.3; sp. activity 800; 3-phosphoglycerate $\rightarrow$ 1,3-diphosphoglycerate) Scopes (1969). Enolase (EC 4.2.1.11; sp. activity 80, 2-phosphoglycerate $\rightarrow$ phosphoenolpyruvate) Czok & Bücher (1960). Pyruvate kinase (EC 2.7.1.40; sp. activity 450, phosphoenolpyruvate $\rightarrow$ pyruvate), was purified by the method of Tietz & Oychoa (1958), followed by CM-cellulose chromatography (Bondar & Pon, 1969); also see below.

The following enzymes were prepared from pig muscle by the methods below: phosphoglucose isomerase (EC 5.3.1.9; sp. activity 1100, fructose 6-phosphate $\rightarrow$ glucose 6-phosphate); phosphoglycerate mutase (EC 2.7.5.3; sp. activity 1400, 3-phosphoglycerate $\rightarrow$ 2-phosphoglycerate); phosphoglucosomutase (EC 2.7.5.1; sp. activity 800, glucose 1-phosphate $\rightarrow$ glucose 6-phosphate); lactate dehydrogenase (EC 1.1.1.27; sp. activity 600, pyruvate $\rightarrow$ lactate); creatine kinase (EC 2.7.3.2; sp. activity 100, creatine $\rightarrow$ phosphocreatine); AMP kinase (EC 2.7.4.3; sp. activity 2200, ATP + AMP $\rightarrow$ 2ADP).

Pig muscle extract (2–3 litres) was fractionated with acetone at pH 6.5, -2°C , by collecting precipitates at 0–35%, 35–45% and 45–55% (v/v) and by dissolving them in ice-cold water. The 0–35% fraction was used for lactate dehydrogenase preparation. Excess of acetone was removed by boiling under reduced pressure by the following procedure. The solution was transferred to a flask which could be evacuated at a filter-pump. The flask was immersed in water at 20°C and as the pressure was lowered boiling commenced. The water was slowly warmed to 35°C and evacuation continued for a further 5–10 min by which time the solution had warmed to about 30°C and most of the acetone had been removed. The solution was now ready for $(\text{NH}_4)_2\text{SO}_4$ fractionation, which was carried out at 20°C , pH 6.0. A 35–55% saturation fraction was taken and heated at pH 5.5 for 10 min at 50°C . After cooling the denatured protein was removed and the solution was made turbid with saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Lactate dehydrogenase crystallized from this solution, and was re-crystallized several times before use.

The second (35–45%) acetone fraction had excess of acetone removed as described above. It could be used for preparation of phosphoglycerate mutase as follows: 3-phosphoglycerate was added (5 mM) and the pH adjusted to 6.0. The temperature was increased to 65°C for 5 min; the fraction was cooled and denatured protein was removed, after which the pH was adjusted to 8.0 with Tris and the fraction was passed through a column ($8\text{cm}^2 \times 4\text{cm}$) of DEAE-cellulose equilibrated with Tris-HCl buffer, pH 8.0, $1/0.01$. The unadsorbed protein was then concentrated by ultrafiltration and remaining impurities were separated on a column ($8\text{cm}^2 \times 90\text{cm}$) of Sephadex G-100.

Other enzymes from the second acetone fraction were obtained as follows. An $(\text{NH}_4)_2\text{SO}_4$ fractionation was carried out at pH 6.0, 20°C . The protein precipitating between 52 and 64% saturation was the most useful, although enzymes (enolase, glyceraldehyde phosphate dehydrogenase) could be isolated in high yield from other fractions.

The 52–64% saturation fraction was extensively dialysed, then applied to a column ($8\text{cm}^2 \times 15\text{cm}$) of CM-cellulose in the cold. The buffer used consisted

of 10 mM-Tris–0.2 mM-EDTA, adjusted to pH 6.5 with cacodylic acid. Creatine kinase was not adsorbed, but on increasing the pH to 8.2 with Tris after passage through the column, it could be adsorbed on DEAE-cellulose (see below). The CM-cellulose column was washed with buffer, pH 6.5, and a linear gradient of 0–0.1 M-KCl in the same buffer was commenced. Two small peaks containing minor components of phosphoglucose isomerase were usually obtained just before the main sharp peak, which contained the major component of this enzyme. This peak was collected, and salted out at 70% saturation of $(\text{NH}_4)_2\text{SO}_4$. This preparation was essentially pure, and crystallized as large truncated prisms (Campbell *et al.*, 1971). Continuation of the KCl gradient produced further peaks from the CM-cellulose, the main ones consisting of pyruvate kinase and aldolase, which were salted out [at 65% and 55% saturation of $(\text{NH}_4)_2\text{SO}_4$ respectively] and crystallized without any further treatment.

