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The Regulation of Glycolysis in Perfused Locust Flight Muscle

By W. C. L. FORD* and D. J. CANDY
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

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Concentrations of glycolytic intermediates, amino acids and possible regulator substances were measured in extracts from locust thoracic muscles perfused under different conditions. The conversion of [^{14}C]glucose into intermediates and CO_2 by muscle preparations was also followed. When muscles perfused with glucose were made anaerobic changes in metabolite concentrations occurred that could be accounted for by an activation of phosphofructokinase and pyruvate kinase. When butyrate and glucose were present in the perfusion medium the rate of glycolytic flux was lower than with glucose alone, and the aldolase reaction appeared to be inhibited. When butyrate alone was supplied to the muscle the concentrations of most glycolytic intermediates were similar to those found when glucose was supplied. Iodoacetate caused changes in concentrations of intermediates that appeared to result from inhibition of glyceraldehyde 3-phosphate dehydrogenase. Fluoroacetate-poisoned muscles showed a high citrate concentration, but no obvious site of inhibition by citrate was apparent in the glycolytic pathway. Mechanisms for control of glycolysis in locust flight muscle are discussed and related to the known properties of isolated enzymes. It is proposed that trehalase, hexokinase, phosphofructokinase, aldolase, and pyruvate kinase may be control enzymes in this tissue.

Locusts use both carbohydrates and fats as energy sources for long-distance flight, but the proportion of carbohydrates used is much higher during the early period of flight than after about 1 h (Weis-Fogh, 1952; Mayer & Candy, 1969). The rate of glycolysis in the muscle must first increase by about 50–100 fold at initiation of flight when carbohydrates are the main substrates, and then decreases as fats replace carbohydrates as the most important fuel. Simultaneously, changes must occur in the rates of the metabolic pathways involved in the metabolism of fats. Amino acids are also oxidized by locust flight muscle (Kirsten *et al.*, 1963; Mayer & Candy, 1969) and the rate of utilization of amino acids may also vary during flight.

This paper describes experiments designed to examine the control of glycolysis in locust flight muscle. Experiments were carried out with perfused working preparations of locust thoracic muscle for which conditions were modified by supplying different substrates, by adding inhibitors or by making the preparations anaerobic. The concentrations of glycolytic intermediates and of some possible regulator substances were measured in extracts from the perfused muscles.

Mechanisms for the control of glycolysis in locust flight muscle suggested from these results are discussed in relation to the known properties of the glycolytic enzymes.

* Present address: Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH9 9AG, U.K.

Materials and Methods

Enzymes and chemicals

Coenzymes and metabolic intermediates were obtained from either Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., or Boehringer Corp. (London) Ltd., London W.5, U.K. Ketose 1-phosphate aldehyde lyase (EC 4.1.2.7), citrate lyase (EC 4.1.3.6) phosphopyruvate hydratase (EC 4.2.1.11), glucose oxidase (EC 1.1.3.4), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), malate dehydrogenase (EC 1.1.1.37), peroxidase (EC 1.11.1.7), phosphoglucomutase (EC 2.7.5.1), phosphoglycerate mutase (EC 5.4.2.1), phosphohexose isomerase (glucose phosphate isomerase, EC 5.3.1.9) triose phosphate isomerase (EC 5.3.1.1) and iodoacetamide were from Sigma (London) Chemical Co. Ltd. L-Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), hexokinase (EC 2.7.1.1), adenylate kinase (EC 2.7.4.3), lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) were from Boehringer Corp. (London) Ltd. Agidex (a 1–4,1–6-amyloglucosidase preparation from *Aspergillus*) was a gift from Glaxo Ltd. (Ulverston, Lancs., U.K.). [U-^{14}C]-Glucose (3.0mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

*scyllo*Inositol, methyl α-D-mannopyranoside and methyl 4,6-*o*-benzylidine α-D-mannopyranoside were synthesized as previously described (Candy, 1967; Candy & Baddiley, 1966). Hexamethyldisilazane and trimethylchlorosilane were from BDH Ltd. (Poole, Dorset, U.K.). Fluorisil and iodoacetate were from Koch–Light Ltd. (Colnbrook, Bucks., U.K.).

Locusts

Insects (*Schistocerca gregaria* Forsk) were obtained from the Anti-Locust Research Centre (London W.8, U.K.) or were bred in our laboratory from the same stock, and were fed on an artificial diet (Howden & Hunter-Jones, 1958) supplemented with young fresh barley shoots. Insects used for experiments were males 13–30 days after the final ecdysis.

Muscle perfusion

Perfusion of locust thoracic muscle preparations was done as described by Candy (1970). The muscle preparation consisted of one side of the thorax with fat-body and gut removed, but with muscles, nerves, tracheal system and cuticle left intact. A stimulation rate of 2 shocks/s and a temperature of 35°C were used for all experiments. The perfusion volume was 1.5ml for the radioactivity incorporation experiments and 2ml for other experiments.

Muscle response to stimulation was monitored by a transducer linked to the dorsal cuticle of the preparation (Candy, 1970). A steady response throughout the course of perfusion was used as a test that the muscle preparation was functioning satisfactorily. When a serious fall in response occurred the experiment was rejected. All substrates used supported similar extents of mechanical response.

Experiments in which metabolite concentrations were to be compared under different perfusion conditions were arranged so that perfusion of each type were done with locusts of the same batch. (The term 'batch' is used here to refer to a group of locusts hatched at the same time and reared together in the same cage until ready for experimentation.) Where applicable, perfusions were done in the order: glucose aerobic (a), other conditions (order varied), glucose aerobic (b). For most purposes the experimental metabolite concentrations were compared with those found for glucose aerobic conditions, so that the latter acted as controls.

Preparation of muscle extracts

At the termination of perfusion the thoracic-muscle preparation (still attached to the electrodes) was removed from the perfusion vessel, quickly but firmly blotted with paper tissue, and immediately clamped with tongs (Wöllenberger *et al.*, 1960), previously cooled to the temperature of liquid N_2. Frozen muscles were stored under liquid N_2 until extracted with $HClO_4$.

