Biochem. J. (1977) **162**, 557–568
Printed in Great Britain

Effect of Acetoacetate on Glucose Metabolism in the Soleus and Extensor Digitorum Longus Muscles of the Rat

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(*Received 5 August* 1976)

1. The effect of acetoacetate on glucose metabolism was compared in the soleus, a slow-twitch red muscle, and the extensor digitorum longus, a muscle composed of 50% fast-twitch red and 50% white fibres. 2. When incubated for 2h in a medium containing 5mM-glucose and 0.1 unit of insulin/ml, rates of glucose uptake, lactate release and glucose oxidation in the soleus were 19.6, 18.6 and 1.47 μmol/h per g respectively. Acetoacetate (1.7mM) diminished all three rates by 25–50%; however, it increased glucose conversion into glycogen. In addition, it caused increases in tissue glucose, glucose 6-phosphate and fructose 6-phosphate, suggesting inhibition of phosphofructokinase. The concentrations of citrate, an inhibitor of phosphofructokinase, and of malate were also increased. 3. Rates of glucose uptake and lactate release in the extensor digitorum longus were 50–80% of those in the soleus. Acetoacetate caused moderate increases in tissue glucose 6-phosphate and possibly citrate, but it did not decrease glucose uptake or lactate release. 4. The rate of glycolysis in the soleus was approximately five times that previously observed in the perfused rat hindquarter, a muscle preparation in which acetoacetate inhibits glucose oxidation, but does not alter glucose uptake or glycolysis. A similar rate of glycolysis was observed when the soleus was incubated with a glucose-free medium. Under these conditions, tissue malate and the lactate/pyruvate ratio in the medium were decreased, and acetoacetate did not decrease lactate release or increase tissue citrate or glucose 6-phosphate. An intermediate rate of glycolysis, which was not decreased by acetoacetate, was observed when the soleus was incubated with glucose, but not insulin. 5. The data suggest that acetoacetate glucose inhibits uptake and glycolysis in red muscle under conditions that resemble mild to moderate exercise. They also suggest that the accumulation of citrate in these circumstances is linked to the rate of glycolysis, possibly through the generation of cytosolic NADH and malate formation.

Studies carried out in cardiac muscle suggest that free fatty acids and ketone bodies replace glucose as a fuel in starvation and diabetes because of their increased availability in these conditions. Randle and his co-workers and others (reviewed by Randle *et al.*, 1966; Ruderman *et al.*, 1969) noted that perfusion of the rat heart with free fatty acids, acetoacetate or 3-hydroxybutyrate decreased glucose uptake, glycolysis and pyruvate oxidation and increased muscle glycogen exactly as did starvation and diabetes. Further, in all of these situations, increases in the concentrations of acetyl-CoA and citrate and in the acetyl-CoA/CoA and NADH/NAD$^+$ ratios occurred in the muscle cell. It was suggested that the latter could account for the observed alterations in glucose metabolism, since pyruvate dehydrogenase is inhibited by acetyl-CoA and NADH,† and citrate is an inhibitor of phosphofructokinase. In addition, the high concentration of glucose 6-phosphate, owing to the block at phosphofructokinase, could decrease glucose phosphorylation by inhibiting hexokinase and could enhance glycogen formation by activating the glucose 6-phosphate-dependent form of glycogen synthase (see Randle *et al.*, 1966). Fatty acids and ketone bodies have also been shown to decrease the transport of glucose into cardiac muscle; however, the mechanism for this has not been determined. In general, the phosphorylation step is rate-limiting when the uptake of glucose is high, owing to the presence of insulin, whereas in other situations the block in transport appears to be dominant (see Morgan & Whitfield, 1974).

Although it is widely believed that free fatty acids and ketone bodies alter glucose metabolism in a similar manner in the large mass of voluntary skeletal

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† More recently shifts in the acetyl-CoA/CoA and NADH/NAD ratios have been demonstrated to affect the conversion of pyruvate dehydrogenase from its inactive into its active form (Cooper *et al.*, 1975; Pettit *et al.*, 1975).

muscle, studies from several laboratories have suggested otherwise (Adrouny, 1969; Beatty & Bocek, 1971; Houghton & Ruderman, 1971; Jefferson *et al.*, 1972; Goodman *et al.*, 1974; see Berger *et al.*, 1976). In experiments with the isolated perfused rat hindquarter, neither glucose uptake nor glycolysis were decreased and glycogen formation was not enhanced as a result of starvation or perfusion with fatty acids or acetoacetate (Berger *et al.*, 1976). On the other hand, lactate oxidation was significantly inhibited by starvation and perfusion with acetoacetate, and, as in heart (Randle *et al.*, 1966; Kerbey *et al.*, 1976), this was associated with both an increase in the concentration of acetyl-CoA and a decrease in the fraction of pyruvate dehydrogenase in its active form (Hagg *et al.*, 1976).

Voluntary skeletal muscle comprises at least three types of fibres, slow-twitch red (intermediate), fast-twitch red (red) and white, which can be readily differentiated on the basis of morphological, functional and biochemical characteristics (Peter *et al.*, 1972; Burke *et al.*, 1971; Close, 1975). Whether glucose metabolism in one of these fibre types more closely resembles that of heart than does a mixed muscle mass such as the hindquarter is not known. Such a notion is attractive, since white fibres make up approximately one-half of the total muscle mass of the hindquarter (see Ariano *et al.*, 1973), whereas the enzymic apparatus and contractile characteristics of the two types of red fibres are more like that of heart (Dawson & Romanul, 1964; see Newsholme & Start, 1973; Close, 1975). For this reason we have compared the metabolism of glucose in the soleus, a slow-twitch red muscle, and the extensor digitorum longus, a muscle composed of approx. 50% white and 50% fast-twitch red fibres (Ariano *et al.*, 1973; Close, 1975), during incubations *in vitro*. The report describes the basic characteristics of the two preparations and the effects of insulin and acetoacetate on glucose uptake and disposition and on tissue concentrations of hexose monophosphates, citrate and malate. A preliminary report of this work has appeared (Maizels *et al.*, 1976). In addition, while this work was in progress, Cuendet *et al.* (1975) reported in preliminary form studies in which ketone bodies and fatty acids altered the uptake and disposition of glucose in the incubated soleus.