For making large amounts of creatine kinase, the following method gave high recovery. The fractions for the original acetone fractionation were different; a 40–48% (v/v) precipitate was collected, dissolved in and dialysed extensively against a buffer consisting of 25 mM-Tris–0.2 mM-EDTA, pH 8.2 (at 25°C) adjusted with HCl, then centrifuged to remove a small precipitate. The fraction was applied to a column of DEAE-cellulose (typically $8\text{cm}^2 \times 30\text{cm}$ for a preparation from 500 g of muscle) in the Tris buffer and after washing the column creatine kinase was eluted by a 0–0.05 M-KCl gradient. The main part of the enzyme peak was concentrated by precipitation with acetone (1 vol.) at 0°C , dissolved in a little 0.1 M-NaCl and applied to a column ($8\text{cm}^2 \times 90\text{cm}$) of Sephadex G-100. The first two-thirds of enzyme activity eluted was collected as the final preparation, which could be crystallized if required.

Phosphoglucosomutase and AMP kinase (myokinase) were made from the 45–55% acetone fraction. For preparation of phosphoglucosomutase, the excess of acetone was boiled off as described above and an $(\text{NH}_4)_2\text{SO}_4$ fraction (0–65% saturation at pH 5.5) was taken and dissolved in a little 50 mM-sodium acetate buffer, pH 5.5. It was then heated at 55°C for 5 min, cooled, and the denatured protein was removed. The solution was made turbid with solid $(\text{NH}_4)_2\text{SO}_4$ and the enzyme crystallized into needles. The preparation was recrystallized with $(\text{NH}_4)_2\text{SO}_4$ in buffer, pH 5.5.

AMP kinase was prepared from the 45–55% acetone fraction by first boiling off excess of acetone as described above. Then $(\text{NH}_4)_2\text{SO}_4$ was added to 25% saturation and the solution was cooled to 5°C . The pH was then adjusted to 2.5 with 2 M- H_2SO_4 , the solution was stirred for 5 min and then the pH was adjusted to 6.0 with 4 M- NH_3 . The slurry so obtained

was warmed at 40°C for 15 min, then centrifuged to remove denatured protein. AMP kinase was collected from the supernatant by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 75% saturation; the precipitate was dissolved in a small amount of 20 mM-sodium succinate buffer, pH 6.0, and was passed through a column (8 cm² × 90 cm) of Sephadex G-75 equilibrated with the succinate buffer. The AMP kinase peak was further purified on a column (4 cm² × 2 cm) of CM-cellulose in the succinate buffer and was eluted by applying a gradient of 20–50 mM buffer. The enzyme from pig muscle was crystallized as illustrated elsewhere (Schirmer *et al.*, 1970).

For the yeast enzymes, the granulated yeast was cytolysed with aq. NH_3 (Scopes, 1971). Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; sp. activity 300–500, glucose 6-phosphate → 6-phosphogluconate) was prepared by the method outlined previously (Newbold & Scopes, 1967), with the following final step: after DEAE-cellulose chromatography, the enzyme fraction was adjusted to pH 5.5 with acetic acid, and adsorbed on to a column (4 cm² × 4 cm) of phosphocellulose (Sigma) equilibrated in sodium acetate buffer, pH 5.5, *I* 0.2. The column was washed with buffer of *I* 0.4, then a gradient of buffer *I* 0.4–1.0 was commenced. The enzyme was eluted at about *I* 0.6 and was concentrated by ultrafiltration and stabilized for storage by adding $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation. Hexokinase (EC 2.7.1.1; sp. activity 400, glucose → glucose 6-phosphate) was prepared by a procedure making use of the $(\text{NH}_4)_2\text{SO}_4$ fractionation method of Darrow & Colowick (1962), followed by dialysis and acetone fractionation at pH 7.0 [38–52% (v/v) acetone, –2°C], adsorption on to a column (4 cm² × 10 cm) of DEAE-cellulose in 20 mM-Tris, pH 7.8, containing 50 mM-KCl, and elution with a 0.05–0.3 M-KCl gradient. The enzyme from the column could be crystallized for further purification. Alcohol dehydrogenase (EC 1.1.1.1; sp. activity 400, ethanol → acetaldehyde) was better prepared from a cytolysate of fresh yeast rather than from the dried variety; the fractionation procedure was essentially that described by Racker (1955). Phosphoglycerate kinase (EC 2.7.2.3; sp. activity 850, 3-phosphoglycerate → 1,3-diphosphoglycerate) was also made from yeast (Scopes, 1971). The yeast enzymes were used for analytical purposes only.

The ATPase (EC 3.6.1.5) used in these experiments was an 'apyrase' from potatoes. The following procedure was used: red-skinned potatoes (4 kg) were homogenized with a little water, the homogenate was strained through butter muslin and then centrifuged. The liquid was fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described by Traverso-Cori *et al.* (1965). After two fractionations with $(\text{NH}_4)_2\text{SO}_4$, further purification was obtained by a combination of gel filtration and CM-cellulose chromatography, as outlined by Traverso-Cori *et al.* (1970).

