Metabolic Adaptation in Phosphorylase Kinase Deficiency

CHANGES IN METABOLITE CONCENTRATIONS DURING TETANIC STIMULATION OF MOUSE LEG MUSCLES

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1. Glycogen, nucleotides and glycolytic intermediates and products were measured before and during tetanus in the hamstrings-muscle groups of normal (C3H) and phosphorylase kinase-deficient (ICR/IAn) mice. 2. Phosphorylase kinase-deficient muscles contained 3–4-fold more glycogen and sustained a larger (approx. 2-fold), more rapid (11 ± 2 ng/s faster) and more prolonged glycogenolysis during 120 s tetanus, despite their lack of phosphorylase *a*. 3. No significant change in total adenine nucleotide contents occurred during tetanus in either strain, but there was a 60–100-fold rise in IMP concentration to approx. 2 mM in both strains. The initial rate of IMP formation was 6-fold more rapid (112 nmol/s per g) in phosphorylase kinase-deficient muscle. 4. Adenylosuccinate content rose to 36 nmol/g in phosphorylase kinase-deficient muscle and to 9 nmol/g in normal muscle at 45 s tetanus, but then fell. 5. In phosphorylase kinase-deficient muscle, glucose 6-phosphate, a powerful phosphorylase inhibitor, was 56% of that in normal muscle. 6. The mass-action ratio of the phosphoglucomutase-catalysed reaction [glucose 6-phosphate]/[glucose 1-phosphate] was markedly lower than K_{eq} . (approx. 17) in relaxed muscle of both strains (approx. 5–7), but rose significantly during tetanus to the value for K_{eq} . 7. The data for IMP satisfy the criteria put forward by Rahim, Perrett & Griffiths [(1976) *FEBS Lett.* **69**, 203–206] for a nucleotide activator of phosphorylase *b*: it should be present at a higher concentration in phosphorylase kinase-deficient muscle, its concentration should rise during muscle work, and it should attain a concentration comparable with its activation constant for phosphorylase *b*.

Muscle glycogen phosphorylase *b* (EC 2.4.1.1) can be activated either by covalent phosphorylation to form phosphorylase *a*, catalysed by phosphorylase kinase (EC 2.7.1.38), or, non-covalently, by adsorptive binding of AMP or IMP. Although non-covalent activation by nucleotides has been known for 40 years (Cori *et al.*, 1938), it is still not clear whether this activation mechanism is of physiological significance. The most clear-cut evidence for non-covalent activation comes from phosphorylase kinase-deficient mice of the I-strain, which are able

to degrade muscle glycogen in response to adrenalectomy administration or electrical stimulation, despite their inability to form muscle phosphorylase *a* (Lyon & Porter, 1963; Danforth & Lyon, 1964; Gross *et al.*, 1976). Fischer *et al.* (1971) concluded that this glycogenolysis was mediated by AMP-activated phosphorylase *b*.

In a previous paper (Rahim *et al.*, 1976) we have suggested that muscle phosphorylase *b* activation in phosphorylase kinase-deficient mice is more probably mediated by IMP formation, since these mice show higher muscle IMP contents when normally active and form sufficient IMP during forced exercise for its intramuscular concentration to approach the activation constant for phosphorylase *b*. We now report more strictly controlled studies on anaesthetized mice in which muscle glycogen,

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nucleotides and glycolytic intermediates and products were measured in an attempt to elucidate the physiological adaptation of these mice to phosphorylase kinase deficiency.

Materials and Methods

Materials

Male C3H mice and ICR/IAn mice, 6 months of age, were taken from colonies maintained in our animal house.

All the reagents were purchased from BDH Chemicals (Poole, Dorset, U.K.) except for the following: AMP, ADP, ATP, glucose 1-phosphate, glucose 6-phosphate, β -NAD⁺, β -NADP⁺, β -NADH and GOD-PERID reagent were purchased from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.); phosphocreatine, dithiothreitol, imidazole, lactic acid, β -mercaptoethanol, pyruvate, bovine serum albumin, triethanolamine hydrochloride, dried baker's yeast (*Saccharomyces cerevisiae*) and Tris base were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.); pentobarbitone sodium (Nembutal) was purchased from the Agricultural and Veterinary Products Division of Abbott Laboratory Ltd. (Queenborough, Kent, U.K.); pH strips (Universalindikator pH 0–14) were obtained from Merck (Darmstadt, Germany); and [8-¹⁴C]inosine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Enzymes were purchased from either Boehringer or Sigma. Diazyme enzyme was kindly given by Professor B. E. Ryman of Charing Cross Hospital Medical School, London. Adenylosuccinase (adenylosuccinate lyase, EC 4.3.2.2) was extracted from dried baker's yeast by the method of Carter & Cohen (1956).

