Biochem. J. (1977) **168**, 161–170
Printed in Great Britain

Inhibition of Glucose Uptake and Glycogenolysis by Availability of Oleate in Well-Oxygenated Perfused Skeletal Muscle

By MICHAEL J. RENNIE* and JOHN O. HOLLOSZY
Department of Preventive Medicine, Washington University School of Medicine, St. Louis, MO 63110, *U.S.A.*

(*Received* 15 *April* 1977)

The effects of exogenous oleate on glucose uptake, lactate production and glycogen concentration in resting and contracting skeletal muscle were studied in the perfused rat hindquarter. In preliminary studies with aged erythrocytes at a haemoglobin concentration of 8g/100ml in the perfusion medium, 1.8mM-oleate had no effect on glucose uptake or lactate production. During these studies it became evident that O_2 delivery was inadequate with aged erythrocytes. Perfusion with rejuvenated human erythrocytes at a haemoglobin concentration of 12g/100ml resulted in a 2-fold higher O_2 uptake at rest and a 4-fold higher O_2 uptake during muscle contraction than was obtained with aged erythrocytes. Rejuvenated erythrocytes were therefore used in subsequent experiments. Glucose uptake and lactate production by the well-oxygenated hindquarter were inhibited by one-third, both at rest and during muscle contraction, when 1.8mM-oleate was added to the perfusion medium. Addition of oleate also significantly protected against glycogen depletion in the fast-twitch red and slow-twitch red types of muscle, but not in white muscle, during sciatic-nerve stimulation. In the absence of added oleate, glucose was confined to the extracellular space in resting muscle. Addition of oleate resulted in intracellular glucose accumulation in red muscle. Contractile activity resulted in accumulation of intracellular glucose in all three muscle types, and this effect was significantly augmented in the red types of muscle by perfusion with oleate. The concentrations of citrate and glucose 6-phosphate were also increased in red muscle perfused with oleate. We conclude that, as in the heart, availability of fatty acids has an inhibitory effect on glucose uptake and glycogen utilization in well-oxygenated red skeletal muscle.

It is well established that the availability of fatty acids inhibits carbohydrate utilization in the well-oxygenated heart (Randle *et al.*, 1963, 1964; Newsholme & Randle, 1964; Garland *et al.*, 1964; Neely *et al.*, 1969; Neely & Morgan, 1974). A number of studies have failed to demonstrate a similar effect in skeletal muscle (Beatty & Bocek, 1971; Jefferson *et al.*, 1972; Goodman *et al.*, 1974; Reimer *et al.*, 1975). We re-evaluated the effect of fatty acids on carbohydrate utilization in exercising rats in which the concentration of fatty acids in plasma was elevated by means of a fat meal followed by heparin injection (Rennie *et al.*, 1976). The rats with increased plasma fatty acids had significantly less depletion of glycogen in muscle and liver than the control animals during the exercise. The glycogen-sparing effect was seen in the red types of skeletal muscle, but not in white muscle.

* Present address: Department of Human Metabolism, University College Hospital Medical School, London WC1E 6JJ, U.K.

These results provided evidence that, as in the heart, the availability of fatty acids inhibits carbohydrate utilization in red skeletal muscle. However, it was not possible to obtain direct information about the effects of fatty acids on glucose uptake by muscle in the exercising rat. Nor was it possible to evaluate the effects of fatty acids on the responses of cofactors and metabolic intermediates in muscle to exercise, because 3–5min elapsed between the end of exercise and sampling of the muscles, while the anaesthesia took effect.

In the present study these problems were overcome by using a perfused rat hindquarter preparation, which makes it possible both to measure glucose uptake and to freeze muscle samples with minimal delay. During the couse of these studies it was found that, in addition to slowing glycogen utilization in contracting red skeletal muscle, the availability of fatty acids decreases glucose uptake in the well-oxygenated rat hindquarter at rest.

Experimental

Animals

Female specific-pathogen-free Wistar rats (Hilltop Laboratory Animals, Scottsdale, PA, U.S.A.) weighing 269±9g were caged individually and fed on a diet of Purina chow *ad libitum*. The animal room was maintained between 18 and 20°C and was lit between 07:00 and 19:00h.

Materials

New England Nuclear Corp. (Boston, MA, U.S.A.) was the source for D-[1-^{14}C]mannitol (50μCi/μmol). Sodium oleate, D-mannitol, adenine and inosine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine serum albumin (Cohn fraction V) was obtained from Pentex Corp. (Kankakee, IL, U.S.A.), dissolved in Krebs–Henseleit (1932) bicarbonate buffer and dialysed twice against 10vol. of the buffer for 24h.

Perfusion medium

The standard perfusion medium was composed of Krebs–Henseleit bicarbonate buffer containing 10m-i.u. of insulin (where 1 i.u. is defined as the activity contained in 0.04167mg of Fourth International Standard preparation) and 4g of bovine serum albumin per 100ml, either 5mM- or 10mM-glucose, 0.15mM-pyruvate and washed human erythrocytes (Hems *et al.*, 1966; Ruderman *et al.*, 1971). The medium also contained 2mM-D-[1-^{14}C]-mannitol (1.25μCi/100ml) which was used to determine the extracellular space. When oleate was included in the perfusion medium, sufficient fatty acid–albumin complex was added to give an oleic acid concentration of 1.8mM in the cell-free perfusate. The solutions used in the preparation of the medium were filtered through Millipore filters (0.45μm pore size; Millipore Corp., Bedford, MA, U.S.A.). The complete cell-free perfusion medium containing the albumin was passed through a filter of 1.0μm pore size before addition of the washed erythrocytes.