The second $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in 20 mM-sodium succinate buffer, pH 6.0, and passed through a column (8 cm² × 90 cm) of Sephadex G-100 with a gel buffer consisting of the succinate buffer containing 0.5 M-KCl. The eluted enzyme was then concentrated 10-fold by ultrafiltration, diluted 10-fold with succinate buffer, and applied to a column (4 cm² × 4 cm) of CM-cellulose equilibrated in succinate buffer containing 50 mM-KCl. The ATPase with highest ATPase/ADPase ratio was adsorbed, and was eluted with a gradient at about 0.15 M-KCl. This preparation had a specific activity of 1000 with Mg^{2+} as the activating cation. The ADPase specific activity was 80 μmol/min per mg of protein, i.e. the same fraction as reported before for the high-ATPase/ADPase-ratio enzyme (Traverso-Cori *et al.*, 1970).

Enzymes were mostly stored as crystalline suspensions in a suitable concentration of $(\text{NH}_4)_2\text{SO}_4$ at +1°C, but phosphorylase, phosphofructokinase and creatine kinase were all stored frozen at low ionic strength, pH 8.0, in the presence of 1 mM-dithiothreitol. The reconstituted glycolytic mixture, after exhaustive dialysis against 50 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA was concentrated by ultrafiltration to 100 mg of protein/ml, then divided into suitable batches and stored frozen in the presence of 1 mM-dithiothreitol. The temperature of the freezer was close to –25°C, and the mixture gave reproducible results even after a year's storage in these conditions. The enzymes phosphorylase and phosphofructokinase were not normally included in the mixture, as different quantities and qualities of these enzymes were used in the final mixes.

The enzyme mixture was thawed and creatine was added to give a suitable concentration, and phosphorylase and phosphofructokinase were added. To start glycolysis, a mixture containing glycogen, ATP, NAD^+ , carnosine, glucose 1,6-diphosphate, 2,3-diphosphoglycerate and magnesium acetate was added. A typical experiment involved adding 1 ml of enzyme+creatine mixture to 1 ml of additional phosphate, creatine, phosphorylase or any other variable being studied, and starting the reaction by adding 0.5 ml of the buffered glycogen-ATP- NAD^+ mixture. The initial pH was about 7.8, and it decreased rapidly owing to the phosphorylation of creatine and parallel stimulation of glycolysis. In the experiments described in the present paper, the pH was allowed to decrease to 7.2 or 7.4 and was then kept at that value by using a pH-stat (Radiometer TTT1c Titrator, with combined electrode GK2021C). The base used to maintain the pH was 0.5 M-Tris.

Glycolysing samples were taken and pipetted into ice-cold HClO_4 . Samples (0.2–0.25 ml) were taken from the mixture at each time, and 6 ml of HClO_4 was used. For following the progress of

reactions in short time-intervals, individual 0.25 ml glycolysing mixtures were prepared and stopped after the desired time by adding 6 ml of cold HClO_4 . Subsequent treatment and analytical methods are described elsewhere (Scopes, 1972a,b).

As in previous papers (Newbold & Scopes, 1967, 1971a,b; Scopes & Newbold, 1968) the glycolytic intermediates are grouped. Thus, 'hexose mono-phosphates' is the sum of glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate, 'fructose diphosphate' is the sum of fructose 1,6-diphosphate and half of the triose phosphates, 'phosphoglycerates' is the sum of 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate, plus 1,3-diphosphoglycerate, which is presumed to have hydrolysed to 3-phosphoglycerate before analysis.

Results

Description of the system

The enzymes were mixed together in proportions that are believed to be similar to the proportions in which they exist naturally in skeletal muscle. The values were taken from those given by Czok & Bücher (1960), Scopes (1970), and other observations of various authors concerning degrees of purification from crude extracts. Small alterations would be unlikely to affect the results significantly, except for the amounts of the controlling enzymes phosphorylase *a* and *b*, and phosphofructokinase. The activities of these were carefully checked before each experiment. The enzyme composition of the mixture is given in Table 1.

The necessary low-molecular-weight compounds include cofactors, buffers and inorganic ions. P_i and K^+ ions were present in the enzyme mixture, together with a little EDTA to complex traces of undesirable heavy metals in the reagents, and dithiothreitol to protect thiol groups further. Creatine was dissolved into the concentrated enzyme mixture just before each experiment. The cofactors ATP, NAD^+ , glucose 1,6-diphosphate and 2,3-diphosphoglycerate were added together with the substrate glycogen, buffers and Mg^{2+} ions. Magnesium acetate was included at the same concentration as ATP. In the present experiments carnosine and Tris were used as additional buffers to the phosphate. Table 2 lists the initial concentrations of low-molecular-weight compounds and glycogen in the mixture at zero time.

The enzymes, additional reagents and the glycogen-cofactor-buffer mixture were preincubated in the water bath before mixing at the start of the experiment. To determine the behaviour during the first minute, experiments were carried out with small quantities (100 μl of enzyme mixture + 100 μl of the extra reagents, started with 50 μl of glycogen-cofactor-buffer), the reactions being stopped after

Table 1. *Enzyme composition of the reconstituted glycolytic system*

Concentrations refer to the values after mixing with all other reagents. Both phosphorylase and phosphofructokinase were added on an activity basis, this activity being checked immediately before each experiment.