Materials and Methods

Animals

Female Sprague–Dawley rats (40–70g), obtained from Charles River Breeding Laboratories, Wilmington, MA, U.S.A., were used. The rats were fed on Purina Laboratory Chow and were allowed water *ad libitum*. Surgery was carried out under anaesthesia with pentobarbital, 5–7mg/100g body wt. administered intraperitoneally.

Materials

Chemicals were reagent grade and were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., Eastman Chemicals, Rochester, NY, U.S.A., or Fisher Scientific Products, Boston, MA, U.S.A. Enzymes were obtained from Sigma or from Boehringer–Mannheim, Indianapolis, IN, U.S.A. Crystalline pig insulin (lot PJ5589) was provided by Dr. W. E. Shaw, Eli Lilly and Co., Indianapolis, IN, U.S.A. Bovine serum albumin (Cohn fraction V, defatted, lot 246) was obtained from Miles Laboratories, Kankakee, IL, U.S.A. Albumin was dissolved in Krebs–Henseleit bicarbonate medium (Krebs & Henseleit, 1932) and dialysed against 2 × 2 litres of the bicarbonate saline for 24h. Sodium acetoacetate was prepared from its ethyl ester by the method of Krebs & Eggleston (1941). [U-^{14}C]Glucose, [3-^{3}H]glucose, [1-^{14}C]inulin, scintillation-grade phenethylamine and butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Before use, the [3-^{3}H]glucose was evaporated to dryness to remove $^{3}H_2O$. All solutions were prepared with water which had been further deionized by passage through an Ultra-pure Bantam demineralizer (Barnstead Co., Boston, MA, U.S.A.).

Dissection procedures

Although the incubated soleus and extensor digitorum longus muscles have been used by others, the operative preparation of each has not been described in detail. Because of this and because the simultaneous removal of both muscles from the same rat is a novel procedure, details of the dissection are described below.

Preparatory dissection. The soleus and extensor digitorum longus muscles of both legs were removed. After removal of the skin to expose the musculature between the mid-thigh and ankle, the fascia overlying the anterior tibialis muscle was stripped along the medial border of the muscle by blunt dissection, in order to expose the entry of its tendon beneath the flexor retinaculum. The retinaculum was then carefully split, with either fine forceps or fine-pointed scissors. This exposes the distal tendons of both the extensor digitorum longus and the anterior tibialis.

Dissection of the soleus. After the preparatory dissection, the hindlimbs were flexed at the hips and anchored to the dissecting tray with adhesive tape so as to expose the achilles tendon and other posterior structures. A tie was then placed around the achilles tendon, the tendon cut distal to the tie and the gastrocnemius and soleus were pulled away from underlying tissue by traction. A second tie was placed around the proximal tendon of the soleus,

the tendon was severed proximal to the tie and the soleus was peeled away from the gastrocnemius by a combination of gentle traction and blunt and sharp dissection. The gastrocnemius was then cut away from the achilles tendon to free the soleus completely. The muscle was placed in ice-cold Krebs–Henseleit solution (Krebs & Henseleit, 1932) that had been gassed with O_2+CO_2 (95:5) immediately after it was freed.

Dissection of the extensor digitorum longus. After removal of the soleus from each limb, the legs were re-extended and a clamp was placed on the exposed tendon of the right anterior tibialis. The tendon was then cut distal to the clamp and the muscle was lifted away from underlying structures to expose the extensor digitorum longus. The proximal attachment of the anterior tibialis was not severed. After this the proximal tendon of the extensor digitorum longus, which is attached to the patella, was exposed by inserting the blade of a pair of fine-pointed scissors along the shaft of the muscle and cutting the overlying musculature. Ties were then placed around the proximal and distal tendons of the extensor digitorum longus and the muscle was excised with the ties attached and placed in Krebs–Henseleit solution. The same procedure was repeated on the other limb.

Immediately after removal, both the soleus and extensor digitorum longus were placed on stainless-steel clips (Hider *et al.*, 1971*a*), in order to maintain the muscles under slight tension during the incubations. The entire procedure, including dissection of all four muscles and their attachment to the clips, takes approx. 15min.

Incubations

Once attached to the clips, the soleus and extensor digitorum longus muscles were transferred to separate 10ml Erlenmeyer flasks, which contained 3ml of Krebs–Henseleit (1932) solution (previously gassed with O_2+CO_2, 95:5), 0.13% albumin and glucose, acetoacetate and radioactive materials as indicated. Two muscles were placed in each flask. After addition of the muscles, the flasks were flushed with O_2+CO_2 (95:5), re-stoppered and placed in a Dubnoff metabolic shaker set at 37–38°C and a shaking rate of 80cycles/min. After 20min, the muscles were transferred to a second set of flasks containing fresh media identical in composition and volume, where they were incubated for an additional 60 or 120min under the same conditions. All measurements were made during this incubation. In a few studies, 0.2ml of medium was taken at 60min, the flasks were re-gassed and the incubations continued for an additional 60min. Six control flasks containing the same incubation media, but not muscle, were simultaneously incubated in all experiments. The initial volume of medium in the control flasks was 3.2ml, of which 0.2ml was withdrawn at the start of each experiment to determine the initial concentration of added substrate. Comparisons of glucose in the medium and other substrates in these flasks at 0 and 120min indicated that evaporation was less than 5%.

At the end of each experiment muscles were removed from the incubation flasks, quickly blotted and dropped into liquid N_2. They were then removed from the clips under liquid N_2 and stored in liquid N_2 until analysis. Samples (usually 2ml) of incubation medium were added to 2ml of ice-cold 6% (v/v) $HClO_4$ for deproteinization.

