The rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in skeletal muscle

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1. Substrate cycling of fructose 6-phosphate through reactions catalysed by 6-phosphofructokinase and fructose-1,6-bisphosphatase was measured in skeletal muscles of the rat *in vitro*. 2. The rate of this cycle was calculated from the steady-state values of the $^3\text{H}/^{14}\text{C}$ ratio in hexose monophosphates and fructose 1,6-bisphosphate after the metabolism of either $[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]\text{glucose}$ or $[3\text{-}^3\text{H}, 2\text{-}^{14}\text{C}]\text{glucose}$. 3. Two techniques for the separation of hexose phosphates were studied; t.l.c. chromatography on poly(ethyleneimine)-cellulose sheets or ion-exchange chromatography coupled with enzymic conversion. These two methods gave almost identical results, suggesting that either technique could be used for determination of rates of fructose 6-phosphate/fructose 1,6-bisphosphate cycling. 4. It was found that more than 50% of the ^3H was retained in the fructose 1,6-bisphosphate; it is therefore probable that previous measurement of cycling rates, which have assumed complete loss of ^3H , have underestimated the rate of this cycle. 5. The effects of insulin, adrenaline and adrenergic agonists and antagonists on rates of fructose 6-phosphate/fructose 1,6-bisphosphate cycling were investigated. In the presence of insulin, adrenaline ($1\text{ }\mu\text{M}$) increased the cycling rate by about 10-fold in epitrochlearis muscle *in vitro*; the maximum rate under these conditions was about $2.5\text{ }\mu\text{mol/h per g}$ of tissue. The concentration of adrenaline that increased the cycling rate by 50% was about 50 nM . This effect of adrenaline appears to be mediated by the β -adrenergic receptor, since the rate was increased by β -adrenergic agonists and blocked by β -adrenergic antagonists. 5. From the knowledge of the precise rate of this cycle, the possible physiological importance of cycling is discussed.

The discovery of fructose-1,6-bisphosphatase (EC 3.1.3.11) activity in a variety of muscles led to the concept of a substrate ('futile') cycle between fructose 6-phosphate and fructose 1,6-bisphosphate in this tissue (Newsholme & Crabtree, 1970). The role of this cycle was considered to be to improve the sensitivity of the flux through the 6-phosphofructokinase reaction to effectors of this enzyme (Newsholme & Crabtree, 1973, 1976). The presence of a very high activity of fructose-1,6-bisphosphatase in bumble-bee flight muscle raised the possibility that, in this particular case, the role of the cycle was to generate heat (Newsholme *et al.*, 1972). This finding prompted an investigation into the effect of ambient temperature on the activity of

this cycle in the bumble bee by Clark *et al.* (1973a). The rate of cycling was determined from changes in the ratio of radioactivity ($^3\text{H}/^{14}\text{C}$) in hexose monophosphates extracted from the flight muscle after the injection of $[5\text{-}^3\text{H}, \text{U-}^{14}\text{C}]\text{glucose}$ into haemolymph. At rest, the cycling rate was increased as the ambient temperature decreased, but it was completely inhibited by flight of the insect. In addition to the bumble bee, a low rate of cycling was observed in muscle of euthermic and hyperthermic pigs (Clark *et al.*, 1973b), but rates of cycling in other muscles have not been reported. Indeed, up to 1978, the rates of substrate cycling that have been reported were low; this led Katz & Rognstad (1978) to suggest that cycling was of little or no physiological importance. However, more recently high rates of cycling between glucose and

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glucose 6-phosphate in insect flight muscle and between triacylglycerol and fatty acids in rat adipose tissue have been reported (Surholt & Newsholme, 1983; Brooks *et al.*, 1982). These findings prompted a re-investigation of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in rat muscle.