Enzyme	Amount (mg/ml)
Phosphorylase (<i>a+b</i>)	2.0 (50 units/ml)
Amylo-1,6-glucosidase	0.1
Phosphoglucumutase	0.6
Phosphoglucose isomerase	0.8
Phosphofructokinase	0.35 (50 units/ml)
Aldolase	6.5
Triose phosphate isomerase	2.0
Glycerol 3-phosphate dehydrogenase	0.3
Glyceraldehyde phosphate dehydrogenase	11.0
Phosphoglycerate kinase	1.4
Phosphoglycerate mutase	0.8
Enolase	2.4
Pyruvate kinase	3.2
Lactate dehydrogenase	3.2
Creatine kinase	5.0
AMP kinase	0.4
Total protein	40

Table 2. *Composition of the glycolysing mixture, excluding enzymes*

At zero time the pH was 7.80 ± 0.05 .

Compound	Concentration (mM)
Glycogen (glucose equivalents)	40
ATP	5
NAD^+	0.5
Glucose 1,6-diphosphate	0.05
2,3-Diphosphoglycerate	0.1
Carnosine	25
Creatine	30*
P_i	30*
K^+	approx. 60
Na^+	approx. 20
Mg^{2+}	5
Acetate	10
EDTA	0.1
Dithiothreitol	0.4
Tris	approx. 15

* Unless otherwise indicated in the text.

predetermined intervals. Thus each point on the time-scale of these experiments represents a separate incubation, though the starting mixtures were identical.

Metabolism during the establishment of the 'resting state'

The results of these experiments are summarized in Figs. 1–3. The points subsequent to 1 min are from samples taken from a bulk mixture which was maintained at pH 7.40.

Fig. 1 gives results from an experiment in which all (i.e. about 99.8%, see below) of the phosphorylase was in the *b* form. The progress of the glycolytic reactions can clearly be seen. By the time of the first sample (5s), creatine had been phosphorylated to the extent of nearly 3mM, with a parallel decrease in [ATP], and increases in both [ADP] and [AMP]. Almost no ATP had yet been resynthesized by the later reactions in the glycolytic sequence; [lactate] was still zero and only a trace of phosphoglycerate had been formed. Moreover, the sum of [ADP] + 2[AMP] at this stage was 3.5mM, compared with 3.4mM for the sum of the concentration of the ATP-consuming compounds, phosphocreatine and fructose diphosphate. The high [AMP] had already stimulated phosphorylase *b*, and caused a peak in the concentration of hexose phosphate (Fig. 1c). During the first 10s, before much pyruvate had been produced, NADH formed by glyceraldehyde phosphate dehydrogenase must have been re-oxidized by dihydroxyacetone phosphate, causing the increase in glycerol 3-phosphate concentration shown in Fig. 1(d). At the same time the intermediates from 1,3-diphosphoglycerate to pyruvate must have accumulated to the same extent; this is also demonstrated in Fig. 1(d).

Between 5 and 10s there was a rapid production of lactate and regeneration of the lost ATP. With the resultant decrease in [AMP] (to values at or below the limits of detection, 10 μ M), phosphorylase and phosphofructokinase activities decreased to very low values, as can be seen from the decrease in hexose phosphate concentrations. This is more clearly illustrated in Fig. 3. Phosphofructokinase was probably not inhibited allosterically at this stage; the limitation on its activity was simply the lack of available fructose 6-phosphate. In the meantime phosphocreatine had been steadily accumulating (Fig. 1b), but after 20s the lack of glycolytic activity slowed the creatine phosphorylation rate to a low value. This persisted slightly even up to 60min (not illustrated), by which time the phosphocreatine concentration was 15mM, i.e. 50% phosphorylated, and there was still 12.5mM- P_i left.

The same mixtures were used except that the phosphorylase was 40% in the *a* form, and a very different picture was obtained (Fig. 2). Because AMP

was not required for phosphorylase activity, hexose monophosphate concentrations increased much faster, and with the greater availability of substrate, phosphofructokinase also acted rapidly, producing an accumulation of fructose diphosphate. This did not decrease until 40s, by which time the pH was 7.4 and phosphofructokinase was subject to inhibition by ATP. Phosphocreatine and lactate accumulated steadily (Fig. 2a), except for a pause between 40s and 1½ min owing to the inhibition of phosphofructokinase. When this inhibition was released (see below), there was another burst of lactate and phosphocreatine formation. The sudden decrease in phosphorylase activity (Fig. 3a) after 20s must have been caused by lack of glycogen in a suitable form, since the P_i concentration was still over 10mM; the amount of amylo-1,6-glucosidase present was probably too low to hydrolyse the limit dextrin fast enough. Later (after 5min), the P_i was limiting, as it decreased almost to zero. The highest concentration of phosphocreatine represented 87% phosphorylation, and it was the lack of P_i that was limiting in this case (the 4mM of phosphate that did not end up in phosphocreatine was trapped in the form of glycerol 3-phosphate and hexose phosphates at 10min).