In our initial experiments, the perfusion medium contained blood-bank time-expired (22-day-old) human erythrocytes, which are subsequently referred to as 'aged' erythrocytes, at a concentration of 8g of haemoglobin/100ml (Hems *et al.*, 1966; Ruderman *et al.*, 1971). In these experiments we found that the perfused muscles were inadequately oxygenated (see the Results section). Measurement of the O_2-dissociation curve revealed an increased O_2 affinity of the aged erythrocytes (i.e. dissociation curve shifted to the left) similar to that reported by Valtis & Kennedy (1954). This effect, which decreases the O_2-delivery function of the erythrocytes, appears to be due to a decrease in ATP and 2,3-bisphosphoglycerate in the cells during storage and can be reversed by incubation of erythrocytes with glucose, adenine and inosine (Valeri, 1974). In subsequent experiments the aged erythrocytes were therefore routinely subjected to this procedure, which has been referred to as 'rejuvenation' (Valeri, 1974).

The rejuvenation procedure involved the addition of 50ml of 0.9% NaCl containing 50mM-isosine, 5mM-adenine, 100mM-glucose, 50mM-sodium pyruvate and 50mM-sodium phosphate, pH7.2, to each unit of aged erythrocytes (Valeri, 1974); one unit of erythrocytes is defined as the erythrocytes from 500ml of whole blood. The mixture of erythrocytes and rejuvenation solution was then incubated in a shaking incubator at 37°C for 60min. The rejuvenated cells were then washed with 0.9% NaCl and Krebs–Henseleit buffer (Hems *et al.*, 1966). Comparison of the dissociation curves of the aged and the rejuvenated erythrocytes showed that rejuvenation caused a decrease in O_2 affinity to normal; the half-saturation pressure of the aged erythrocytes was 15.5mmHg, compared with 25.5mmHg for the rejuvenated cells. The rejuvenated erythrocytes were added to the perfusion medium at a concentration of 12g of haemoglobin/100ml.

Perfusion procedure

The surgical preparation of the hindquarter was performed as described by Ruderman *et al.* (1971). The rats were anaesthetized with sodium pentobarbital (5mg/100g body wt.). A tracheal cannula was inserted and respiration maintained with a small animal respirator (Phipps and Bird, Richmond, VA, U.S.A.). During surgery the animals were kept warm with a heating pad. The perfusion apparatus was similar to that of Ruderman *et al.* (1971) with minor modifications. A Silastic tube oxygenator (Hamilton *et al.*, 1974) was substituted for the multibulb glass oxygenator, obviating the need for a second circuit of the medium through the peristaltic pump. Flow was monitored with a Gilmont flowmeter (Curtin Scientific Co., St. Louis, MO, U.S.A.), which was frequently calibrated by a timed collection of the perfusion medium as it flowed out of the venous catheter. In the initial series of experiments with aged erythrocytes the perfusion medium was recirculated after passage through the hindlimbs; the flow rate was 7ml/min, except during sciatic-nerve stimulation, when it was increased to 10ml/min (i.e. 5ml/min per stimulated hindlimb).

In subsequent experiments, with rejuvenated erythrocytes, a single-flow-through perfusion was used; the flow rate through the hindquarter was kept at 7ml/min at a pressure of 8–9kPa (70–80mmHg) when resting muscle was perfused. In the experiments in which the sciatic nerve was stimulated, perfusate flow was limited to the stimulated hindlimb by ligation of the contralateral iliac artery. Perfusate flow through the hindlimb was maintained at 3.5ml/min during the preliminary equilibrium period.

At the start of the experiment, the flow rate was increased to 15ml/min, which caused pressure to increase to 18–20kPa. Stimulation of the muscles was begun at the time the flow was increased. Each perfusion began with a 7min-long washout and equilibration period during which the perfusate was discarded. Perfusate volume was 200ml.

Stimulation of muscles to contract

The skin of one hindlimb was carefully removed, leaving the surface blood vessels intact. The sciatic nerve was exposed in the gluteal region, but not cut until later. The achilles tendon was severed and tied to a 5mm-diameter stainless-steel ring. The skinned hindlimb was covered with paper tissue, which was kept moist with Krebs–Henseleit buffer. The abdomen was then opened for insertion of the arterial and venous catheters. After preparation of the animal for hindlimb perfusion (Ruderman *et al.*, 1971) the animal was placed lying on its ventral surface in an acrylic plastic cabinet maintained at 37°C. The leg was fixed in an acrylic plastic cradle with a steel pin through the ankle joint. The animal was taped to the perfusion platform to ensure immobility.

During the initial 7min equilibrium period of the perfusion, the hindlimb was prepared for stimulation and measurement of muscle contraction. The sciatic nerve was cut approx. 5mm distal from the point at which it emerged from between the gluteal muscles. The distal end of the nerve was drawn into a suction electrode. The gastrocnemius–plantaris–soleus muscle group was connected to a Statham model UC3 force transducer (Honeywell, Test Instruments Division, Denver, CO, U.S.A.) by means of a rigid wire hooked to a stainless-steel ring which was sutured to the severed achilles tendon. The resting tension on the muscles was adjusted to give the optimal length at which active twitch tension was maximal. Contractions were elicited by stimulating the sciatic nerve with supramaximal (approx. 0.5V) square-wave pulses of 0.5ms duration, which were administered with a Grass S48 Stimulator (Grass Instruments, Quincy, MA, U.S.A.).

