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The Contents of Adenine Nucleotides, Phosphagens and some Glycolytic Intermediates in Resting Muscles from Vertebrates and Invertebrates

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1. The lowest contents of ATP and the lowest ATP/AMP concentration ratios are observed in the molluscan muscles that have very low rates of energy expenditure during contraction. The highest contents of ATP are observed in the extremely aerobic insect flight muscle and the extremely anaerobic pectoral muscle of the pheasant and domestic fowl. In general, the lowest ATP/AMP concentration ratios are observed for muscle in which the variation in the rate of energy utilization is small (e.g. some molluscan muscles, heart muscle); the highest ratios are observed in muscles in which this variation is large (lobster abdominal muscle, pheasant pectoral muscle, some insect flight muscles). This finding is consistent with the proposed role of AMP and the adenylate kinase reaction in the regulation of glycolysis. However, in the flight muscle of the honey-bee the ATP/AMP ratio is very low, so that glycolysis may be regulated by factors other than the variation in AMP concentration. 2. The variation in the contents of arginine phosphate in muscle from the invertebrates is much larger than the variation in creatine phosphate in muscle from the vertebrates. 3. The contents of hexose monophosphates and pyruvate are, in general, higher in the muscles of vertebrates than in those of the invertebrates. The contents of phosphoenolpyruvate are similar in all the muscles investigated, except for the honey-bee in which it is about 4–10-fold higher. 4. The mass-action ratios for the reactions catalysed by phosphoglucoisomerase and adenylate kinase are very similar to the equilibrium constants for these reactions. Further, the variation in the mass-action ratios between muscles is small. It is concluded that these enzymes catalyse reactions close to equilibrium. However, the mass-action ratios for the reactions catalysed by phosphofructokinase and pyruvate kinase are much smaller than the equilibrium constants. The variation in the ratios between different muscles is large. It is concluded that these enzymes catalyse non-equilibrium reactions. Since the variation in the mass-action ratios for the reactions catalysed by the phosphagen kinases (i.e. creatine and arginine phosphokinases) is small, it is suggested that these reactions are close to equilibrium.

In this paper the contents of adenine nucleotides, creatine phosphate (or arginine phosphate), creatine (or arginine) and some glycolytic intermediates in a large number of muscles from different animals are reported together with the calculated mass-action ratios for the following reactions: adenylate kinase (EC 2.7.4.3), arginine phosphokinase (EC 2.7.3.3), creatine phosphokinase (EC 2.7.3.2), phosphofructokinase (EC 2.7.1.11), phosphoglucoisomerase (EC 5.3.1.9) and pyruvate kinase (EC 2.7.1.40). Under resting conditions (or as near resting as possible), muscles were rapidly frozen by the freeze-clamping technique, the frozen muscles were extracted and the contents of the intermediates measured by specific enzymic techniques. A recent survey of the literature for contents of the glycolytic and energy intermediates in muscle *in vivo* indicates that such data are available only for tissues from the rat, mouse, guinea pig and, in some cases, the frog (Williamson & Brosnan, 1974). The present findings are discussed in relation to the function and/or control of these enzymes in the muscle.

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Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: EDTA (disodium salt) and all inorganic reagents were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and were of the highest purity available. Nembutal

was obtained from Abbott Laboratories, Arge-Vet Division, Queenborough, Kent, U.K. Sandoz-MS222 was obtained from Thompson and Joseph, Castle House, Castle Meadow, Norwich NOR 41D, U.K. Arginine kinase was prepared in this laboratory from lobster muscle by Dr. A. R. Leech.

Animals

The sources of the animals were as given by Newsholme & Taylor (1969) except for the addition of the following: rats were obtained from Charles River (U.K.) Ltd., Cherry Garden Lane, Ash, Canterbury, Kent, U.K., and kept in the animal house of the Department of Biochemistry; mice were bred in this Department; lobsters were obtained from Fisher Bros., 12 Billingsgate Street, London E.C.3, U.K.; frogs and snails were obtained from Gerrard and Haig, East Preston, Sussex, U.K. Locusts were used 7–14 days after the final moult. Flies were used between 7 and 14 days after emerging from pupae. All other insects were of undetermined age, but they were known to be capable of flight. Apart from rats and mice, for which only male animals were used, muscle tissue was obtained from male and female animals indiscriminately.

Freeze-clamping of muscle

All muscle tissues used in the measurements of contents of adenine nucleotides and intermediates were frozen rapidly with the aid of aluminium tongs cooled in liquid N_2 (see the Results section). The method of removing and freeze-clamping the muscle depended on the animal and the muscle under investigation. In each case, the method that was finally adopted represented a compromise between rapidity of freezing, selection of a specific muscle and freezing the muscle under resting conditions (see also the Results section). For the snail, a piece of the foot was rapidly dissected and immediately freeze-clamped. For the other molluscs, the shell was opened by cutting through the muscle with a scalpel and a piece of muscle was rapidly dissected and freeze-clamped. From opening the shell to freezing the muscle, the procedure was completed in 5–10s. For the lobster, the animal was 'anaesthetized' by placing in ice for 20min, then the tail was cut off and a piece of abdominal muscle was cut away and freeze-clamped within 15s from removal of the tail. For the insects, the whole animal was freeze-clamped while at rest (i.e. non-flying). This procedure resulted in much of the flight muscle being squeezed out of the thorax, so that the muscle could be easily separated from cuticle and other tissues. In some insects, the muscle was dissected away from the cuticle at liquid-N_2 temperatures. For frogs, the animals were anaesthetized with Sandoz-MS222 (0.1g/litre of water) and the skin was removed from the hind limb, the gastrocnemius muscle was separated from the other muscles and freeze-clamped; the dissection was completed within 30s. For dogfish, the animals were anaesthetized by intravenous (caudal-vein) injection of Nembutal (0.1ml/kg), and a piece of white muscle was rapidly dissected and freeze-clamped; the dissection was completed within 30s. For the birds, the animals were anaesthetized by an intraperitoneal injection of Nembutal, feathers and skin were rapidly removed from above the pectoral muscles and a piece of pectoral muscle was dissected and immediately freeze-clamped. From the initial incision to the freeze-clamping of the muscle, the procedure was completed within 5s. For rats and mice, the animals were anaesthetized with diethyl ether, the skin was dissected from the hind limb and the exposed muscle was freeze-clamped *in situ*. The muscle was dissected away from the bone at liquid-N_2 temperatures.