The theoretical analysis of the role of substrate cycles in improving sensitivity indicates that the improvement is proportional to the rate of cycling (Newsholme & Crabtree, 1976). One prediction of this hypothesis is that the cycling rate will vary from one condition to another and that some hormones will specifically increase the rate of substrate cycling. In order to test this prediction, it is necessary to measure the precise rate of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate. To do this, it is necessary to measure the change in the ratios of radioactivity ($^3\text{H}/^{14}\text{C}$) in both hexose monophosphates and fructose 1,6-bisphosphate; if ^3H is retained in fructose 1,6-bisphosphate, measurement of the ratio only in the hexose monophosphates will underestimate the cycling rate (Katz & Rognstad, 1976; Newsholme & Crabtree, 1976; see the Appendix). Since methods for such a separation have been described (Conyers *et al.*, 1976; Hammerstedt, 1980), the precise rate of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate can now be measured. This has been done in isolated preparations of rat muscles, and the effect of catecholamines on the rate of this cycle has been studied.

Materials and methods

Animals

Male Wistar rats were obtained from Bantin and Kingman, Hull, U.K., and were maintained in the Department of Biochemistry animal house for at least 5 days before use. Rats of body wt. 60–80 g were used for obtaining hemidiaphragms and those of 160–180 g for obtaining epitrochlearis muscles and preparation of stripped soleus.

Chemicals and enzymes

All enzymes and biochemicals were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K., except bovine serum albumin (fraction V), Dowex resins and 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD), which were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. 2-(Biphenyl-4-yl)-6-phenylbenzoxazole (PBBO) was obtained from Fluorochem, Glossop, Derbyshire, U.K. All chemicals were from Fisons Scientific Apparatus, Loughborough, Leics., U.K., and were of A.R. grade. Poly(ethyleneimine)-cellulose thin-layer plates were obtained from Macherey Nagel and Co., Düren, West Germany.

Radiochemicals

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Because of the importance of radiochemical purity and specificity of labelling, new batches of [^3H]glucose were routinely analysed to assess their purity. The method of Postle & Bloxham (1980) was used; in brief, D-[^3H]glucose purity was assessed by adding a trace amount of label (10000 d.p.m.) to carrier glucose (5 μmol). Initially any ^3H at C-2 of glucose is removed by conversion into glucose 6-phosphate and equilibration through phosphoglucose isomerase (Rose & O'Connell, 1960); the $^3\text{H}_2\text{O}$ produced was removed by freeze-drying (the complete conversion of glucose into glucose 6-phosphate was shown spectrophotometrically by the fact that free glucose could not be detected after 30 min of incubation). After freeze-drying, the [^3H]glucose 6-phosphate/[^3H]fructose 6-phosphate was converted into glycerol 3-phosphate and the coupling enzymes were inactivated by heating to 70°C for 5 min. ^3H derived from C-5 was then exchanged with water of the medium through the action of triose phosphate isomerase (Rieder & Rose, 1959). This two-step procedure prevented loss of ^3H from C-3 (which was trapped as [^3H]glycerol 3-phosphate) and the extent of conversion of NADH into NAD^+ indicated that the conversion of hexose monophosphate into glycerol 3-phosphate was 95% complete. The purity of a variety of batches varied from 90.3 to 95.1%.

Incubation techniques

Animals were killed by cervical dislocation, epitrochlearis muscles were rapidly removed and pairs preincubated for 30 min in 2 ml of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) supplemented with 1.5% bovine serum albumin (defatted by the method of Chen, 1967), 4 mM-pyruvate, 5 mM-succinate, 4 mM-glutamate and 5 mM-glucose in an oscillating (100 rev./min) water bath at 37°C. During this time, flasks were gassed continually with O_2/CO_2 (19:1). Muscles were then transferred to incubation flasks containing 2 ml of Krebs–Ringer bicarbonate buffer supplemented with 1.5% albumin and 5 mM-glucose containing appropriately ^3H - and ^{14}C -labelled glucose (2 μCi of both ^3H and ^{14}C /ml). Flasks were incubated for 60 min at 37°C, with gassing for the initial 15 min period. After 60 min muscle pairs were removed, rapidly blotted and freeze-clamped. Preliminary experiments established that the concentrations of ATP, ADP and AMP in the epitrochlearis muscle during the incubation were similar to those measured in the muscle freeze-clamped *in situ* (Challiss, 1983). Samples of incubation media were taken, depro-

teinized by acidification with HClO_4 , neutralized and stored at -20°C for subsequent analysis.