At the end of the equilibration period the perfusate flow rate was adjusted to 15ml/min and electrical stimulation was begun. The muscles were stimulated indirectly through the nerve with 100ms trains of 10Hz. The trains were delivered at a rate of 30/min. The isometric tension developed by the gastrocnemius–plantaris–soleus muscle group was recorded on a Sanborn recorder, model 301 (Hewlett–Packard Co., Palo Alto, CA, U.S.A.). In addition to the gastrocnemius–plantaris–soleus muscle group, stimulation of the sciatic nerve caused contraction of the hamstring muscles, the deep portion of the crural muscles, and the lateral crural muscles; the total mass of the muscles stimulated to contract averaged approx. 4.5g.

Sampling of perfusate and tissue

Arterial and venous samples of perfusate were taken at 0, 5 and 10min, placed in ice-cold tubes containing 17.5mg of NaF/2ml of perfusate, and centrifuged at 1500*g* for 10min. The cell-free supernatants were kept frozen until they were analysed. At the end of the perfusion, samples of muscle were frozen with Wollenberger tongs (Wollenberger *et al.*, 1960) cooled in liquid N_2. The entire soleus muscle, which consists predominantly of slow-twitch red fibres (Baldwin *et al.*, 1972), was frozen *in situ*. Samples of the superficial portion of the gastrocnemius, which consists mainly of fast-twitch white fibres, and of the deep portion of the medial head of the gastrocnemius, which contains predominantly fast-twitch red fibres (Hickson *et al.*, 1976), were cut out and then frozen. Cutting out a sample of gastrocnemius muscle and clamp-freezing it took less than 10s. Muscle samples were kept at −70°C until they were analysed.

Analytical methods

Frozen muscle samples were pulverized and homogenized in $HClO_4$ (Lowry & Passonneau, 1972). A portion of the $HClO_4$ homogenate was used for measurement of glycogen content (Keppler & Decker, 1974) and the remainder was centrifuged at 3000*g* for 15min at 4°C. The supernatant was removed, neutralized with KOH and used for measurement of citrate (Passonneau & Brown, 1974), glucose 6-phosphate (Lang & Michal, 1974), glucose (Bergmeyer *et al.*, 1974), ATP (Lamprecht & Trautschold, 1974), phosphocreatine (Lamprecht *et al.*, 1974) and lactate (Gutmann & Wahlefeld, 1974). The $HClO_4$-insoluble pellet was used for protein determination (Gornall *et al.*, 1949). In addition, glucose and lactate were measured on the arterial and venous samples of the perfusate. Haemoglobin was measured both by the cyanmethaemoglobin method of Drabkin & Austin (1935) and by using the Instrumentation Laboratories CO-oximeter (see below). The procedure for determining the extracellular space with D-[1-^{14}C]mannitol was essentially the same as that used previously in studies on frog sartorius muscle (Narahara & Ozand, 1963; Holloszy & Narahara, 1965). The concentration of D-[1-^{14}C]mannitol in the perfusion medium was 2mM (1.25μCi/100ml). The radioactivity of the cell-free perfusate and neutralized muscle extracts was determined with a Packard Tri-Carb AAA liquid-scintillation counter (Packard Instrument Co., Downers Grove, IL, U.S.A.).

Perfusate pO_2 and pH and the O_2 saturation of haemoglobin were measured by using an Instrumentation Laboratories blood gas analyser and CO-oximeter (Lexington, MA, U.S.A.). The O_2 content of the perfusate was calculated from the

pO_2 values, the percentage saturation of haemoglobin and the concentration of haemoglobin in the perfusate.

Rates of glucose and O_2 uptake, and of lactate release, were calculated from the arteriovenous differences and the perfusate flow rate, and are expressed as μmol/min per 15g of muscle (which was the average weight of muscle perfused in one hindlimb).

Results

Weight of perfused muscle

At the end of 12 perfusions, with seven preparations at rest and five stimulated, Bromophenol Blue (Mallinckrodt Chemicals, St. Louis, MO, U.S.A.) was injected into the arterial circulation. The stained muscles were dissected out and weighed. The average weight of the perfused muscles was 15.0±0.6g (mean±S.D.) per hindlimb. To permit comparison of results obtained from perfusions of the whole hindquarter (resting) and of one hindlimb (stimulation of sciatic nerve), results are expressed per 15g of muscle, i.e. the muscles of one hindlimb.

Studies in the unmodified hindquarter perfusion system

The presence of 1.8mM-oleate in the perfusion medium had no significant effect on glucose uptake when the rat hindquarter was perfused with medium containing aged erythrocytes at a concentration of 8g of haemoglobin/100ml (Table 1). Stimulation of the sciatic nerve increased glucose uptake of the stimulated hindlimb by 23%. Only about 4.5g, or about 30%, of the total perfused muscle contracts in response to stimulation of the cut sciatic nerve (see the Experimental section). If the increase in glucose uptake occurred only in the contracting muscles, it can be calculated that the stimulation caused an increase in glucose uptake of approx. 75% in the involved muscles. Lactate release into the perfusion medium was increased by approx. 10μmol/min by the stimulation. Inclusion of oleate in the perfusate had no significant effect on lactate production (Table 1).

The perfused gastrocnemius–plantaris–soleus muscle group fatigued very rapidly, with developed tension decreasing from approx. 1000g initially to 10–50g after 5min of stimulation. This finding contrasted with our observations in anaesthetized rats with intact circulations, in which the contractile force of the stimulated muscles falls to about 40–50% of initial values after 2–3min, as a result of fatigue of the white muscle fibres, and then levels off and remains constant for a prolonged period (M. J. Rennie & J. O. Holloszy, unpublished work). The rapid decrease in contractile force to very low values in the perfused muscles suggested to us that O_2 supply to the red muscle fibres, which are dependent on aerobic metabolism during prolonged contractile activity, might be inadequate. The finding that the O_2 consumption of the perfused muscles increased little with contractile activity supported this possibility (Table 2).

