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The metabolic state of muscle in the isolated perfused rat hemicorpus in relation to rates of protein synthesis

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1. Measures of perfusion adequacy in perfused rat hemicorpus preparations were investigated as potential indices of tissue function during studies of muscle protein metabolism. 2. Perfusion under normal conditions for up to 80 min resulted in rates of protein synthesis and concentrations of ATP in muscle that were similar to those *in vivo*, but phosphocreatine in muscle gradually decreased and muscle lactate increased. Hypoxic conditions led to lower rates of protein synthesis, lower phosphocreatine and raised lactate contents in muscle compared with normal perfusions, and ATP was slightly decreased. Hypoxic preparations also released more lactate and K^+ into the medium and had higher perfusion pressures, but glucose uptake and muscle water content were not altered. In totally ischaemic muscle, concentrations of ATP and phosphocreatine were even lower than in hypoxic muscle, and that of lactate was higher. 3. From 11 preparations perfused for 60 min under normal conditions, three were selected on the basis of lower muscle ATP content than the others. Preparations with low ATP also showed lower muscle phosphocreatine concentrations, O_2 uptake and CO_2 output, as well as higher perfusion pressure and muscle lactate concentrations than in the remaining preparations, but muscle water, ADP and AMP concentrations and lactate and K^+ flux were no different. 4. In perfusions extended to 3 h, deterioration of function was more apparent. There were significant correlations between rates of protein synthesis and the concentrations of ATP, phosphocreatine and lactate in two different muscles ($r = 0.756$–0.929), but not with any of the other indices investigated. 5. Taken overall, these experiments showed that concentrations of ADP, AMP and water in muscle, rates of lactate and glucose metabolism, K^+ output, perfusion pressure and blood gas parameters were unsuitable for distinguishing unsound from sound preparations, because they did not consistently demonstrate differences, or could not be ascribed to only muscle metabolism. 6. It was found that ATP, phosphocreatine and lactate concentrations in muscle were the best indicators of impaired metabolic state in studies of protein synthesis. Measurements of these could be used on a routine basis for rejecting unsatisfactory preparations.

Isolated muscle preparations, such as the perfused rat hemicorpus, have been used to investigate a variety of metabolic processes, including the regulation of protein turnover (see, e.g., Jefferson, 1980; Preedy & Garlick, 1981, 1983). However, the removal of an organ from the donor animal must result in some interruption of the tissue's homoeostatic mechanisms and alterations in its normal metabolism. Despite this, only a few studies have attempted to investigate the integrity of the tissue after isolation. Although one can never be certain that the performance of an isolated tissue is exactly the same as *in vivo*, it is important to know whether the metabolism of major metabolites such as ATP is altered, and the degree to which the isolation procedure has altered the particular system under study. For example, low cellular concentrations of

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ATP may not only alter the rates of ATP-requiring processes such as protein synthesis, but may also influence the binding of hormones (Draznin *et al.*, 1980), so altering the response of the tissue to hormonal stimuli. Furthermore, excessive variability of experimental results observed *in vitro* may occur not only because of variations within the groups of donor animals: tissue dysfunction may arise in a proportion of the preparations as a result of the isolation or perfusion procedures. It is therefore likely that in any group of preparations some will be less satisfactory than the rest, and it is important to be able to distinguish these by means of physiological or biochemical measurements.

This paper describes a systematic investigation of tissue integrity in an isolated perfused muscle preparation. Our first approach (Expt. 1) was to compare muscle perfused normally with that from similar rats *in vivo*. Tissue dysfunction was then deliberately induced by hypoxia and total ischaemia, in order to identify biochemical changes indicative of unsatisfactory perfusions. These parameters were then examined in a second set of perfusions (Expt. 2) prepared normally, but separated into two groups on the basis of their ATP concentrations, to see which measurements could be used for identifying unsatisfactory perfusions which had occurred routinely, rather than by deliberate intervention. Finally, in Expt. 3, an attempt was made to find a relationship between the selected criteria and rates of muscle protein synthesis. As in a previous study (Preedy & Garlick, 1983), we were able to show that muscles perfused for short periods were in general able to synthesize protein at a rate comparable with that *in vivo*. However, when the period of perfusion was extended, correlations between the rate of protein synthesis and some of the selected criteria were observed. These particular criteria were then used on a routine basis for determining whether the experimental results associated with a particular preparation should be rejected, on the grounds that it could not be expected to display the same behaviour as other preparations in the group.

Materials and methods

Animals

Three separate groups of male Wistar rats were obtained from Charles River (Margate, Kent, U.K.), and were housed singly in wire-bottomed cages in a humidified temperature-controlled environment on a 12h-light/12h-dark cycle. Animals were fed *ad lib.* on a 18% (w/w) protein diet (Payne & Stewart, 1972) for a minimum of 5 days, until they weighed 175–215g. Two rats were used for perfusions daily, between 12:00 and 17:00h. When appropriate, measurements *in vivo* were obtained from rats killed at the same time of day. All rats were allowed free access to food and water until removed from the cage for measurement.

Materials

Biochemicals were obtained from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.), except for tyrosine decarboxylase and heparin, which were from Sigma Chemical Co. (Poole, Dorset, U.K.). Radioisotopes were from Amersham International (Amersham, Bucks., U.K.). Materials for electron-microscopic studies were obtained from Emscope Laboratories (Ashford, Kent, U.K.) and remaining chemicals were from B.D.H. Ltd. (Poole, Dorset, U.K.).