Collection of $^{14}CO_2$

In some experiments [U-^{14}C]glucose (0.03μCi/ml of medium) was added to the initial medium and $^{14}CO_2$ was collected. When this was done, the muscles were rapidly removed from the flask at 120min and the stopper was replaced with a rubber vial closure, from which was suspended a plastic centre well containing a strip of filter paper wetted with 0.2ml of phenethylamine. Then 1ml of 1M-HCl was injected into the incubation medium through the vial closure to liberate CO_2. After 1h, the centre wells were removed and dropped directly into vials containing 10ml of Butler's (1961) solution [naphthalene, 120g; 2,5-diphenyloxazole, 4g; 1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.5g; *p*-dioxan, 1000ml]. Samples were counted for radioactivity in a Nuclear–Chicago model 300 Isocap liquid-scintillation counter. Counts were corrected for quenching by using an external standard.

Separation of [3-^{3}H]glucose from ^3H_2O

The magnitude of glycolysis was assessed in some studies from the production of ^3H_2O from [3-^{3}H]-glucose (Katz & Rognstad, 1966). The ^3H_2O was separated from glucose by ion-exchange chromatography (Hallen, 1960) by using columns (4.5cm× 1.2cm) of Dowex 1 (X2; borate form). A volume of incubation medium was mixed with an equal volume of 0.3M-borate and 0.2ml was applied to the column. The ^3H_2O was eluted with 4.5ml of water. Glucose remained on the resin. The eluate was counted for radioactivity in a scintillation fluid composed of butyl-PBD (6g/l), naphthalene (80g/l), 40% (v/v) methoxyethanol and 60% (v/v) toluene.

Analytical methods

Perfusate and tissue specimens were deproteinized with 6% (v/v) $HClO_4$. The extracts were neutralized with a solution of 2M-KOH containing 0.5M-triethanolamine and the precipitated $KClO_4$ was removed by centrifugation at 1000*g* for 10min. Metabolites in tissue and medium were measured

spectrophotometrically by standard enzymic assays as described previously (Ruderman & Berger, 1974; Goodman *et al.*, 1974; MacDonald *et al.*, 1976). All glucose determinations were carried out in duplicate and the mean of the two values was used. Changes in acetoacetate in the medium, and, in the presence of insulin, of glucose, averaged 10–15% over 120min. Tissue metabolites were determined with a Perkin–Elmer model 356 double-beam spectrophotometer. Radioactivity of [1-^{14}C]inulin was counted in deproteinized samples of tissue and medium as described previously (Ruderman *et al.*, 1971). Incorporation of [3-^{3}H]glucose into glycogen was determined in KOH hydrolysates of muscle to which carrier glycogen (1 mg) had been added. The glycogen was precipitated and washed once with 66% (v/v) ethanol and then dissolved in water (Stauffacher & Renold, 1969).

Calculations

Rates of utilization and production were calculated from measured differences in concentration or radioactivity between control and experimental flasks. In general, the mean value for three or six control flasks was used as a basis for comparison. Thus glucose utilization would be calculated by using the following expression:

$$\text{Glucose utilization } (\mu\text{mol/h g}) = [\text{glucose}_C] - [\text{glucose}_E] \times \frac{V}{T} \times \frac{1000}{W}$$

where $[\text{glucose}_C]$ is mean concentration of glucose in control flasks at 120min (μmol/ml), $[\text{glucose}_E]$ is glucose concentration in experimental flask at 120 min, V is volume of incubation medium (ml), T is duration of incubation (h) and W is the weight of the incubated muscles in (mg). No correction was made for the small volume change caused by evaporation.

The weight of the incubated muscles was proportional to body weight in rats weighing between 37 and 75g. It was therefore possible to calculate the weights of the soleus and extensor digitorum longus muscles to be used in determining flux rates from body weight. Computation of muscle weight on this basis allowed us to store frozen tissues for metabolite assays until processed (usually within 1–2 months). In addition, it probably provided a more accurate assessment of the weight of the incubated muscle, because of the variability inherent in cutting the muscles off the clips and in weighing milligram quantities of frozen tissue. The ratio of body weight to the weight of two soleus muscles was 1450 ± 20 (mean $\pm$ S.E.M.; $n = 108$). For the extensor digitorum longus, the ratio was 1370 ± 10 (mean $\pm$ S.E.M.; n=122). Student's t test was used for statistical analysis of data.

Results

Perfusion conditions

Rats weighing less than 75g were used to eliminate metabolic changes in the incubated muscles attributable to inadequate diffusion (see Chaudry & Gould, 1969; Goldberg *et al.*, 1975). As shown in Table 1, rates of glucose uptake (cf. Chaudry & Gould, 1969) and lactate release were substantially diminished in rats weighing 150 and 200g, as were the concentrations of ATP and phosphocreatine.

Fat-free albumin was added to the incubation medium to bind unesterified fatty acids released from the muscles and to diminish insulin binding to the surface of the flask (Hider *et al.*, 1971*b*). Pain & Manchester (1970) found it necessary to include 0.13% albumin in media in which the extensor digitorum longus was incubated, to prevent gross swelling; however, swelling was not evident in the absence of albumin in the present study. In accordance with this, tissue concentrations of phosphocreatine and ATP and rates of glucose uptake and lactate release were similar in the presence and absence of albumin (Table 1).

Viability of the preparations

In agreement with the findings of others (see Keul *et al.*, 1972), the concentrations of phosphocreatine and ATP were higher in the extensor digitorum longus, which contains a high proportion of white muscle, than in the soleus (Table 1). The concentrations of both high-energy phosphate compounds were maintained during incubations, although a moderate decrease in ATP was evident in the extensor digitorum longus at the end of the preincubation period. In addition, the ratio of lactate/pyruvate in the medium after 120min of incubation ranged between 5 and 8 (see Table 3). In both muscles, rates of glucose uptake and lactate release were similar in incubations carried out for 60 and 120min.