For the metabolite-assay experiments extracts were made from groups of three or four muscle preparations that had been perfused under identical conditions on the same day. The tissues were crushed to a fine powder in a stainless-steel percussion mortar at the temperature of liquid N_2. The material was transferred to 7.2% (w/v) $HClO_4$ (2.5ml for four muscle preparations) at 0°C and the mixture was immediately ground thoroughly. The mixture was diluted to 4ml and centrifuged at 2000*g* for 10min at 4°C. The sediment was washed with 3×1ml of water and stored at −70°C until analysed for bound glycogen. The supernatant and washing were combined and carefully neutralized to pH7.0 with 20% (w/v) KOH. The precipitate of $KClO_4$ was removed by centrifugation at 0°C. Samples were stored at −70°C until required.

A similar extraction procedure was used for the radioactive-tracer experiments, except that only one muscle preparation at a time was extracted and volumes were scaled down accordingly.

Assay of metabolic intermediates

In preliminary experiments attempts were made to assay intermediates by dehydrogenase-linked reactions in which NADH oxidation or NAD^+ reduction were measured fluorimetrically. However, it was found that locust muscle extracts contained materials that fluoresced at a similar wavelength to that of NADH. Attempts to remove the interfering fluors with ion-exchange resins, organic solvents, or Fluorisil columns were unsatisfactory. Typically, a proportion of the interfering compounds (up to 80% with Fluorisil) was removed, but the remaining fluorescence was still too intense to allow assays to be done. Because of this problem less sensitive spectrophotometric assays were used for most metabolite assays.

Reactions were done in glass cuvettes of 0.4ml capacity and 1.0cm light-path. Extinction at 340nm was recorded by using a Gilford 2000 spectrophotometer (with a Unicam SP.500 monochromater) equipped with a chart recorder and thermostatically controlled cell-holder. The general procedure was to add 0.01ml samples of appropriate enzyme to a mixture of buffer, coenzymes, coupling enzymes and muscle extract at 25°C. Any change in extinction was followed to a new steady state and then a further addition of enzyme was made to check that the reaction had reached completion or equilibrium. A known sample of authentic substrate was then added, and the extinction change produced was used to check whether the system was giving the appropriate response.

Methods used were modified from those described by Hohorst (1963) for glucose 6-phosphate, fructose 6-phosphate and ATP, Sacktor & Wormser-Shavit (1966) for fructose 1,6-diphosphate, dihydroxyacetone phosphate plus glyceraldehyde phosphate, glycerol 3-phosphate, lactate and malate, Czok & Eckert (1963) for phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate, Dagley (1963) for citrate and Adam (1963) for pyruvate, ADP and AMP. Glycogen was assayed in extracts as previously described (Mayer & Candy, 1969). However, not all of the glycogen of locust muscle was extracted by $HClO_4$ treatment, but some remained bound to the protein precipitate. A similar observation was made for rat heart-muscle extracts (Bartley & Dean, 1968). For measurement of this 'protein-bound glycogen', samples of the insoluble residue sedimented during $HClO_4$ extraction of the muscle were treated with 2ml of a 10mg/ml solution of Agidex in 0.2M-sodium acetate buffer, pH4.5, for 2h at 50°C. Glucose released was then determined by the glucose oxidase procedure as described by Krebs *et al.* (1963).

Trehalose, glucose and *scyllo*inositol were measured by g.l.c. of their trimethylsilyl derivatives by using a modification of the method of Sweeley *et al.* (1963). Samples (0.05ml) of extract were mixed with 0.05ml each of internal standards (0.2mg of methyl α-D-mannopyranoside/ml and 0.2mg of methyl 4,6-*o*-benzylidine α-D-mannopyranoside/ml). The mixture was evaporated to dryness at room temperature and 0.05ml of a mixture of anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane (10:2:1, by vol.) was added. After 30min at room temperature a 0.01ml sample was chromatographed on a column (1.7m×3.2mm) of 10% (w/w) SE30 on Supasorb AW (BDH) in a Pye 104 gas chromatograph equipped with a flame ionization detector. The initial temperature of the column was 180°C and this was increased at the rate of 1.5°C/min to 250°C and then kept at 250°C for 20min. The sugar derivatives emerged in the following sequence: methyl α-D-mannopyranoside, 194°C; α-D-glucopyranose, 201°C; β-D-glucopyranose, 208°C; *scyllo*inositol, 212°C; methyl 4,6-*o*-benzylidine α-D-mannopyranoside, 216°C; and trehalose after 13min at 250°C. Sugar concentrations were calculated from the ratios of recorded peak areas after allowing for different response factors found for trehalose, glucose and *scyllo*inositol compared with those for the internal standards.

Amino acids were measured with an automatic analyser as described by Mayer & Candy (1969). When the radioactivity of the amino acids was to be determined duplicate samples of extracts were separated on the same analyser columns, but eluate fractions were collected for counting and not passed through the ninhydrin reaction system.

Inorganic phosphate was measured as described by Lowry *et al.* (1964).

Radioactivity measurements

Radioactivity was measured with either of the Nuclear–Chicago liquid-scintillation systems 725 or Unilux 2. Non-aqueous samples were mixed with 10ml of toluene containing 2,5-diphenyloxazole (0.5%, w/v) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.01%, w/v). Aqueous samples (1ml) were counted for radioactivity in a mixture of 6.7ml of the above toluene scintillator and 3.3ml of Triton X-100. Efficiencies were determined by the channels-ratio procedure and were normally in the range 60–70%. Radioactivity on chromatography paper was measured by rolling the paper into a cylinder and placing it in a vial containing 10ml of the toluene scintillator. Trial experiments showed that the efficiency was about 50% and, within the range used, was nearly independent of paper size and quantity of radioactivity. Where accurate measurement of the radioactivity of chromatographic spots was required, the compound was eluted from the paper with water and the radioactivity counted as described for aqueous samples.