Table 1. *Absence of an effect of oleate on glucose uptake and lactate production in the perfused rat hindquarter with aged erythrocytes in the perfusion medium*

The hindquarter was perfused with standard medium containing aged erythrocytes at a concentration of 8g of haemoglobin/100ml. Glucose concentration was 5mM. Perfusate flow rate was 3.5ml/min per hindlimb at rest and 5ml/min per hindlimb during stimulation. A 7min washout and equilibrium period during which the perfusate was discarded preceded the experiment; during the experiment the perfusate was recycled. Isometric muscle contractions were elicited by stimulating the sciatic nerve with 100ms trains of 100Hz at a rate of 30/min. Values (μmol/min per 15g of muscle) are means±S.E.M. for four animals.

Oleate (1.8mM)	Glucose uptake		Lactate production	
	Resting	Stimulated	Resting	Stimulated
–	3.3±0.4	4.1±0.3	1.8±0.5	12.2±1.4
+	3.2±0.3	3.9±0.4	1.2±0.3	11.6±0.8

Modification of the perfusate to improve O_2 delivery

To obtain information on the 'normal' O_2 consumption by the rat hindquarter, animals were anaesthetized and surgically prepared as for perfusion, except that the aorta and vena cava were left intact. Small samples of blood (0.75ml) were drawn from the abdominal aorta and inferior vena cava with a needle and syringe for determination of O_2 content. Blood flow was then estimated by means of a timed collection of 1.0ml of blood into a calibrated catheter with an internal diameter of 1.65mm, which was inserted into the vena cava after the blood samples were obtained. O_2 consumption was calculated from the arteriovenous O_2 difference and the flow rate. Measurements on six rats gave an average O_2 uptake of 11.8±2.2μmol of O_2/min per 15g of muscle; this was more than twice the value of 4.8μmol of O_2/min obtained during perfusion with aged erythrocytes. In view of this further evidence that O_2 delivery to the muscles was inadequate when the medium contained aged human erythrocytes, it was decided to modify the perfusate to try to improve O_2 delivery.

As shown in Table 2, perfusion with either fresh or rejuvenated human erythrocytes (see the Experimental section for rejuvenation procedure), at a haemoglobin concentration of 12g/100ml, resulted in an O_2 uptake approximately twice that obtained with aged erythrocytes and similar to that found in anaesthetized rats with intact circulations. Stimulation of the sciatic nerve resulted in a greater than

Table 2. *O_2 consumption by the hindquarter perfused with aged, fresh or rejuvenated human erythrocytes*

The perfusate flow rate was 3.5ml/min per hindlimb at rest, 5ml per hindlimb during stimulation in the experiments with aged erythrocytes and 15ml/min per hindlimb during stimulation in the experiments with fresh or rejuvenated erythrocytes. Isometric muscle contractions were elicited by stimulating the sciatic nerve with 100ms trains of 100Hz at a rate of 30/min. The rates of O_2 uptake measured during the 5th and 10th min of the experiment were averaged. Values are means±S.E.M., with the numbers of experiments given in parentheses. Abbreviation: Hb, haemoglobin.

Human erythrocytes in perfusate		O_2 consumption by hindlimb (μmol/min per 15g of muscle)	
		Resting	Stimulated
Aged (8g of Hb/100ml)		4.8±0.4 (8)	7.3±0.8 (8)
Fresh (12g of Hb/100ml)		10.2 (2)	Not determined
Rejuvenated (12g of Hb/100ml)	No oleate	9.4±0.4 (16)	31.5±3.2 (16)
	1.8mM-oleate	10.3±0.5 (16)	32.7±4.6 (16)

Table 3. *Effects of perfusion with oleate (1.8mM) and of muscle contraction on glucose uptake and lactate production in the perfused rat hindquarter with rejuvenated erythrocytes in the perfusion medium*

The hindquarter was perfused with standard medium containing rejuvenated erythrocytes at a concentration of 12g of haemoglobin/100ml. Glucose concentration was 10mM. A single-flow-through perfusion was used. Each experiment lasted 10min and was preceded by a 7min washout and equilibration period during which the flow rate was 3.5ml/min per hindlimb. During the experiments, perfusate flow rate was 3.5ml/min per hindlimb at rest, and 15ml/min per hindlimb during sciatic-nerve stimulation. Isometric muscle contractions were elicited by stimulating the sciatic nerve with 100ms trains of 100Hz at a rate of 30/min. In experiments in which the sciatic nerve was stimulated, perfusate flow was limited to the stimulated hindlimb. The rates of glucose uptake and lactate production are averages of the values obtained during the 5th and 10th minutes of the experiments. Values (μmol/min per 15g of muscle) are means±S.E.M. for 16 animals.

Oleate (1.8mM)	Glucose uptake		Lactate production	
	Resting	Stimulated	Resting	Stimulated
−	5.45±0.25	11.9±0.8	1.43±0.26	15.9±1.0
+	3.67±0.20*	8.1±0.9†	1.22±0.17	11.5±0.8*

* The value with oleate is significantly different from that without oleate, $P<0.01$ (Student's t test).

† As for *, $P<0.05$.

3-fold increase in O_2 consumption, compared with an increase of only 50% when aged erythrocytes were used. As only about 30% of the total perfused muscle mass (about 4.5g) contracts in response to sciatic-nerve stimulation, it can be calculated that the O_2 consumption of the contracting muscles was increased roughly 8-fold over the resting value.