Methods

Anaesthetization. The mice were anaesthetized by a modification of the method described by Lyon & Porter (1963). They were injected intraperitoneally with 0.15 ml of 0.5% sodium pentobarbitone/10g body wt. [0.5% sodium pentobarbitone was made up in 10% (v/v) ethanol and 20% (v/v) propylene glycol and stored at 4°C]. Within 15 min, another dose of 0.05 ml/10g body wt. was given intraperitoneally. At this concentration of sodium pentobarbitone, deep anaesthesia for about 60 min was obtained in both ICR/IAn and C3H mice.

Stimulation of the hamstrings-muscle group. When the mice were deeply anaesthetized (approx. 30 min after the injection) the hamstrings muscle was rapidly exposed. The sciatic nerve was then crushed to prevent transmission of electrical pulses to the other tissues and the leg was tied by a thread to a strain gauge. The animal was kept undisturbed in a

warm environment for about 10 min. The sciatic nerve was then stimulated electrically, distally to the crushed position, by platinum electrodes at 40 V, with 25 Hz 0.3 ms square-wave pulses. Isometric contractions were induced for periods of 5, 10, 15, 20, 30, 45, 60, 90 and 120 s, followed by rapid freeze-clamping of the hamstrings muscle with liquid-N₂-precooled tongs. The muscle extracts were prepared as described previously (Rahim *et al.*, 1976; Lush *et al.*, 1979).

Metabolite analysis. Glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate were assayed fluorimetrically by a modification of the method of Lang & Michal (1974). The reaction mixture contained 280 μ mol of triethanolamine buffer, pH 7.4, 1 μ mol of NADP⁺, 1 μ mol of dithiothreitol, 1 μ mol of EDTA, 0.1 μ mol of MgCl₂, 0.15 μ mol of ATP and extract in a total volume of 1.6 ml.

Lactate was assayed by measuring the formation of NADH fluorimetrically by a modification of the method described by Edwards *et al.* (1975). The reaction mixture contained buffer made up of 750 μ mol of glycine, 300 μ mol of hydrazine sulphate, 36 μ mol of EDTA at pH 9.5, 2.5 μ mol of NAD⁺ and extract in a total volume of 1.5 ml.

Pyruvate was determined by the method of Passonneau & Lowry (1974). Phosphocreatine was assayed by a modification of the combined methods of Lamprecht *et al.* (1974). The assay mixture contained 70 μ mol of triethanolamine buffer, pH 7.6, 0.35 μ mol of NADP⁺, 5 μ mol of MgCl₂, 25 μ mol of glucose, 0.22 μ mol of ADP and extract in a total volume of 1.6 ml.

Nucleotides were quantified as described by Perrett (1976).

Glycogen was extracted from muscle with 30% (w/v) KOH and precipitated with ethanol and 10% (w/v) Na₂SO₄. It was then quantified by a modification of the method by Huijing (1970). The assay mixture contained 40 μ mol of sodium acetate, pH 4.8, 500 μ g of Diazyme enzyme and extract containing less than 200 μ g of glycogen in a total volume of 1 ml. The mixture was incubated at 37°C overnight and the glucose produced was analysed by the GOD-PERID reagent. Glycogen (less than 200 μ g/ml) and glucose were also run as standards.

Adenylosuccinate was measured by using adenylosuccinase, as described by Schultz & Lowenstein (1978).

All metabolite concentrations are expressed in terms of wet weight of tissue. Where molar concentrations of metabolites are given it is assumed that they are uniformly distributed throughout the cell water and that this constitutes 0.58 of the wet weight (Narahara *et al.*, 1960).

Assay of phosphorylase. Phosphorylase was extracted from the muscle by a modification of the method of Stull & Mayer (1971): 20 mM-triethanol-

amine buffer, pH 6.7, was used in the second extraction instead of 20 mM-glycerophosphate.

Phosphorylase *a* was assayed in the absence of AMP, and total phosphorylase in the presence of 1 mM-AMP. The assay mixture contained 1% oyster glycogen, 16 mM-glucose 1-phosphate, 100 mM-KCl and 5.6 mM-EDTA in 50 mM-triethanolamine buffer, pH 6.8. The activity of the phosphorylase was assayed by measuring the amount of phosphate released from glucose 1-phosphate by an auto-analyser method (Birkett, 1970).

Results and Discussion

Glycogen

The data for resting muscles in Fig. 1 show that phosphorylase kinase-deficient muscles contain 3–4-fold more glycogen than do normal muscles. Previous studies on phosphorylase kinase-deficient muscle (e.g. Lyon & Porter, 1963) have given similar results, demonstrating that this is indeed a glycogen-storage disease. Despite this, as Fig. 1 shows, the muscle glycogen is degraded rapidly during muscle contraction and the abnormal muscle was found to liberate almost twice as many glucosyl units over 120 s. The rate of glycogenolysis in the abnormal muscle was significantly higher throughout the experiment. A linear regression was performed on the differences between the two glycogen contents over the period 0–120 s, and it showed an excess glycogen depletion in the ICR/IAn-mouse muscle of 11 ± 2 ng/s. The correlation coefficient was 0.92, $P < 0.001$.