Fig. 3 represents the accumulated activities of phosphorylase and phosphofructokinase for these two experiments. Phosphorylase activity was calculated as: $\frac{1}{2}$ (lactate + pyruvate + phosphoglycerates + glycerol 3-phosphate) + hexose phosphates. Phosphofructokinase activity was calculated as phosphorylase activity minus hexose monophosphates. Fig. 3 demonstrates that in the presence of no more than 0.2% of phosphorylase *a*, inhibition of both enzymes was almost complete after 10s; the small throughput afterwards was probably due to the small amount of phosphorylase *a* present. In the presence of 40% phosphorylase *a*, phosphorylase activity was rapid for 20s, then decreased, but did not stop until 3–4min. The decrease should not theoretically have occurred until P_i was limiting, but as indicated above, may have been caused by lack of correct glycogen structure. Phosphofructokinase activity decreased similarly and in fact stopped after 20s, because the low [fructose 6-phosphate] allowed ATP inhibition. This resulted in a slow increase in the concentration of hexose monophosphates, leading to release of the ATP inhibition at about 1½ min.

Confirming that there were no unexpected side-reactions or loss of transferable phosphoryl groups, the final concentrations of phosphocreatine were in each experiment 1.5 ± 0.1 times the concentration of lactate.

Effect of temperature

The rates of all the reactions depend on temperature. A set of glycolysing mixtures at various

Fig. 1. Metabolite concentrations during development of the steady-state (Experiment 29) in which phosphorylase *a* is 0.2% of total phosphorylase

Initial [creatine] = initial [phosphate] = 30mM. (a) $\circ$, [ATP]; Δ , [ADP]; $\square$, [AMP]. AMP was undetectable after 20s. (b) $\bullet$, [Phosphocreatine]; $\blacktriangle$, [lactate]. The values for [phosphocreatine] in Figs. 1, 2 and 4 are taken as the mean of measured [phosphocreatine] and initial [creatine] minus measured [creatine]. (c) ∇ , [Hexose monophosphates]; $\triangledown$, [fructose diphosphate]. Fructose diphosphate was undetectable after 20s. (d) $\bullet$ [Glycerol 3-phosphate]; $\circ$ sum of [phosphoglycerates] plus [pyruvate]. For the first 10s, nearly all the latter was phosphoglycerates, but after 20s, all was pyruvate.

Fig. 2. Metabolite concentrations during development of the steady-state (Experiment 29) in which phosphorylase *a* is 40% of total phosphorylase

Initial [creatine] = initial [phosphate] = 30 mM. (a) ●, [Phosphocreatine]; ▲, [lactate]; ■, [P_i]. (b) ▼, [Hexose monophosphates]; ▽, [fructose diphosphate].

temperatures was studied; phosphorylase was 20% in the *a* form. The maximum rates of lactate production are shown in Table 3. At the higher temperatures, the rate factor for lactate production (i.e. the rate compared with the rate at 10°C lower) was 2.4, but at the lower temperatures the rate-factor values were much higher. From an investigation of the detailed glycolytic behaviour, it was clear that the discontinuity in the temperature behaviour was due to phosphorylase. Values for the maximum phosphorylase activity during the initial burst when there was sufficient AMP to activate the *b* form (confirmed by measurement) (cf. Fig. 1*a*) are shown in Table 3. This activity was extremely low at 0°C, but increased sharply as the temperature increased to 20°C. At the higher temperatures the apparent rate factor was about 1.9, a typical value for an enzyme (the actual value for phosphorylase for the direction of glycogenolysis has not been reported). The behaviour at low temperatures implies either an abnormally high

Fig. 3. Accumulative enzyme activities (from Expt. 29, Figs. 1 and 2)

(a) Accumulative phosphorylase activity; ○, 0.2% phosphorylase in *a* form; ●, 40% phosphorylase in *a* form. (b) Accumulative phosphofructokinase activity; ○, 0.2% phosphorylase in *a* form; ●, 40% phosphorylase in *a* form.

activation energy, or more probably partial inactivation due to aggregation (Graves *et al.*, 1965).

Extent of creatine phosphorylation

A number of experiments were carried out to determine the extent of creatine phosphorylation. Both the total amount of creatine and, most important, the amount of P_i available initially were varied. It became clear that one of the major factors in determining the amount of creatine phosphorylated was the amount of phosphorylase *a* activity. In earlier experiments, a phosphorylase *b* preparation was used which had an *a* content of about 0.2%; this small quantity was sufficient to affect the results significantly. At high phosphorylase *a* activities, within a short

Table 3. *Effect of temperature on maximum glycolytic rates*

Maximum rates of lactate production were determined from a plot of [lactate] against time. Similarly the maximum throughput of phosphorylase was calculated graphically; this was always in the early stages where [AMP] was in the range 0.4–0.7 mM, sufficient to stimulate phosphorylase *b* fully. Rate factors are the rates compared with rate at 10°C lower. Rates are expressed as $\mu\text{mol}/\text{min}$ per ml.