Ketone-body metabolism

The activities of succinyl-CoA transferase (EC 2.8.3.5) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) are higher in slow-twitch red than in fast-twitch red muscle, and both have markedly higher activities than in white muscle (Winder *et al.*, 1974). In accordance with this, Kark *et al.* (1971) have demonstrated that soleus muscle fibres of the guinea pig oxidize 3-hydroxybutyrate at a rate 20 times greater than do comparable fibres of white muscle. As shown in Table 2, the uptake and net utilization of acetoacetate were similar in the soleus and extensor digitorum longus; however, conversion of acetoacetate into 3-hydroxybutyrate was much greater in the soleus. Presumably, net acetoacetate utilization

Table 1. *Effect of duration of incubation, rat size and presence of albumin on metabolism in the isolated soleus and extensor digitorum longus muscles*

Results are means ± S.E.M. with numbers of observations in parentheses. Fed rats, weighing 40–70g, were used unless otherwise noted. Incubation media contained 5mM-glucose, 0.13% albumin and 0.1 unit of insulin/ml. Unincubated tissue was taken directly from rats anaesthetized with pentobarbital.

Duration of incubation (min)	Glucose uptake (μmol/h per g)	Lactate release (μmol/h per g)	Tissue concentration (μmol/g)	
			Phosphocreatine	ATP
Soleus				
No incubation	—	—	8.9±0.5 (12)	3.5±0.1 (12)
Preincubation	—	—	9.0±1.1 (4)	2.9±0.4 (4)
60	14.8±2.3 (6)	20.3±3.3 (6)	10.8±0.5 (7)	3.2±0.2 (7)
120	19.6±0.8 (18)	18.6±0.5 (21)	9.4±1.3 (9)	3.3±0.2 (10)
120 (no albumin)	17.3±1.6 (9)	16.5±0.7 (9)	9.1±0.6 (14)	2.4±0.2 (16)
120 (150g rats)	9.9 (2)	9.2 (2)	5.9 (2)	2.2 (2)
120 (200g rats)	4.9 (2)	5.5 (2)	3.5 (2)	1.6 (2)
Extensor digitorum longus				
No incubation	—	—	15.4±1.5 (11)	5.5±0.5 (12)
Preincubation	—	—	13.4±0.9 (5)	4.0±0.4 (5)
60	17.5±8.2 (6)	11.7±0.7 (6)	15.1±2.6 (3)	4.6±0.6 (3)
120	15.0±0.8 (16)	11.7±0.4 (22)	14.6±0.7 (9)	4.9±0.4 (7)
120 (no albumin)	11.8±2.0 (6)	13.2±1.8 (7)		
120 (150g rats)	8.1 (2)	8.3 (2)	9.3 (2)	2.8 (2)
120 (500g rats)	4.1 (2)	4.5 (2)	4.6 (1)	2.3 (1)

Table 2. *Metabolism of acetoacetate in the soleus and extensor digitorum longus muscles*

Results are means ± S.E.M. with numbers of observations in parentheses, and were determined during a 120min incubation. Additions were present in both the incubation and preincubation media. Initial concentrations of glucose and acetoacetate were 5mM and 1.7mM respectively. Net acetoacetate uptake is calculated by subtracting 3-hydroxybutyrate release from acetoacetate uptake.

Insulin (0.1 unit/ml)	Acetoacetate uptake (μmol/h per g)	3-Hydroxybutyrate release (μmol/h per g)	Net acetoacetate utilized (μmol/h per g)
Soleus			
−	8.3±0.05 (8)	0.57±0.07 (7)	7.7
+	8.3±0.4 (9)	0.65±0.08 (7)	7.7
Extensor digitorum longus			
−	7.0±0.05 (6)	0.03±0.03 (9)	7.0
+	6.6±0.8 (6)	0.05±0.92 (5)	6.6

and 3-hydroxybutyrate formation by the extensor digitorum longus were primarily due to its fast red component.

Glucose metabolism

Effect of insulin. Rates of glucose uptake, lactate and pyruvate release and glucose oxidation by the

Table 3. *Effects of insulin and acetoacetate on glucose metabolism in the soleus and extensor digitorum longus muscles*
Results are means ± S.E.M. with numbers of observations in parentheses. Incubations were for 120 min. Initial glucose concentration was 5 mM. Glucose oxidation was calculated from $^{14}CO_2$ generation from [U-^{14}C]glucose and glycolysis from the sum of the mean values for lactate+pyruvate+alanine release (results not shown)/2 and glucose oxidation. N.D., Not determined. * indicates values significantly different from the comparable group perfused without acetoacetate, $P<0.01$.

Additions to incubation medium						
Acetoacetate (1.7 mM)	Insulin (0.1 unit/ml)	Glucose uptake (μmol/h per g)	Lactate release (μmol/h per g)	Pyruvate release (μmol/h per g)	Glucose oxidation (μmol/h per g)	Glycolysis (μmol of glucose h per g)
Soleus						
−	−	9.8±1.1 (18)	10.3±0.7 (18)	N.D.	0.66±0.13 (3)	
−	+	19.6±0.8 (18)	18.6±0.5 (21)	2.5±0.6 (3)	1.47±0.11 (12)	12.1
+	−	9.2±1.6 (10)	12.6±1.1 (10)	N.D.	0.60±0.03 (3)	
+	+	14.9±1.4* (10)	14 ±0.7* (10)	3.0±0.2 (4)	0.66±0.07* (9)	9.2
Extensor digitorum longus						
−	−	7.9±1.2 (14)	5.3±0.5 (19)	N.D.	0.35 (2)	
−	+	15 ±0.8 (16)	11.7±0.4 (20)	2.1±0.2 (3)	1.24±0.12 (3)	8.2
+	−	10.6±1.2 (8)	7.9±0.2 (11)	N.D.	0.45±0.10 (3)	
+	+	14.4±1.3 (8)	11.4±0.4 (10)	N.D.	0.57 (2)	7.3

soleus and extensor digitorum longus are presented in Table 3. In the soleus, insulin stimulated both the uptake of glucose and the release of lactate approximately twofold. In addition, it enhanced the oxidation of glucose, as assessed by the generation of $^{14}CO_2$ from [U-^{14}C]glucose. Since the great majority of the lactate and pyruvate released by the soleus, in the absence as well as in the presence of insulin, is derived from exogenous glucose (see below), the increase in glucose oxidation cannot be explained solely by an increase in the specific radioactivity of muscle pyruvate. Presumably, insulin activated pyruvate dehydrogenase.