Fig. 1. Changes in the amount of glycogen in mouse muscle in response to electrical stimulation

The mice (O, C3H; ●, ICR/IAn) were treated as described in the Materials and Methods section. The experimental points represent means $\pm$ S.E.M. ($n = 6$).

This enhanced glycogenolysis accounts for the observation by Lyon & Porter (1963) that phosphorylase kinase-deficient I-strain mice can swim twice as long before exhaustion as can normal C57 mice. The data in Fig. 1 are at variance, however, with those of Danforth & Lyon (1964), who found that glycogenolysis occurred more slowly in muscle of I-strain phosphorylase kinase-deficient mice and that less glycogen was degraded during 15 s tetanus. Their results are usually quoted as showing an impaired glycogenolysis in phosphorylase kinase deficiency.

This discrepancy may be due to differences in experimental methods. Danforth & Lyon (1964) tetanized isolated superfused gastrocnemius muscles under anoxic conditions. They did not measure glycogenolysis directly, but assayed two of its products, glucose 6-phosphate and lactate, and calculated the glucosyl units consumed, a method that can only give a lower limit on the rate of glycogenolysis, since other products were neglected. The experiments in Fig. 1, on the other hand, were performed on oxygenated hamstrings muscle *in situ* and glycogenolysis was calculated directly from the loss of glycogen. Contents of glucose 6-phosphate, lactate and other metabolites were measured in the present study, and the significance of these data in relation to those of Danforth & Lyon (1964) is considered below.

Fig. 2. Percentage of phosphorylase *a* in the muscle of C3H mice in response to electrical stimulation

The amounts of phosphorylase *a* are expressed as a percentage of total phosphorylase activity. See the Materials and Methods section for experimental procedures. The experimental points represent means $\pm$ S.E.M. ($n = 8$). For ICR/IAn mice, no significant formation of phosphorylase *a* was observed.

Fig. 3. Changes in concentration of phosphocreatine in the muscle of mice in response to electrical stimulation. Mice: ○, C3H; ●, ICR/IA. See Table 1 for experimental details. The experimental points represent means $\pm$ S.E.M. ($n = 10$ at 0 s, 6 at 5, 10 and 60 s and 4 at 30 s).

Phosphorylase activation

Despite their marked glycogenolytic response, no detectable formation of phosphorylase *a* was observed in the muscle of ICR/IA mice, though an increase in phosphorylase *a* was observed in the muscle of C3H mice, reaching a maximum of 50–60% in the 10th second of tetanus, after which it declined (see Fig. 2). The decline in phosphorylase *a* after 10 s tetanus was in accord with the results reported by Danforth *et al.* (1962), Danforth & Helmreich (1964) and Piras & Staneloni (1969). The total phosphorylase in the muscle of ICR/IA mice was 45% higher than that in C3H mice, confirming earlier observations by Lyon & Porter (1963), Lyon & Mayer (1969) and Gross & Mayer (1973).

Phosphocreatine

Fig. 3 shows the fall in the content of phosphocreatine, which was similar in both mouse strains. Almost 80% of the phosphocreatine was dephosphorylated after 60 s tetanus.

Purine nucleotides

Table 1 shows the total adenine nucleotide contents in the muscle of ICR/IA and C3H mice.

There were no significant changes in response to stimulation. It should be remembered, however, that much of the muscle ADP (Seraydarian *et al.*, 1962) and AMP (Rahim *et al.*, 1976; Griffiths & Rahim, 1978) are sequestered, and may not participate in intracellular reactions. Changes in the remaining free

ADP and AMP pools could be masked by these large concentrations of bound nucleotides.

The ATP measured was probably free in the cell water, since there is no evidence for large concentrations of tight-binding sites for ATP in muscle. The reason for the stability of total ATP concentration is probably therefore the buffering effect of the creatine kinase (ATP-creatine *N*-phosphotransferase, EC 2.7.3.2) equilibrium and the loss of phosphocreatine (Fig. 3).

The most dramatic effect of tetanus on nucleotide composition is the 60–100-fold rise in IMP concentration (Fig. 4), from 0.04 to 2.5 mM in ICR/IA mouse muscle and from 0.017 to 1.8 mM in C3H mouse muscle. This rise was six times more rapid in the phosphorylase kinase-deficient mouse muscle (112 nmol/s per g in ICR/IA mouse; 16.7 nmol/s per g in C3H mouse), and since IMP is an activator of phosphorylase *b* it is reasonable to suppose that these mice have adapted to their inability to produce phosphorylase *a* by enhancing the rate at which they accumulate IMP. We have previously shown (Rahim *et al.*, 1976) that mice from this phosphorylase kinase-deficient strain have higher resting IMP concentrations when normally conscious and form more IMP during swimming than do normal mice from the SAS/4 strain. In further experiments on nucleotide contents after swimming (Rahim *et al.*, 1978) we have found

Fig. 4. Changes in the concentration of IMP in the muscle of mice in response to electrical stimulation. Mice: ○, C3H; ●, ICR/IA. See Table 1 for experimental details. The experimental points represent means $\pm$ S.E.M. ($n = 14$ at 0 s and 6 for the remaining periods of stimulation).