Temp. (°C)	Rates ($\mu\text{mol}/\text{min}$ per ml)		Rate factors	
	Lactate	Phosphorylase	Lactate	Phosphorylase
0	0.24	0.12	—	—
10	2.4	1.8	10	15
20	10	11	8.5	6
30	23	21	2.3	1.9
40	59	37	2.5	1.8

Table 4. *Amount of creatine phosphorylated after 30 min*

The pH was kept at 7.40 (7.30 in Experiment 45) by means of a pH-stat. In Experiments 27–29, the phosphorylase *a* content of the phosphorylase *b* used was not checked directly, but was probably close to 0.2%. In experiment 45, it was not detectable at the 0.1% level. The values given for phosphocreatine are the averages of the measured phosphocreatine and of the initial creatine minus the measured creatine.

Experiment no. and temp. (°C)	Phosphorylase <i>a</i> (% of total phosphorylase)	Initial concentrations (mM)		Concentration after 30 min (mM)	
		P _i	Creatine	Phosphocreatine	P _i
27 (25°C)	0.2	20	30	11.7	8.2
		30	30	12.9	16.5
		40	30	17.1	23.0
		20	40	11.2	7.4
		30	40	16.3	12.8
		40	40	21.2	19.3
28 (25°C)	0.2	30	30	14.4	13.1
		30	30	25.9	1.7
		30	30	26.4	0.5
29 (30°C)	0.2	30	30	16.5	15.3
	40	30	30	25.9	approx. 0.1
45 (37°C)	<0.1	35	30	10.0	23.6
	4	35	30	27.0	2.8

time, the phosphocreatine concentration reached values up to 90% of that of the total creatine; moreover, in most of these cases it was the low availability of P_i which was the limiting factor. In later experiments, a phosphorylase *b* preparation containing no detectable (less than 0.1%) *a* form was used; this resulted in the least amount of phosphocreatine after 30 min and is described in greater detail below. The various results obtained in these experiments, showing the amount of creatine phosphorylated after 30 min, are given in Table 4.

Effect of small amounts of phosphorylase a and of an ATPase on creatine phosphorylation

As indicated in the last section, the phosphorylation of creatine is greatly dependent on the phosphorylase *a* content of the mixture. Experiment 45, in which the phosphorylase *a* content of the phosphorylase *b* preparation was less than 0.1%, is now described in greater detail. The mixture, which was maintained at pH 7.30 (pH-stat) and 37°C, was allowed to react for 40 min, then some pure phosphorylase *a* was added, amounting to 0.4% of the

Fig. 4. Progressive changes in concentrations of compounds with addition of small quantities of phosphorylase *a* (Expt. 45)

Initially phosphorylase *a* activity was 0.1% of the total phosphorylase activity. Indicated by solid arrows, at 40 min, phosphorylase *a* was added to 0.4%; at 80 min, more was added, to 1.0%; at 100 min, more was added, to 4%. The broken arrow indicates that at 110 min, ATPase (5 units/ml) was added. Initial [creatine] = 30 mM; initial [phosphate] = 35 mM. (a) ■, $[P_i]$; ●, [phosphocreatine]. (b) ▼, [Hexose monophosphates].

total (i.e. 0.2 unit/ml at 30°C, or about 0.3 unit/ml at the temperature of the experiment). This immediately speeded up the rate of phosphorylation of creatine (Fig. 4a). After another 40 min, more phosphorylase *a* was added, making the total to 1%; this gave a further slight stimulus, especially to the hexose monophosphate concentration (Fig. 4b). Further phosphorylase *a* (to 4%) was added 20 min later, which quickly resulted in phosphorylation of most of the remaining creatine. Hexose monophosphate concentrations (Fig. 4b), which were negligible in the absence of phosphorylase *a*, increased during the

Table 5. Effect of adding ATPase on the steady-state concentration of phosphocreatine

The mixture was at pH 7.20 (pH-stat) and 30°C. Initial $[P_i]$ = initial [creatine] = 30 mM. The percentage of phosphorylase *a* was approx. 0.1.

ATPase activity (units/ml)	[Phosphocreatine] at 30 min (mM)
0	13.1
0.5	10.2
1	6.5
2	<4.4*

* At 15 min after addition of extra ATPase to the 1 unit/ml mixture; [phosphocreatine] was still decreasing.

experiment, but finally decreased owing to lack of P_i (Fig. 4a).

At the end of this period, 5 units of potato ATPase/ml was added; the rapid decrease in phosphocreatine could not be stabilized before virtually all had disappeared. This experiment is analogous to the situation in contracting muscle, in which the ATP is initially regenerated from phosphocreatine.

Another experiment with ATPase present is shown in Table 5; the phosphorylase *a* content this time was about 0.1%. With no ATPase, the phosphocreatine after 30 min had reached a steady value of 13.1 mM; at this point 0.5 unit of ATPase/ml was added. The phosphocreatine concentration then decreased slightly to a value just over 10 mM after another 30 min, by which time it was constant again. Other mixtures, to which 1 unit of ATPase/ml was added either after the initial phosphocreatine accumulation, or at zero time, settled out to a steady phosphocreatine concentration of 6.2–6.8 mM. Addition of still more ATPase resulted in a greater loss. Thus, at least in the virtual absence of phosphorylase *a*, the system cannot maintain such high phosphocreatine concentrations in the presence of an ATPase.