Preparation of extracts and separation of intermediates

Weighed muscle pairs were powdered under liquid N_2 , HClO_4 was added to a final concentration of 6% (w/v), and 0.25 μmol of glucose 6-phosphate, 0.05 μmol of fructose 6-phosphate and 0.20 μmol of fructose 1,6-bisphosphate were added to act as 'carriers' for the labelled intermediates. The frozen powder was transferred to an Eppendorf tube and thawed on ice for 1 h. Samples were processed by the method described by Conyers *et al.* (1976) and Surholt & Newsholme (1983).

Two techniques for the separation of hexose monophosphates and fructose 1,6-bisphosphate were used: poly(ethyleneimine)-cellulose t.l.c. (Conyers *et al.*, 1976) and Dowex-2 (formate form) anion-exchange chromatography (Hammerstedt, 1980). In the t.l.c. method, the positions of the sugar phosphates on the plate were localized with a thin-layer radioactivity scanner (model LB 2723; Berthold, Wildbad, West Germany) in combination with $[^3\text{H}]$ - and $[^{14}\text{C}]$ -glucose 6-phosphate and $[^{14}\text{C}]$ fructose 1,6-bisphosphate standards. In the Hammerstedt (1980) method the ion-exchange column was used to achieve an initial separation, but the specificity of the technique relied on enzymic conversion of each metabolite of interest such that the selective affinity (i.e. the concentration of formic acid/ammonium formate required to displace the metabolite from the anion-exchange resin) was altered. This technique caused some loss of ^3H from hexose monophosphates (see Table 1), so that it was necessary to run $[5\text{-}^3\text{H}, \text{U-}^{14}\text{C}]$ glucose 6-phosphate standards to control for non-metabolic ^3H loss. No such loss occurs with the t.l.c. method.

Radioactivity in isolated intermediates was determined by addition of a high-performance scintillation cocktail (19 g of butyl-PBD, 1.19 g of PBBO, 500 ml of Triton X-100, 1750 ml of toluene). Samples were stored in the dark for 24 h, and ^3H and ^{14}C radioactivities were determined in a Beckman LS 7500 scintillation counter previously programmed with the appropriate quench-correction parameters by using $[^3\text{H}]$ - and $[^{14}\text{C}]$ -hexadecane standards.

Measurement of rate of glycolysis

Initial experiments established that lactate was a major end-product of glycolysis in epitrochlearis muscles incubated *in vitro* (Challiss *et al.*, 1983). Thus in all treatments described in the present paper 85–95% of glucose + glycogen utilized by epitrochlearis muscle was metabolized to lactate (results not shown). It was therefore considered

satisfactory to use the rate of lactate production as an index of glycolytic rate.

Measurement of cycling rate

The rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate can be measured by use of $[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$ - or $[3\text{-}^3\text{H}, 2\text{-}^{14}\text{C}]$ -glucose. Some of the ^3H on C-3 or C-5 is lost in the triosephosphate isomerase reaction, which is known to catalyse a near-equilibrium reaction in muscle (see Newsholme & Leech, 1983). Assuming that aldolase catalyses a near-equilibrium reaction, some ^3H will also be lost from fructose 1,6-bisphosphate, so that the rate of fructose-1,6-bisphosphate in incubated epitrochlearis muscle can be calculated from the formula:

$$\text{cycling} = \frac{(J + B)(1 - S_1)}{(S_1 - S_2)}$$

where J is the rate of glycolysis, B is the rate of glycogen synthesis, S_1 is the ratio of radioactivities ($^3\text{H}/^{14}\text{C}$) in hexose monophosphates/ $(^3\text{H}/^{14}\text{C})$ in original glucose and S_2 is the ratio of radioactivities ($^3\text{H}/^{14}\text{C}$) in fructose 1,6-bisphosphate/ $(^3\text{H}/^{14}\text{C})$ in original glucose (for derivation see the Appendix).