Identification of labelled products from [^{14}C]glucose

In some experiments perfusion of the muscle was done by using 20μCi of 0.08M-[U-^{14}C]glucose in a perfusion volume of 1.5ml and the muscle preparation was extracted with $HClO_4$ as described above. The extract was passed through a column (8mm diam.) containing 2g of Dowex-50 (H^+ form) resin and the column was washed with 10ml of water. The combined effluent and washings were concentrated and chromatographed on Whatman no. 3 paper with solvent (1) [propan-1-ol–pyridine–acetic acid–water (8:8:1:4, by vol.) (Gordon *et al.*, 1956)]. Whatman no. 1 paper was used for subsequent chromatography. Radioactive material remaining at the origin was treated with Agidex to hydrolyse any glycogen present to glucose, which was then separated by paper chromatography in solvent (2) [propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Baar & Bull, 1953)]. Other radioactive compounds with R_{Glc} values of less than 0.65 in solvent (1) were separately eluted and chromatographed in solvent (3) [methanol–1M-ammonium acetate (7:3, v/v) (Cole & Ross, 1966)]. Further resolution of these compounds was done by paper electrophoresis (Runeckles & Krotkov, 1957). Radioactive compounds with R_{Glc} values greater than 0.65 in solvent (1) were further chromatographed with solvent (2) and then solvent (4) [butan-1-ol–acetic acid–butyl acetate–water (120:24:9:49,

by vol.) (James *et al.*, 1968)]. Each apparently homogeneous radioactive compound was subjected to further chemical or enzymic identification tests.

Statistical treatment of results

Results obtained from metabolite assay experiments were usually expressed as means ± S.D., with the number of different extracts assayed indicated in parentheses. The results were examined for significant differences by the two-way analysis of variance procedure to test variation between all batches and all perfusion conditions. In addition, differences between individual perfusion conditions were tested by the procedure of Tukey as described by Snedecor (1956). For one experiment only (Table 3) significance was tested by using Student's '*t*' test.

Calculations

Results obtained from the metabolite assay experiments were examined by plotting the results in the form of a cross-over diagram according to the principles first proposed by Chance *et al.* (1958). An alternative approach was to calculate the mass-action ratios of the glycolytic reactions and to compare these with the calculated equilibrium constants. Any reaction where the mass-action ratio was far removed from the equilibrium position was regarded as being a potentially rate-limiting, and therefore control, reaction (Bücher & Rüssmann, 1964).

In such calculations certain assumptions have been made. (a) The cytosol pH was 7.0. No information is available for insect muscle, but the mean values for 17 other types of muscle is 6.93 ± 0.27 (calculated from information collected by Waddell & Bates, 1969). (b) Metabolites were present only in the muscle of the preparations and were confined to the cytosol, which occupied 50% of the muscle liquid volume (the remainder consisting of 30% mitochondria and 20% extracellular space). The latter assumption is probably incorrect for certain metabolites such as glucose and citrate, but in many cases the assumptions allow valid comparisons to be made between different perfusion conditions even though the calculated values are not absolute. For some reactions the mass-action ratio of a single reaction could not be determined because the concentration of either substrate or product was not known. In such cases the overall mass-action ratio of sequential reactions was calculated such that the unknown compound was the product of one reaction and the substrate of another.

The $[NAD^+]/[NADH]$ ratio of a cell compartment can be found indirectly from the concentrations of substrates of a dehydrogenase reaction for which reactants are at equilibrium (Bücher & Klingenberg, 1958). For locust flight muscle a suitable enzyme for this calculation is glycerol 3-phosphate dehydrogenase, which occurs in the cytosol in high activity that is much greater than that required to account for the glycolytic rate (Vogell *et al.*, 1959; Crabtree & Newsholme, 1972). The $[NAD^+]/[NADH]$ ratios shown in Table 4 have been calculated from the concentrations of glycerol 3-phosphate and triose phosphates after making the assumptions described above, and assuming that the ratio of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate was 15:1.

Results and Discussion

Oxidation rates of exogenous substrates

With [^{14}C]glucose as substrate the production of $^{14}CO_2$ at a steady rate (usually after more than 30–40min of perfusion) was taken as a measure of glycolytic flux. This measure depends on the assumptions that exogenous glucose was the main substrate for glycolysis under these conditions, and that endogenous metabolite pools in the muscle became equilibrated with labelled metabolites from [^{14}C]-glucose so that the rate of glucose catabolism through glycolysis approached the rate of $^{14}CO_2$ production. The first assumption is probably correct since the amount of readily available endogenous substrate in the muscle is only able to support muscle activity for 30–40min (Candy, 1970). The good agreement found between radiochemical and direct assay of intermediates (see below) is the evidence for the validity of the second assumption.

The steady-state production of $^{14}CO_2$ from [^{14}C]-glucose without other substrate corresponded to 48nmol of glucose metabolized/min per 100mg fresh wt. of muscle. When 0.08M-butyrate (unlabelled)

Table 1. *Radioactive compounds derived from [U-^{14}C]-glucose in locust flight muscle*

The muscle preparation was perfused with 0.08M-[U-^{14}C]glucose for 60min under aerobic conditions. Radioactive products were separated and measured as described in the Materials and Methods section. Concentrations are expressed as nmol/100mg fresh wt. of muscle and are minimum values calculated from radioactivity measurements as described in the text.

Compound	Concentration
Glycogen (soluble)	136
Trehalose	240
Glucose	1000
Glucose 6-phosphate	20
Glycerol 3-phosphate	16
Glycerol	21

was also present this rate was decreased to 25nmol/min per 100mg. When 0.08M-proline was present as a mixture with 0.08M-[^{14}C]glucose the rate of glucose oxidation was 31nmol/min per 100mg.