Perfusion of the hemicorpus

This procedure was based on the methods of Ruderman *et al.* (1971) and Jefferson (1975), and has previously been described in detail (Preedy & Garlick, 1981, 1983). Briefly, after anaesthesia with pentobarbitone (50mg/kg body wt.) and heparin treatment (5000i.u./kg body wt.) of the rat, the blood vessels to non-muscle tissues were ligated and the internal organs removed. After aortic cannulation, the preparation with the skin attached was hemisected above the diaphragm and flushed (10ml/min) with 50ml of non-recirculating perfusion medium gassed (300ml/min) with O_2/CO_2 (19:1). The entire preparation was then transferred to a temperature-controlled cabinet maintained at 37.5°C, and perfused with recirculating medium (usually 100ml). Perfusions were timed from this point. In one study (Expt. 1) intermediate values were obtained by ligating the left common iliac vessels, decreasing the flow rate accordingly, and removing the entire left leg from above the upper thigh. This procedure did not cause overt degenerative changes in the remaining perfused muscle. In a controlled study we found that neither ATP nor phosphocreatine concentrations, nor rates of protein synthesis, were affected by this technique. The perfusate, in bicarbonate buffer, pH7.45 (Krebs & Henseleit, 1932), contained 45% (v/v) human erythrocytes (approx. 12–16g of haemoglobin/100ml of perfusate), 5.25% (w/v) bovine serum albumin (Cohn Fraction V), 0.043mM-pyruvate, 11mM-glucose, 1i.u. of heparin/ml and normal plasma concentrations of amino acids as described previously (Preedy & Garlick, 1983). Insulin (25m-i.u./ml) was added to all perfusions. In hypoxic preparations the perfusate was gassed with N_2, which displaced some, but not all, of the O_2. These preparations continued to remove O_2 from the perfusate, but at 20–30% of the control rates. Thus, whereas the arterio-venous difference in the percentage O_2 saturation of haemoglobin in normal perfusions was $45.5 \pm 4.4\%$ (4), $52.2 \pm 5.2\%$

(4) and 54.4±6.5% (4) at 0, 40 and 80min respectively, corresponding values for hypoxic perfusions were 13.0±3.0% (5), 9.8±2.8% (4) and 10.5±1.6% (4). In the totally ischaemic group, preparations were transferred to the perfusion cabinet (at 37.5°C) after cannulation, but no medium was passed through the aorta.

Measurement of protein synthesis in the hemicorpus and in vivo

Rates of protein synthesis in the hemicorpus were estimated after a preliminary period of perfusion (15min), after which 38ml of recirculating perfusate was replaced by a fresh batch containing 4μCi (28μmol) of L-[U-^{14}C]tyrosine, as described previously (Preedy & Garlick, 1983). At the end of the incorporation period, muscles were rapidly dissected out and plunged into liquid N_2. Fractional rates of protein synthesis were then calculated from the specific radioactivity of tyrosine in protein (S_B) and that of the free tyrosine in muscle homogenates (S_A), at time t (days) after introduction of the label, from the formula:

$$k_s = (S_B \times 100)/(S_A \times t)$$ (Preedy & Garlick, 1983)

where k_s is the fractional rate of synthesis, expressed as a percentage of the protein synthesized per day. To measure rates of protein synthesis *in vivo*, rats were given constant infusions of L-[U-^{14}C]tyrosine as described by Garlick *et al.* (1975). Fractional rates of protein synthesis were calculated from the relationship between specific radioactivities of protein-bound and tissue-free tyrosine in samples taken at the end of 3h infusion (Garlick *et al.*, 1973). The labelled amino acid, tyrosine, was the same for measurements both *in vivo* and *in vitro*, to eliminate possible differences arising from the use of different amino acids. A detailed discussion of the accuracy of these measurements is given by Preedy & Garlick (1983). The techniques for preparation of samples and measurement of tyrosine specific radioactivity have been described previously (Preedy & Garlick, 1983).

Measurement of tissue metabolites in vivo and in vitro

After anaesthesia by intraperitoneal injection of pentobarbitone, samples from intact animals *in vivo* were taken 5min after intraperitoneal injection of heparin. Pre-perfusion samples were removed after ligation of blood vessels and hemisection, and immediately before aortic cannulation. During the period between injection of heparin and removal of muscle, animals were given O_2 via a cranial hood. In both cases muscles were quickly exposed and freeze-clamped between aluminium blocks kept in liquid N_2. After storage (−196°C), muscles were ground to a fine powder under liquid N_2, precipitated with 4vol. of 0.9M-$HClO_4$, and the supernatants neutralized to pH7.4±0.1 with 4M-KOH, and stored at −196°C until analysis.

Plasma samples for K^+ determination were obtained by centrifuging (3000*g*, 15min, 0–4°C) small samples of perfusate removed during the experiments. For the measurement of rates of lactate and glucose metabolism by the hemicorpus, whole perfusate was transferred directly into ice-cold 0.9M-$HClO_4$ and 0.16% (w/v) uranyl acetate in 0.9% (w/v) NaCl respectively, and the supernatants were stored at 0–4°C. In these experiments a portion of the medium used to perfuse each hemicorpus was kept aside in the perfusion cabinet at 37.5°C, and sampled concurrently with the recirculating medium from the hemicorpus. This enabled the rates of K^+, lactate and glucose metabolism by the erythrocytes in each perfusion to be estimated, in order to give a more accurate measurement of the metabolism by the hemicorpus alone. To make our results comparable with other perfusion studies, rates of K^+, glucose and lactate metabolism (also O_2 uptake; see below) were expressed in terms of the amount of skeletal muscle in each hemicorpus (see Preedy & Garlick, 1981).

Metabolites were assayed as follows: ATP and phosphocreatine by the method of Lamprecht *et al.* (1974); ADP and AMP by the method of Jaworek & Gruber (1974); lactate by the method of Gutman & Wahlefeld (1974); glucose with the GOD–Perid kit, from Boehringer; K^+ by the method of Collins & Polkinhorne (1952); tissue water by heating at 110°C to constant weight.