The rate of glycolysis (J) is assumed to be equal to the rate of lactate production; lactate is measured spectrophotometrically (Gutman & Wahlefeld, 1974). The rate of glycogen synthesis (B) is calculated from the rate of $[^{14}\text{C}]$ lactate production from $[^{14}\text{C}]$ glucose (λ), measured by separation of lactate on ion-exchange columns (Hammerstedt, 1980), and the rate of $[^{14}\text{C}]$ glucose incorporation into glycogen (γ), measured by the method of Cuendet *et al.* (1976), as follows:

$$B = J \times \gamma/\lambda$$

(for derivation see the Appendix).

Results

Two techniques have been described in the literature for separation of hexose phosphates, particularly for the determination of the rate of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle: use of t.l.c. on poly(ethyleneimine)-cellulose (Conyers *et al.*, 1976) and ion-exchange chromatography followed by enzymic conversion (Hammerstedt, 1980). The two methods were directly compared: under two different incubation conditions, almost identical results were obtained (Table 1). That two totally different methods of separation give rise to such similar results suggests that either method can be used for the separation of the hexose monophosphates and fructose 1,6-bisphosphate from muscle and measurement of the cycling

Table 1. Radioactivities and ratios of radioactivities in hexose monophosphate and fructose biphosphate and the calculated rates of cycling between fructose 6-phosphate and fructose biphosphate in isolated epitrochlearis muscle of the rat after separation of hexose monophosphates by t.l.c. or ion-exchange chromatography. Incubations, separations and measurement of radioactivities and calculation of cycling rates were performed as described in the Materials and methods section: results are presented as means $\pm$ S.E.M. for six separate incubations. The t.l.c. technique was adopted from Conyers *et al.* (1976) (see the Materials and methods section) and the ion-exchange technique was as described by Hammerstedt (1980). The latter procedure causes some loss of ^3H during the separation from both hexose monophosphates and fructose biphosphate: in this experiment 4% of ^3H was lost during separation, and this was corrected for during the calculation of S_1 . The mean glycolytic rates were 3.80 and 6.20 $\mu\text{mol/h}$ per g wet wt. of muscle in control and insulin incubations respectively.

Addition to medium	Chromatographic procedure	Radioactivity (d.p.m.)						Cycling rate ($\mu\text{mol/h}$ per g wet wt. of muscle)		
		Hexose monophosphate		Fructose biphosphate						
		^3H	^{14}C	^3H		^{14}C			S_1	S_2
None	T.l.c.	2218 $\pm$ 173	2393 $\pm$ 190	582 $\pm$ 79	869 $\pm$ 94	0.983 $\pm$ 0.005	0.710 $\pm$ 0.013	0.24 $\pm$ 0.05		
	Ion-exchange	3655 $\pm$ 227	4094 $\pm$ 239	1476 $\pm$ 96	2219 $\pm$ 107	0.985 $\pm$ 0.005	0.705 $\pm$ 0.012	0.20 $\pm$ 0.04		
Insulin	T.l.c.	3532 $\pm$ 274	3826 $\pm$ 300	957 $\pm$ 49	1425 $\pm$ 96	0.981 $\pm$ 0.007	0.713 $\pm$ 0.008	0.44 $\pm$ 0.07		
	Ion-exchange	5601 $\pm$ 413	6300 $\pm$ 434	1528 $\pm$ 90	2347 $\pm$ 184	0.982 $\pm$ 0.005	0.690 $\pm$ 0.009	0.40 $\pm$ 0.06		

rate; all further work reported in this paper used the t.l.c. method of separation.

Time-courses of the changes in $^3\text{H}/^{14}\text{C}$ ratios in hexose monophosphate and fructose 1,6-bisphosphate isolated from control and adrenaline-treated epitrochlearis muscles are presented in Fig. 1. The results demonstrate that a steady state is established in the muscle, under control conditions, within the first 20 min of incubation. The situation is more complex in adrenaline-treated muscles, as the stimulation of fructose 6-phosphate/fructose 1,6-bisphosphate cycling by adrenaline decreases with time of exposure to the hormone (Lefkowitz *et al.*, 1980).