Absolute rates of glucose uptake and lactate release were somewhat lower in the extensor digitorum longus; however, the stimulatory effects of insulin were very similar to those observed in the soleus. The listed rates for glucose oxidation are less reliable than for the soleus, since a considerable portion of the pyruvate generated by the extensor digitorum longus is derived from a source other than exogenous glucose (see below).

The rate of glucose oxidation in the soleus was very similar to that reported for the perfused hindquarter (see Berger *et al.*, 1976). On the other hand, the basal rates of glucose uptake and glycolysis were more than five times greater and insulin caused a relatively smaller increase in glucose uptake. In addition, insulin, which has only a minimal effect on lactate and pyruvate release in a hindquarter perfused with 5 mM-glucose, nearly doubled the rate of glycolysis. Since similar changes occurred in the extensor digitorum longus, these findings appear to reflect some fundamental difference between incubated and perfused muscles at rest, rather than some unique characteristic of slow-twitch red muscle.

Effect of acetoacetate. In the presence of insulin, acetoacetate caused a 25% decrease in the uptake of glucose and the release of lactate by the soleus (Table 3). In addition, glucose oxidation was decreased by more than 50%. In contrast, in the absence of insulin, acetoacetate inhibited neither glucose uptake nor lactate release. The data are too limited to determine with certainty whether it also failed to inhibit glucose oxidation. In the extensor digitorum longus, acetoacetate did not decrease the uptake or the release of lactate, even when insulin was present. Further, in the absence of insulin, the release of lactate was significantly increased.

Glycolysis and glycogen formation. The disposition of glucose was also investigated by studying the fate of [3-^{3}H]glucose added to the incubation medium. As shown in Table 4, acetoacetate enhanced the incorporation of label into glycogen, but decreased glycolysis, in the soleus incubated with insulin. The

Table 4. *Effect of acetoacetate on glycolysis and glucose conversion into glycogen by the soleus muscle*

Results are means ± S.E.M. of seven or eight observations in each group. Glucose (5 mM) and [3-^{3}H]glucose (1.7 μCi/ml) were added to both the incubation and preincubation media. Calculated rates of glycolysis were determined from the measured values for lactate and pyruvate release and glucose oxidation shown in Table 3 and from rates of alanine release (results not shown). * indicates value significantly different from muscle incubated with insulin in the absence of acetoacetate, $P<0.01$.

Additions to incubation medium		From [3-^{3}H]glucose			
Acetoacetate (1.7 mM)	Insulin (0.1 unit/ml)	Glycolysis (μmol/h per g)	Glucose conversion into glycogen (μmol/h per g)	Net glucose uptake (μmol/h per g)	Calculated rate of glycolysis (μmol/h per g)
−	−	8.1 ± 0.6*	0.72 ± 0.08*	8.9	6.9
−	+	14.4 ± 0.6	3.3 ± 0.2	17.1	12.4
+	+	8.5 ± 0.4*	4.2 ± 0.1*	12.7	8.9

Table 5. *Effects of insulin and acetoacetate on tissue concentrations of hexose phosphates, citrate and malate in the soleus and extensor digitorum longus*

Incubation conditions are described in the legend to Table 2 and the Materials and Methods section. Metabolites were measured in tissue frozen in liquid N_2 after 120 min of incubation. Results are means ± S.E.M. of five to seven determinations in each group. The symbols * and † indicate values significantly different from those of muscles incubated in the absence of acetoacetate and insulin respectively, $P<0.05$ or greater.

Additions to incubation media		Tissue concentration (nmol/g wet wt.)			
Acetoacetate (1.7 mM)	Insulin (0.1 unit/ml)	Glucose 6-phosphate	Fructose 6-phosphate	Citrate	Malate
Soleus					
−	−	195 ± 39	39 ± 5	167 ± 10	185 ± 18
−	+	398 ± 44†	85 ± 11†	188 ± 20	290 ± 30†
+	−	224 ± 19	40 ± 7	309 ± 30*	259 ± 25*
+	+	707 ± 36*†	146 ± 23*†	419 ± 40*†	402 ± 26*†
Extensor digitorum longus					
−	−	163 ± 16	26 ± 3	238 ± 35	
−	+	372 ± 30†	63 ± 9†	323 ± 40	
+	−	252 ± 14*	41 ± 8	313 ± 23	
+	+	504 ± 47*†	84 ± 9†	402 ± 32	

magnitude of glycolysis, as determined from the formation of ^3H_2O, was very similar to that calculated from the release of lactate, pyruvate and alanine plus glucose oxidation. This strongly suggests that very little of the substrate entering the glycolytic pathway in the soleus was derived from muscle glycogen. In addition, it suggests that the great majority of the alanine (net release approx. 5% that of lactate; results not shown), lactate and pyruvate released into the incubation medium was derived from exogenous glucose.

Tissue metabolites. Insulin caused twofold increases in concentrations of glucose 6-phosphate and fructose 6-phosphate in the soleus (Table 5). Presumably this was secondary to an increased rate of glucose transport into the cell, since glycolysis and glycogen synthesis were increased (see Tables 3 and 4). In support of this view, free glucose did not accumulate in muscle cell water (Table 6), indicating that transport was still rate-limiting for glucose uptake. When acetoacetate, which inhibits glucose uptake and glycolysis, was added to the medium, the concentrations of glucose 6-phosphate and fructose 6-phosphate increased another twofold, suggesting inhibition of phosphofructokinase, and the concentration of citrate, an inhibitor of phosphofructokinase (Garland *et al.*, 1963), was markedly increased. The concentration of citrate was increased to a lesser extent when the soleus was incubated with acetoacetate in the absence of insulin. Like citrate, the concentration of malate was increased by the addition of acetoacetate to the incubation medium, and to an even greater extent by addition of acetoacetate plus insulin. In contrast with that of citrate, however, the concentration of malate was also increased by the addition of insulin alone.