Labelled products from [^{14}C]glucose

When muscles were perfused for 60min with [^{14}C]glucose about 10μmol of substrate was completely oxidized to $^{14}CO_2$, and about 2.7% of the total radioactivity was present in the muscle preparation. The identity of the radioactive compounds present in the perfusion medium was not studied in detail, but preliminary experiments showed that over 90% of the radioactivity corresponded to glucose and about 1% to trehalose. Table 1 shows muscle extract components identified on the basis of chromatography in at least three solvent systems and by chemical or enzymic tests. Minimal values for concentrations were calculated on the assumption that the compounds had the same specific radioactivity (per carbon atom) as the glucose substrate. For glycogen this assumption is incorrect, since any glycogen formed from radioactive glucose was diluted with the large pool of endogenous glycogen. A low apparent value was also obtained for glycerol 3-phosphate, where some loss occurred during purification. However, concentrations of the other compounds calculated in this way show reasonable agreement with concentrations found by direct assay (Table 3).

The considerable amount of radioactive labelling of trehalose was somewhat unexpected, since muscle is mainly responsible for trehalose breakdown. The main site for trehalose synthesis in locust is the fat-body (Candy & Kilby, 1961), and it is possible that the observed incorporation could be accounted for by fat-body contamination of the perfused muscle preparation. However, in other experiments individual muscles were isolated and carefully freed from all fat-body. Trehalose synthesis occurred when such preparations were incubated with 0.08M-glucose, and this synthesis, but not trehalose hydrolysis, was strongly inhibited by anaerobic conditions, iodoacetate or fluoride. This suggests that trehalose synthesis in the muscle occurs by an energy-dependent system, perhaps involving UDP-glucose as in the fat-body system. However, the possibility that synthesis was due to individual fat-body or haemolymph cells trapped in the muscle cannot be entirely ruled out.

The compounds listed in Table 1 accounted for about 70% of the total radioactivity of the extract, and a further 10% was absorbed during the Dowex-50 treatment. Elution of the Dowex-50 column with 1M-HCl followed by paper chromatography of the eluate showed that this radioactivity was present in a mixture of amino acids. In other experiments the nature of the labelled amino was studied by ion-exchange chromatography of extracts on the amino acid analyser. The results (Table 2) show that aspartate, glutamate and alanine had specific radioactivities which approached that of glucose. Transaminases for these amino acids are presumably active in flight muscle. Glutamine was present in high concentrations but had a lower specific radioactivity, suggesting that the rate of its formation and breakdown is relatively slow. Glycine was present at high concentration but at very low specific radioactivity. This suggests that free glycine in muscle is derived from an extramuscular source.

Effects of anaerobic conditions

Table 3 shows the results from metabolite assays done on extracts of muscle preparations perfused with 0.08M-glucose as sole substrate. For the aerobic controls the perfusion was done with a constant stream of oxygen for 1h. In the anaerobic experiments perfusion was done with oxygen for 1h and then the gas supply was changed to nitrogen. After

Table 2. *Radioactive amino acids derived from [U-^{14}C]glucose in locust flight muscle*

The muscle preparation was perfused with 0.08M-[U-^{14}C]glucose for 60min under aerobic conditions. Radioactive amino acids produced were separated by ion-exchange chromatography and determined as described in the Materials and Methods section. Each value is the mean of two determinations.

Amino acid	$\frac{\text{Specific radioactivity of amino acid}}{\text{Specific radioactivity of glucose}}$ (%)	Concn. (nmol/100mg fresh wt. of muscle)
Alanine	71.0	57
Asparagine	13.0	74
Aspartate	66.0	46
Glutamate	52.0	102
Glutamine	11.0	1150
Glycine	0.5	1000

Table 3. *Metabolite concentrations in muscles perfused with glucose under aerobic and anaerobic conditions*

Muscles were perfused, freeze-clamped and the metabolite concentrations were measured as described in the Materials and Methods section. Results are expressed as nmol/100mg fresh wt. of muscle±S.D. The number of different extracts assayed is indicated in parentheses.

Metabolite	Concentration		Probability that difference due to chance alone (%)
	Anaerobic	Aerobic	
Glycogen (soluble)	480±27 (4)	440±81 (7)	90
Trehalose	208±44 (4)	219±47 (7)	90
Glucose	1021±297 (4)	854±99 (7)	90
Glucose 6-phosphate	22.3±3.3 (10)	23.8±2.3 (13)	10–20
Fructose 6-phosphate	4.5±1.5 (4)	4.6±1.5 (7)	90
Fructose diphosphate	9.8±2.5 (4)	4.8±2.1 (7)	0.1
Triose phosphates	17.6±5.8 (4)	14.4±0.9 (7)	5–10
Glycerol 3-phosphate	253±45.6 (10)	62.5±18 (13)	0.1
3-Phosphoglycerate	9.3±4.0 (4)	9.3±5.4 (7)	90
Phosphoenolpyruvate	2.9±1.3 (7)	5.4±2.1 (10)	1–5
Pyruvate	63.5±13.0 (7)	11.0±3.0 (10)	0.1
Lactate	101.6±15.2 (10)	85.6±16.8 (13)	5
Citrate	41.3±7.4 (4)	35.1±6.4 (7)	10
Malate	28.0±7.1 (10)	10.8±2.7 (13)	0.1
ATP	303±25 (4)	292±27 (8)	90
ADP	77.2±18.2 (7)	76.4±9.6 (10)	90
AMP	17.6±3.9 (3)	10.0±9.7 (5)	30

about 100s delay the mechanical response of the muscle preparation decreased rapidly and with considerable irregularity, until no response was obtained at about 180s. At this point the muscle was freeze-clamped. Three muscle preparations were pooled for each extract.

Anaerobic conditions produced significantly higher concentrations of fructose 1,6-diphosphate, glycerol 3-phosphate, pyruvate, lactate and malate, and significantly lower concentrations of phosphoenolpyruvate, than aerobic controls. Higher concentrations of triose phosphate and citrate were also found for anaerobic conditions, but these differences had only a low level of statistical significance. The major end-products of anaerobic glycolysis in the locust flight muscle were glycerol 3-phosphate and pyruvate, as was found for living *Locusta migratoria* made anoxic (Bücher *et al.*, 1959). Only a small increase in lactate concentration occurred, and even this could be accounted for by the presence of tissues other than flight muscle in the preparation.