Under basal conditions, the cycling rate in muscle is 0.22 $\mu\text{mol/h}$ per g of muscle, which is 6% of the glycolytic flux and represents about 1% of the maximal activity of fructose-1,6-bisphosphatase *in vitro* in epitrochlearis muscle (Challiss, 1983). The rate of cycling was increased 2-fold by insulin and about 10-fold by adrenaline (1 μM) (Table 2). An increase in cycling rate was observed at 10 nM-adrenaline, and the maximum effect

Fig. 1. Radioactivity ratios ($^3\text{H}/^{14}\text{C}$) in hexose monophosphates (○, Δ) and fructose 1,6-bisphosphate (●, ▲) in epitrochlearis muscles incubated in the presence (Δ, ▲) and absence (○, ●) of 1 μM -adrenaline.

The $^3\text{H}/^{14}\text{C}$ ratio in initially added glucose was set at 1.000 ± 0.003 (36). All points represent means $\pm$ S.E.M. for at least six muscle pairs. Statistically significant differences between S_1 values for basal and adrenaline-stimulated conditions are indicated by ** $P < 0.01$ and * $P < 0.05$. All values of S_1 and S_2 are statistically significantly different ($P < 0.05$) from the $^3\text{H}/^{14}\text{C}$ ratio in the initially added glucose as judged by Student's *t* test.

Table 2. *Effect of catecholamines on the ratios of radioactivities in hexose monophosphate and fructose biphosphate, the rates of total glycolysis and the calculated cycling rate between fructose 6-phosphate and fructose biphosphate in isolated epitrochlearis, soleus and hemidiaphragm muscles of the rat*

Procedure for the incubations, extraction of muscle, separation of glycolytic intermediates and measurement of radioactivities and rates of glycolysis are described in the Materials and methods section. Glucose (5 mM) was present in all incubations. In all incubations except those in Expt. 1, insulin was present at a concentration of 1 munit/ml. Results are presented as means $\pm$ S.E.M., with the numbers of separate incubations given in parentheses. The cycling rate is calculated as glycolytic rate $\times (1 - S_1)/(S_1 - S_2)$.

Expt. no.	Muscle	Radiolabels in glucose	Hormone	S_1	S_2	Rates ($\mu\text{mol/h}$ per g of muscle)		
						Glycolysis	Cycling	
1	Epitrochlearis	$[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	None	0.985 ± 0.004 (12)	0.704 ± 0.010	3.80 ± 0.20	0.22 ± 0.05	
			Insulin	0.981 ± 0.006 (13)	0.704 ± 0.008	6.20 ± 0.20	$0.42 \pm 0.05^*$	
2	Epitrochlearis	$[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	None	0.983 ± 0.003 (6)	0.684 ± 0.015	5.29 ± 0.23	0.30 ± 0.03	
			Adrenaline (0.01 μM)	0.951 ± 0.009 (5)	0.699 ± 0.012	5.36 ± 0.30	$0.90 \pm 0.18^*$	
			Adrenaline (0.1 μM)	0.950 ± 0.007 (5)	0.687 ± 0.012	8.54 ± 0.73	$1.69 \pm 0.32^*$	
			Adrenaline (1 μM)	0.936 ± 0.012 (5)	0.695 ± 0.019	9.40 ± 0.68	$2.43 \pm 0.45^*$	
			Adrenaline (10 μM)	0.956 ± 0.008 (5)	0.735 ± 0.015	10.35 ± 1.01	$2.22 \pm 0.51^*$	
3	Epitrochlearis	$[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	None	0.965 ± 0.007 (6)	0.665 ± 0.015	4.18 ± 0.23	0.49 ± 0.13	
			Phenylephrine (10 μM)	0.954 ± 0.004 (5)	0.663 ± 0.021	4.72 ± 0.38	$0.75 \pm 0.08^*$	
			Phenylephrine (10 μM) plus propranolol (10 μM)	0.969 ± 0.008 (5)	0.674 ± 0.011	4.37 ± 0.19	0.46 ± 0.06	
			Isoprenaline (10 μM)	0.932 ± 0.008 (5)	0.704 ± 0.021	6.65 ± 0.41	$1.98 \pm 0.41^*$	
			Isoprenaline (10 μM) plus propranolol (10 μM)	0.959 ± 0.010 (5)	0.684 ± 0.015	4.79 ± 0.36	0.71 ± 0.17	
4	Epitrochlearis	$[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	Insulin	0.978 ± 0.004 (6)	0.683 ± 0.009	6.53 ± 0.33	0.49 ± 0.06	
	Soleus		Insulin	1.001 ± 0.003 (4)	0.626 ± 0.016	9.92 ± 0.38	-0.03 ± 0.10	
	Hemidiaphragm	$[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	Insulin	0.995 ± 0.008 (7)	0.794 ± 0.030	12.88 ± 0.50	0.34 ± 0.13	
	Hemidiaphragm		Insulin plus adrenaline	0.994 ± 0.006 (7)	0.863 ± 0.014	15.31 ± 0.32	$0.67 \pm 0.14^*$	
	Epitrochlearis		Insulin	0.979 ± 0.006 (7)	0.707 ± 0.010	6.39 ± 0.28	0.48 ± 0.11	
	Epitrochlearis	$[3\text{-}^3\text{H}, 2\text{-}^{14}\text{C}]$	Insulin	0.981 ± 0.002 (11)	0.731 ± 0.008	5.42 ± 0.25	0.46 ± 0.08	
	Epitrochlearis	$[6\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]$	Insulin	0.995 ± 0.006 (7)	0.997 ± 0.34	6.33 ± 0.34	—	