Table 6. *Effect of incubation with acetoacetate on tissue glucose in the incubated soleus*

Results are means ± S.E.M. with numbers of observations in parentheses. Incubation media contained [1-^{14}C]inulin, 0.25 μCi/ml (sp. radioactivity 2.5 μCi/mg), glucose (5 mM) and insulin (0.1 unit/ml). Inulin and glucose spaces were calculated from content per g of tissue divided by content per μl of incubation medium. Glucose in cell water was calculated from observed values for glucose in medium and tissue and inulin space. It was assumed that cell water comprises 75% of tissue after correction for fluid in the extracellular space. See legend to Table 3 and the Materials and Methods section for further details.

Additions	Inulin space (μl/g)	Glucose in medium at 120 min (μmol/ml)	Tissue glucose (μmol/g)	Glucose space (μl/g)	Glucose in cell water (mM)
None (10)	197 ± 20	4.34 ± 0.06	0.7 ± 0.09	204 ± 9	~0
Acetoacetate, 1.7 mM (8)	195 ± 22	4.53 ± 0.09	1.47 ± 0.09	323 ± 14	0.92 ± 0.12

Table 7. *Effect of incubation with a glucose-free medium on lactate and pyruvate release and tissue concentrations of glucose 6-phosphate, citrate and malate in the soleus and extensor digitorum longus*

Incubation conditions were as described in Table 2 except that glucose was not added to the incubation or preincubation medium.

Additions to incubation medium		Net release (μmol/h per g)		Tissue concentration (nmol/g)		
Acetoacetate (1.7 mM)	Insulin (0.1 unit/ml)	Lactate	Pyruvate	Glucose 6-phosphate	Citrate	Malate
Soleus						
−	−	1.7 ± 0.4 (8)		103 ± 11 (3)	113 ± 19 (3)	126 ± 11 (3)
−	+	2.2 ± 0.4 (9)		52 ± 0.7 (3)	160 ± 9 (3)	96 ± 12 (3)
+	−	3.0 ± 0.1 (3)	0.79 ± 0.06 (4)	118 ± 16 (3)	144 ± 28 (3)	100 ± 27 (3)
+	+	2.1 ± 0.2 (14)	0.48 ± 0.06 (10)	39 ± 8.4 (3)	116 ± 14 (8)	106 ± 11 (8)
Extensor digitorum longus						
−	−	4.8 ± 0.2 (7)		113 ± 5 (3)	139 ± 17 (3)	
−	+	3.0 ± 0.6 (8)		81 ± 27 (3)	203 ± 46 (3)	
+	−	3.0 ± 1.1 (3)		134 ± 15 (3)	179 ± 37 (3)	
+	+	4.6 ± 0.7 (3)		119 ± 19 (3)	281 ± 39 (3)	

Substantial increases in glucose 6-phosphate and fructose 6-phosphate were also noted in the extensor digitorum longus after the addition of insulin to the incubation medium. Addition of acetoacetate caused modest increases in the concentrations of glucose 6-phosphate, fructose 6-phosphate and citrate, in both the presence and the absence of insulin. As noted above, these changes were not associated with a decrease of lactate release or glucose uptake.

Inhibition of glucose uptake in the soleus by acetoacetate was associated with an increase in intracellular glucose to 0.92 mM (Table 6), a concentration approximately tenfold greater than the overall K_m of muscle hexokinase(s) for glucose (Ozand *et al.*, 1962). Although this suggests that the uptake of glucose was inhibited at the hexokinase step, it does not rule out the possibility that glucose transport was also inhibited. Against this notion, however, is the observation that the uptake of glucose was not inhibited by acetoacetate in the extensor digitorum longus. Therefore, unless the transport of glucose into the cell is differentially affected by acetoacetate in the two muscles, such a block seems unlikely. This conclusion is also supported by the finding by Cuendet *et al.* (1975) that the uptake of non-metabolized sugars such as 3-*O*-methylglucose and 2-deoxyglucose is not decreased when the soleus is incubated with 3-hydroxybutyrate.

Incubation with a glucose-free medium. The release of lactate (cf. Tables 7 and 3) and the con-

centrations of malate and glucose 6-phosphate in tissue (cf. Tables 7 and 5) were markedly diminished when the soleus was incubated in a glucose-free medium. Under these conditions, acetoacetate failed to inhibit the release of lactate or increase tissue concentrations of glucose 6-phosphate, citrate or malate, even though rates of acetoacetate utilization were the same as when glucose was present (results not shown). Where it was measured, the release of pyruvate was less diminished than that of lactate, suggesting that the cytosol was more oxidized (cf. Tables 3 and 7).

Incubation with a glucose-free medium caused a relatively smaller decrease in the release of lactate in the extensor digitorum longus, presumably reflecting the higher glycogen content (see Peter *et al.*, 1972; Close, 1975) and phosphorylase activity (see Peter *et al.*, 1972; Baldwin *et al.*, 1973) of white and fast-twitch red muscle (cf. Tables 3 and 7). In addition, the concentration of glucose 6-phosphate tended to be less diminished. As with the soleus, acetoacetate did not significantly decrease glycolysis when added to the glucose-free medium, although it may have slightly increased tissue citrate.

It is noteworthy that insulin diminished the concentration of glucose 6-phosphate in both muscles. This did not occur in the presence of glucose (Table 5) and is probably related to the fact that insulin stimulates glycogen synthesis and inhibits glycogenolysis. Why insulin did not also diminish glycolysis in these experiments is not clear.

Discussion

Acetoacetate and 3-hydroxybutyrate are major oxidative substrates of skeletal muscle during starvation, in both man (Gammeltoft, 1949–1950; Owen & Reichard, 1971) and experimental animals (Ruderman & Goodman, 1973). There is general agreement that they displace glucose as a fuel in voluntary skeletal muscle by inhibiting pyruvate dehydrogenase (see Berger *et al.*, 1976); however, it is not clear whether they also inhibit glucose uptake and glycolysis (reviewed by Ruderman *et al.*, 1969, 1977; see Berger *et al.*, 1976). The present study suggests that this, in part, may be related to the heterogeneity of muscle fibres in voluntary muscle. Thus glucose uptake and glycolysis were decreased and glycogen was enhanced when a slow-twitch red muscle such as the soleus was incubated with acetoacetate, whereas neither was decreased in the extensor digitorum longus, which is composed of 50% white and 50% fast-twitch red fibres. Whether the moderate increases in the concentrations of citrate and hexose monophosphates in the extensor digitorum longus were solely due to changes in its fast-red component and whether inhibition of glycolysis in these fibres was missed because of the presence of white muscle remains to be determined. This would not be surprising, since fast-twitch red muscle resembles heart more closely than does white muscle, in that it has a far higher activity of the enzymes of ketone-body utilization (Winder *et al.*, 1974) as well as a greater overall capacity for oxidative metabolism (see Dawson & Romanul, 1964; Peter *et al.*, 1972; Newsholme & Start, 1973; Close, 1975).