Measurement of blood gases, haemoglobin and perfusion pressure

Samples for the determination of blood gases, haemoglobin or pH were collected into silicone-treated glass syringes, via the aortic or venous cannulae as described by Preedy (1981). Blood gas parameters [the partial pressure of dissolved O_2 (pO_2), percentage O_2 saturation of haemoglobin (sO_2), partial pressure of dissolved CO_2 (pCO_2), total CO_2 (tCO_2), pH and haemoglobin] were measured on an ABL blood-gas analyser (Selman & Tait, 1976), except in Expt. 3, where the sO_2 was measured with a Kipp Haemoreflector. Rates of O_2 uptake were calculated from the sO_2, haemoglobin concentrations and perfusate flow rates. As perfusate flow was induced by a peristaltic pump, arterial perfusion pressure was calculated from the mean of the minimum and maximum, obtained from an in-line pressure gauge (Preedy, 1981).

Microscopic studies

Plantaris muscles were rapidly dissected out and bisected longitudinally. One half, for light microscopy, was plunged into buffered formaldehyde for fixation, staining and sectioning by standard pro-

cedures. The remaining half, for electron microscopy, was rapidly fixed in buffered glutaraldehyde and cacodylate solution, then quickly cut into 0.5mm cubes, before staining and sectioning by standard procedures. Sections of tissue areas were observed at random, until a longitudinal section containing myofibrils without associated nuclei was present. At least three samples were examined for each treatment.

Statistics

Results are presented as means ± S.E.M., with the numbers of observations in parenthesis. Differences between groups were assessed by Student's *t* test.

Results

In Expt. 1 a single batch of rats was divided into five groups, which were designated: *in vivo*, preperfusion, normal perfusion, hypoxic perfusion and total ischaemia (for further details see Table 1 and the Materials and methods section).

Muscles were first examined morphologically. This approach has been used previously to assess muscle integrity during perfusion (Ruderman *et al.*, 1971; Strohfeldt *et al.*, 1974; Reimer *et al.*, 1975). In the present study, visual examination of the hindquarters of the totally ischaemic preparations revealed that the feet were usually darker in tone than those of intact rats, probably reflecting the contents of reduced haemoglobin in totally ischaemic tissue. The visual appearance of the limbs in the normal and hypoxic perfusions was not noticeably different from that *in vivo*. On light-microscopic examination (magnification ×480), haematoxylin- and eosin-stained myofibrils from totally ischaemic tissues at 40min and 80min of perfusion were similar to those from intact rats. Although there were signs of pyknotic nuclei in these totally ischaemic preparations, they were not easy to distinguish. The myofibrils from normally perfused tissues at 40min and 80min were similar to those in intact animals. Electron micrographs (magnification ×8000) showed marked differences between totally ischaemic specimens and those *in vivo*. There was an abundance of swollen mitochondria in the totally ischaemic samples at 40 and 80min, which was associated with a loss of fine structure. The cristae were invariably disrupted and many mitochondria were ruptured. However, in some specimens *in vivo* a few of the mitochondria also fitted this description. This may have been due to inadequacies of the conventional fixing techniques (Minassian & Huang, 1979). There did not appear to be any obvious difference between micrographs taken from specimens *in vivo* and those perfused normally for 40 or 80min. Hypoxic preparations perfused for 40 and 80min were inconsistent, displaying characteristics of both normally perfused and totally ischaemic preparations. The appreciable degree of variability in the appearance of the electron micrographs from preparations both *in vivo* and *in vitro* may have reflected the heterogeneity of different regions of the muscle. On the basis of these studies, we concluded that microscopic examination was excessively time-consuming, involved an element of subjectivity and was not sufficiently reproducible to warrant routine use as a measure of tissue integrity.

Neither normal nor hypoxic perfusion resulted in changes in water content, but the perfusion pressure was lower in hypoxic perfusions (Table 1). Biochemical analysis of the tissue (Table 1) demonstrated that immediately after the isolation procedure (pre-perfusion), ATP, phosphocreatine, and to a lesser extent lactate, were maintained at concentrations close to those *in vivo*. However, during normal perfusion there was a progressive decrease in phosphocreatine and a progressive increase in lactate, but ATP remained at values *in vivo*. Despite the changes in phosphocreatine and lactate, rates of protein synthesis in normal perfusions were comparable with those *in vivo*. Both hypoxia and total ischaemia further decreased phosphocreatine and elevated lactate concentrations. The ATP concentration was also lowered by hypoxia, but not significantly. With total ischaemia ATP content was maintained at 40min, presumably at the expense of phosphocreatine (Haljamae & Enger, 1975), but was significantly decreased after 80min. Protein synthesis was not measured in the totally ischaemic group, but in the hypoxic perfusions it was decreased to about half the rate seen either *in vivo* or in normal perfusions (Table 1).

The rates of lactate and K^+ release and of glucose uptake were also measured in normal and hypoxic perfusions (Table 2). Hypoxia increased the release of lactate, but did not alter glucose uptake. K^+ release was also significantly increased during the latter half of the perfusions, when expressed either as μmol/min per g of muscle or as a percentage of total muscle K^+.