occurred at $1\ \mu\text{M}$; when the cycling rate was plotted against the concentration of adrenaline, the concentration that stimulated cycling half-maximally was observed to be about 50 nM (plot not shown).

The β -agonist isoprenaline increased the rate of cycling 4-fold, but this effect was abolished by the presence of the β -antagonist propranolol; the α -agonist phenylephrine increased the cycling rate by 50%, but this effect was also abolished by the β -antagonist (Table 2). This suggests that the α -agonist at the concentration used ($10\ \mu\text{M}$) exhibited some β -agonist activity. This is supported by the fact that the effects of phenylephrine and isoprenaline were not modified by the presence of the α -antagonist phentolamine (results not shown).

Cycling between fructose 6-phosphate and fructose 1,6-bisphosphate was also observed in the incubated hemidiaphragm preparation. However, in the incubated stripped soleus-muscle preparation the rate of cycling was zero; that is, the $^3\text{H}/^{14}\text{C}$ radioactivity ratio was decreased in fructose 1,6-bisphosphate, but was not different from that of the administered glucose in hexose monophosphate (Table 2, Expt. 4). These results are predicted by the distribution of fructose 1,6-bisphosphatase in muscle; activity is present in epitrochlearis and diaphragm muscles, but it is not detectable in soleus muscle (Opie & Newsholme, 1967; Challiss, 1983).

Identical rates of cycling were obtained when epitrochlearis muscle was incubated with either $[5\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]\text{glucose}$ or $[3\text{-}^3\text{H}, 2\text{-}^{14}\text{C}]\text{glucose}$ (Table 2). This is expected, since ^3H on either C-3 or C-5 of glucose is lost in the triose phosphate isomerase reaction after the conversion of glucose into triose phosphates. This is an important finding, since it provides evidence to support the view that the decrease in ^3H in the hexose monophosphate is due to fructose-1,6-bisphosphatase activity. It has been suggested that loss of ^3H in hexose monophosphate from $[5\text{-}^3\text{H}]\text{glucose}$ could be due to transaldolase activity. However, the latter enzyme would not lead to a loss of ^3H in hexose monophosphates if the ^3H in the triose phosphates had arisen from $[3\text{-}^3\text{H}]\text{glucose}$ (Hue & Hers, 1974).

To demonstrate that the loss of ^3H in both the hexose monophosphate and fructose bisphosphate pools was due to specific reactions, intermediates were analysed after muscles were incubated with $[6\text{-}^3\text{H}, 6\text{-}^{14}\text{C}]\text{glucose}$ (Table 2). No loss of ^3H occurred in either hexose monophosphates or fructose 1,6-bisphosphate, indicating that non-specific deamination was not the cause of the decrease in ^3H radioactivity.