Discussion

The present experiments were designed to investigate the behaviour of a reconstituted glycolytic system in fully controlled conditions. The results reported in the present paper describe the development of a 'resting-state' metabolite situation comparable with that existing in resting muscle, from the unphysiological starting conditions. However, it must be borne in mind that resting muscle is aerobic, and so has additional potential for ATP synthesis through oxidative phosphorylation, as well as the ability to utilize glucose and fatty acids as substrates.

The results show that anaerobic glycolysis from glycogen is fully capable of establishing high con-

centrations of phosphocreatine, implying high ATP/ADP ratios. In the presence of small amounts of phosphorylase *a*, up to 90% of the creatine was phosphorylated at temperatures from 25°C to 37°C (see Table 4), a higher value than has been reported in mammalian muscles *in vivo*. However, this was in the absence of any ATPase; even the low resting-state turnover due to ATPase-type systems in muscle results in a depletion of phosphocreatine, at least in the absence of any phosphorylase *a* (Table 5). (Approximately 0.5 μ mol of ATP split/min per g is the rate immediately *post mortem* in muscle from immobilized animals.) Thus, although anaerobic glycolysis is potentially capable of leading to phosphorylation of at least 90% of the creatine, the actual steady-state amount is critically dependent on the presence of small amounts of phosphorylase *a* on the one hand, and of ATP-splitting systems on the other. It should also be noted that these experiments were done in the absence of citrate, an anion that further complicates the situation *in vivo* because of its inhibitory effect on phosphofructokinase (Parmeggiani & Bowman, 1963).

The effective concentrations of adenine nucleotides in intact muscle are difficult to estimate, particularly because most of the ADP present in an HClO₄ extract of muscle originates from actin, and so was not freely available in solution. Attempts to use the measured concentrations of ATP, ADP, creatine, phosphocreatine and H⁺ to relate these to the equilibrium constant of creatine kinase lead to nonsensical results because of this effective compartmentalization of at least the ADP. The reconstituted glycolytic system described here eliminates all possibility of compartmentalization and except for small allowances for specific and non-specific binding between enzymes and the nucleotides, it should be possible to compare the measured ADP and AMP with theoretical values derived from known equilibrium constants. (Apart from during the initial burst of activity, it can safely be assumed that the relevant enzymes were capable of maintaining equilibrium between their reactants.) It is necessary to use several constants, and unfortunately not all of these are known with great certainty. They are: the equilibrium constants for both the creatine kinase reaction (0.01 at pH 7.40; Kuby & Noltmann, 1962), the AMP kinase reaction (0.09 for [MgADP⁻]/[ADP³⁻]/[MgATP²⁻]/[AMP²⁻]; R. K. Scopes, unpublished work), and the affinity constants for both MgATP²⁻ and MgADP⁻. In the presence of approximately 80 mM (Na⁺ + K⁺), as in the present experiments, these affinity constants are apparently lowered; values of 40000 M⁻¹ and 2000 M⁻¹ respectively have been used for the following calculations (cf. O'Sullivan & Perrin, 1964). The results of the first experiment (Fig. 1) after 15 min incubation were that the measured ADP concentration was 0.18 mM, and that of AMP was near the limit of detection, 0.01 mM.

Calculated concentrations are: MgADP⁻, 44 μ M; ADP³⁻, 80 μ M; AMP²⁻, 9 μ M. In addition it can be calculated that [HADP²⁻] would be 20 μ M, making the total [ADP] 0.14 mM, in good agreement with the measured value. The comparable values from the 5 min point (Fig. 2) in the experiment with 40% of phosphorylase *a* are: measured [ADP], 70 μ M; [AMP], zero. Calculated [MgADP⁻], 7.2 μ M; [ADP³⁻], 12 μ M; [HADP²⁻], 3 μ M; [AMP²⁻], 0.22 μ M; total [ADP], 22 μ M. Thus the difference between calculated and measured ADP concentration is in both cases about 0.05 mM, which may be the concentration of 'protein-bound' ADP in these experiments. It should also be noted that the concentration of AMP in the 'phosphorylase *b* only' experiment, 9 μ M, would be sufficient to cause a rapid glycogenolysis were it not for the inhibition by ATP (Madsen, 1964; Morgan & Parmeggiani, 1964). These calculations also demonstrate that the effective AMP concentration would increase by a factor of 40 if ATPase activity caused the phosphocreatine concentration to decrease from 26 mM to 15 mM. Because of compartmentalized AMP in whole muscle, this large change is masked in normal estimations.

