The Activities of Some Enzymes Concerned with Energy Metabolism in Mammalian Muscles of Differing Pigmentation

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1. Extractable hexokinase activity was measured in the red and white skeletal muscles of the rabbit and in the hearts and diaphragms of four animal species differing markedly in size. Activities vary over a 40-fold range, being least in white skeletal muscle of the laboratory rabbit and greatest in mouse heart. 2. Hexokinase activities correlate approximately with capacities to undertake reactions of the tricarboxylic acid cycle as determined by succinate oxidase assays. Both enzyme activities seem best related to the average contractile-energy expenditure per unit weight of muscle over an extended period, rather than to the rapidity of individual contractions. 3. Hexokinase and succinate oxidase activities cannot be related to a muscle's content of soluble pigment. They display an inverse relationship with activities of phosphorylase and glycolytic enzymes, but only within the group of rabbit skeletal muscles whose oxidative capacities are at the lower end of the observed range. 4. Total glycogen-UDP glucosyltransferase activities do not vary significantly between rabbit skeletal muscles, although those of hexokinase differ by about sixfold. On the average, glucose 6-phosphate is probably oxidized directly. However, observations cited in the literature suggest that muscles with an active hexokinase may well preferentially accumulate glycogen when glucose is present in excess of the fibres' capacity to oxidize it. 5. When considered with published results obtained *in vivo*, the present findings indicate that phosphorylase has a minor role in the energy expenditure of muscles with a predominantly oxidative metabolism. In these, the major substrates appear to be blood glucose, fatty acids and possibly lipids. 6. The histochemical criteria by which muscle fibres are commonly described as red or white are inadequate.

The nature of muscle pigmentation and its physiological significance have been studied since the late Nineteenth Century. Earlier work was reviewed by Needham (1926) and more recently some aspects have been discussed by Perry (1960).

To some extent the degree of pigmentation correlates with the content of myoglobin and mitochondrial enzymes, with slowness of contraction and with the proportion of its time a muscle spends in the contracted state. Heart muscle also contains blood haemoglobin in considerable quantities and its dark appearance is said to be enhanced by a relatively high post-mortem pH value (see Lawrie, 1950).

Extractable glycogen phosphorylase (EC 2.4.1.1) and enzymes of the glycolytic pathway are most abundant in white (phasic) muscles, which contract relatively infrequently, and histochemical staining

commonly shows that the glycogen phosphorylase and the tricarboxylic acid-cycle activities of different fibres vary inversely. Heart muscle and red forms of skeletal muscle have more lipase (EC 3.1.1.3) and enzymes for fatty acid degradation than does white skeletal muscle (George & Scaria, 1957; George & Talesara, 1961; Pette, 1966).

In preliminary studies, we found the ratio of extractable glycogen phosphorylase to hexokinase (EC 2.7.1.1) activity was about 100-fold greater in white (phasic) muscles of the laboratory rabbit than in red (tonic) muscles; the corresponding ratio in diaphragm was of an intermediate value (Burleigh & Schimke, 1968). Dr D. Pette has informed us of similar results he has obtained on rabbit soleus (tonic) and adductor magnus (phasic) muscles and on rat heart. The ratio of hexokinase to glycogen phosphorylase activity was greatest in the rat heart muscle, and in the three muscles he examined hexokinase activity correlated well with that of

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the citrate synthase (EC 4.1.3.7) enzyme of the tricarboxylic acid cycle (Pette, 1966, 1968). Hexokinase is also more active in red-muscle fibres of the guinea pig than in fibres of white muscle (Peter, Jeffress & Lamb, 1968).

There appears to be no comprehensive and systematic survey of the relative activities of hexokinase and glycogen phosphorylase in mammalian muscles that differ in pigmentation, tricarboxylic acid-cycle activity and frequency of use. Our original observations have therefore been extended.

We have particularly studied diaphragm, since the size and mitochondrial content of its fibres varies markedly and systematically with the size, species and breathing rate of the animal of origin (Gauthier & Padykula, 1966).

Given the tendency for glycogen phosphorylase and terminal respiratory activities to be inversely related in muscle fibres, it seemed pertinent to ask whether the ratio of glycogen phosphorylase to hexokinase activity in diaphragm varies in animal species of different sizes and whether similar trends are apparent in heart muscle. Adequate comparison between species has inevitably required that we estimate the degree to which these parameters vary with an individual animal's age.

MATERIALS AND METHODS

Sources of muscle. White laboratory mice (Swiss-Webster), rats (Sprague-Dawley) and rabbits (New Zealand) were supplied by Simonsen Laboratories, Gilroy, Calif., U.S.A. Deer mice (*Peromyscus maniculatus*) were caught locally. Ox diaphragms and hearts were obtained from a slaughterhouse; they were transported to the laboratory in ice, and homogenization was begun 1½–2½ hr. after removal of the muscles from the animals.