Discussion

In previous measurements of the rate of cycling between fructose 6-phosphate and fructose 1,6-bis-

phosphate (e.g. Clark *et al.*, 1973a,b) by the dual-label technique, it had been assumed that all of the ^3H is lost from fructose 1,6-bisphosphate, so that it was not necessary to separate this compound and measure its radioactivity. The present work demonstrates, for the first time in mammalian systems, that a considerable proportion of ^3H is retained in fructose 1,6-bisphosphate, so that, to measure the precise rate of this cycle, fructose 1,6-bisphosphate must be separated and its radioactivity measured. It is unclear from the present work whether the retention of ^3H in fructose 1,6-bisphosphate is due to a failure of the triose-phosphate isomerase reaction to cause a complete loss of ^3H label or a failure of aldolase to catalyse a reaction sufficiently close to equilibrium.

The substrate-cycling data presented in this paper represent the first report of quantitative evidence in support of the existence of a fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle in mammalian skeletal muscle. Rates of substrate cycling have been precisely determined and the effect of adrenaline was investigated. Adrenaline (in the presence of insulin) markedly increases the rate of cycling in the isolated epitrochlearis muscle. The maximum increase in the cycling rate (about 10-fold) was observed in the presence of $1\ \mu\text{M}$ -adrenaline.

Furthermore, it has been unequivocally demonstrated in this work that the stimulation of the cycling rate by adrenaline is via a β -adrenergic mechanism; thus a β -agonist has a much larger effect on cycling rates than an α -agonist, and the effect is abolished by a β -antagonist, but not by an α -antagonist (Table 2). This is suggestive of a role for cyclic AMP in activation of fructose-1,6-bisphosphatase (and possibly 6-phosphofructokinase) in the epitrochlearis muscle, but there is at present no indication of the molecular mechanism for this activation.

It has been shown previously that catecholamines and β -adrenergic agonists increase the rate of the triacylglycerol cycle in white and brown adipose tissue (Brooks *et al.*, 1982, 1983). These effects are entirely consistent with the role that catecholamines are thought to play in the anticipatory phase of exercise (Newsholme, 1980). However, doubt can be cast on whether the data presented here are supportive of the postulated role of this substrate cycle in improvement of sensitivity in skeletal muscle (Newsholme & Crabtree, 1976), since the cycling rate/flux ratio, which governs the improvement in sensitivity, is not dramatically increased in epitrochlearis muscle *in vitro*. Thus under basal incubation conditions the cycling rate is 5–10% of the glycolytic flux and, although the cycling rate increases by about 10-fold on addition of adrenaline, the concurrent increase in glycolytic

flux results in only about a 4-fold increase in the cycling rate/flux ratio. If it is assumed that cycling rates measured in epitrochlearis muscle *in vitro* are representative of those occurring in all skeletal muscle *in vivo*, then the energy cost of this cycle can be calculated. Newsholme & Crabtree (1976) have shown that if the fructose 6-phosphate/fructose 1,6-bisphosphate cycle were maximally active in skeletal muscle of the rat (i.e. a rate of fructose 1,6-bisphosphate hydrolysis of $1\text{ }\mu\text{mol/min per g}$ of tissue), the heat generated would account for about 12% of the basal metabolic rate; substitution of measured rates of fructose 1,6-bisphosphate hydrolysis reported in the present work into this calculation shows that the energy cost of this cycle does not exceed 0.5% of the basal metabolic rate.