The high rate of glycolysis in incubated muscles is of interest, since the ability of acetoacetate to inhibit glycolysis in the soleus appear to be related to the rate of glycolysis. In the present study, this was evident from the fact that incubation of the soleus with a glucose-free medium diminished glycolysis by

Table 8. *Inhibition of glycolysis by ketone bodies in various tissues*

Results are means and except for brain were based on studies carried out *in vitro* or in perfused organs. Rates of glycolysis in brain were calculated from arteriovenous differences for glucose measured *in vivo* in anaesthetized fed rats and rats with diabetic ketoacidosis induced by streptozotocin. It was assumed that blood flow to brain was 1 ml/min per g of brain (Miller *et al.*, 1975) and that disposition of glucose into other pathways was negligible. Although the degree to which acetoacetate inhibited glycolysis was variable, in all tissues with a high glycolytic rate it caused an increase in citrate and a block at phosphofructokinase.

Tissue	Experimental conditions	Rate of glycolysis (μmol/g per h) Control	Ketone bodies	Reference
(*a*) Tissues with high glycolytic rate				
Soleus	Insulin + glucose	12.4	8.6	The present paper
Perfused heart	Insulin + glucose	64	37	Randle *et al.* (1966)
Submaxillary gland	Glucose	12	10.5	Thompson & Williamson (1975)
Brain	*In vivo*	29	15	Ruderman *et al.* (1974)
(*b*) Tissues with low glycolytic rate				
Perfused hindquarter	Insulin + glucose, resting muscle	2.6	2.3	Berger *et al.* (1976)
Soleus	Insulin, no glucose	2.1	2.0	The present paper

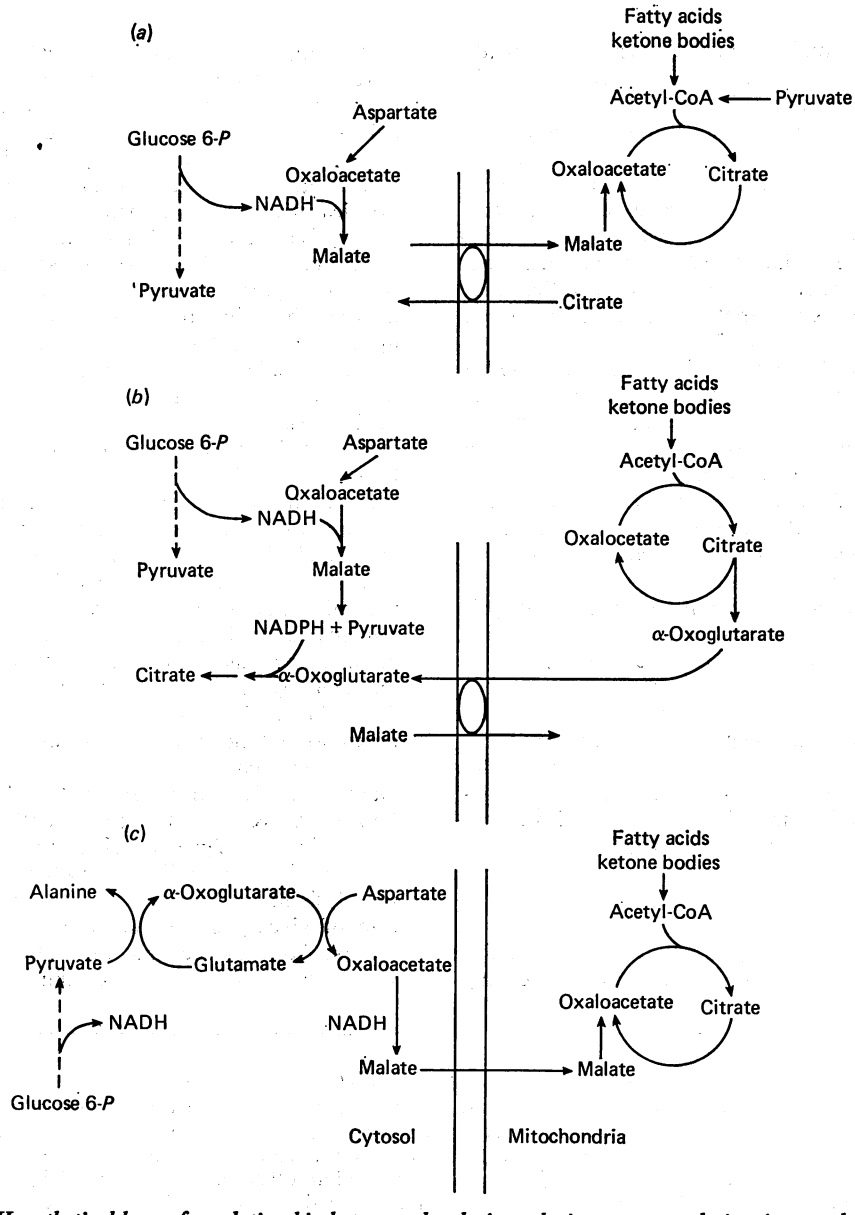

Fig. 1. *Hypothetical bases for relationship between glycolysis and citrate accumulation in muscle cytosol*
(*a*) NADH generated during glycolysis is used for the formulation of malate, in which form the carbon skeletons of aspartate and oxaloacetate can enter the mitochondria. Malate may also be the antiport for citrate efflux from muscle mitochondria. (*b*) Conversion of malate into pyruvate provides NADPH for the conversion of α-oxoglutarate into citrate in the cytosol. (*c*) Aspartate in the cytosol must be converted into malate in order to enter the mitochondria. In addition to generating NADH for this process, glycolysis provides pyruvate, which accepts the amino group of aspartate.