Table 1. Amounts of nucleotides in the muscle of C3H and ICR/1An mice in response to tetanic electrical stimulation

Mouse sciatic nerves were stimulated with 40V, 0.3 ms pulses at 25 Hz to produce an isometric tetanus of approx. 5 g. The muscles were freeze-clamped at the indicated times and metabolites were extracted with 10% (w/v) trichloroacetic acid/20% (v/v) methanol mixture. See the text for full details. Concentrations are expressed in nmol/g and are the means $\pm$ s.e.m. of the numbers in parentheses.

Mice	ICR/ 1An	Nucleotides	Period of stimulation (s) ... 0						90	120
			AMP	ADP	ATP	Total adenylates	Total purines	AMP	ADP	ATP
C3H		AMP	36.4 $\pm$ 3.7 (14)	43.5 $\pm$ 8.9 (6)	31 $\pm$ 5.2 (6)	33.4 $\pm$ 10.5 (6)	49 $\pm$ 16 (6)	37 $\pm$ 6 (6)	37 $\pm$ 6 (6)	37 $\pm$ 6 (6)
		ADP	930.7 $\pm$ 63.8 (14)	890.5 $\pm$ 80 (6)	1173 $\pm$ 130 (6)	903 $\pm$ 42 (6)	773 $\pm$ 86.7 (6)	997.6 $\pm$ 101 (6)	995 $\pm$ 80 (6)	995 $\pm$ 80 (6)
		ATP	7553 $\pm$ 486 (14)	6765 $\pm$ 675 (6)	9227 $\pm$ 462 (6)	7001 $\pm$ 446 (6)	6314 $\pm$ 635 (6)	7099 $\pm$ 1015 (6)	7069 $\pm$ 427 (6)	7069 $\pm$ 427 (6)
		Total adenylates	8544 $\pm$ 667 (14)	6799.8 $\pm$ 750 (6)	10431 $\pm$ 581 (6)	7933 $\pm$ 471 (6)	7136 $\pm$ 675 (6)	8133 $\pm$ 1119 (6)	8101.5 $\pm$ 470 (6)	8101.5 $\pm$ 470 (6)
		Total purines	10461 $\pm$ 668 (14)	8151.7 $\pm$ 799 (6)	11908 $\pm$ 789 (6)	8987 $\pm$ 461 (6)	8573 $\pm$ 702 (6)	9063 $\pm$ 932 (6)	9201 $\pm$ 492 (6)	9201 $\pm$ 492 (6)
		AMP	54.3 $\pm$ 5.7 (14)	39.5 $\pm$ 5 (6)	52.8 $\pm$ 19.5 (6)	49.4 $\pm$ 10.3 (6)	32.8 $\pm$ 8.1 (6)	33.8 $\pm$ 8.1 (6)	34.3 $\pm$ 3.8 (6)	34.3 $\pm$ 3.8 (6)
		ADP	930 $\pm$ 119 (14)	683 $\pm$ 39 (6)	942 $\pm$ 84 (6)	868 $\pm$ 57 (6)	805.5 $\pm$ 104 (6)	792 $\pm$ 52 (6)	717 $\pm$ 43 (6)	717 $\pm$ 43 (6)
		ATP	6989 $\pm$ 580 (14)	6036 $\pm$ 452 (6)	7305 $\pm$ 663 (6)	6576 $\pm$ 558 (6)	6433 $\pm$ 604 (6)	5499.8 $\pm$ 282 (6)	5135 $\pm$ 352 (6)	5135 $\pm$ 352 (6)
		Total adenylates	7641 $\pm$ 688 (14)	6599 $\pm$ 476 (6)	8286 $\pm$ 746 (6)	7388 $\pm$ 497 (6)	7697 $\pm$ 1319 (6)	6326 $\pm$ 282 (6)	5887 $\pm$ 390 (6)	5887 $\pm$ 390 (6)
		Total purines	7651 $\pm$ 650 (14)	6919 $\pm$ 436 (6)	8463 $\pm$ 783 (6)	8052 $\pm$ 620 (6)	8308 $\pm$ 763 (6)	7007 $\pm$ 393 (6)	6500 $\pm$ 407 (6)	6500 $\pm$ 407 (6)

similar but more marked effects when ICR/IAN mice are compared with C3H mice, the strain used as controls in the present paper.