When the results are expressed as a cross-over diagram (Fig. 1) it is apparent that anaerobic conditions caused a change in the steady-state concentration of metabolites to give an apparent positive cross-over at the reactions catalysed by phosphofructokinase and pyruvate kinase and a negative cross-over at the overall reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate phosphokinase. The apparent activation of phosphofructokinase must be treated with caution as the main effect was an increase in concentration of the product of a virtually irreversible reaction, but such activation could have been caused by a change in the concentration of any of several possible effectors. One of these, AMP, does increase in concentration, although the statistical significance of this is low. Krebs (1964) pointed out that in the presence of adenylate kinase, AMP may be a sensitive indicator of the phosphorylation state of the adenine nucleotides, so that an activating effect of AMP on phosphofructokinase could be an effective way of altering the glycolytic rate in response to changes in energy metabolism of the flight muscle. The cross-over at pyruvate kinase could have occurred by activation of the enzyme by an effector, or could have been an indirect effect of pyruvate accumulation owing to possible inhibition of pyruvate oxidation, perhaps by inhibition of pyruvate dehydrogenase. The negative cross-over seen at the glyceraldehyde 3-phosphate dehydrogenase region could have been a result of the low $[NAD^+]/[NADH]$ ratio (Table 4) exerting an effect on the equilibrium position of this enzyme.

The mechanism by which anaerobic conditions stop muscular activity is not apparent from the assay results. Thus although muscle activity had completely ceased as a result of the anaerobic conditions no major decrease in the concentration of ATP was found. It is possible that the muscle stimulation-

Table 4. *Calculated cytoplasmic redox potential and [NAD⁺]/[NADH] ratio in muscles perfused under different conditions*

Values were calculated from the glycerol 3-phosphate and triose phosphate concentrations listed in Tables 3, 5 and 7 and making the assumptions discussed in the text.

Perfusion condition	[NAD⁺]/[NADH] ratio
Glucose, aerobic (Control series A, Table 5)	1430
Glucose, aerobic (Control series B, Table 5)	1920
Glucose, anaerobic (Table 3)	787
Glucose+butyrate (Table 5)	4000
Butyrate (Table 5)	2660
Iodoacetate 'to stop' (Table 7)	9960
Iodoacetate '77 min' (Table 7)	8540
Fluoroacetate (Table 7)	2160

system through the nerves is more sensitive to anoxia than the muscles themselves.

Perfusions with glucose plus butyrate

Metabolite concentrations in muscles perfused with 0.08 M-glucose plus 0.08 M-butyrate are shown in Table 5. Compared with the glucose-perfused controls the muscles perfused with glucose plus butyrate show a significantly higher concentration of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and citrate. At a lower level of statistical significance the glycogen and trehalose concentration are also higher in the perfusions with glucose plus butyrate.

The presence of butyrate apparently spared the carbohydrate available to the muscle, as shown by the higher values of the glycogen and trehalose reserves after perfusion and by the lower rate of oxidation of [^{14}C]glucose in the presence of butyrate. The calculated decrease in glycolytic flux of about 50% could be accompanied by a negative cross-over in the pathway. Such a cross-over occurs at the aldolase reaction (Fig. 1), and in addition there appears to be a positive cross-over at the pyruvate kinase reaction. Because this apparent effect on aldolase was at a low level of statistical significance four further perfusions were done with glucose plus butyrate together with four controls. Only concentrations of fructose 1,6-diphosphate and triose phosphates were measured. When the results were pooled with those shown in Table 5, the calculated value for log [fructose 1,6-diphosphate]/[triose phosphate]^2 was significantly higher ($P<0.01$) for the perfusions with glucose plus butyrate. The cross-over

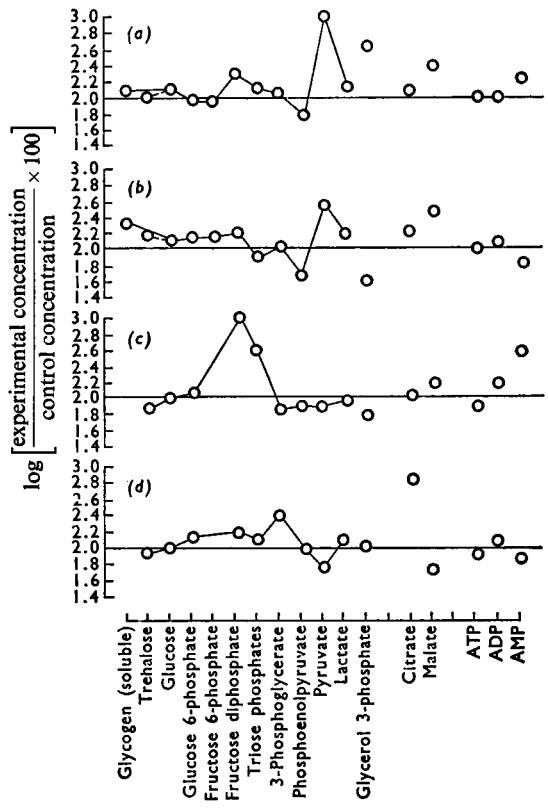

Fig. 1. *Comparison of metabolite concentrations in muscle perfused under different conditions*

Concentrations are expressed relative to those found for the 'aerobic glucose' conditions of perfusion and are calculated from the results shown in Tables 3, 5 and 7. Perfusion conditions were: (*a*) anaerobic, (*b*) butyrate+glucose, (*c*) iodoacetate, (*d*) fluoroacetate. In (*a*) and (*b*) --- denotes route from alternative substrate

at aldolase is rather unexpected since this enzyme is not regarded as being an important control enzyme in most tissues. However, aldolase from rabbit skeletal muscle (but not liver) is inhibited by adenine nucleotides and fructose 6-phosphate (Spolter *et al.*, 1965) and so may be involved in the regulation of mammalian muscle glycolysis under some conditions. Rat heart perfused with acetate in addition to glucose shows a cross-over at the phosphofructokinase reaction (Williamson, 1965). The mechanism for this effect of fatty acids on glycolysis in mammalian systems may be an inhibition of phosphofructokinase by citrate (Newsholme & Randle, 1964).