The tension developed by the gastrocnemius–plantaris–soleus muscle group during the 100ms long isometric contractions averaged 1050±40g (mean±S.E.M. for 32 animals). Contractile force decreased by 50–60% during the first 2–3min of stimulation and then stabilized, remaining constant until the end of the 10min stimulation period. The final tension averaged 465±15g. The initial large fall in tension occurred regardless of the flow rate. However, contractile force stabilized at 40–50% of the initial value only if a high perfusate flow rate was maintained; if the muscles were not adequately oxygenated, tension continued to fall rapidly. The initial rapid decrease in tension is probably due to fatigue of the white fibres; the soleus, which consists entirely of red fibres, does not show any decrease in contractile force under these experimental conditions (i.e. perfusion with medium containing rejuvenated erythrocytes at a flow rate of 15ml/min per hindlimb).

As it appeared that O_2 delivery is adequate with this system, perfusion medium containing rejuvenated erythrocytes at a haemoglobin concentration of 12g/100ml was routinely used in subsequent experiments.

Effects of oleate and sciatic-nerve stimulation

Glucose uptake and lactate production (*Table* 3). The effects of a high concentration of fatty acid on glucose uptake were re-examined in the rat hindquarter perfused with medium containing rejuvenated erythrocytes. In these studies a single flow-through, instead of recycling, of the perfusate was used to ensure that the concentrations of glucose and fatty acids available to the muscles were constant throughout the experiment. Glucose uptake by resting muscle was decreased by approximately one-third when 1.8mM-oleate was included in the perfusion medium. Stimulation of the sciatic nerve, together with an increase in the flow rate to 15ml/min, resulted in a slightly greater than 2-fold rise in glucose uptake and roughly a 10-fold increase in lactate production. The increases in glucose uptake and

lactate production associated with stimulation of muscle contraction were both inhibited by approx. 30%, whereas contractile force was unaffected, when 1.8mM-oleate was included in the perfusion medium.

Muscle glycogen and lactate (*Table* 4). The decrease in glycogen concentration and the increase in lactate in response to the contractile activity were greatest in white muscle, intermediate in fast-twitch red muscle and smallest in the slow-twitch red type of muscle. Addition of 1.8mM-oleate to the perfusion medium significantly protected against glycogen depletion in the red types of muscle, so that almost 50% more glycogen remained after 10min of stimulation in red muscles perfused with oleate than in the controls. In contrast, the availability of oleate did not significantly affect glycogen utilization in the white type of muscle.

Lactate concentration was slightly but significantly higher in resting slow-twitch red muscle when oleate was included in the medium; no significant effects of the fatty acid were seen in the fast-twitch red or white types of muscle at rest. Addition of oleate to the perfusion medium resulted in a significantly smaller increase in lactate concentration in response to contractile activity in the red types of muscle, but not in white muscle.

Intracellular accumulation of free glucose (*Table* 5). In the absence of added fatty acid from the perfusion medium, glucose was confined to the extracellular space in all three types of skeletal muscle at rest. Contractile activity caused considerable intracellular accumulation of glucose. This finding, which confirms previous reports (Kipnis *et al.*, 1959; Berger *et al.*, 1975), provides evidence that when muscle is stimulated to contract in the presence of insulin and glucose the site of regulation of the rate of glucose uptake shifts from transport across the cell membrane to glucose phosphorylation.

There were rather large differences in intracellular glucose accumulation in the three types of muscle after stimulation. The concentration of glucose attained in white muscle was roughly three times as great as in slow-twitch red muscle and 1.7 times as great as in fast-twitch red muscle. It seems probable that these variations in intracellular glucose concentration are a consequence of some of the major differences that exist between the skeletal-muscle types in the activities of various of the enzymes that are involved in, or can affect, glucose metabolism. Among these may be the differences in hexokinase and phosphorylase activities. Total hexokinase activity is roughly three times as great in the red types of muscle as in white muscle, whereas total phosphorylase activity in white muscle is about seven times as great as in slow-twitch red, and twice as great as in fast-twitch red muscle in sedentary rats (Baldwin *et al.*, 1973).

When oleate was added to the perfusion medium, glucose accumulated intracellularly in the slow-twitch red and fast-twitch red types of muscle, but not in white muscle, at rest. Perfusion with oleate also resulted in significantly higher intracellular glucose concentrations in the stimulated fast-twitch red and slow-twitch red types of muscle. These results indicate that the availability of fatty acids results in an inhibition of glucose phosphorylation in well-oxygenated red skeletal muscle.

Citrate, ATP, phosphocreatine and glucose 6-phosphate. Perfusion with medium containing oleate resulted in a significant rise in citrate concentration in the slow- and fast-twitch red types of muscle both at rest and during sciatic-nerve stimulation (Table 5). No significant changes in citrate concentration occurred in white muscle.

Table 4. *Effects of perfusion with oleate* (1.8mM) *and of muscle contraction on the concentrations of glycogen and lactate in the different types of rat skeletal muscle*

At the end of the experiments described in Table 3, the soleus muscle (slow-twitch red) and samples of the deep red (fast-twitch red) and superficial white (fast-twitch white) portions of the gastrocnemius muscle were clamp-frozen. Values are means±S.E.M. for 14 resting or 16 stimulated muscles.