In evaluating the above observations, it is important to consider that during the course of normal experimentation tissue dysfunction will occur unintentionally rather than by deliberate induction of hypoxia or ischaemia. In Expt. 2, therefore, we perfused a series of hemicorpus preparations in the normal way, and then looked for relationships between the various parameters (Tables 3–5). On the basis of ATP concentrations in three different muscles, we provisionally divided the preparations into two groups: the three preparations assigned to Group I had low concentrations

Table 1. *Effect of the isolation procedure (pre-perfusion), normal perfusion, hypoxic perfusion and total ischaemia on muscle concentrations of ATP, phosphocreatine and lactate, water content, arterial perfusion pressure and rates of muscle protein synthesis in the isolated rat hermicorpus preparations in Expt. 1*

Groups were as follows. *In vivo:* rats were anaesthetized, heparin treated and given O_2 as in preparation for perfusion, before exposure and freeze-clamping of muscles. Pre-perfusion: rats were prepared for perfusion and muscles were taken immediately before aortic cannulation. Normal perfusion: perfused as described in the Materials and methods section, but with no preliminary perfusion period. Hypoxic perfusion: prepared and perfused as in normal perfusions, but the perfusate was gassed with N_2 to decrease O_2 uptake by 70–80%. Total ischaemia: rats were prepared and cannulated as in normal perfusion, but no medium was perfused. Fractional rates of protein synthesis (percentage of the tissue protein renewed per day by synthesis) in gastrocnemius muscle were measured *in vivo* by constant infusion of [^{14}C]tyrosine for 3 h and in the hemicorpus by incorporation of [^{14}C]tyrosine between 0–40 min and 0–80 min as described in the Materials and methods section. Values for muscle water were measured in tibialis anterior, and ATP, phosphocreatine and lactate concentrations were measured in quadriceps at 40 min and 80 min. The intermediate values at 40 min were obtained by removal of one leg, with decrease of perfusate flow (see the Materials and methods section). The statistical significance of differences from values obtained in normal perfusions at the corresponding time point are indicated as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. None of the differences between values *in vivo* and pre-perfusion were statistically significant.

Treatment	Perfusion time (min)	Arterial pressure (mmHg/preparation)	Water (%, w/w)	Content (μmol/g wet wt. of muscle)			Protein synthesis, k_s (%/day)
				ATP	Phosphocreatine	Lactate	
In vivo	–	–	76.9±0.2 (4)	6.1±0.4 (7)	9.6±0.7 (7)	4.2±0.3 (8)	12.4±0.6 (5)
Pre-perfusion	–	–	–	6.9±0.6 (5)	9.3±1.5 (5)	6.0±1.9 (5)	–
Normal perfusion	40	86±3 (8)	76.4±0.3 (8)	5.8±0.3 (6)	8.7±0.6 (7)	14.8±2.0 (8)***	15.9±0.9 (7)
	80	86±6 (8)	77.4±0.2 (8)	6.5±0.3 (8)	5.5±0.5 (8)***	24.2±2.5 (7)***	12.8±1.6 (8)
Hypoxic perfusion	40	71±6 (6)*	77.3±0.2 (6)	4.9±0.4 (7)	4.3±1.6 (7)*	31.2±5.4 (5)**	7.0±1.1 (4)***
	80	69±1 (5)*	77.7±0.5 (4)	5.5±0.5 (5)	4.2±0.9 (4)	37.9±10.6 (3)	6.9±1.6 (6)*
Total ischaemia	40	–	–	6.4±1.1 (4)	1.3±0.4 (4)***	42.3±2.8 (4)***	–
	80	–	–	2.8±0.4 (4)***	0.5±0.3 (4)***	62.0±2.2 (4)***	–

Table 2. *Effect of hypoxia on lactate release, glucose uptake and K^+ release in the perfused rat hemicorpuses in Expt. 1*

Normal and hypoxic perfusions were prepared as described in the Materials and methods section and in the legend to Table 1. For the measurement of lactate release, glucose uptake and K^+ release by the hemicorpus, corrections were made for the metabolism by the erythrocytes alone. To make rates comparable with other studies, rates are based on the assumption that the contribution of other non-muscle tissues was negligible. The percentage loss of K^+ from muscle was derived from the measurement of muscle K^+ content (Preedy, 1981). Significance of difference between normal and hypoxic perfusions: $^{**}P < 0.01$; $^{***}P < 0.001$.

Parameter	Perfusion time (min)	Normal perfusion	Hypoxic perfusion
Lactate release	7–40	0.013±0.018 (8)	0.164±0.023 (6)***
(μmol/min per g of muscle)	40–80	0.075±0.022 (8)	0.256±0.039 (3)**
Glucose uptake	7–40	0.310±0.022 (8)	0.241±0.028 (6)
(μmol/min per g of muscle)	40–80	0.238±0.038 (8)	0.222±0.035 (4)
K^+ release	7–40	0.021±0.010 (7)	0.036±0.009 (5)
(μmol/min per g of muscle)	40–80	0.038±0.008 (7)	0.088±0.009 (3)**
K^+ loss (cumulative loss as % of	7–40	0.86±0.42 (7)	1.49±0.38 (5)
total muscle K^+)	40–80	1.93±0.43 (7)	4.52±0.44 (3)**

Table 3. *Concentrations of muscle ATP, ADP, AMP, phosphocreatine and lactate in vivo, and in Groups I and II of the perfused rat hemicorpuses in Expt. 2*