Preparation of homogenates. Muscles were excised, chilled, freed of extraneous tissue and blood and cut into small pieces with scissors. By using a Servall Omni-Mixer at power setting no. 7, they were homogenized for 30 sec. in ice-cold phosphate-glucose buffer, pH 7.1. The buffer contained (final concn.) potassium phosphates (10 mM with respect to phosphate), 10 mM-glucose, 5 mM-EDTA and 5 mM-2-mercaptoethanol. As a routine, 25 ml. of extraction fluid/g. wet wt. of muscle was used, and where necessary homogenates were appropriately diluted in 50 mM-potassium phosphate buffer, pH 7.4. Mouse diaphragm was homogenized in 50 vol. (v/w) of medium and dilutions were altered accordingly. With rat diaphragm it was checked that hexokinase and glycogen phosphorylase specific activities were identical when based on 1:25 and 1:50 homogenates. Results on rabbit muscles where the muscles were homogenized in 20 vol. of fluid have been included.

Enzyme assays. Except for hexokinase, all enzyme assays were performed on appropriate dilutions of whole homogenates. In rabbit muscle about 40–60% of the hexokinase activity sediments after centrifugation at 30000 g for 20 min. at 2°. Assay of uncentrifuged homogenates was made inconvenient by high background absorption and a

tendency for sedimentation in the cuvette to mask the low reaction rates of white muscle. In accord with observations by Wilson (1968) on particulate hexokinase from brain, it was found that the enzyme sedimenting at 30000 g for 20 min. could be solubilized by treating ice-cold homogenates with Triton X-100 (final concn. 1%, v/v). When time-courses were followed in semitendinosus, diaphragm and adductor magnus muscles, solubilized enzyme activity was maximal and constant between 30 and 90 min. of incubation. Mouse heart gave a similar result. Rates given by centrifuged detergent-treated extracts were not measurably less than those of whole homogenates when these were measured in a variety of muscles covering the extremes of hexokinase content. The muscles were mouse heart and rabbit heart, diaphragm, semitendinosus and adductor magnus. Unlike findings on brain hexokinase (Wilson, 1968), there was no activation of enzyme by the detergent and there was no further enzyme release or activation when homogenates were incubated in the presence of Triton X-100 plus 10 mM-ATP.

As a routine, total hexokinase was measured in muscle homogenates preincubated with Triton X-100 (1%, v/v) at 0° for 45–60 min. and centrifuged at 40000 g for 20 min. at 2°. When soluble enzyme was measured, the detergent treatment was omitted and the extracts were centrifuged at 30000 g for 20 min. at 2°.

The assay methods for hexokinase, glycogen phosphorylase, glucose phosphate isomerase (EC 5.3.1.9) and fructose diphosphate aldolase (EC 4.1.2.13) were given by Burleigh & Schimke (1968). Units of hexokinase, glycogen phosphorylase and glucose phosphate isomerase activity were μ moles of hexose phosphate formed at 37°/hr./g. wet wt. Fructose diphosphate aldolase activity was expressed as μ moles of fructose 1,6-diphosphate cleaved at 37°/hr./g. wet wt.

Succinate oxidase was measured by using an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The assay medium contained 50 mM-potassium phosphate buffer, final pH 7.6, 60 mM-succinic acid and 0.06 mM-cytochrome c. The final volume was 5 ml. and measured rates of consumption of O₂ were in the range 2–5 μ moles/hr. In all muscles investigated at least 90% of the activity was found in the 35000 g pellet obtained in the process of determining myoglobin and haemoglobin. This pellet was resuspended in 30 vol. of 50 mM-potassium phosphate buffer, pH 7.6, homogenized again in the Servall Omni-Mixer for 15 sec. and further dispersed by 15 sec. of ultrasonic treatment with a Branson Sonifier (Heat Systems Inc., Melville, N.Y., U.S.A.) at minimal output. Trebling the duration of ultrasonic treatment did not alter the observed activities. Consumption of O₂ was expressed as μ moles of O₂ consumed at 37°/hr./g. wet wt. of tissue.

Glycogen-UDP glucosyltransferase (EC 2.4.1.11) was measured in the presence of glucose 6-phosphate by the method of Leloir, Olavarria, Goldemberg & Carminatti (1959). Muscles were homogenized in 6 vol. of 50 mM-tris-maleate buffer, pH 8.5. Uncentrifuged extracts were expressed through acid-washed glass wool. A portion was diluted twofold with extraction fluid and samples (10 μ l.) were incubated with 40 μ l. of reaction medium at 37° for 0, 10 and 20 min. The complete assay mixture contained (final concn.) 8 mM-UDP-glucose, 10 mM-glucose 6-phosphate, 5 mM-EDTA and rabbit liver glycogen (8 mg./ml.).