It is noteworthy that the phosphofructokinase from *S. gregaria* flight muscle differs from the enzyme

Table 5. *Metabolite concentrations in muscles perfused with glucose, glucose plus butyrate and butyrate*

Muscles were perfused, freeze-clamped and the metabolite concentrations were measured as described in the Materials and Methods section. Results are expressed as nmol/100mg fresh wt. of muscle±S.D. Unless otherwise stated (in parentheses) results are the means of duplicate assays on four different extracts for each perfusion condition except for glucose series B where three extracts were used.

Metabolite	Glucose series A	Glucose series B	Glucose+ butyrate	Butyrate	Differences significant at the 5% level
Glycogen (insoluble)	614 (2)	555 (2)	788 (1)	382 (2)	
Glycogen (soluble)	471±89	405±68	1120±640	233±255	Glucose+butyrate> butyrate
Trehalose	222±39	215±80	303±33	142±26	Glucose+butyrate>rest
Glucose	909±120	800±48	1053±158	26±12	Butyrate<rest
Glucose 6-phosphate	25±4	25±3	35±2	12±2	Glucose+butyrate> glucose>butyrate
Fructose 6-phosphate	5.4±1.6	3.7±1.4	7.5±1.8	3.3±1.5	Glucose+butyrate>rest
Fructose diphosphate	4.1±2.5	5.6±1.8	8.8±2.2	3.2±0.8	Glucose+butyrate>rest
Triose phosphates	16.6±0.4	12.2±1.4	11.2±3.3	4.8±3.1	Glucose series A>butyrate
Glycerol 3-phosphate	62±21	68±18	31±1.0	20±11	None
3-Phosphoglycerate	11.2±7.3	7.3±3.5	10.1±4.8	6.3±3.4	None
Phosphoenolpyruvate	4.8±1.9	5.9±2.3	2.6±1.0	3.3±3.6	None
Pyruvate	14.0±5.7	8.4±0.3	14.9±7.6	7.2±3.3	None
Lactate	59±22	75±33	97±43	90±46	None
Citrate	34±6	37±7	59±10	46±10	Glucose+butyrate> glucose
Malate	7.2±1.6	3.8±0.3	15.6±11.9	4.6±1.7	None
ATP	302±26	283±28	300±36	288±27	None
ADP	80±12	73±7	100±17	92±22	None
AMP	15±9 (3)	—	16±16 (3)	16±9	None

of mammalian muscle in not being inhibited by citrate (Walker & Bailey, 1969). Similarly, increased citrate concentrations in the present experiments (with butyrate and fluoroacetate) are not reflected in a cross-over at phosphofructokinase. An alternative explanation for these results is that the triose phosphate isomerase reaction was displaced from equilibrium in the muscles perfused with butyrate plus glucose. However, aldolase is present in much lower activities than triose phosphate isomerase in locust flight muscle (Vogell *et al.*, 1959) and is thus more likely to be rate-limiting.

The amino acids found in muscle extracts (Table 6) must be endogenous or the amino group must have been derived from endogenous sources, since no amino acids were added to the perfusion medium. In the presence of glucose plus butyrate the concentrations of alanine, glutamate and especially proline were significantly higher than in muscles perfused with glucose alone. Proline may provide tricarboxylic acid-cycle intermediates by conversion into 2-oxoglutarate, as occurs in the blowfly (Sacktor & Childress, 1967). In the locust muscle preparation butyrate is presumably metabolized through the tricarboxylic acid cycle, yet the utilization of proline is considerably decreased. It seems that either the tricarboxylic acid-cycle intermediates do not require replenishment at as high a rate with butyrate as substrate or that butyrate has a sparing effect on the use of proline as an addition substrate at the oxidation rates that occurred during perfusion. These results suggest that there are controls existing for reactions of amino acid utilization in flight muscle. One such reaction may be the first step in proline oxidation catalysed by proline dehydrogenase, and this would be in agreement with the situation in other insects. Thus the proline dehydrogenase of blowfly flight muscle is activated by ADP (Hansford & Sacktor, 1970), and measurements of the proline dehydrogenase activities in flight muscles from *Locusta* and other insects have lead to a suggestion by Crabtree & Newsholme (1970) that this enzyme is rate limiting (and therefore a likely control) for proline oxidation in many insects.

Perfusions with butyrate

Muscles perfused in the presence of 0.08 M-butyrate as sole exogenous substrate contained a much lower concentration of glucose than muscles perfused with

Table 6. *Amino acid concentrations in muscles perfused with glucose (aerobically and anaerobically), glucose plus butyrate and butyrate*

Muscles were perfused, freeze-clamped and the amino acid concentrations were measured as described in the Materials and Methods section. Results are expressed as nmol/100mg fresh wt. of muscle±S.D. and are the mean of determinations of four extracts per perfusion condition except for glucose aerobic series B, where three extracts were used.

Amino acid	Glucose aerobic series A	Glucose aerobic series B	Glucose anaerobic	Glucose+ butyrate	Butyrate	Differences significant at the 5% level
Alanine	62±20	50±5	103±24	156±19	77±15	Glucose+butyrate, and glucose anaerobic>rest
Glycine	763±110	979±153	889±253	1085±332	989±165	None
Proline	<20	<20	<20	262±89	157±62	Glucose+butyrate, and butyrate>rest
Serine	114±18	110±18	110±26	134±62	106±24	None
Threonine	95±38	—	69±38	80±39	90±39	None
Glutamine	523±193	432±85	531±157	742±292	435±170	None
Glutamate	80±60	72±19	82±41	162±24	92±39	Glucose+butyrate> glucose aerobic
Aspartate	54	26	33	38	33	None

glucose (Table 5). This difference was reflected in a twofold lower concentration of glucose 6-phosphate in the muscles perfused with butyrate. Triose phosphates were also present in significantly lower concentrations in the muscles perfused with butyrate, and other glycolytic intermediates were present in generally slightly lower concentrations. These results show that control of the glycolytic pathway in locust flight muscle is such that the concentrations of glycolytic intermediates are maintained at a fairly normal value even in the presence of very low concentrations of glucose. Bücher & Rüssmann (1964) have proposed that maintenance of the concentrations of intermediates of a metabolic pathway at a reasonable value when the flux through the pathway is low is a way of ensuring the functional readiness of the system to respond to rapid changes in the demands on that pathway. This may be particularly important for glycolysis in insect flight muscle, where sudden and large changes occur in the metabolic flux at the initiation of flight.