At the end of 60 min perfusion, muscles were rapidly exposed and freeze-clamped for subsequent analysis of tissue metabolites. Samples *in vivo* were obtained as described in the Materials and methods section. Perfusions were divided into Groups I and II on the basis of ATP concentrations in all three muscles (see the text). Significance of difference from values for Group II: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Muscle	*In vivo*	Group I	Group II
	ATP (μmol/g wet wt.)		
Gastrocnemius	4.7±0.8 (6)	3.5±0.8 (3)*	5.7±0.5 (8)
Quadriceps	5.7±0.5 (6)	3.6±0.3 (3)*	5.5±0.4 (8)
Quadratus lumborum	6.4±1.4 (6)	3.5±0.3 (3)*	5.8±0.5 (8)
	ADP (μmol/g wet wt.)		
Gastrocnemius	0.76±0.08 (6)	0.86±0.13 (3)	0.83±0.04 (8)
Quadriceps	0.72±0.09 (5)	0.65±0.07 (3)	0.75±0.06 (8)
Quadratus lumborum	0.83±0.04 (5)	0.79±0.07 (3)	0.91±0.10 (8)
	AMP (μmol/g wet wt.)		
Gastrocnemius	0.074±0.009 (6)	0.100±0.046 (3)	0.091±0.008 (8)
Quadriceps	0.059±0.008 (4)	0.131±0.053 (3)	0.084±0.011 (8)
Quadratus lumborum	0.082±0.004 (5)	0.109±0.030 (3)	0.093±0.013 (8)
	Phosphocreatine (μmol/g wet wt.)		
Gastrocnemius	10.5±1.7 (6)	3.7±2.5 (3)*	11.7±1.3 (8)
Quadriceps	12.8±1.5 (5)	5.1±2.4 (3)*	11.3±1.1 (8)
Quadratus lumborum	16.5±1.0 (5)**	4.1±1.6 (3)*	9.4±1.1 (8)
	Lactate (μmol/g wet wt.)		
Gastrocnemius	1.7±0.4 (6)**	13.7±4.6 (3)*	6.1±0.9 (8)
Quadriceps	1.6±0.3 (6)***	10.9±6.6 (3)	6.2±0.7 (8)
Quadratus lumborum	2.1±0.4 (5)**	11.1±4.9 (3)	11.8±2.0 (8)

of ATP relative to the remaining eight preparations, which were assigned to Group II. Subsequent analysis of preparations perfused for 3h demonstrated the importance of ATP, as correlations were observed between ATP and rates of muscle protein synthesis (see below).

Table 3 shows that gastrocnemius and quadriceps muscles in Group II had concentrations of ATP, ADP, AMP and phosphocreatine that were not significantly different from those *in vivo*, but lactate was increased. Quadratus lumborum muscle behaved similarly, except that phospho-

Table 4. *Lactate uptake, K^+ release, muscle water content and arterial perfusion pressure in Group-I and Group-II perfusions in Expt. 2*

Perfusions were prepared and separated into Groups I and II as described in the text and in the legend to Table 3. Rates of lactate and K^+ flux were obtained by sampling the recirculating perfusate at 0 and 60 min. At the same time a separate vessel of perfusate in the absence of the hemicorpus was sampled to make allowances for the metabolism by erythrocytes alone. Lactate uptake and K^+ release are expressed in terms of the amount of skeletal muscle in the hemicorpus (Preedy & Garlick, 1981). The percentage loss of K^+ was calculated from the known concentration of K^+ in skeletal muscle (Preedy, 1981). Muscle water concentration was obtained in quadriceps. Significance of difference between Group I and Group II: $^{**}P < 0.01$.

Parameters	Perfusion time (min)	Group I	Group II
Lactate uptake (μmol/min per g of muscle)	0–60	0.083±0.033 (3)	0.064±0.023 (8)
K^+ release (μmol/min per g of muscle)	0–60	0.049±0.018 (3)	0.066±0.015 (7)
K^+ loss (cumulative loss as % of total muscle K^+)	0–60	3.8±1.4 (3)	5.1±1.1 (7)
Muscle water content (% of wet wt.)	60	76.0 (2)	74.8±0.5 (7)
Arterial perfusion pressure (mmHg/preparation)	0	131±8 (3)	77±8 (7)**
	10	143±47 (3)	78±6 (7)
	30	146±50 (3)	75±7 (6)
	60	172±59 (3)	95±10 (6)

creatine content fell below that *in vivo*. The lower concentrations of ATP in Group I compared with Group II were accompanied by substantial decreases in the concentration of phosphocreatine in all the muscles. ADP and AMP concentrations, however, were not significantly different between Groups I and II. The concentration of lactate in gastrocnemius and quadriceps of Group I perfusions was higher than in Group II, but the difference was only statistically significant for the gastrocnemius, and there was no difference in quadratus lumborum.

Table 4 shows that arterial perfusion pressure was elevated in Group I, which is the opposite of that found in the hypoxic perfusions in Table 1. This could have been because increased vascular resistance in Group I was a primary defect, possibly a cause of many of the other changes, whereas the lowered vascular resistance in hypoxic perfusions was a result of hypoxia itself, which has been documented by Cain & Chapler (1980). Table 4 also shows that lactate uptake and K^+ efflux by the hemicorpus were the same in two groups.

It is not clear why there was an efflux of lactate in the normal perfusions in Expt. I (Table 2), but an uptake in Expt. 2 (Table 4). However, the muscle lactate concentrations in the donor rats (i.e. the values *in vivo*) were much lower in Expt. 2, and this may have been responsible for the uptake rather than the efflux. In addition the erythrocytes were of different age, and the albumin was from a different batch. It is well recognized that the albumin can contain many contaminants which can modify the metabolism of the perfused tissue (see, e.g., Li & Jefferson, 1978; Dobbs & Lee, 1979; Lee & Holland, 1979). This may also explain other differences (i.e. rate of K^+ efflux or loss of phosphocreatine) between the two sets of normal perfusions in Expts. 1 and 2.

Measurements of arterio-venous differences (Δ) in blood gases in Groups I and II are shown in Table 5. No difference in ΔpO_2 could be detected at any time during perfusion, but, when O_2 content was expressed as the percentage saturation of haemoglobin (sO_2), Group I had a significantly lower arterio-venous difference, indicating a lower O_2 uptake during the last 15 min of perfusion. The values of ΔsO_2 in Group II, expressed in terms of O_2 uptake, were 27.5±3.6 (6), 27.1±3.9 (7), 34.5±2.1 (7) and 32.8±2.8 (7) μmol/h per g of muscle at 5, 25, 45 and 60 min respectively. These compare favourably with values of 13–40 μmol/h per g of muscle in comparable perfusions with insulin, quoted in a review by Ruderman *et al.* (1980). The perfusate CO_2 was expressed both as pCO_2 and as total CO_2 (tCO_2). The arterio-venous differences in both were significantly lower in Group I during the latter 35 min of perfusion. Measurements of pH showed a fall between artery and vein in Group II, whereas in Group I there was a rise. This difference was statistically significant throughout the perfusion.