For each animal, the problem of whether to use an anaesthetic to obtain resting muscle was considered. In some animals, experiments were performed to compare anaesthetic methods or to compare the results obtained with and without anaesthetization (see the Results section and Table 1).

Measurement of adenine nucleotides and intermediates

The frozen muscle was powdered in a percussion mortar at −70°C, and the powdered muscle was extracted by the addition of 4–5vol. of frozen $HClO_4$ (6%, w/v). The extraction took place in a mortar, and continual mixing with the pestle thawed the mixture of $HClO_4$ and frozen muscle powder. The precipitated protein was removed by centrifugation and the extract was neutralized with 3M-$KHCO_3$. The metabolic intermediates were measured by enzymic techniques, which are simple, sensitive and, in particular, specific for the compounds under investigation. Glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate and adenine nucleotides were determined by using the methods described by Gevers & Krebs (1966), and pyruvate was determined in the same assay as phosphoenolpyruvate (Czok & Eckert, 1963). Creatine phosphate and arginine phosphate were determined in the same assay as that for ATP after the addition of creatine or arginine phosphokinase and ADP (Lamprecht & Stein, 1963).

Results

Investigations into use of anaesthetics and extraction techniques

Effects of anaesthetics on the contents of energy metabolites. In the present investigation the amounts

Table 1. *Effects of anaesthetization on contents of adenine nucleotides, creatine phosphate and creatine in different muscles*

Animals were anaesthetized as follows. Dogfish were anaesthetized in three ways; they were rapidly removed from the tank and immediately stunned by a sharp blow on the head above the eyes. They were placed in a tank of sea water and Sandoz-MS222 was added (2.5 g/kg of dogfish in 25–50 litre of sea water). Nembutal (0.1 ml/kg) was injected into the caudal vein. Frogs were anaesthetized by the addition of Sandoz-MS222 to the water (0.1 g/litre). The insects were anaesthetized with CO_2, and muscles from four or five insects were pooled for each analysis reported in the Table. Values are means ± S.E.M. for the number of determinations in parentheses.

			Contents (μmol/g fresh wt.)				
Animal	Muscle	Condition of animal	ATP	ADP	AMP	Creatine phosphate	Creatine
Locust (*Schistocerca gregaria*)	Flight	Not anaesthetized	5.83 ± 0.27 (9)	0.48 ± 0.06 (5)	0.05 ± 0.03 (5)	—	—
		Anaesthetized with CO_2	6.29 (2)	0.50 (2)	0.02 (2)	—	—
Rosechafer (*Pachnoda ephippiata*)	Flight	Not anaesthetized	8.51 ± 0.16 (6)	1.09 ± 0.04 (6)	0.06 ± 0.01 (6)	—	—
		Anaesthetized with CO_2	8.05 (2)	1.14 (2)	0.10 (2)	—	—
Dogfish (*Scylliorhinus canicula*)	White	Anaesthetized by blow on the head	4.18 ± 0.41 (4)	0.73 ± 0.12 (4)	0.07 ± 0.02 (4)	4.9 ± 3.0 (3)	33.14 ± 7.9 (3)
		Anaesthetized by intravenous injection of Nembutal	5.14 ± 0.62 (6)	0.81 ± 0.03 (6)	0.029 ± 0.01* (6)	8.8 ± 2.4 (6)	36.6 ± 7.7 (6)
		Anaesthetized with Sandoz-MS222	2.50 ± 0.22* (6)	0.72 ± 0.11 (6)	0.32 ± 0.07* (6)	4.8 ± 0.7 (6)	16.4 ± 2.9* (6)
Frog (*Rana temporaria*)	Gastrocnemius	Not anaesthetized	3.42 ± 0.27 (8)	0.56 ± 0.03 (8)	0.03 ± 0.01 (8)	10.82 ± 1.81 (8)	11.56 ± 2.7 (8)
		Anaesthetized with Sandoz-MS222	4.47 ± 0.13* (4)	0.70 ± 0.03 (4)	0.03 ± 0.01 (4)	19.9 ± 0.94* (4)	7.8 ± 0.63 (4)

* Difference from the immediate controls is statistically significant ($P < 0.05$).

of metabolic intermediates in certain muscles or muscle groups were measured so that, in some cases, preparation of the animal and/or dissection of the muscle before freeze-clamping (see below) was necessary. It was considered that such procedures might cause sufficient trauma to modify the resting-state metabolism in the muscle and the problem arose as to the use of anaesthetics. The danger in using an anaesthetic is that it might interfere either directly or indirectly in metabolism and modify the concentrations of the metabolites under investigation. The problem of not using an anaesthetic is that the animal or the muscle may respond to the preparative procedure in such a way that the muscle can no longer be described as resting. To examine if anaesthetics were necessary, selected animals were investigated. The dogfish, frog and insects were chosen. The dogfish was anaesthetized by stunning, intravenous injection of Nembutal (caudal vein) or addition of Sandoz-MS222 to the sea water containing the fish. The frog was anaesthetized by the addition of Sandoz-MS222 to the water. The insects were anaesthetized with CO_2. The contents of creatine phosphate, creatine, ATP, ADP and AMP were measured and are reported in Table 1. In the dogfish the ATP/AMP concentration is lowest (8) when Sandoz-MS222 was used as an anaesthetic and highest when Nembutal was used (170); there was, however, little difference in the contents of creatine or creatine phosphate. In the frog, the ATP/AMP content ratio and the creatine phosphate content were slightly higher in the anaesthetized animals (Table 1). In the two insects studied, there was no effect of the anaesthetic on the ATP/AMP content ratios. On the basis of the results in Table 1, it was decided to use an anaesthetic to obtain muscle from all vertebrate species, but anaesthetics were not used for the invertebrates except for the lobster. It was necessary to cool the lobster in ice to immobilize the animal sufficiently to dissect the muscle.