The maximal activity, under optimal conditions *in vitro*, of AMP deaminase in unstimulated muscle of ICR/IAN mice was 60% of that of C3H-mouse muscle ($378 \pm 20 \mu\text{mol}$ of NH_4^+ /min per g compared with $569 \pm 53 \mu\text{mol}$ of NH_4^+ /min per g in ICR/IAN and C3H mice respectively), so the enhanced rate of IMP accumulation is not due simply to a higher activity of the enzyme forming it. Alternatively, the accumulation of IMP in the early stages of tetanus (0–10s in ICR/IAN mice and 0–60s in C3H mice) could be due to an imbalance between the rate of IMP synthesis (which is at least 112 nmol/s per g) and its utilization. The main pathway of IMP utilization is via adenylosuccinate synthetase (EC 6.3.4.4), whose maximum velocity, under optimum conditions, is 4 nmol/s per g in ICR/IAN mice. IMP accumulates more slowly in C3H mice (17 nmol/s per g), but this minimal rate of synthesis is still higher than the maximal rate of IMP utilization by adenylosuccinate synthetase, which is 5 nmol/s per g in these mice. However, the rate-limiting enzyme in the purine nucleotide cycle, at least in terms of maximal velocity, appears to be adenylosuccinase, whose maximal velocity is 1.8 nmol/s per g in both mouse strains.

Fig. 5 shows the variation in muscle adenylosuccinate concentration during tetanus. This metabolite is of interest, since, with AMP and IMP, it makes up the purine nucleotide cycle (Lowenstein, 1972).

The intramuscular adenylosuccinate concentration in both groups of muscles rose to a maximum at

Fig. 5. Changes in the concentration of adenylosuccinate in the muscle of mice in response to electrical stimulation. Mice: O, C3H; ●, ICR/IAN. See Table 1 for experimental details. The experimental points represent the average adenylosuccinate concentration in six mice.

45 s (although the peak was lower in the C3H mice) and then fell steadily for the remainder of the tetanus. Because of the low concentrations of this metabolite, it was necessary to pool the groups of six muscle extracts from each period of tetanus. Thus the data points represent the average adenylosuccinate concentrations in six mice but do not include standard errors. There was no detectable ($<0.2 \text{ nmol/g}$) adenylosuccinate in any of the control samples taken from the contralateral legs of stimulated animals.

These data may be compared with those of Goodman & Lowenstein (1977) and Schultz & Lowenstein (1978), who found a maximum value of approx. 4 nmol/g in normal rat muscle after 15 min stimulation at 5 Hz and in rat brain after 40 s stimulation at 60 Hz respectively. This fall in adenylosuccinate concentration coincided with the rise in IMP concentration, and in the latter phase of the experiment there was a strong negative correlation between adenylosuccinate and IMP concentration, which they attributed to the known inhibitory effect of IMP on adenylosuccinate synthetase (Tornheim & Lowenstein, 1972).

The data in Figs. 4 and 5 are qualitatively similar to those of Goodman & Lowenstein (1977), in that the adenylosuccinate content fell after the IMP content reached its peak. However, in ICR/IAN-mouse muscle, the peak IMP concentration was reached much earlier (10 s tetanus) than the point at which adenylosuccinate concentration began to fall (60 s tetanus), and there was no correlation when adenylosuccinate concentration was plotted against AMP concentration in either mouse strain.

From Fig. 5, the rates of adenylosuccinate accumulation in ICR/IAN-mouse muscle was 0.2 nmol/s per g in the first 30 s tetanus and rose to 2.1 nmol/s per g in the next 15 s tetanus, whereas in C3H-mouse muscle the rates were 0.4 nmol/s per g in the first 15 s tetanus, which fell to 0.2 nmol/s per g in the next 30 s tetanus.

The transitory accumulation of adenylosuccinate in the first 15 s tetanus could be due to a lag in adenylosuccinase activation (Schultz & Lowenstein, 1978).

Glycolytic metabolites

Table 2 shows the changes in the contents of glycolytic metabolites in the muscle of ICR/IAN and C3H mice during tetanus. In resting muscle there was no significant difference in the concentrations of glucose 6-phosphate, but there was a highly significant difference ($P < 0.001$) throughout the tetanus, the concentration in C3H-mouse muscle being $1.09 \pm 0.06 \mu\text{mol/g}$ (79% higher).

The formation of fructose 6-phosphate, which depends on the availability of glucose 6-phosphate, followed the same pattern at a much lower concen-

Table 2. *Amounts of glycolytic intermediates in the muscle of C3H and ICR/IAn mice in response to periods of electric stimulation*
 The concentrations are expressed in $\mu\text{mol/g}$ and are the means ($\pm$ S.E.M.) for the numbers of determinations given in parentheses. See Table 1 for experimental details.