		Tissue concentration (μmol/100mg of muscle protein)			
		Glycogen		Lactate	
Type of muscle	Oleate (1.8mM)	Resting	Stimulated	Resting	Stimulated
Slow-twitch red	−	14.9±0.7	8.2±0.5	0.89±0.13	4.34±0.25
	+	15.0±1.2	12.2±0.9*	1.35±0.14†	3.14±0.19†
Fast-twitch red	−	17.8±0.8	6.5±0.4	1.21±0.09	7.30±0.70
	+	17.0±0.8	9.4±0.6*	1.47±0.09	4.54±0.59*
Fast-twitch white	−	18.2±1.2	4.8±0.6	2.00±0.14	11.3±0.8
	+	17.6±1.1	6.1±0.4	2.34±0.32	10.4±1.4

* Value with oleate is significantly different from that without oleate, $P<0.01$.
† As for *, $P<0.05$.

Table 5. *Effects of perfusion with oleate* (1.8mM) *and of muscle contraction on the concentrations of intracellular glucose, and of citrate and glucose 6-phosphate in the different types of rat skeletal muscle*

At the end of the experiments described in Table 3, the soleus muscle and samples of the deep red and superficial white portions of the gastrocnemius were clamp-frozen. Values are means±S.E.M. for 14 resting or 16 stimulated muscles. n.d., None detected; glucose distributed in a volume less than, or equal to, the extracellular ([^{14}C]mannitol) space.

		Tissue concentration (μmol/100mg of muscle protein)					
		Intracellular glucose		Citrate		Glucose 6-phosphate	
Type of muscle	Oleate (1.8mM)	Resting	Stimulated	Resting	Stimulated	Resting	Stimulated
Slow-twitch red	−	n.d.	0.53±0.14	0.085±0.003	0.108±0.007	0.172±0.019	0.320±0.035
	+	0.52±0.11	0.88±0.11†	0.112±0.009†	0.144±0.009*	0.259±0.016*	0.422±0.036
Fast-twitch red	−	n.d.	0.90±0.23	0.107±0.010	0.104±0.011	—	0.352±0.022
	+	0.23±0.04	1.35±0.17†	0.148±0.010†	0.163±0.013*	—	0.462±0.036*
Fast-twitch white	−	n.d.	1.56±0.15	0.045±0.004	0.049±0.006	—	0.734±0.067
	+	n.d.	1.83±0.12	0.062±0.005	0.050±0.005	—	0.833±0.085

* Value with oleate significantly different from that without oleate $P<0.01$.
† As for *, $P<0.05$.

Table 6. *Effects of muscle contraction on concentrations of ATP and phosphocreatine*

At the end of the experiments described in Table 3, the soleus muscle and samples of the deep red and superficial white portions of the gastrocnemius muscle were clamp-frozen. Values are means±S.E.M. for 28 resting or 32 stimulated muscles.

	Tissue concentration (μmol/100mg of muscle protein)			
	ATP		Phosphocreatine	
	Resting	Stimulated	Resting	Stimulated
Slow-twitch red	1.79±0.04	1.80±0.05	5.82±0.14	4.24±0.20
Fast-twitch red	2.92±0.08	2.43±0.09	7.71±0.22	3.53±0.16
Fast-twitch white	2.90±0.07	2.25±0.08	7.68±0.26	3.10±0.48

The soleus muscle was clamp-frozen *in situ*, whereas the fast-twitch red and fast-twitch white muscle samples had first to be cut from the gastrocnemius before they could be frozen. Cutting a resting skeletal muscle stimulates conversion of phosphorylase from the *b* into the *a* form, and this results in a burst of glycogenolysis with a large increase in glucose 6-phosphate concentration (R. K. Conlee, M. J. Rennie, W. W. Winder & J. O. Holloszy, unpublished work). Because of this phenomenon, it was not possible to obtain accurate measurements of glucose 6-phosphate concentration in samples of fast-twitch red and fast-twitch white muscle at rest. Significantly higher concentrations of glucose 6-phosphate were found in resting slow-twitch red and in contracting slow-twitch red and fast-twitch red muscle perfused with oleate than in the controls (Table 5).

The availability of fatty acid had no significant effect on the concentrations of ATP or phosphocreatine in any of the muscle types. The values obtained on the control and oleate-perfused muscles have therefore been combined in Table 6, which shows the effects of the contractile activity on ATP and phosphocreatine concentrations.

Discussion

The term 'glucose–fatty acid cycle' was proposed by Randle *et al.* (1963) to describe an inverse relationship between fatty acid oxidation and carbohydrate utilization, which, they suggested, was responsible for the decreased carbohydrate tolerance found in a variety of conditions in which plasma free (non-esterified) fatty acids are increased. Much of the evidence that oxidation of fatty acids inhibits carbohydrate utilization was obtained from studies on the isolated perfused rat heart, in which availability of fatty acid inhibits glucose uptake, glycolysis, glycogenolysis and pyruvate oxidation (Randle *et al.*, 1963, 1964; Garland *et al.*, 1964; Newsholme & Randle, 1964; Neely *et al.*, 1969; Neely & Morgan, 1974).

Randle *et al.* (1964) and Garland *et al.* (1964) were also able to show a smaller, but significant, inhibitory effect of fatty acids on carbohydrate

utilization in rat diaphragm. However, a number of subsequent investigations have failed to demonstrate an inhibiting effect of fatty acids on glucose metabolism in a variety of preparations of voluntary muscle, including rat diaphragm (Schonfeld & Kipnis, 1968), teased muscle fibres (Beatty & Bocek, 1971) and the perfused rat hindquarter (Jefferson *et al.*, 1972; Goodman *et al.*, 1974; Reimer *et al.*, 1975). These negative findings led Reimer *et al.* (1975) to conclude that the glucose–fatty acid cycle is confined to heart muscle and does not operate in skeletal muscle. However, studies in dogs (Seyfert & Madison, 1967) and in man (Balasse & Neef, 1974) have shown that elevation of plasma fatty acid concentration decreases peripheral glucose uptake. Furthermore, during prolonged exercise, the respiratory quotient decreases progressively (Edwards *et al.*, 1934; Costill *et al.*, 1971*a*; Paul, 1971) as plasma fatty acid concentration rises (Costill *et al.*, 1971*b*; Paul, 1971; Rennie & Johnson, 1974), indicating a change in respiratory substrate from carbohydrate to fat; this decrease in respiratory quotient occurs long before glycogen stores are depleted. These findings, which argued against the conclusion that the glucose–fatty acid cycle is limited to heart muscle, led us to re-evaluate the effects of fatty acids on carbohydrate metabolism in skeletal muscle.