Effects of extraction procedure. To interpret the amounts of metabolic intermediates measured in any tissue in relation to the metabolic flux and/or the existence of near-equilibrium or non-equilibrium reactions in the steady-state, it is important that the extraction procedure causes minimum changes in the concentrations of the metabolites. The procedure used in the present work is very similar to that used in many other laboratories (for general description of technique, see Newsholme, 1962; Hess, 1965; Start, 1969). The muscle is clamped between plates of aluminium cooled in liquid N_2 (freeze-clamping); this produces almost instantaneous cooling of the tissue to very low temperatures (see Wollenberger *et al.*, 1960). The frozen muscle is pulverized in a percussion mortar at liquid-N_2 temperatures and the frozen muscle powder is extracted with $HClO_4$ at 0°C. [The recovery of added glycolytic intermediates and adenine nucleotides in this extraction process has been shown to be almost 100% (Newsholme, 1962; Start, 1969)]. Further, after neutralization of the extract, the amounts of the intermediates were measured by specific enzymic techniques, which permit the precise measurement of individual metabolites without the necessity of separation procedures that could result in loss of metabolites.

In view of the precautions taken in this work, it is considered that statistically significant variations in amounts of metabolic intermediates in various muscles are indicative of metabolic and physiological differences within the muscles. Some possible metabolic bases for the variations are discussed in this paper.

Amounts of metabolites

The amounts of ATP, ADP, AMP, creatine (or arginine) and creatine phosphate (or arginine phosphate)/g of tissue in the muscles that have been investigated together with the calculated mass-action ratios for adenylate kinase and arginine or creatine phosphokinase, are presented in Table 2. The amounts of glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate, phosphoenolpyruvate and pyruvate/g of muscle, together with the calculated mass-action ratios of phosphoglucoisomerase, phosphofructokinase and pyruvate kinase, are presented in Table 3. For ease of comparison, amounts of adenine nucleotides, phosphagens and glycolytic intermediates (and calculated mass-action ratios) from heart and tissues other than muscle, which were obtained from the literature, are reported in Tables 2 and 3.

Amounts of adenine nucleotides and phosphagens. For the 21 muscles investigated (from 21 different animals) the variation in amount of the adenine nucleotides/g of tissue was remarkably small. This ranged from 0.76 to 8.51, 0.43 to 1.57 and 0.02 to 0.28 μmol/g for ATP, ADP and AMP respectively. The largest differences in the extreme values are observed for both ATP and AMP; for each nucleotide it is about 11-fold. If, however, the ATP content in the foot of the snail is excluded from consideration, the variation in content of ATP is only about fourfold (2.02–8.51). The amounts of ATP/g in the insects studied are remarkably similar, 5.35–8.51 μmol/g. In the vertebrate muscles, the range is slightly greater, 4.47–8.05 μmol/g. The range of the ratios of the amounts of ATP/AMP (and similarly ATP/ADP) is very much larger than for the range of the amounts/g *per se*; the ATP/AMP concentration ratio ranges from 8.8 to 402.5 (i.e. about 45-fold). From a comparison with the contents of adenine nucleotides in other tissues, the contents of ATP and especially the ATP/AMP content ratio are much higher in muscle tissue.

Phosphagen (i.e. arginine phosphate or creatine phosphate) was detected in all the muscles studied. The amounts of arginine phosphate in invertebrate muscle ranged from 0.48 to 52.16 μmol/g (i.e. more than 100-fold); this is much greater than the variation in the contents of creatine phosphate in vertebrates (i.e. about fivefold). The highest phosphagen amounts were observed in the snap muscles of *Pecten*, the abdominal muscles of the lobster and the pectoral muscles of the domestic fowl (52, 33 and 27 μmol/g respectively). The concentration ratio of creatine phosphate/creatine (or arginine phosphate/arginine) ranges from 0.2 to 9.0 approximately (white muscle of the dogfish and snap muscle of *Pecten* respectively).

Amounts of glycolytic intermediates. A large variation in the amounts of hexose phosphates/g was found in the muscles investigated; the contents of glucose 6-phosphate, fructose 6-phosphate and fructose diphosphate range from 0.02 to 1.59, 0.01 to 0.35 and 0.01 to 0.41 μmol/g respectively. There is also a large variation in the content of pyruvate (0.02–0.48 μmol/g), but if the value for the honey-bee is omitted the variation in the content of phosphoenolpyruvate is small (0.01–0.09 μmol/g). The lowest contents of hexose monophosphates are observed in some of the invertebrate muscles. It is noteworthy that, except for the honey-bee, insect flight muscles which contain low amounts of hexose monophosphates also contain low activities of the glycolytic enzymes, and they probably rely on compounds other than carbohydrates for energy provision (see Crabtree & Newsholme, 1972*a*,*b*).

The flight muscle of the honey-bee appears to be metabolically unusual in a number of respects. Thus, although the contents of hexose monophosphates are very low, those of pyruvate and phosphoenolpyruvate are high; indeed, the content of the latter is the highest observed in the present work. Further, of the insects studied, the flight muscle of the honey-bee has the lowest ATP/AMP concentration ratio (see Table 2).