Differences in the ratios of glucose to trehalose and of glucose to glucose 6-phosphate for the different perfusion conditions could indicate that trehalase was greatly inhibited and hexokinase was activated in the presence of butyrate. However, it is probable that the distribution of glucose between extracellular space and muscle cells was different in the two perfusion conditions, and in particular it is likely that there was a large contribution of extracellular glucose to total muscle glucose in the muscles perfused with glucose. The concentration of glucose at the sites of action of the two enzymes is therefore not known with any certainty.

The concentrations of intermediates in muscles perfused with butyrate could be maintained by activation of hexokinase and perhaps glycogen phosphorylase. In addition inhibition of pyruvate utilization may occur since the concentrations of pyruvate (and alanine) in such muscles were similar to those found in muscles perfused with glucose.

Effects of iodoacetate

Muscle preparations were perfused for 60min with 0.08M-glucose as substrate, and then iodoacetate (0.3mM final concentration) was added. Little change occurred in the mechanical response of the muscle for about 10min after inhibitor addition, but then the response declined until contractions ceased, usually by 30min after addition. Iodoacetate inhibited the production of $^{14}CO_2$ from [^{14}C]glucose with about the same time-course as that for the decrease in mechanical response.

In other experiments, iodoacetamide caused a decrease in mechanical response after a rather longer delay than for iodoacetate. However, $^{14}CO_2$ production increased up to about double the previous steady-state value after 25min, and then declined. This stimulation of $^{14}CO_2$ production in the absence of an increased mechanical response suggests that iodoacetamide may act as an uncoupler of oxidative phosphorylation. A similar effect on $^{14}CO_2$ production was also found when dinitrophenol was added

Table 7. *Metabolite concentrations in muscles perfused with glucose and inhibited with fluoroacetate or iodoacetate*

Perfusions were done for 60min in the presence of glucose and then inhibitor was added. For the Iodoacetate 'to stop' experiments perfusion was continued until contractions ceased. For the iodoacetate '77min' experiments perfusion was continued for 17min after addition of inhibitor. For the fluoroacetate experiments perfusion was continued for 50min after addition of inhibitor. Muscles were then freeze clamped and metabolite concentrations were measured as described in the Materials and Methods section. Results are expressed as nmol/100mg fresh wt. of muscle±S.D. and are the means of determinations on three extracts per perfusion condition.

Metabolite	Control series A	Control series B	Iodoacetate 'to stop'	Iodoacetate '77min'	Fluoro-acetate	Differences significant at the 5% level
Trehalose	183±57	211±46	138±38	165±45	265±82	None
Glucose	706±144	760±179	728±135	709±74	727±103	None
Glucose 6-phosphate	36±8	28±8	36±6	28±4	41±1	Fluoroacetate > control B
Fructose di-phosphate	11±4	11±4	113±12	75±3	19±10	Iodoacetates > controls
Triose phos-phates	12±12	12±5	47±7	39±8	17±10	Iodoacetates > controls
Glycerol 3-phosphate	91±31	68±35	52±43	50±18	84±31	Iodoacetates < control series A
3-Phospho-glycerate	13±2	12±5	8±5	8±3	36±25	None
Pyruvate	20±10	16±8	14±5	13±9	11±7	None
Lactate	62±16	85±34	71±14	60±14	97±19	None
Citrate	25±6	24±6	29±2	32±23	190±38	Fluoroacetate > controls
ATP	367±58	340±16	286±44	293±23	308±27	None
ADP	95±11	86±28	140±44	139±38	111±21	Iodoacetates > controls
AMP	7±7	6±9	25±2	10±8	4±3	Iodoacetate 'to stop' > controls
Alanine	47±13	65±10	59±18	65±1	116±34	Fluoroacetate >controls
Glycine	1133±180	1054±112	922±65	844±4	988±45	Iodoacetate '77min' < control series A
Proline	<20	<20	<20	<20	<20	
Serine	72±25	119±58	100±14	109±31	140±19	None
Threonine	54±24	58±10	80±6	98±7	92±26	Iodoacetate '77min' > controls; Fluoro-acetate >control series A
Glutamine	412±122	501±115	491±77	521±117	474±106	None
Glutamate	45±11	57±20	59±2	43±12	50±8	None
Asparagine	36±8	43±13	47±12	51±6	53±22	None
Aspartate	41±8	41±8	70±18	65±12	38±4	Iodoacetate > controls

during perfusion. Lehninger (1951) has reported that iodoacetamide, but not iodoacetate, would uncouple the respiration of rat liver mitochondria.

Iodoacetate caused a significant increase in the concentrations of fructose 1,6-diphosphate, triose phosphates, ADP and AMP and a decrease in the concentration of glycerol 3-phosphate in the muscle (Table 7). These results suggest that in flight muscle it is glyceraldehyde phosphate dehydrogenase that is most strongly inhibited by iodoacetate. Examination of the mass-action ratios of the reactions shows that the product of the glyceraldehyde phosphate dehydrogenase and phosphoglycerate phosphokinase reactions moves about 100-fold away from equilibrium, whereas the phosphofructokinase reaction moves several times nearer to equilibrium.