In Expt. 3 perfusion was extended to 3 h, since it would be expected that variability would increase if some degree of tissue dysfunction were occurring. Fig. 1 shows the relationships between protein-synthesis rates and concentrations of ATP, phosphocreatine and lactate in gastrocnemius and

Table 5. *Comparison of arterio-venous differences in perfusate pO_2, sO_2, pCO_2, tCO_2 and pH in Group-I and -II perfusions in Expt. 2*

Perfusions were prepared as described in the text and in the legend to Table 3. Arterio-venous differences (Δ) were obtained by simultaneously withdrawing perfusate into silicone-treated glass syringes. Blood-gas parameters (pO_2, partial pressure of dissolved O_2; sO_2, percentage oxygen saturation of haemoglobin; pCO_2, partial pressure of dissolved CO_2; tCO_2, total CO_2) and pH were measured on an automated blood-gas analyser. A negative prefix signifies a mean decrease in values from arterial to venous side, and a positive prefix signifies an increase. Significance of difference between Group I and II: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Arterio-venous difference	Perfusion time (min)	Group I	Group II
ΔpO_2 (mmHg)	5	−184 (2)	−189±45 (7)
	25	−176±88 (3)	−158±42 (7)
	45	−180±93 (3)	−145±104 (7)
	60	−156±92 (3)	−142±38 (7)
ΔsO_2 (%)	5	−10.9 (2)	−22.3±2.5 (7)
	25	−18.1±11.9 (3)	−30.4±1.7 (7)
	45	−13.1±10.1 (3)	−28.0±1.5 (7)*
	60	−8.9±9.6 (3)	−26.7±2.1 (7)*
ΔpCO_2 (mmHg)	5	+0.25 (2)	+8.2±0.7 (7)
	25	−1.3±3.0 (3)	+7.2±0.3 (7)***
	45	−0.1±2.6 (3)	+6.4±0.4 (7)***
	60	−1.6±3.0 (3)	+6.8±0.6 (7)***
ΔtCO_2 (mmol/ml)	5	+0.3 (2)	+2.0±0.2 (6)
	25	+0.2±0.9 (3)	+1.9±0.1 (7)*
	45	+0.1±0.6 (3)	+1.6±0.2 (7)*
	60	+0.5±0.7 (3)	+1.8±0.2 (7)**
ΔpH	5	+0.031 (2)	−0.027±0.004 (6)**
	25	+0.035±0.009 (3)	−0.027±0.003 (7)***
	45	+0.015±0.013 (3)	−0.031±0.003 (7)***
	60	+0.018±0.022 (3)	−0.031±0.004 (7)**

quadriceps muscles. The correlation coefficients range between 0.756 and 0.929, and all are statistically significant ($P<0.02$). Similar correlations were attempted for perfusate K^+ and lactate concentrations, ΔpO_2, ΔsO_2, ΔtCO_2, ΔpH and rates of O_2 consumption, but the values of r were not significant for any of these. When preparations were perfused for periods shorter than 3h (60–90min, as in all previous and subsequent studies), significant correlations between protein synthesis and ATP, phosphocreatine and lactate could not be obtained, because the degree of variation in the individual parameters was much less and there were smaller numbers in the groups.

Discussion

The objective of these studies was to select parameters that could be used for routinely assessing the performance of the perfused rat hemicorpus preparations used for studies on protein metabolism in muscle. The results confirmed that normal perfusions of less than 90min duration (Table 1) were able to maintain rates of protein synthesis that were similar to those *in vivo*, as observed previously (Preedy & Garlick, 1983). However, in some preparations there was evidence of metabolic insufficiency, e.g. a decrease in phosphocreatine and an increase in lactate concentration. As the concentrations of these metabolites in pre-perfusion samples (i.e. immediately after the operation) were similar to those *in vivo*, it appears that the deterioration of the tissue was taking place during the actual perfusion. This was usually evident when perfusion was continued for as long as 3h, but was occasionally observed in preparations perfused for only 60min (e.g. Group I, Table 3). The aim of the study was therefore to find a way of detecting those preparations that had deteriorated excessively during the perfusion and might have given rise to results that were atypical of the group in general. In previous muscle-perfusion studies, measurements such as arterial perfusion pressure (Jefferson *et al.*, 1972), oedema or water content (Mehl *et al.*, 1964; Barak *et al.*, 1971), ATP, ADP, AMP and phosphocreatine in muscle (Rookledge, 1971; O'Donovan *et al.*, 1975; Dohm *et al.*, 1980), lactate concentration in muscle (Caldwell *et al.*, 1978), lactate release (Ruderman *et al.*, 1971), glucose uptake (Hillgartner *et al.*, 1982), O_2 consumption (Rennie & Holloszy, 1977), K^+ efflux (Ward & Buttery, 1979*a*), light-microscopic examination (Miller *et al.*, 1954) and electron-microscopic examination (Strohfeldt *et al.*, 1974) have been