Mass-action ratios. The mass-action ratios for adenylate kinase and creatine phosphokinase (or arginine phosphokinase) reactions, which were calculated from the data obtained in this work, are presented in Table 2. For 21 different muscles the mass-action ratios for the adenylate kinase reaction range from 0.22 to 1.26 (i.e. about a fivefold variation). The range of the mass-action ratios for the other tissues (reported from the literature) is from 0.40 to 0.78, if adipose tissue is excluded from consideration. The mass-action ratios for the arginine phosphokinase reaction ranges from 1.0 to 7.7, and those for the creatine phosphokinase reaction range from 3.2 to 8.5 (excluding the value for the dogfish; see below). For both phosphokinase reactions the ratios vary less than 10-fold.

The mass-action ratios for the reactions catalysed by phosphoglucoisomerase, phosphofructokinase and pyruvate kinase are reported in Table 3. The ratios for the phosphoglucoisomerase reaction range from 0.08 to 0.77 (i.e. about a 10-fold variation). The mass-action ratios for the tissues other than muscle range from 0.25 to 0.33 (Table 3). The mass-action ratios for the phosphofructokinase reaction range from 0.03 to 3.58 (i.e. more than a 100-fold variation). The mass-action ratios for the tissues other than muscle range from 0.09 to 2.3 (Table 3) and those for the pyruvate kinase reaction range from 1.44 to 103.6 (i.e. more than a 70-fold variation). The mass-action ratios for the tissues other than muscle range from 0.7 to 5.4 (Table 3).

Discussion

In the interpretation of the results, no attempt has been made to distinguish between free and bound adenine nucleotides or between cytoplasmic and mitochondrial concentrations of metabolites. At the present time, satisfactory methods for measurement of intermediates within these different cellular compartments are not available and any interpretation that involves the use of a precise concentration of a metabolic intermediate must be made with caution. In this discussion, interpretations are based primarily on concentration ratios and, since it is possible that compartmentation will affect, for example, the concentrations of ATP, ADP and AMP to similar extents, the danger of misinterpretation of data is decreased. Further, provided that compartmentation is similar in different muscles, comparison of a qualitative or semiquantitative nature between muscles should not be meaningless.

Amounts of adenine nucleotides and phosphagens

Rather low concentrations of ATP were observed in two types of molluscan muscle, the foot of the snail and the posterior adductor muscle of the sea mussel. This finding is consistent with the low rate of energy expenditure by these muscles during contraction (see Rüegg, 1971). The other molluscan muscle, the snap muscle of *Pecten*, has a higher ATP content and it is known to utilize ATP at a high rate during contraction (Rüegg, 1971). In all of the other muscles studied, the contents of ATP are very similar. Although the maximum rates of energy utilization in these muscles are different, it is likely that the rates are high in comparison with the two molluscan muscles described above (see Crabtree & Newsholme, 1972*a*). The highest amounts of ATP/g are present in two distinct types of muscle, the flight muscle of an insect (the rosechafer) and the pectoral muscles of the domestic fowl (about 8 μmol/g). This finding is noteworthy since the latter muscle contains very few

Table 2. *Contents of adenine nucleotides, creatine, arginine and the phosphagens in muscles from vertebrates and invertebrates*

Muscles were extracted and compounds measured as described in the Materials and Methods section. The results are reported as means ± S.E.M. with the number of individual muscles investigated given in parentheses. Arginine and arginine phosphate were measured and are reported for muscles from invertebrates whereas creatine and creatine phosphate were measured and are reported for muscles from vertebrates. The mass-action ratios for adenylate kinase, arginine phosphokinase and creatine phosphokinase are calculated in the direction of ATP formation. The data for the mouse brain are taken from Lowry *et al.* (1964), for guinea-pig cerebral-cortex slices from Rolleston & Newsholme (1967), for rat liver and kidney from Hems & Brosnan (1970), for perfused rat heart from Opie *et al.* (1971) and for the rat epididymal fat-pad from Saggerson & Greenbaum (1970).