Effects of fluoroacetate

Muscle preparations were perfused with 0.08M-glucose for 60min and then fluoroacetate (final concentration 20mM) was added. This caused a steady loss in mechanical response, but the time taken for contractions to stop were more variable than for

iodoacetate. The rate of $^{14}CO_2$ production from [^{14}C]glucose declined steadily after addition of inhibitor.

To inhibit the metabolism of the perfused locust muscle a high concentration of fluoroacetate had to be used, compared with the 0.2–1.0mM used for perfused rat heart by Williamson (1967). This lack of sensitivity was also apparent in the living insects where injection of an amount of fluoroacetate equivalent to ten times the LD_{50} per unit weight for a rat caused no apparent change in behaviour of the insects.

Fluoroacetate caused a considerable accumulation of citrate in the perfused muscle (Table 7). In many tissues fluoroacetate is metabolized to fluorocitrate, which is a competitive inhibitor of aconitase (see Peters, 1957). The inhibition of aconitase can produce an elevation of citrate concentrations, which in rat heart strongly inhibits phosphofructokinase (Bowman, 1964; Williamson, 1967). Such an effect of citrate on phosphofructokinase may not occur in the locust system, since no cross-over occurred at this site. However, a cross-over was observed between 3-phosphoglycerate and pyruvate, although the difference in metabolite concentrations had only a low level of statistical significance. It is possible that in the locust muscle a late enzyme of glycolysis is inhibited by citrate. In muscles inhibited with fluoroacetate the concentration of glucose 6-phosphate was significantly higher than in controls, and other early intermediates of glycolysis were at a generally higher concentration.

scylloInositol in muscle

*scyllo*Inositol was present in all of the muscle extracts at an average concentration of 0.26μmol/100mg fresh wt. of muscle. The concentration of this compound did not vary significantly with different perfusion conditions, nor did it become labelled in the [^{14}C]glucose-incorporation experiments. It has not previously been reported in muscle, although its presence in insect haemolymph was established by Candy (1967). Its function in insects is not known, but it could be present in muscle as a fairly inert solute to help maintain the osmotic pressure of the muscle cell in equilibrium with the relatively high osmotic pressure of the haemolymph.

Control of glycolysis in locust flight muscle

Calculations of the mass-action ratios of the glycolytic reactions show that in the locust muscle phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate phosphokinase, and pyruvate kinase were considerably displaced from equilibrium. The trehalase and hexokinase reactions also appear to be displaced from equilibrium, but calculations for these reactions are unreliable because of uncertainty about the intracellular concentrations of glucose and trehalose.

It is likely that several control points for glycolysis operate in locust flight muscle, although not all of the control points exert a controlling influence on the rate of glycolysis under all conditions. Enzymes which may be involved in such control include trehalase, hexokinase, phosphorylase, phosphofructokinase, aldolase and pyruvate kinase. It should be noted that the rate of glucose oxidation during perfusion was probably about 10% of the maximal rate during flight and it is possible that some control effects found could be changed in emphasis during flight.

As the first enzyme in the utilization of the main blood sugar, trehalase would seem to be a likely and important control enzyme. Locust muscle has an active trehalase with a K_m of about 3mM (D. J. Candy, unpublished work), so that at rest some mechanism must operate to prevent wasteful hydrolysis of haemolymph trehalose. Either the trehalase must be considerably inhibited at rest or trehalose must be separated from the enzyme by a permeability barrier. During flight the rate of trehalose utilization increases considerably, so that hydrolysis of trehalose must be activated in some way. Sacktor & Wormser-Shavit (1966) found that the thoracic concentration of trehalose in blowflies decreased within a few seconds after the initiation of flight and suggested that trehalase is a control point in this insect. At the present time there is no direct evidence whether the control is at the enzyme or at a permeability barrier. There have been no reports of control properties of isolated trehalase from insect tissues despite experiments designed to reveal such properties (D. J. Candy, unpublished work) and it seems equally likely that trehalose utilization is controlled by changes in the permeability of the muscle to trehalose.

Hexokinase could be a control enzyme in locust muscle. Its substrate, glucose, is only present in fairly low concentrations in the haemolymph (Mayer & Candy, 1969), and in the muscle probably arises mainly as an intermediate in the metabolism of trehalose. Kerly & Leaback (1957) showed that the hexokinase from locust flight muscle is inhibited by glucose 6-phosphate, and this effect may operate *in vivo* to control the rate of glucose phosphorylation.

Childress & Sacktor (1970) showed that glycogen phosphorylase is a control enzyme in the blowfly muscle, and in locusts phosphorylase activity may be subject to control, although there is no direct evidence for this.

Another probable control enzyme in locust flight muscle is phosphofructokinase. Regulatory properties have been found for phosphofructokinase isolated from locust flight muscle (Walker & Bailey, 1969). On theoretical grounds regulation of phosphofructokinase through changes in AMP concentrations is a

more sensitive control than through changes in other adenine nucleotides (Krebs, 1964). The results presented here are compatible with such a control operating in locust muscle, since no significant differences were detected in ATP or ADP concentrations with different perfusion conditions, whereas some differences were found in AMP concentrations. Regulation of the rate of conversion of fructose 6-phosphate into fructose 1,6-diphosphate could also involve fructose diphosphatase (Newsholme & Gevers, 1967), an enzyme found in flight muscles of locusts (Newsholme & Crabtree, 1970).

It is possible that a late reaction of the glycolytic sequence is an additional control point. By analogy with mammalian systems the most likely control enzyme here would be pyruvate kinase, but evidence for this was not conclusive because of the difficulty of obtaining reliable estimates of the amounts of phosphoenolpyruvate in the extracts. Bailey & Walker (1969) have shown that the pyruvate kinase of locust flight muscle is inhibited by ATP and requires ADP for maximal activity.

The rate of glycolysis in locust flight muscle seems to be controlled by mechanisms similar to those of mammalian muscle, although the relative importance of some of the control sites may be different. One difference may be the signal that co-ordinates the tricarboxylic acid cycle and glycolysis. In mammalian muscle this operates through the effect of citrate on phosphofructokinase, but such an effect does not seem to occur in the locust muscle.

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