We (Rennie *et al.*, 1976) have found that glycogen depletion during exercise was slower in leg muscles of rats with increased plasma free fatty acids than in controls. This inhibitory effect of fatty acids was limited to the red types of skeletal muscles. White muscle, which constitutes about 50% of the muscle mass of the rat hindlimb and has a very low capacity for fatty acid oxidation (Baldwin *et al.*, 1972), was not protected against glycogen depletion. On the basis of these findings and the negative results of previous studies, we assumed that the demonstrable effects of fatty acid on carbohydrate utilization would be limited to a slowing of glycogen utilization and lactate production in contracting red muscle in the present study. This supposition proved incorrect, as oleate resulted in a significant decrease in glucose uptake at rest, as well as in glucose uptake and lactate production during muscle contraction, in the well-oxygenated rat hindlimb.

Like Goodman *et al.* (1974), we found that fatty acids had no effect on glucose uptake in the rat hindquarter perfused with aged erythrocytes. It seems reasonable that the difference in response to fatty acids between muscles perfused with aged erythrocytes and those perfused with rejuvenated erythrocytes relates to the difference in oxygenation. Our initial attempts to study the effects of muscle contraction were frustrated by the very rapid development of fatigue. A rapid fall in contractile force is, in our experience, a sensitive indicator of inadequate oxygenation of red skeletal muscle. The rapid decrease in force, together with the lack of an appropriate increase in O_2 consumption during muscle contraction, alerted us to the possibility that O_2 delivery was inadequate. This suspicion was confirmed when substitution of fresh or rejuvenated erythrocytes for aged ones, together with raising haemoglobin concentration by 50%, resulted in a 2-fold increase in O_2 consumption in resting hindquarter. O_2 uptake by one hindlimb perfused with rejuvenated erythrocytes was as great as that of the whole hindquarter perfused with aged erythrocytes (Table 2; Ruderman *et al.*, 1971; Goodman *et al.*, 1974). It seems reasonable, in this context, that availability of fatty acid does not inhibit utilization of carbohydrate in muscle perfused with aged erythrocytes, because the rate of fatty acid oxidation is low owing to limited availability of O_2.

It is at present not clear whether the same explanation applies to the lack of an effect of fatty acids in the preparations used by Jefferson *et al.* (1972), in which the perfusion medium contained 25% bovine erythrocytes, or by Reimer *et al.* (1975), in which the hindquarter preparation was perfused with erythrocyte-free medium.

An important difference in the experimental procedures is that we used a single-flow-through perfusion, whereas Jefferson *et al.* (1972) and Reimer *et al.* (1975) used a recirculating system in which the perfusate was recycled through the hindquarter for 1 h or longer. During prolonged reperfusion the hindquarter releases fatty acids, probably from adipose tissue, into the perfusate; as a result, fatty acid concentration can rise to high values in the initially fatty acid-free medium (Jefferson *et al.*, 1972). Also, during prolonged reperfusion, glucose in the medium can fall from an initially high concentration to a very low value by the end of the experiment (Jefferson *et al.*, 1972).

The present results show that, as in the heart (Garland *et al.*, 1963; Parmeggiani & Bowman, 1963; Garland & Randle, 1964; Newsholme & Randle, 1964; Randle *et al.*, 1964; England & Randle, 1967), oxidation of fatty acids results in an increase in citrate concentration with inhibition of phosphofructokinase, accumulation of glucose 6-phosphate, inhibition of hexokinase, and slowing of glucose uptake in the red types of skeletal muscle. However, the increase in citrate concentration in red skeletal muscle is considerably smaller than that in the heart. For example, in the present study citrate concentration increased from about 240 to 375 nmol/g wet wt. in contracting fast-twitch red muscle when oleate was included in the perfusion medium. In the perfused rat heart, citrate concentration has been reported to increase from about 220 nmol/g wet wt. when glucose is the only substrate in the medium to as high as 700 nmol/g when fatty acids or ketones are available (Garland & Randle, 1964). Another

difference is that, although the availability of fatty acids significantly slows the decrease in glycogen concentration, there is still considerable glycogen depletion in red skeletal muscle during contractile activity (Table 4; Rennie *et al.*, 1976). In contrast, there is normally no decrease, or an increase, in glycogen concentration in contracting rat heart muscle when fatty acids or ketones and glucose are available (Randle *et al.*, 1964; Neely *et al.*, 1969). It seems likely that these differences in response are a consequence of the more than 3-fold greater mitochondrial content and respiratory capacity of the heart as compared with red skeletal muscle in sedentary rats (Winder *et al.*, 1974; Holloszy *et al.*, 1975).

In conclusion, the present results, together with our previous findings in exercising rats (Rennie *et al.*, 1976), show that the glucose–fatty acid cycle functions in red skeletal muscle both at rest and during contractile activity. Although there are quantitative differences in response, which are probably a consequence of the differences in mitochondrial content and respiratory capacity, the mechanisms by which fatty acid oxidation inhibits carbohydrate utilization in skeletal muscle appear to be the same as in the heart.