The buffer was 7.5 mM-tris-maleate, pH 8.5. The reaction was stopped by boiling for 1 min. At each time, UDP formation was measured in the complete reaction mixture and in a control without UDP-glucose. Rates were expressed as μ moles of UDP formed/hr. at 37°/g. wet wt. of muscle. At the two dilutions used, the rates were proportional to the amount of added extract.

All enzymes were stable over the period required to complete the assays. For each enzyme investigated it was established that low activity in muscle extracts was not due to the presence of inhibitor. Rates were always additive when equal portions of extracts showing marked differences in activity were mixed in the same reaction vessel. Carry-over of glucose from the extraction medium did not inhibit glycogen phosphorylase even when extracts were used undiluted, and in no instance was phosphogluconate dehydrogenase present in measurable amounts under the conditions of the hexokinase assay.

Myoglobin and haemoglobin. These were determined as the reduced carbonyl derivatives essentially by the procedure of Reynarfarje (1963). The extraction fluid was ice-cold 50 mM-potassium phosphate buffer, pH 6.6, and muscles were homogenized in 9.25 ml. or 19.25 ml. of fluid/g. wet wt. of tissue, depending on the expected pigment concentration. Particular care was taken to free muscle surfaces of all visible adhering blood. Minor deviations from the published method were that (a) the homogenizer was a Servall Omni-Mixer used for two periods of 10 sec. at power setting no. 7 and (b) the homogenate was centrifuged at 35 000 g for 15 min. at 2°. Doubling the extraction time did not increase yields of soluble pigment and re-extracting the pellet invariably yielded less than 10% or else an immeasurably small proportion of the total pigment in the first supernatant. No losses of horse heart myoglobin were observed when a solution was homogenized by the standard procedure at a concentration in the range found in muscle extracts. Extinctions were read at 538, 568 and 576 nm. By using the extinction coefficients of Biörck (1949), concentrations of myoglobin and haemoglobin were

calculated from the observed extinctions at two different pairs of wavelengths and the results were averaged.

Chemicals. Substrates and auxiliary enzymes used in assays were of the highest available purity from the Sigma Chemical Co., St Louis, Mo., U.S.A., or from Calbiochem, Los Angeles, Calif., U.S.A. Succinic acid, NaF and $MgCl_2$ were obtained from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.; $Na_2S_2O_4$, tris and potassium phosphates were from the J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A., and glucose was from Allied Chemicals, Morristown, N.J., U.S.A. 2-Mercaptoethanol was a product of Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and myoglobin was from Mann Research Laboratories, New York, N.Y., U.S.A.

RESULTS

Hexokinase and glycogen phosphorylase activities in rabbit skeletal muscles. In rabbit up to 60% of the hexokinase activity in extracts of skeletal muscle sediments after 20 min. at 30 000 g and is completely solubilized by incubating the homogenates in ice with Triton X-100. After appropriate centrifugation, soluble and total hexokinase activities were measured in the supernatants from untreated and detergent-treated homogenates respectively.

Fig. 1 illustrates a typical experiment in which amounts of total and soluble hexokinase activity were compared with that of glycogen phosphorylase (a plus b) in rabbit muscles that differ in visible redness. Both total and soluble hexokinase activities vary inversely with that of phosphorylase, which is most active in white muscles. The relative proportions of soluble and particulate hexokinase do not vary systematically between the different muscles. Results from replicate experiments

Table 1. *Glycogen phosphorylase, hexokinase and succinate oxidase activities of rabbit skeletal muscles differing in pigmentation and of hearts and diaphragms in different animal species.*

Methods of assay and units of activity are described in the Materials and Methods section. Mean values $\pm$ S.D. are given with the numbers of determinations in parentheses. N.M., Not measured.

	$10^{-3} \times$ Glycogen phosphorylase (units)	Hexokinase (units)	Succinate oxidase (units)	Succinate oxidase/ hexokinase ratio	Phosphorylase/ hexokinase ratio
Laboratory mouse heart	1.2 ± 0.3 (3)	1070 ± 50 (4)	1690 ± 190 (3)	1.6	1.1
Rat heart	1.5 ± 0.1 (3)	623 ± 93 (4)	1880 ± 75 (3)	2.9	2.4
Rabbit heart	1.1 ± 0.2 (3)	471 ± 74 (3)	1210 ± 46 (3)	2.6	2.2
Ox heart	0.6 ± 0.1 (4)	248 ± 33 (3)	670 ± 33 (3)	2.6	2.5
Deer-mouse diaphragm	1.6 (1)	590 (1)	N.M.		2.7
Laboratory mouse diaphragm	1.7 ± 0.3 (8)	632 ± 29 (9)	597 ± 79 (4)	0.9	2.7
Rat diaphragm	2.7 ± 0.6 (11)	241 ± 35 (12)	520 ± 44 (3)	2.1	11.2
Rabbit diaphragm	3.2 ± 0.5 (12)	118 ± 39 (12)	300 ± 58 (5)	2.3	27.1
Ox diaphragm	2.6 ± 0.4 (4)	48 ± 2 (4)	254 ± 19 (4)	5.3	53.7
Rabbit semitendinosus	0.4 ± 0.2 (6)	158 ± 33 (6)	105 ± 17 (4)	0.7	2.5
Rabbit soleus	0.8 ± 0.4 (5)	129 ± 22 (5)	N.M.		6.2
Rabbit semimembranosus	5.6 ± 0.5 (5)	47 ± 15 (5)	N.M.		119
Rabbit adductor magnus	6.6 ± 0.9 (6)	27 ± 4 (6)	33 ± 4 (4)	1.2	245