Estimates of phosphorylase *a* percentages *in vivo* are usually no less than about 5% (see, e.g., Stull & Mayer, 1971), yet if all phosphorylase activity was due to the small fraction of *a* form, the resting rate of metabolism would theoretically be satisfied with only 0.5% of *a* form; the present results demonstrate that such small amounts do indeed have a significant effect. The high activities measured *in vivo* can only be explained by either an unknown inhibitor of phosphorylase *a* restricting its activity, or by steric factors associated with the quasi-soluble state of the enzyme in association with glycogen, or by assuming that methods for arresting conversion of form *b* into form *a* during sampling are not adequate. The substantial effects of as little as 0.2% of the *a* form (Table 4 and Fig. 4) point to the likelihood of a very low effective percentage *in vivo*, at least in muscles containing several μ mol of P_i/g. In the conditions used in the present experiments, pure phosphorylase *b* exhibited less than 0.1% of *a*-type activity.

References

- Beisenherz, G., Bücher, T. & Garbade, K. H. (1955) *Methods Enzymol.* **1**, 391-397
- Bendall, J. R. (1960) *Struct. Funct. Muscle* **3**, 227-274
- Bondar, R. J. L. & Pon, N. G. (1969) *Biochim. Biophys. Acta* **191**, 743-747
- Brown, D. H. & Brown, B. I. (1966) *Methods Enzymol.* **8**, 515-524
- Campbell, J. W., Duée, E., Hodgson, G., Mercer, W. D., Stammers, D. K., Wendell, P. L., Muirhead, H. & Watson, H. C. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 165-170

- Czok, R. & Bücher, T. (1960) *Advan. Protein Chem.* **15**, 315-415
- Darrow, R. A. & Colowick, S. P. (1962) *Methods Enzymol.* **5**, 226-235
- DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. & Krebs, E. G. (1968) *J. Biol. Chem.* **243**, 2200-2208
- Fischer, E. H. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65-71
- Gilbert, C., Kretzschmar, K. M., Wilkie, D. R. & Woledge, R. C. (1971) *J. Physiol. (London)* **218**, 163-193
- Goldfarb, A. R., Saidel, L. J. & Mosovich, E. (1951) *J. Biol. Chem.* **193**, 397-404
- Graves, D. J., Sealock, R. W. & Wang, J. H. (1965) *Biochemistry* **4**, 290-296
- Hohorst, H. J., Reim, M. & Bartels, H. (1962) *Biochem. Biophys. Res. Commun.* **7**, 142-146
- Kuby, S. A. & Noltmann, E. A. (1962) *Enzymes*, 2nd edn., **6**, 515-596
- Ling, K. H., Marcus, F. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1893-1899
- Lister, D., Scopes, R. K. & Bendall, J. R. (1969) *Anim. Prod.* **11**, 288, abstr. 41
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Madsen, N. B. (1964) *Biochem. Biophys. Res. Commun.* **15**, 390-395
- Maréchal, G. & Beckers-Bleukx, G. (1966) *Biochem. Z.* **345**, 286-299
- Morgan, H. E. & Parmeggiani, A. (1964) *J. Biol. Chem.* **239**, 2435-2439
- Nelson, T. E., Kolb, E. & Larner, J. (1968) *Biochim. Biophys. Acta* **167**, 212-215
- Newbold, R. P. & Lee, C. A. (1965) *Biochem. J.* **97**, 1-6
- Newbold, R. P. & Scopes, R. K. (1967) *Biochem. J.* **105**, 127-136
- Newbold, R. P. & Scopes, R. K. (1971a) *J. Food Sci.* **36**, 209-214
- Newbold, R. P. & Scopes, R. K. (1971b) *J. Food Sci.* **36**, 215-218
- O'Sullivan, W. J. & Perrin, D. D. (1964) *Biochemistry* **3**, 18-26
- Parmeggiani, A. & Bowman, R. H. (1963) *Biochem. Biophys. Res. Commun.* **12**, 268-273
- Racker, E. (1955) *Methods Enzymol.* **1**, 500-503
- Schirmer, I., Schirmer, R. H., Schulz, G. E. & Thuma, E. (1970) *FEBS Lett.* **10**, 333-338
- Scopes, R. K. (1969) *Biochem. J.* **113**, 551-554
- Scopes, R. K. (1970) in *The Physiology and Biochemistry of Muscle as a Food* (Briskey, E. J., Cassens, R. G. & Marsh, B. B., eds.) vol. 2, pp. 471-492, University of Wisconsin Press, Madison
- Scopes, R. K. (1971) *Biochem. J.* **122**, 89-92
- Scopes, R. K. (1972a) *Anal. Biochem.* **49**, 73-87
- Scopes, R. K. (1972b) *Anal. Biochem.* **49**, 88-94
- Scopes, R. K. & Newbold, R. P. (1968) *Biochem. J.* **109**, 197-202
- Stull, J. T. & Mayer, S. E. (1971) *J. Biol. Chem.* **246**, 5716-5723
- Tietz, A. & Ochoa, S. (1958) *Arch. Biochem. Biophys.* **78**, 477-493
- Traverso-Cori, A., Chaimovich, H. & Cori, O. (1965) *Arch. Biochem. Biophys.* **109**, 173-185
- Traverso-Cori, A., Traverso, S. & Reyes, H. (1970) *Arch. Biochem. Biophys.* **137**, 133-142