		Muscle contents (μmol/g of frozen muscle)					Concentration ratios		Mass-action ratios	
Animal	Muscle	ATP	ADP	AMP	Creatine or arginine phosphate	Creatine or arginine	ATP/ADP	ATP/AMP	Adenylate kinase	Arginine or creatine phosphokinase
Mollusca										
Snail (*Helix pomatia*)	Foot	0.76 ± 0.09 (6)	0.43 ± 0.04 (5)	0.06 ± 0.02 (6)	1.44 ± 0.28 (6)	3.69 ± 1.14 (4)	1.8	12.7	0.24	4.6
Scallop (*Pecten maximus*)	Snap	6.63 ± 0.19 (6)	0.73 ± 0.04 (6)	0.03 ± 0.01 (6)	52.16 ± 2.11 (4)	5.78 ± 0.79 (4)	9.1	221.0	0.42	1.0
Sea mussel (*Mytilus edulis*)	Posterior adductor	2.02 ± 0.09 (3)	1.08 ± 0.05 (3)	0.23 ± 0.02 (3)	1.99 ± 0.21 (3)	—	1.9	8.8	0.39	—
Crustacea										
Lobster (*Homarus vulgaris*)	Abdominal	6.95 ± 0.24 (9)	0.56 ± 0.07 (10)	0.02 ± 0.01 (9)	33.36 ± 2.66 (9)	18.41 ± 4.74 (5)	12.3	347.5	0.51	6.8
Insecta										
Locust (*Schistocerca gregaria*)	Flight	5.83 ± 0.27 (9)	0.48 ± 0.06 (5)	0.05 ± 0.03 (5)	8.77 ± 0.22 (7)	2.85 ± 0.26 (6)	12.1	116	1.26	3.9
Cockroach (*Periplaneta americana*)	Flight	6.03 ± 0.32 (8)	0.81 ± 0.05 (6)	0.04 ± 0.04 (6)	9.39 ± 1.03 (8)	9.59 ± 1.86 (4)	7.4	150.7	0.37	7.5
Cockroach (*Blaberus discoidalis*)	Flight	6.11 ± 0.54 (8)	0.78 ± 0.08 (8)	0.04 ± 0.01 (5)	11.23 ± 0.89 (8)	7.85 ± 1.2 (5)	7.8	152.7	0.41	5.3
Water bug (*Lethocerus cordofanus*)	Flight	7.15 ± 0.19 (8)	0.81 ± 0.03 (8)	0.04 ± 0.01 (8)	4.38 ± 0.40 (7)	1.66 ± 0.15 (5)	8.8	178.7	0.42	3.3
Cockchafer (*Melolontha melolontha*)	Flight	6.18 ± 0.22 (5)	0.86 ± 0.03 (5)	0.08 ± 0.01 (5)	6.03 ± 0.69 (5)	—	7.1	77.2	0.69	—
Rosechafer (*Pachnoda ephippiata*)	Flight	8.51 ± 0.16 (6)	1.09 ± 0.04 (6)	0.06 ± 0.01 (6)	4.55 ± 0.13 (6)	4.50 ± 1.11 (6)	7.8	141.8	0.44	7.7
Dung-beetle (*Heliocopris sp.*)	Flight	5.66 ± 0.42 (6)	0.98 ± 0.12 (6)	0.13 ± 0.01 (6)	4.36 ± 0.27 (6)	2.50 ± 0.35 (6)	5.8	43.5	0.76	3.3
Honey-bee (*Apis mellifera*)	Flight	5.35 ± 0.50 (5)	1.57 ± 0.21 (5)	0.28 ± 0.04 (5)	0.48 (2)	0.43 (1)	3.4	19.1	0.61	3.0
Blowfly (*Calliphora vicinia*)	Flight	7.72 ± 0.39 (5)	0.89 ± 0.13 (5)	0.06 ± 0.02 (5)	2.41 ± 0.28 (5)	—	8.7	128.6	0.60	—
Pisces										
Dogfish (*Scylliorhinus canicula*)	White	5.14 ± 0.62 (6)	0.81 ± 0.03 (6)	0.029 ± 0.01 (6)	8.8 ± 2.4 (6)	36.6 ± 7.7 (6)	6.3	177.2	0.22	26.4
Amphibia										
Frog (*Rana temporaria*)	Gastrocnemius	4.47 ± 0.13 (4)	0.70 ± 0.03 (4)	0.03 ± 0.01 (4)	19.9 ± 0.94 (4)	7.8 ± 0.63 (4)	6.4	149	0.27	2.5
Aves										
Pigeon (*Columba livia*)	Pectoral	7.04 ± 0.40 (6)	1.02 ± 0.08 (6)	0.05 ± 0.01 (5)	14.23 ± 1.3 (6)	17.51 ± 1.6 (6)	6.9	141.0	0.34	8.5
Starling (*Sturnus vulgaris*)	Pectoral	6.51 (2)	1.15 (2)	0.02 (1)	16.2 (2)	12.75 (2)	5.7	325.0	0.88	4.4
Domestic fowl (*Gallus gallus*)	Pectoral	8.05 ± 0.41 (5)	0.76 ± 0.02 (5)	0.02 ± 0.01 (5)	27.2 ± 0.8 (5)	11.03 ± 0.6 (5)	10.5	402.5	0.31	8.4
Pheasant (*Phasianus colchicas*)	Pectoral	7.23 ± 0.34 (5)	0.77 ± 0.04 (5)	0.03 ± 0.01 (5)	—	—	9.3	241.0	0.38	—
Mammalia										
Laboratory rat	Thigh	6.23 ± 0.34 (6)	0.72 ± 0.04 (7)	0.05 ± 0.01 (7)	23.11 ± 2.4 (6)	8.78 ± 0.6 (6)	8.6	124.0	0.62	3.3
Laboratory mouse	Thigh	4.99 ± 0.32 (5)	0.55 ± 0.05 (5)	0.03 ± 0.01 (5)	17.17 ± 1.2 (6)	6.04 ± 0.52 (4)	9.4	166.3	0.51	3.2
Laboratory mouse	Intact brain	2.3	0.9	0.2	2.4	8.9	2.5	11.5	0.6	6.9
Guinea pig	Cerebral-cortex slices	2.09	0.49	0.09	4.33	3.37	4.3	23.2	0.78	3.5
Laboratory rat	Liver (*in situ*)	2.74	1.34	0.26	—	—	2.0	10.5	0.40	—
	Kidney (*in situ*)	1.71	0.96	0.25	—	—	1.8	6.8	0.46	—
	Perfused heart	4.48	0.85	0.09	7.2	—	5.3	49.8	0.56	—
	Incubated epididymal fat-pad	89.8*	21.7*	22.2*	—	—	4.1	4.0	4.66	—

* Results expressed in nmol/mg of tissue N.

Table 3. *Contents of some glycolytic intermediates in muscles from vertebrates and invertebrates*

Muscles were extracted and intermediates measured as described in the Materials and Methods section. The results are reported as means ± S.E.M. with the number of individual muscles given in parentheses. The mass-action ratios for the enzymes were calculated in the direction of glycolysis. The data for tissues other than muscle were obtained from the sources given in Table 2. Data for the perfused rat heart were obtained from Williamson (1965).