Mice	Metabolites	Period of stimulation (s) ... 0					
		5	10	30	60	90	120
ICR/ IAn	Glucose 1-phosphate	0.03 $\pm$ 0.008 (4)	0.103 $\pm$ 0.02 (6)	0.084 $\pm$ 0.022 (6)	0.116 $\pm$ 0.034 (5)	0.06 $\pm$ 0.02 (5)	0.18 $\pm$ 0.08 (6)
	Glucose 6-phosphate	0.16 $\pm$ 0.007 (4)	0.74 $\pm$ 0.14 (6)	0.76 $\pm$ 0.17 (6)	1.7 $\pm$ 0.25 (5)	1.26 $\pm$ 0.32 (5)	3.03 $\pm$ 0.7 (6)
	Fructose 6-phosphate	0.04 $\pm$ 0.02 (4)	0.14 $\pm$ 0.01 (6)	0.27 $\pm$ 0.11 (6)	0.37 $\pm$ 0.04 (5)	0.28 $\pm$ 0.06 (5)	0.57 $\pm$ 0.17 (6)
	Pyruvate	0.06 $\pm$ 0.008 (4)	0.176 $\pm$ 0.037 (6)	0.24 $\pm$ 0.02 (6)	0.17 $\pm$ 0.02 (5)		
	Lactate	2.58 $\pm$ 0.11 (14)	5.7 $\pm$ 0.4 (6)	6 $\pm$ 0.5 (6)	7.4 $\pm$ 0.5 (5)	7.9 $\pm$ 1.1 (5)	6.22 $\pm$ 0.2 (6)
C3H	Glucose 1-phosphate	0.043 $\pm$ 0.019 (4)	0.13 $\pm$ 0.03 (6)	0.046 $\pm$ 0.014 (4)	0.275 $\pm$ 0.078 (6)	0.19 $\pm$ 0.046 (4)	0.18 $\pm$ 0.036 (6)
	Glucose 6-phosphate	0.18 $\pm$ 0.03 (4)	1.42 $\pm$ 0.43 (6)	1.5 $\pm$ 0.5 (4)	3.63 $\pm$ 0.9 (6)	2.96 $\pm$ 0.6 (4)	4.24 $\pm$ 0.73 (6)
	Fructose 6-phosphate	0.08 $\pm$ 0.02 (4)	0.27 $\pm$ 0.07 (6)	0.34 $\pm$ 0.12 (4)	0.9 $\pm$ 0.22 (6)	0.6 $\pm$ 0.05 (4)	0.82 $\pm$ 0.18 (6)
	Pyruvate	0.048 $\pm$ 0.009 (4)	0.17 $\pm$ 0.03 (6)	0.28 $\pm$ 0.05 (4)	0.18 $\pm$ 0.06 (6)		
	Lactate	2.6 $\pm$ 0.08 (14)	4.9 $\pm$ 0.3 (6)	7.4 $\pm$ 0.4 (4)	7.2 $\pm$ 0.5 (6)	10.3 $\pm$ 0.5 (4)	7.6 $\pm$ 0.6 (6)

tration. The concentration of the first product of glycogenolysis, glucose 1-phosphate, rose at a comparable rate in the muscle of both mouse strains.

There was a marked rise in total lactate concentration in muscle from both mouse strains during the first 90 s tetanus, followed, in both cases, by a fall at 120 s tetanus. This fall could reflect a decrease in the rate of lactate formation from glycolysis (although glucose 6-phosphate concentration continues to rise in this period) or an increase in the rate of its removal, consequent on such factors as blood flow. In this context, it is noteworthy that no significant increase in lactate concentration occurred in the muscle of mice from these strains after 15–30 min swimming (Z. H. A. Rahim & J. R. Griffiths, unpublished work).

The pyruvate content of muscle from both mouse strains was lower than that of lactate, but followed a similar pattern during tetanus. However, pyruvate fell from its peak value in both sets of muscle in the 30–60 s period, whereas the fall in lactate occurred between 90 and 120 s tetanus. Again, no attempt was made to differentiate between intracellular and extracellular pyruvate.

Accumulation of lactate was used by Danforth & Lyon (1964) in their calculation of the rate of glycogenolysis in anaerobic, superfused muscles. In the present study, however, it would not be safe to assume that all the lactate (or pyruvate) formed would have been retained in the muscle (although there is evidence that the translocation of lactate to the blood during tetanus is slow in human muscle; Karlsson, 1970) or that some oxidation of pyruvate had not taken place. It is also likely that some lactate measured was extracellular rather than intracellular, and no attempt was made to allow for this. Nevertheless, the rates of increase in lactate concentration in the kinase-deficient animals were similar. Lactate accumulation in ICR/IAN-mouse muscle (Table 2) during the first 10 s tetanus was $0.32 \mu\text{mol/s}$ per g, compared with approx. $0.3 \mu\text{mol/s}$ per g in Fig. 1 of Danforth & Lyon (1964) for I-strain mouse muscle. The rates of lactate accumulation in the normal muscle were quite different: $0.2 \mu\text{mol/s}$ per g in C3H-mouse muscle *in situ* (Table 2) and approx. $0.6 \mu\text{mol/s}$ per g in isolated C57-mouse muscle (Danforth & Lyon, 1964; Fig. 2).

The sum of the concentrations of lactate, pyruvate, glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate can be compared (allowing for the stoichiometry of two lactate or pyruvate molecules per molecule of hexose) with the loss of glycogen glucosyl groups: $1.16 \mu\text{mol}$ of glucosyl groups/s per g was liberated from glycogen in the first 10 s tetanus from ICR/IAN-mouse muscle and $0.25 \mu\text{mol}$ of glucosyl groups/s per g as glycolytic intermediates were formed. For normal C3H-mouse muscle, the values were $0.6 \mu\text{mol}$ of glucosyl groups/s

per g liberated from glycogen and $0.27 \mu\text{mol}$ of glucosyl groups/s per g formed as glycolytic intermediates. It is clear that most of the glucosyl residues liberated by the muscle *in situ* are not accounted for, either because the lactate and pyruvate were lost into the circulation, or because pyruvate was oxidized, or because other glycolytic intermediates have been neglected.