The technical assistance of Mrs. May Chen is gratefully acknowledged. This research was supported by U.S. Public Health Service Research Grant AM18986 and by a Research Center Grant from the Muscular Dystrophy Associations of America. M. J. R. was the recipient of a Research Fellowship from the Muscular Dystrophy Associations of America.

References

Balasse, E. O. & Neef, M. A. (1974) *Eur. J. Clin. Invest.* **4**, 247–252

Baldwin, K. M., Klinkerfuss, G. H., Terjung, R. L., Molé, P. A. & Holloszy, J. O. (1972) *Am. J. Physiol.* **222**, 373–378

Baldwin, K. M., Winder, W. W., Terjung, R. L. & Holloszy, J. O. (1973) *Am. J. Physiol.* **225**, 962–966

Beatty, C. H. & Bocek, R. M. (1971) *Am. J. Physiol.* **220**, 1928–1934

Berger, M., Hagg, S. & Ruderman, N. B. (1975) *Biochem. J.* **146**, 231–238

Bergmeyer, H. U., Bernt, E., Schmidt, F. & Stork, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1196–1201, Academic Press, New York and London

Costill, D. L., Sparks, K., Gregor, R. & Turner, C. (1971*a*) *J. Appl. Physiol.* **31**, 353–356

Costill, D. L., Bowers, R., Branam, G. & Sparks, K. (1971*b*) *J. Appl. Physiol.* **31**, 834–838

Drabkin, D. L. & Austin, J. H. (1935) *J. Biol. Chem.* **112**, 51–65

Edwards, H. T., Margaria, R. & Dill, D. B. (1934) *Am. J. Physiol.* **108**, 203–209

England, P. J. & Randle, P. J. (1967) *Biochem. J.* **105**, 907–920

Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **93**, 678–687

Garland, P. B., Randle, P. J. & Newsholme, E. A. (1963) *Nature (London)* **200**, 169–170

Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 665–678

Goodman, M. N., Berger, M. & Ruderman, N. B. (1974) *Diabetes* **23**, 881–888

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Gutmann, I. & Wahlefeld, A. W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1464–1468, Academic Press, New York and London

Hamilton, R. L., Berry, M. N., Williams, M. C. & Severinghaus, E. M. (1974) *J. Lipid Res.* **15**, 182–186

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292

Hickson, R. C., Heusner, W. W., Van Huss, W. D., Taylor, J. F. & Carrow, R. E. (1976) *Eur. J. Appl. Physiol.* **35**, 251–259

Holloszy, J. O. & Narahara, H. T. (1965) *J. Biol. Chem.* **240**, 3493–3500

Holloszy, J. O., Booth, F. W., Winder, W. W. & Fitts, R. H. (1975) in *Metabolic Adaptation to Prolonged Physical Exercise* (Howald, H. & Poortmans, J. R., eds.), pp. 438–447, Birkhäuser Verlag, Basel

Jefferson, L. S., Koehler, J. O. & Morgan, H. E. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 816–820

Keppler, D. & Decker, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1127–1131, Academic Press, New York and London

Kipnis, D. M., Helmreich, E. & Cori, C. F. (1959) *J. Biol. Chem.* **234**, 165–170

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 2101–2110, Academic Press, New York and London

Lamprecht, W., Stein, P., Heinz, F. & Weisser, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1777–1781, Academic Press, New York and London

Lang, G. & Michal, G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1238–1242, Academic Press, New York and London

Lowry, O. H. & Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis*, pp. 123–124, Academic Press, New York and London

Narahara, H. T. & Ozand, P. (1963) *J. Biol. Chem.* **238**, 40–49

Neely, J. R. & Morgan, H. E. (1974) *Annu. Rev. Physiol.* **36**, 413–459

Neely, J. R., Bowman, R. H. & Morgan, H. E. (1969) *Am. J. Physiol.* **216**, 804–811

Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 641–651

Parmeggiani, A. & Bowman, R. H. (1963) *Biochem. Biophys. Res. Commun.* **12**, 268–273

Passonneau, J. V. & Brown, J. G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1565–1568, Academic Press, New York and London

Paul, P. (1971) in *Muscle Metabolism during Exercise* (Pernow, B. & Saltin, B., eds.), pp. 225–247, Plenum Press, New York and London

Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963) *Lancet* **i**, 785–789

Randle, P. J., Newsholme, E. A. & Garland, P. B. (1964) *Biochem. J.* **93**, 652–665

Reimer, F., Löffler, G., Hennig, G. & Wieland, O. H. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1055–1066

Rennie, M. J. & Johnson, R. H. (1974) *Eur. J. Appl. Physiol.* **33**, 215–226

Rennie, M. J., Winder, W. W. & Holloszy, J. O. (1976) *Biochem. J.* **156**, 647–655

Ruderman, N. B., Houghton, C. R. S. & Hems, R. (1971) *Biochem. J.* **124**, 639–651

Schonfeld, G. & Kipnis, D. M. (1968) *Am. J. Physiol.* **215**, 513–522

Seyfert, W. A. & Madison, L. L. (1967) *Diabetes* **16**, 765–776

Valeri, C. R. (1974) in *The Red Blood Cell* (Surgener, D. M., ed.), pp. 528–532, Academic Press, London and New York

Valtis, D. J. & Kennedy, A. C. (1954) *Lancet* **i**, 119–124

Winder, W. W., Baldwin, K. M. & Holloszy, J. O. (1974) *Eur. J. Biochem.* **47**, 461–467

Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270**, 399–412