		Muscle contents (μmol/g of frozen muscle)					Mass-action ratios		
Animal	Muscle	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate	Phosphoenol-pyruvate	Pyruvate	Phospho-gluco-isomerase	Phospho-fructo-kinase	Pyruvate kinase
Mollusca									
Snail (*Helix pomatia*)	Foot	0.07 ± 0.011 (9)	0.02 ± 0.003 (8)	—	0.05 ± 0.010 (5)	0.04 ± 0.014 (4)	0.28	—	1.44
Scallop (*Pecten maximus*)	Snap	0.05 ± 0.010 (5)	0.01 ± 0.001 (5)	0.013 ± 0.002 (3)	0.05 ± 0.008 (3)	0.06 ± 0.014 (6)	0.20	0.13	10.67
Sea mussel (*Mytilus edulis*)	Posterior adductor	0.06 ± 0.007 (3)	0.01 (2)	0.04 ± 0.01 (3)	0.05 ± 0.007 (3)	0.06 ± 0.012 (3)	0.16	3.38	1.94
Crustacea									
Lobster (*Homarus vulgaris*)	Abdominal	0.73 ± 0.08 (6)	0.15 ± 0.03 (5)	0.11 ± 0.05 (3)	0.03 ± 0.004 (5)	0.08 ± 0.014 (5)	0.20	0.06	34.2
Insecta									
Locust (*Schistocerca gregaria*)	Flight	0.14 ± 0.02 (7)	0.02 ± 0.004 (6)	0.05 ± 0.014 (6)	0.04 ± 0.006 (5)	0.02 ± 0.003 (3)	0.14	0.21	5.0
Cockroach (*Periplaneta americana*)	Flight	0.23 ± 0.03 (7)	0.05 ± 0.015 (6)	0.16 ± 0.014 (6)	0.013 ± 0.001 (3)	0.18 ± 0.05 (6)	0.21	0.45	103.6
Cockroach (*Blaberus discoidalis*)	Flight	0.36 ± 0.08 (8)	0.03 ± 0.004 (3)	0.075 ± 0.005 (3)	0.03 ± 0.006 (5)	0.18 ± 0.08 (6)	0.08	0.35	54.8
Waterbug (*Lethocerus cordofanus*)	Flight	0.02 ± 0.003 (3)	0.01 ± 0.001 (3)	0.01 (1)	0.02 ± 0.002 (6)	0.04 ± 0.012 (5)	0.50	0.17	19.3
Cockchafer (*Melolontha melolontha*)	Flight	0.03 ± 0.006 (5)	0.02 ± 0.006 (4)	0.02 (2)	0.05 ± 0.003 (3)	0.07 ± 0.016 (4)	0.66	0.17	10.6
Rosechafer (*Pachnoda ephippiata*)	Flight	0.21 ± 0.031 (6)	0.02 ± 0.005 (6)	0.12 ± 0.02 (6)	0.09 ± 0.004 (6)	0.17 ± 0.037 (6)	0.09	0.73	13.7
Dung-beetle (*Heliocopris sp.*)	Flight	0.02 ± 0.008 (4)	—	—	0.04 ± 0.013 (6)	0.08 ± 0.019 (6)	—	—	11.5
Honey-bee (*Apis mellifera*)	Flight	0.07 ± 0.005 (5)	0.03 ± 0.002 (5)	0.05 ± 0.011 (5)	0.21 ± 0.06 (4)	0.21 ± 0.07 (3)	0.42	0.44	3.4
Blowfly (*Calliphora vicinia*)	Flight	0.37 ± 0.04 (5)	0.06 ± 0.015 (5)	0.24 ± 0.02 (5)	0.04 ± 0.004 (5)	0.15 ± 0.023 (5)	0.16	0.45	31.2
Pisces									
Dogfish (*Scylliorhinus canicula*)	White	1.59 ± 0.31 (3)	0.23 ± 0.067 (3)	0.09 ± 0.023 (3)	0.02 ± 0.003 (3)	0.27 ± 0.112 (3)	0.14	0.06	85.6
Amphibia									
Frog (*Rana temporaria*)	Gastrocnemius	0.91 ± 0.11 (8)	0.18 ± 0.026 (8)	0.04 ± 0.009 (8)	0.03 ± 0.005 (8)	0.12 ± 0.028 (8)	0.20	0.03	25.5
Aves									
Domestic pigeon (*Columbia livia*)	Pectoral	1.45 ± 0.21 (6)	0.35 ± 0.055 (5)	0.25 ± 0.055 (5)	0.06 ± 0.015 (4)	0.16 ± 0.023 (4)	0.24	0.10	18.1
Starling (*Sturnus vulgaris*)	Pectoral	0.40 (2)	0.31 (2)	0.26 (2)	0.05 (2)	0.08 (2)	0.77	0.14	9.5
Domestic fowl (*Gallus gallus*)	Pectoral	0.69 ± 0.12 (5)	0.12 ± 0.025 (5)	0.41 ± 0.05 (5)	0.09 ± 0.013 (5)	0.27 ± 0.054 (5)	0.17	0.32	31.5
Mammalia									
Laboratory rat	Thigh	1.25 ± 0.26 (5)	0.18 ± 0.038 (3)	0.10 ± 0.035 (3)	0.03 ± 0.008 (3)	0.14 ± 0.020 (6)	0.14	0.06	35.7
Laboratory mouse	Thigh	0.37 ± 0.06 (5)	0.07 ± 0.008 (6)	0.03 ± 0.017 (3)	0.03 (2)	0.04 ± 0.009 (4)	0.19	0.05	11.9
Laboratory mouse	Intact brain	0.08	0.02	0.12	—	—	0.25	2.3	—
Guinea pig	Cerebral-cortex slices	0.18	0.06	0.04	0.06	0.06	0.33	0.13	5.4
Laboratory rat	Liver (*in situ*)	0.16	0.05	<0.01	0.10	0.03	0.31	0.09	0.7
	Kidney (*in situ*)	0.07	0.02	<0.01	0.05	0.05	0.28	0.33	1.8
	Perfused heart	630†	154†	42†	19†	88†	0.24	0.03	40.0
	Incubated epididymal fat-pad	6.01*	2.10*	1.30*	—	—	0.35	0.16	—

* Results expressed in nmol/mg of tissue N.
† Results expressed in nmol/g dry wt.

mitochondria (see George & Berger, 1966), whereas insect flight muscle contains many mitochondria (see Smith, 1966).