Non-covalent activation of phosphorylase b

Previously (Rahim *et al.*, 1976) we suggested three criteria that a potential nucleotide activator of phosphorylase *b* should satisfy: (i) it should be present at higher concentration in phosphorylase kinase-deficient than in normal muscle; (ii) its concentration should rise during muscle work; and (iii) it should attain a concentration comparable with its activation constant for phosphorylase *b*.

All these criteria are satisfied by IMP. Its maximum intracellular concentration was approx. 2 mM in normal and phosphorylase kinase-deficient muscle, whereas the activation constant for IMP, determined by using phosphorylase *b* extracts from phosphorylase kinase-deficient muscles, was 1.2 mM. It is thus likely that IMP activation of phosphorylase *b* occurred.

An alternative mechanism by which phosphorylase *b* activity could be enhanced during muscle work would be a decrease in the concentration of one of its inhibitors. This possibility can be discounted, since all the major inhibitors assayed either showed no change in total concentration (ATP or ADP), or, in the case of glucose 6-phosphate, increased in concentration. Nevertheless, the concentration of glucose 6-phosphate was lower in the phosphorylase kinase-deficient mouse muscle. Since there is evidence that ADP is extensively protein-bound (Seraydarian *et al.*, 1962), it is theoretically possible that this large inert pool could mask a fall in the concentration of free ADP. We have calculated (Z. H. A. Rahim, R. J. Shuttlewood & J. R. Griffiths, unpublished work), on the contrary, that free ADP rises markedly in concentration during tetanus (see also Goodman & Lowenstein, 1977). There is therefore no evidence that non-covalently induced phosphorylase *b* activity is enhanced in tetanized phosphorylase kinase-deficient muscle by a decrease in the concentration of any important inhibitor.

There remains the possibility that AMP is compartmented in the muscle (Griffiths & Rahim, 1978) and that, although the total AMP concentration is unchanged, the AMP pool in contact with phosphorylase *b* rises in concentration during muscle contraction. Although there is no direct way to determine the concentration in this pool, indirect evidence suggests that AMP-induced activation could also be significant (Z. H. A. Rahim & J. R. Griffiths, unpublished work).

Mass-action ratios for the phosphoglucomutase- and phosphoglucoisomerase-catalysed reactions

It is usually assumed that the reactions catalysed by phosphoglucomutase and phosphoglucoisomerase are close to equilibrium *in vivo*, so that their mass-action ratios (the ratios [glucose 6-phosphate]/[glucose 1-phosphate] and [fructose 6-phosphate]/[glucose 6-phosphate] respectively) would approximate to their equilibrium constants [17 (Busby & Radda, 1976) for phosphoglucomutase and 0.3 (Beis & Newsholme, 1975; Banks *et al.*, 1976) for phosphoglucoisomerase]. Fig. 6 shows that the mass-action ratio of phosphoglucomutase differs greatly from the equilibrium constant at rest, both in C3H and in ICR/IAn mice, but rises to the normal value during exercise. In C3H mice, the mass-action ratio rose much more rapidly towards the equilibrium constant during tetanus, reaching 18 after 5 s, whereas in ICR/IAn mice the same value was attained between 40 and 60 s. Both rises were statistically significant ($P < 0.05$). In ICR/IAn mice there was a linear increase in mass-action ratios with time from 5 to 120 s ($0.13 \pm 0.02 \text{ s}^{-1}$; $r = 0.95$, $P < 0.005$).

Fig. 6. Mass-action ratios for phosphoglucomutase-catalysed reaction in the muscle of mice at the different periods of stimulation

Mice: O, C3H; ●, ICR/IAn. The mass-action ratio is defined as the ratio of the concentration of glucose 6-phosphate to the concentration of glucose 1-phosphate. The experimental points represent means $\pm$ S.E.M. ($n = 4$ at 0, 30 and 90 s, 6 at 5, 10, 60 and 120 s).

The mass-action ratio of the phosphoglucoisomerase reaction was significantly raised in resting C3H-mouse muscle ($P < 0.05$). After 10 s tetanus it fell to approx. 0.2 and remained there for a further 110 s tetanus; in ICR/IAn mice the mass-action ratio remained in the range 0.25–0.33 throughout the tetanus (results not shown).

The data in Fig. 6 can be accounted for on two hypotheses: either the phosphoglucomutase-catalysed reaction is not at equilibrium in resting muscle and attains equilibrium during tetanus, or there is a pool of glucose 1-phosphate, at least in relaxed muscle, which is separate from the pool in equilibrium with glucose 6-phosphate. In the latter case, the reaction would tend towards equilibrium during contraction either because of the elimination of the separate glucose 1-phosphate pool or because of an increase in the pool in equilibrium with glucose 6-phosphate. There have been many suggestions that glucose 6-phosphate exists in more than one pool, particularly in liver (see the review by Ryman & Whelan, 1971) and also in muscle (Dawson *et al.*, 1977), but this could not explain the present observation.