Although this study presents data on the stimulation of fructose 6-phosphate/fructose 1,6-bisphosphate cycling by adrenaline which are qualitatively consistent with the hypothesis of Newsholme & Crabtree (1976), it can be argued that the increase in the cycling rate/flux ratios observed are not large enough to support the role of substrate cycles in improving sensitivity. However, two caveats must be added. Firstly, although the incubated epitrochlearis muscle has been used extensively in physiological and biochemical investigations (Garber *et al.*, 1976; Nesher *et al.*, 1980a,b; Challiss *et al.*, 1983), it is necessarily denervated in removal from the donor animal and therefore lacks a route for neural stimulation. A concerted effect of neural and hormonal stimulation *in vivo* may result in a higher ratio of cycling rate/flux than was observed in the present work. It is noteworthy that in flight muscle of the death's-head moth (*Acherontia atropos*) *in vivo* initiation of flight results in a cycling flux through glucose-6-phosphatase which approaches the maximal activity of this enzyme measured *in vitro* (Surholt & Newsholme, 1981, 1983). Secondly, all of the cycling rates presented were measured after 60 min of incubation *in vitro*, a time necessary to allow radioisotopic steady state to be established and sufficient metabolic end-products to accumulate to permit their determination. It is known that the effects of catecholamines decrease with exposure time (Lefkowitz *et al.*, 1980) and therefore stimulations of cycling rates presented may not be optimal. This is borne out by the decrease in the value of S_1 observed with time of adrenaline exposure (Fig. 1).

Thus, although the present work represents a significant advance in the investigation of substrate cycling, it is likely that data obtained *in vivo* on changes in the rates of substrate cycling in skeletal muscle will be required before the hypothesis of Newsholme & Crabtree (1976) can be accepted or rejected.

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APPENDIX

Calculation of the rate of fructose 6-phosphate/fructose 1,6-bisphosphate cycling in a tissue with active glycogenolysis and/or glycogen synthesis

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It has been shown by Newsholme & Crabtree (1976) that the rate of fructose 6-phosphate/fructose 1,6-bisphosphate cycling (C) is given by the equation

$$C = \frac{T(1 - S_1)}{(S_1 - S_2)}$$

where T is the glycolytic rate, $S_1 = (^3\text{H}/^{14}\text{C})$ ratio of hexose monophosphates/ $(^3\text{H}/^{14}\text{C})$ ratio of glucose and $S_2 = (^3\text{H}/^{14}\text{C})$ ratio of fructose 1,6-bisphosphate/ $(^3\text{H}/^{14}\text{C})$ ratio of glucose.

This formula is derived for a system such as that described in Scheme 1. However, in the main paper (Challiss *et al.*, 1984), it is clear that glycogenolysis and glycogen synthesis may occur under the various conditions investigated. Thus the system investigated is better described by Scheme 2. It is therefore necessary to derive new equations to take glycogen/glucose 1-phosphate cycling into account.

From Scheme 2, let J denote the rate of glycolysis, F and C the fluxes through 6-phosphofructokinase and fructose-1,6-bisphosphatase respectively, A the rate of glycogenolysis and B the

rate of glycogen synthesis. Let the specific radioactivities of glucose, hexose monophosphate (HMP) and fructose 1,6-bisphosphate (FBP) be S_g , S_h and S_f for ^3H and R_g , R_h and R_f for ^{14}C respectively.

It is assumed that glycogen acts as a 'sink' (i.e. no ^{14}C flux comes from glycogen to enter the hexose monophosphate pool).

Considering ^{14}C fluxes in the steady state:

Flux to HMP = flux from HMP (i)

$$GR_g + CR_f = R_h (F + B)$$

Since the ^{14}C label is not diluted between HMP and FBP,

$$R_h = R_f$$

and eqn. (i) becomes

$$GR_g + CR_h = R_h (F + B)$$

$$GR_g = R_h (J + B)$$

$$R_h/R_g = \frac{G}{(J + B)} \quad (1)$$

Scheme 1. Model for substrate cycling at the level of fructose 6-phosphate/fructose 1,6-bisphosphate, assuming that the hexose monophosphate pool is undiluted by glycogenolysis

HMP represents hexose monophosphate, FBP represents fructose 1,6-bisphosphate, and F , C and T represent the fluxes through 6-phosphofructokinase, fructose 1,6-bisphosphatase and glycolysis respectively.

Scheme 2. Model for substrate cycling at the level of fructose 6-phosphate/fructose 1,6-bisphosphate, taking account of an active glycogen/glucose 1-phosphate cycle
 HMP represents hexose monophosphate, FBP represents fructose 1,6-bisphosphate, G , F , C and J represent the fluxes through glucose phosphorylation, 6-phosphofructokinase, fructose 1,6-bisphosphatase and glycolysis respectively, and A and B represent the fluxes between glycogen and HMP.