The highest amounts of phosphagen/g of tissue were observed in the abdominal muscle of the lobster and the snap muscle of *Pecten*. This finding is consistent with the function of phosphagen in provision of a significant proportion of the total energy required during the short violent bursts of mechanical activity that these muscles are known to perform. The phosphagen content is generally rather low in the insect flight muscle, despite the fact that the rate of energy utilization is very high during flight. Further, insect flight can be sustained for long periods of time so that the concentration of phosphagen would provide a negligible proportion of the total energy required in these muscles. The amounts of phosphagen/g of muscle are similar in the different vertebrate muscles examined, including that of the rat heart, despite the fact that the physiological roles of some of these muscles are different. This suggests that the role of the phosphagen in these muscles, and perhaps in the insect flight muscles also, is to provide a very transient buffer of the concentration of the ATP in the muscle.

A noteworthy observation arising from this work is the high content of creatine and a low creatine phosphate/creatine content ratio in the dogfish muscle. This low ratio is found in the well-anaesthetized animals despite a high ATP/AMP content ratio. This suggests that the high concentration of creatine is not due to degradation of creatine phosphate. Perhaps creatine is used in the dogfish muscle to provide intracellular osmoregulation.

Ratio of the contents of ATP/AMP

The variation in the ratios of the contents of ATP/AMP in the muscles studied was about 45-fold (8.8–402.5). In general, for muscle in which the change in the rate of energy expenditure from minimum to maximum values is very large, the ATP/AMP concentration ratio is large (e.g. the snap muscle of *Pecten*, some of the insect flight muscles, pectoral muscles of the birds, leg muscles of the rat and mouse; see Table 1). For muscle in which the change in rate of energy utilization is small, the ATP/AMP concentration ratio is small (e.g. the foot muscle of the snail, the posterior adductor muscle of the sea mussel, the pectoral muscle of the pigeon, in comparison with those of the pheasant and domestic fowl, and the heart muscle of the rat, see Table 2). Similarly, in tissues other than muscle, in which the change in the rate of glycolysis is probably not very large, the ATP/AMP concentration ratios are very low (a range of 4–23, see Table 2). These relationships are consistent with the theory of control of glycolysis in which small changes in ATP concentration produce much larger changes in that of AMP through the reaction catalysed by adenylate kinase (see Newsholme, 1970). In this theory, the higher the initial resting ATP/AMP concentration ratio is, the greater the change in concentration of AMP for a given change in that of ATP (see Newsholme & Start, 1973). Muscles that require a large change in the rate of glycolysis to satisfy the energy demands of the contractile apparatus should possess a high resting ATP/AMP concentration ratio; mechanical activity will cause a decrease in the concentration of ATP and a large increase in AMP, which will stimulate glycolysis and hence energy production. Some insect flight muscles provide exceptions to the relationship (cockchafer, dung-beetle and honey-bee); they have lower ATP/AMP concentration ratios than the other muscles. However, the flight muscles of the cockchafer and dung-beetle may depend more on proline or fat oxidation than on carbohydrate oxidation for energy production for flight (see Crabtree & Newsholme, 1970, 1972*a*,*b*) so that changes in the content of AMP may be much less important in the control of energy formation in the flight muscles of these particular insects. Nonetheless, the flight muscles of the honey-bee are known to depend on carbohydrate metabolism for energy production (Crabtree & Newsholme, 1972*a*,*b*) and it is anticipated that glycolysis will be controlled in relation to the energy demand of the contractile process in the muscle. The very low ATP/AMP concentration ratio suggests that changes in the concentration of AMP are unimportant in the regulation of the activity of phosphofructokinase and the rate of glycolysis in the flight muscle of the honey-bee. Another observation consistent with this suggestion is that the flight muscle of the honey-bee does not possess any detectable activity of fructose diphosphatase (Newsholme & Crabtree, 1973), and consequently a substrate cycle catalysed by fructose diphosphatase and phosphofructokinase does not operate in this muscle. [The role of this cycle is to amplify the response of fructose 6-phosphate phosphorylation, and hence glycolytic flux, to changes in the concentration of AMP (see Newsholme & Crabtree, 1973)]. The metabolic mechanism for control of glycolysis in this muscle is unknown.

Mass-action ratios of some reactions

The mean values of mass-action ratios for the phosphoglucoisomerase reaction and the adenylate kinase reaction are 0.3 and 0.5 respectively, for the muscles used in this investigation. The equilibrium constants for these reactions are 0.3 (Kahana *et al.*, 1960) and 0.44 (Eggleston & Hems, 1952) respectively. Thus the values for the mass-action ratios and the equilibrium constants are very similar, which indicates that these reactions are close to equilibrium

in all the muscles that have been investigated (under conditions of rest). The near-equilibrium nature of the adenylate kinase reaction is important, since this indicates that it can play an amplification role in the regulation of glycolysis (Newsholme, 1970) in all of these muscles.

The results also indicate that, for all the muscles studied, the variation in the ratios of these two reactions is small, whereas the variation in the contents of the individual metabolites is large (e.g. the mass-action ratios for the adenylate kinase reaction vary about 11-fold whereas the ATP/AMP concentration ratio varies about 45-fold). On the other hand the mass-action ratios for the reactions catalysed by phosphofructokinase and pyruvate kinase are very much smaller than the equilibrium constants (the equilibrium constants are approx. 1×10^3 and 1×10^4 respectively), which indicates that, in all the muscle studied, these enzymes catalyse non-equilibrium reactions. Moreover, for both reactions the variation in the mass-action ratios between the different muscles is very large; it is more than 100-fold for the phosphofructokinase reaction and more than 70-fold for the pyruvate kinase reaction. The concentration ratios of the products/substrates (i.e. mass-action ratio) is constrained in a near-equilibrium reaction by the value of the equilibrium constant; any large variation in the substrate concentration must be 'balanced' by a similar variation in the product. No such constraint need apply to non-equilibrium reactions. These considerations predict a much smaller variation in the mass-action ratios between different tissues for a near-equilibrium reaction compared with a non-equilibrium reaction. The importance of this for the creatine phosphokinase and arginine phosphokinase reactions is discussed below.