Alternatively, the phosphoglucomutase-catalysed reaction could be kept from attaining equilibrium in resting muscle by the absence of glucose 1,6-bisphosphate, the essential cofactor. It would be necessary to postulate a very low phosphoglucomutase activity if the mass-action ratio were to remain far from equilibrium in relaxed muscle, since one would expect very little glycogenolysis.

Postulated role of 'crippled' phosphorylase b kinase in phosphorylase kinase-deficient muscle

There has been a controversy about the presence of an abnormal enzyme protein in the muscle of phosphorylase kinase-deficient mice. Cohen & Cohen (1973) found that antibodies against rabbit phosphorylase kinase formed a precipitin line in Ouchterlony double-diffusion experiments with extracts of phosphorylase kinase-deficient muscle, and this observation was confirmed by Gross & Mayer (1974) and by Gross *et al.* (1975), who also studied the properties of the very low (approx. 0.3% of normal) phosphorylase kinase-like activity in these extracts and concluded that a 'crippled' enzyme was present at normal concentrations. Cohen *et al.* (1976) drew the opposite conclusion. They found that antibodies to purified subunits of phosphorylase kinase did not precipitate with extracts of phosphorylase kinase-deficient muscle, and they attributed their earlier observations (Cohen & Cohen, 1973) to a trace contaminant. These later results have been confirmed by Daegelen-Proux *et al.* (1978). Cohen *et al.* (1976) suggested that the X-chromosome gene, which is defective in phosphorylase kinase deficiency, is a

control gene and that none of the three phosphorylase kinase subunits can be synthesized in its absence.

Studies by Varsanyi *et al.* (1978) suggest an explanation for the 0.3% phosphorylase kinase-like activity in the abnormal muscles. They found that the sarcoplasmic reticulum of normal and phosphorylase kinase-deficient mouse muscle contains a Ca^{2+} -dependent protein kinase which can convert phosphorylase *b* into *a*. Since this enzyme would be present in homogenates of phosphorylase kinase-deficient muscle and in extracts of protein-glycogen complexes ('glycogen particles'), which always contain sarcoplasmic-reticulum elements (Meyer *et al.*, 1970), it could account for their ability to catalyse the phosphorylation of phosphorylase *b* to phosphorylase *a*. This Ca^{2+} -dependent kinase would not, however, activate phosphorylase *b* to phosphorylase *a* in intact muscle, since it is situated on the sarcoplasmic reticulum and is not, like true phosphorylase kinase, bound to glycogen particles (Varsanyi *et al.*, 1978).

These observations account for the anomaly that intact skeletal muscles from phosphorylase *b* kinase-deficient animals form no detectable phosphorylase *a* on stimulation (Danforth & Lyon, 1964; Fig. 1), whereas broken-cell preparations show phosphorylase *a* formation (Gross & Mayer, 1974). They do not, however, explain the isoproterenol-induced glycogenolysis observed in intact diaphragm muscle of I-strain mice (Gross *et al.*, 1976, 1978), which occurs without any change in nucleotide concentrations (Gross *et al.*, 1978; J. R. Griffiths & Z. H. A. Rahim, unpublished work). These effects are not found in limb muscles (Gross *et al.*, 1976; J. R. Griffiths & Z. H. A. Rahim, unpublished work), on which the present studies were performed.

Physiological role of non-covalent phosphorylase b activation

In view of the data presented by Rahim *et al.* (1976) and in the present paper, it seems likely that the glycogenolysis in working phosphorylase kinase-deficient muscles is mediated non-covalently by nucleotides, since they form IMP (activator) much more rapidly and glucose 6-phosphate (inhibitor) rather less rapidly than do normal muscles. Both effects would tend to enhance non-covalently mediated glycogenolysis, and they suggest a metabolic adaptation to the condition. Similar IMP concentrations are also formed in normal muscle, though more slowly, and probably cause glycogenolysis. In view of the likely compartmentation of AMP within muscle, one cannot exclude the possibility that AMP-induced activation has a role. Indeed, the very high rate of glycogenolysis in ICR/IAN-mouse muscle from 0 to 10 s, when IMP concentrations are relatively low, suggest an additional activation mechanism such as AMP.

The data in Fig. 2 and previous studies by Danforth *et al.* (1962), Danforth & Helmreich (1964) and Piras & Staneloni (1969) show that phosphorylase *a* contents decline markedly during sustained muscle work. Since the muscle glycogen is almost entirely degraded in exhausted muscle (Terjung *et al.*, 1972, 1973; Hickson *et al.*, 1977; Conlee *et al.*, 1978), it seems possible that non-covalent phosphorylase *b* activation could be responsible for this terminal phase of glycogenolysis.

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