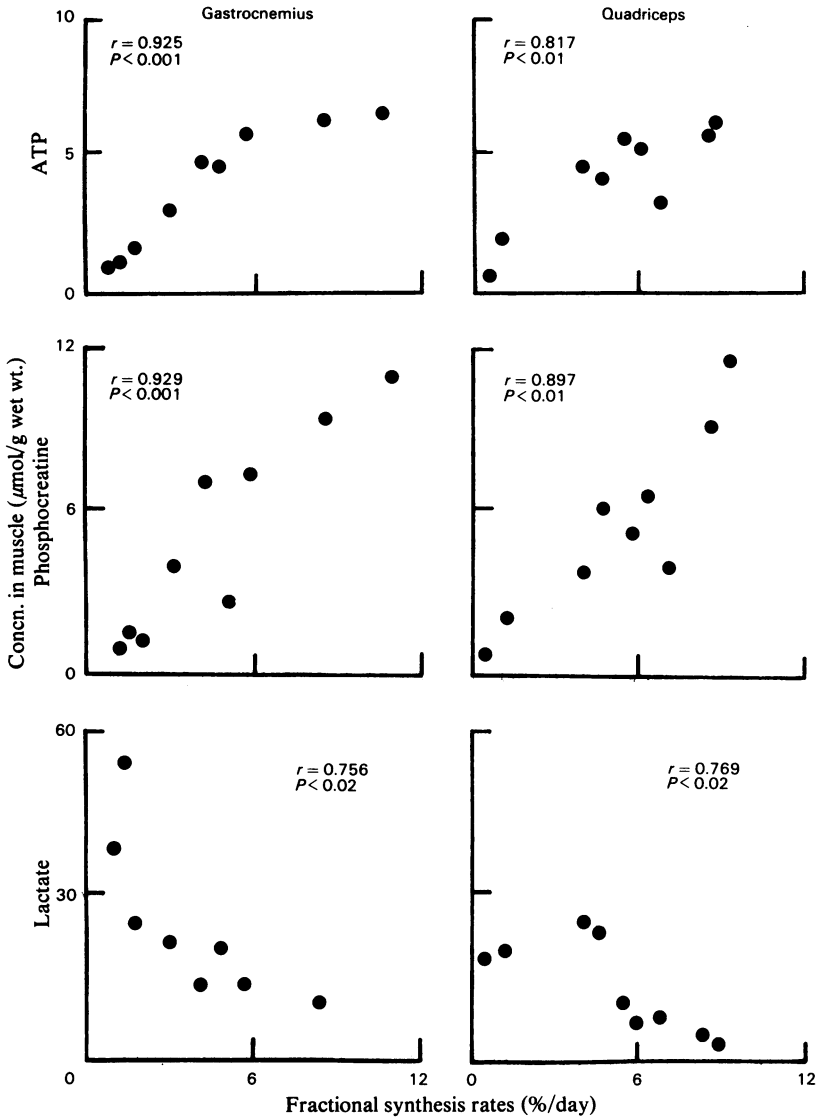

Fig. 1. *Correlations between rates of protein synthesis and concentrations of ATP, phosphocreatine and lactate in gastrocnemius and quadriceps muscles*

Preparations from fed rats were perfused as described in the text, and rates of protein synthesis were obtained by adding L-[U-^{14}C]tyrosine 15min after the start of perfusion. At the end of 180min, muscles were rapidly removed for determination of tissue metabolites and tyrosine specific radioactivities.

used for assessing the preparation, but there appears to be no generally accepted measure of tissue dysfunction. Indeed, there is no reason *a priori* to suppose that there is a single indicator of overall dysfunction that is applicable to all types of study on isolated muscle. Our approach has therefore been to examine a wide range of parameters in relation to rates of protein synthesis, using preparations perfused under normal conditions, together with others subjected deliberately to metabolic abuse. The intention was then to select a few parameters that could be used routinely as a basis for detection and rejection of unsatisfactory preparations.

In Expt. 1, in which tissue dysfunction was induced artificially by hypoxia and ischaemia, unacceptable preparations could be distinguished by several parameters (Table 1 and 2). This seems to indicate that the concept of a 'bad' perfusion (as indicated by microscopic studies, arterial perfusion pressure, concentrations of ATP, phosphocreatine and lactate, efflux of lactate and K^+) does

indeed exist. In particular, in hypoxic perfusions many of these changes were associated with a decrease in the rate of muscle protein synthesis. However, it must be emphasized that, in the normal course of perfusion, dysfunction of tissue metabolism is unintentional rather than deliberate. We were not surprised, therefore, that some of the results from Expt. 2, in which dysfunction occurred unintentionally, were inconsistent with those from Expt. 1 (e.g. perfusion pressure, lactate and K^+ efflux), since the dysfunction could have been caused by mechanisms other than ischaemia or hypoxia.

In Expt. 2 the rationale for dividing the preparations into two groups (Tables 3, 4 and 5) on the basis of ATP concentrations was that ATP is the fundamental or basic unit of energy, without which many processes, such as the binding of hormones (Draznin *et al.*, 1980) and protein synthesis (Ayuso-Parrilla & Parrilla, 1975), cannot occur at the optimum rate. It was also evident that in three preparations muscle ATP content was unusually low. This approach of dividing perfusions into two groups was also used by Ruderman *et al.* (1971), who separated perfused rat hindquarters partially on the basis of muscle phosphocreatine content, and Bloxam (1971), who divided perfused rat livers on the basis of K^+ uptake. We also included in the comparison of Groups I and II the measurements of ADP and AMP, in addition to arterio-venous differences in blood gas parameters, as possible additional indicators of tissue dysfunction. These results (Tables 3, 4 and 5) show that, when hemicorpuses are prepared routinely, apparent anomalies sometimes occur, manifested as a decrease in the concentration of ATP in muscle. This is associated with decreased phosphocreatine and elevated lactate content in muscle, increased arterial perfusion pressure, decreases in O_2 uptake and CO_2 output, as well as increases in pH across the hemicorpus. Although the causes of these differences were not investigated, it is probable that the primary cause was not hypoxia or ischaemia alone, otherwise marked differences in the concentrations of ADP or AMP, such as those reported by Sabrourova & Berezov (1975) and Reimer *et al.* (1975), may have been evident. However, for reasons of economy, one cannot routinely measure all those parameters that showed differences between control and hypoxic or totally ischaemic preparations in Expt. I, or those that showed differences between Group-I and -II perfusions in Expt. 2. From the results of this study, we concluded that the most suitable indicators of tissue integrity for our particular requirements are the muscle concentrations of ATP, phosphocreatine and lactate. These values showed marked differences between the situation *in vivo* and that in perfusions subjected to hypoxic or ischaemic conditions (Table 1). Furthermore, they showed a lesser, though still co-ordinated, response to the suboptimal conditions that arose in the Group-I preparations in Expt. 2 (Table 3). Concentrations of ADP and AMP in muscle, and tissue water content, appeared relatively unresponsive to metabolic state. Measurements of changes in concentration of metabolites in the perfusion medium were complicated by the need to correct for the effects of erythrocyte metabolism, and also by the contribution of non-muscle tissues in the hemicorpus preparation itself, since muscle comprises only 56% of the total weight (Preedy & Garlick, 1981). This is also a problem in the interpretation of the blood-gas profiles for individual preparations, although these studies are useful indicators of the general metabolic and physiological characteristics of the hemicorpus perfusion system.