The mean mass-action ratio for the arginine phosphokinase and creatine phosphokinase reactions from this investigation is 6.0. The equilibrium constant for the creatine phosphokinase reaction is 100 (Noda *et al.*, 1954); it is assumed that the equilibrium constant for the arginine phosphokinase reaction is similar. The difference between the equilibrium constant and mass-action ratios (about 16-fold) might indicate that the reaction is non-equilibrium in the muscle (see Rolleston, 1972). However, the variation between the ratios in 18 different muscles (and in brain, see Table 2) is very small. If the dogfish muscle is excluded, the variation is about eight fold, whereas the variation in the content of arginine phosphate between different muscles is greater than 100-fold. Further, variation in the mass-action ratios for the 'phosphagen' kinase reaction is, as indicated by the S.E.M. values for the results presented in Table 2, similar to that for adenylate kinase and is much less than for those for pyruvate kinase or phosphofructokinase (see Table 4). These considerations suggest that the phosphagen kinase reaction is close to equilibrium. The discrepancy between the mass-action ratios and the apparent equilibrium constant for this reaction may be explained by a difference between the value of the equilibrium constant as measured *in vitro* and that which applies to this reaction intracellularly. Factors such as the ionic strength, pH or Mg^{2+} concentration could be different in the cell from those that were used in the measurement of the constant *in vitro*. It is important to know whether the 'phosphagen kinase' enzymes catalyse a reaction close to equilibrium, since the accepted role of phosphagens in buffering small changes in the ATP/ADP ratio requires the reaction to be near equilibrium.

Table 4. *Mean mass-action ratios and* S.E.M. *values for the reactions catalysed by adenylate kinase, phosphofructokinase, 'phosphagen' kinase and pyruvate kinase*

The mean values ± S.E.M. are calculated from the data in Tables 2 and 3. The numbers of individual results used in the calculation are given in parentheses.

Reaction	Mean mass-action ratio
Adenylate kinase	0.48 ± 0.037 (21)
Phosphofructokinase	0.42 ± 0.19 (18)
Phosphagen kinase	4.8 ± 0.52 (17)
Pyruvate kinase	24.8 ± 5.60 (20)

This work has provided a further method for discovering whether reactions are near equilibrium or far-removed from equilibrium; mass-action ratios for the reaction are measured in a given tissue in a large number of animals and a small variation in the ratios as indicated by the value of the S.E.M. suggests that the reaction is near equilibrium.

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References

Crabtree, B. & Newsholme, E. A. (1970) *Biochem. J.* **117**, 1019–1021

Crabtree, B. & Newsholme, E. A. (1972*a*) *Biochem. J.* **126**, 49–58

Crabtree, B. & Newsholme, E. A. (1972*b*) *Biochem. J.* **130**, 697–705

Czok, R. & Eckert, L. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 229–233, Academic Press, New York and London

Eggleston, L. V. & Hems, R. (1952) *Biochem. J.* **52**, 156–160

George, J. C. & Berger, A. J. (1966) *Avian Myology*, pp. 30–32, Academic Press, New York and London

Gevers, W. & Krebs, H. A. (1966) *Biochem. J.* **98**, 720–735

Hems, D. A. & Brosnan, J. T. (1970) *Biochem. J.* **120**, 105–111

Hess, B. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 43–55, Academic Press, New York and London
Kahana, S. E., Lowry, O. H., Schulz, D. W., Passonneau, J. V. & Crawford, E. J. (1960) *J. Biol. Chem.* **235**, 2179–2184
Lamprecht, W. & Stein, P. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 610–616, Academic Press, New York and London
Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) *J. Biol. Chem.* **239**, 18–30
Newsholme, E. A. (1962) Ph.D. Thesis, University of Cambridge
Newsholme, E. A. (1970) in *Essays in Cell Metabolism* (Bartley, W., Kornberg, H. L. & Quayle, J. R., eds.), pp. 189–223, Wiley-Interscience, London
Newsholme, E. A. & Crabtree, B. (1973) *Symp. Soc. Exp. Biol.* **26**, 429–460
Newsholme, E. A. & Start, C. (1973) *Regulation in Metabolism*, pp. 111–114, Wiley, London
Newsholme, E. A. & Taylor, K. (1969) *Biochem. J.* **112**, 465–474
Noda, L., Kuby, S. A. & Lardy, H. A. (1954) *J. Biol. Chem.* **210**, 83–95
Opie, L. H., Mansford, K. R. L. & Owen, P. (1971) *Biochem. J.* **124**, 475–490
Rolleston, F. S. (1972) *Curr. Top. Cell. Regul.* **5**, 47–73
Rolleston, F. S. & Newsholme, E. A. (1967) *Biochem. J.* **104**, 524–533
Rüegg, J. C. (1971) *Physiol. Rev.* **51**, 201–248
Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J* **119**, 193–219
Smith, D. S. (1966) *Prog. Biophys. Mol. Biol.* **16**, 107–142
Start, C. (1969) D.Phil. Thesis, University of Oxford
Williamson, J. R. (1965) *J. Biol. Chem.* **240**, 2308–2321
Williamson, D. H. & Brosnan, J. J. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 2266–2302, Academic Press, New York and London
Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tieren* **270**, 399–412