80–90%, and eliminated the effect of acetoacetate on both the release of lactate and the tissue concentration of citrate. Likewise, during incubation with glucose but no insulin (glycolysis less by 40%), acetoacetate caused a smaller rise in citrate and failed to inhibit glycolysis. In keeping with these observations, a review of the literature reveals that tissues in which ketone bodies and fatty acids diminish glycolysis (owing to inhibition of phosphofructokinase by citrate) are characterized by a high rate of glycolysis (Table 8).

The link between glycolysis and citrate accumulation in muscle incubated with acetoacetate remains to be determined. It is unlikely that a high rate of glucose metabolism increases muscle citrate simply

by increasing the generation of acetyl-CoA, since glucose oxidation accounted for less than 10% of the acetyl-CoA formed in the soleus incubated with acetoacetate (cf. Tables 2 and 3), and the utilization of acetoacetate was not increased by glucose. Other possibilities are depicted in Fig. 1. One of these is that glycolysis affects citrate metabolism by generating NADH and secondarily malate in the cytosol. Malate, unlike oxaloacetate, can enter the mitochondria, where it is oxidized to oxaloacetate. Thus it could be vital for citrate formation when oxaloacetate is rate-limiting. Also, malate is a counterion for the transport of citrate out of mitochondria in cardiac muscle (Cheema-Dhadli *et al.*, 1975). Therefore the flow of citrate into the cytosol could be diminished if the cytosolic malate concentration is low. Finally, malate could be necessary for citrate accumulation if the latter was synthesized in large part from α-oxoglutarate in the cytosol, as suggested by Safer & Williamson (1973).

A final possibility stems from the observation that, in a rat heart perfused with acetate, the oxaloacetate, utilized in the synthesis of citrate, is derived from aspartate, and pyruvate serves as a nitrogen acceptor (Randle *et al.*, 1970). Our data did not address the possibility that pyruvate was limiting for aspartate deamination. However, in other studies (E. Maizels, N. B. Ruderman & M. N. Goodman, unpublished work) we failed to observe a significant decrease in either the release or the tissue concentration of alanine when the soleus was incubated with a glucose-free medium. Whether a decrease in alanine formation was masked by a compensatory increase in proteolysis (owing to the absence of glucose; see Fulks *et al.*, 1975) remains to be determined, however.

Except for the fact that glucose oxidation was not increased, the metabolism of the soleus and extensor digitorum longus is very similar to that of muscle *in situ*, during sustained exercise of a mild to moderate intensity; i.e. the uptake of glucose and glycolysis are accelerated, but not to the extent that the ratio lactate/pyruvate is increased (see Felig & Wahren, 1971) or tissue phosphocreatine and ATP are diminished. In this regard, it is noteworthy that an increase in tissue citrate has been observed in fast-red muscle (obtained from the vastus lateralis), as well as in the soleus, of rats given a meal of fat plus heparin to raise plasma unesterified fatty acids, and then exercised on a treadmill for 30min (Rennie *et al.*, 1976). These investigators also noted a smaller depletion of muscle glycogen in the two types of red muscle. Similar changes were not observed in white-muscle fibres obtained from the vastus lateralis.

It is less likely that the uptake of glucose and glycolysis are substantially inhibited by lipid substrates in red muscle of an intact animal at rest, since their rates of glycolysis are proably much lower than those observed in incubated muscle (cf. Ruderman *et al.*, 1971). In addition, we have not observed increases in citrate, glucose 6-phosphate or fructose 6-phosphate in the soleus of starved or diabetic rats biopsied *in situ* (E. Maizels, M. N. Goodman & N. B. Ruderman, unpublished work) and Rennie *et al.* (1976) have not observed substantial changes in these metabolites or in muscle glycogen in rats fed on a meal of fat. Less clear is whether fatty acids and ketone bodies inhibit glucose uptake and glycolysis during severe exhaustive exercise. In studies with the perfused rat hindquarter, Berger *et al.* (1976) noted that acetoacetate inhibited glucose oxidation during isometric exercise induced by bilateral sciatic-nerve stimulation at a rate of 5 per s. On the other hand, neither glucose uptake nor glycolysis was decreased, although tissue citrate was moderately increased. Whether inhibition of glycolysis was missed because of a predominance of white muscle in the exercising portion of the hindquarter or because of other metabolic alterations (e.g. ATP was decreased) is not known.

In man, glucose is usually the major fuel of skeletal muscle during the early phases of moderately intense exercise (see Felig & Wahren, 1975). However, if the exercise is sustained, glucose is partially replaced by unesterified fatty acids and, if their concentration is high, by ketone bodies (Havel *et al.*, 1967; Felig & Wahren, 1975; Wahren *et al.*, 1975). Similar changes probably occur in experimental animals (see Rennie *et al.*, 1976). Since it is predominantly red muscle that is working at a high rate during sustained exercise, we would speculate that inhibition of glycolysis by free fatty acids and ketone bodies would be most readily demonstrable in this circumstance and possibly during the recovery period after exercise, when the concentrations of unesterified fatty acids and ketone bodies in plasma are high (Johnson *et al.*, 1969) and muscle glycogen is being repleted (Terjung *et al.*, 1974).

We thank Dr. Mary Jane Spiro and Dr. Robert Spiro for their advice in setting up the chromatographic method for separating glucose and ^3H_2O. The technical assistance of Mrs. Zenta Skulte and Mrs. Ludmilla Klavins is also gratefully acknowledged. These studies were supported in part by U.S. Public Health Service grants AM 15272 and AM 19514 and by a grant from the American Diabetes Association. N. B. R. was the recipient of a Career Research and Development Award from the National Institutes of Health (AM 15729) and M. N. G. of a Research and Development Award from the American Diabetes Association.

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