The choice of measurements of ATP, phosphocreatine and lactate concentrations as indicators of the metabolic state of individual preparations for studies of the regulation of protein synthesis is supported by the close correlations demonstrated in Fig. 1. In this experiment perfusions were continued for 3h, to accentuate the effects of deteriorating performance. It should be noted that in our usual studies of protein synthesis we never exceed a total perfusion time of 90min.

Having adopted a set of parameters suitable for use as criteria of metabolic state, it is necessary to decide on a strategy for their use. One possibility is to consider rejection on the basis of metabolite concentrations rising above or falling below an absolute value, as has been used by others (e.g. Ruderman *et al.*, 1971; Ward & Buttery, 1979*b*). The main objection is the real possibility that the selected parameters would alter as a result of the treatment of the animal *in vivo*, or as a result of the experimental variation of perfusion conditions *in vitro*. For example, though Ruderman *et al.* (1971) set a value of 0.13μmol/min per g for lactate release, their subsequent studies (Goodman *et al.*, 1978) demonstrated that in certain conditions it was possible to obtain preparations that took up lactate from the medium, as in our study (Table 4). This approach was therefore considered unsuitable.

Another method is to reject preparations if the metabolite concentration falls outside a fixed number of standard deviations from the mean e.g. in liver perfusions Krone *et al.* (1974) used 2 standard deviations. However, this technique is very insensitive when variability is high. For example, in some of our studies it would only be possible to reject preparations on the basis of ATP and phosphocreatine contents deviating by more than 2 standard deviations if their concentrations had fallen below zero.

The approach that we have finally adopted is to

reject preparations if metabolite concentrations are a certain percentage above or below the mean value for that group. The appropriate percentage was derived by examining the variation in intact animals. Five groups of data *in vivo* were examined in which the lowest ATP or phosphocreatine, or highest lactate, content in muscle was expressed as a percentage of the mean value obtained from the remaining animals in that group. For ATP this ranged from 55% to 88%, for phosphocreatine 55% to 87%, and for lactate 147% to 257%. Thus, individual hemicorpuses are rejected if muscle ATP or phosphocreatine content falls below 50%, or if lactate content rises above 275%, of the mean values for the remaining preparations in that group. If any of the metabolites fail to obey the criteria, that hemicorpus and all associated results are discarded.

The advantage of this technique is that it uses the variability observed in the intact animal. It thus takes account of inter-animal variability and measurement error. It does not use the variability of that individual group's data *in vitro*, as this would tend to be inaccurate because of small group sizes. Deviations from the mean values observed *in vitro* were used as rejection criteria, because this takes into account the possibility that a particular treatment, either *in vivo* or *in vitro*, may alter tissue concentrations of metabolites as part of the normal physiological response, without causing tissue dysfunction. Such alterations in concentration may be important to recognize functionally, but should not be the cause of rejection of individual preparations.

The proportion of preparations rejected by these criteria declined steadily, as the operator gained in skill. In early perfusions as many as 25% of preparations were rejected, whereas in the most recent experiment the rejection rate was only 3%.

When these criteria for rejection were applied to Groups I and II, the three perfusions in Group I were found to be unsatisfactory. In addition, one perfusion in Group II was found to have low enough phosphocreatine in the quadratus lumborum to merit rejection. This was not surprising, in view of the inability of this muscle, compared with gastrocnemius and quadriceps, to maintain phosphocreatine concentrations, and quadratus lumborum has not been used in subsequent studies. Also, as three separate muscles were examined in this study, the chances of a single erroneous measurement leading to a rejected preparation must have been appreciable. In subsequent experiments rejection of unsatisfactory preparations was based on measurements in one muscle only.

Application of the criteria to subsequent experiments also demonstrated their suitability. Although we were not able to detect significant correlations between protein synthesis and ATP, phosphocreatine or lactate in perfusions lasting less than 3h (see the Results section), rates of synthesis in rejected hemicorpuses from seven groups of perfusions of 60–90min duration were on average 20% lower than those from the remaining preparations in the same group. This difference may be an appreciable proportion of that resulting from the experimental manipulation under study. For example, the effect of lack of insulin in preparations from starved rats was to decrease synthesis by 30% (Preedy & Garlick, 1983).

When we first began these studies, we attempted to measure nearly all of the parameters that others had used to characterize their perfused preparations. In summary, therefore, we have been able to show that most of these parameters are unsuitable for indicating the metabolic state of the preparation as regards its ability to synthesize protein. Our results demonstrate, however, that measurements of ATP, phosphocreatine and lactate concentrations in muscle can be used for assessing the performance of the perfused hemicorpus, and we believe that our approach might also be useful for other preparations *in vitro*.

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