display this inverse relationship between total hexokinase and glycogen phosphorylase as well as a positive correlation of hexokinase with pigmentation as measured by myoglobin content (Burleigh & Schimke, 1968). Hexokinase and phosphorylase activities are consistent with those of Pette (1968), who apparently only measured soluble hexokinase.

Hexokinase and phosphorylase activities in heart and diaphragm of different animal species. Gauthier & Padykula (1966) found that the proportion of so-called red and white muscle fibres in mammalian diaphragm varies with animal size, red fibres predominating in smaller animals.

Since glycogen phosphorylase is commonly held to be more active in white muscles, we measured glycogen phosphorylase (α plus β) and total hexokinase activities in diaphragms of the mouse, rat, rabbit and ox. Similar measurements were made on heart muscles from the same species and mean values are given in Table 1. Ox muscles were obtained from adult animals of unspecified age and the mice, rats and rabbits were 6 weeks old or older.

In diaphragm the ratio of glycogen phosphorylase to hexokinase activity increases progressively from a value of about 2.5 in mice to more than 50 in the ox (Table 1). Hexokinase activity decreases about 14-fold from approx. 630 units to 48. Superficially this resembles the pattern in the red and white muscles of the rabbit; however, in the diaphragms the differences in hexokinase account for most of the variation in the phosphorylase/hexokinase ratios. Glycogen phosphorylase is about half as active in mouse diaphragm as in rat diaphragm, but is not progressively more active in rabbit and ox (Table 1).

Succinate oxidase activities are also shown in Table 1. They decrease with increasing animal size, and this confirms the histological observations of mitochondrial content made by Gauthier & Padykula (1966). The observed respiration rates in rabbit heart and diaphragm also agree well with known values for α -oxoglutarate oxidation (see Slater, 1960). Our measurements of succinate oxidase activity in rabbit semitendinosus and adductor magnus muscles are also consistent with those quoted by Slater (1960) for rabbit soleus and white back muscle.

The differences in succinate oxidase between the various diaphragms are less marked than those in hexokinase and those between mouse and rat and between rabbit and ox are only significant at $P < 0.2$. However, the differences between mouse and rabbit, rat and ox, and mouse and ox are significant at $P < 0.001$. The mean ratio of succinate oxidase to hexokinase activity increases fivefold between mouse and ox.

Since the rate of breathing is faster in small animals than in large, Gauthier & Padykula (1966)

attributed the preponderance of small 'red' fibres with abundant mitochondria in the diaphragms of small animals to greater frequency of use. Heart rate shows a similar trend to greater values in small animals (Table 5). Table 1 shows that hexokinase and succinate oxidase activities in heart diminish with increasing animal size, though hexokinase only varies by about fourfold compared with a 14-fold range in diaphragm. In heart, the succinate oxidase/hexokinase activity ratio does not vary significantly between species, nor does the glycogen phosphorylase/hexokinase ratio. These represent qualitative differences from diaphragm. Glycogen phosphorylase activities are uniformly low in hearts of all species and, if anything, decrease with greater animal size.

Variation in pigmentation and enzyme activities of diaphragm with age. As a control for the above experiments, it was necessary to establish the degrees to which the enzyme activities of interest vary with age and growth of an individual species of animal. These experiments were confined to mice, rats and laboratory rabbits. Diaphragms of at least the first two species become visibly redder with age.

Pertinent results are given in Figs. 2 and 3 and Table 2.

Fig. 1. Soluble and total hexokinase activities and glycogen phosphorylase activities in extracts of rabbit skeletal muscles. Assay methods and units of activity are described in the text. Abbreviations: SN, semitendinosus; SL, soleus; D, diaphragm; MG, medial gastrocnemius; SM, semimembranosus; AM, adductor magnus. ●, Total hexokinase; ○, soluble hexokinase; ■, phosphorylase.

Fig. 2. Age-dependent variation in total hexokinase and glycogen phosphorylase activities and in the glycogen phosphorylase/hexokinase activity ratio of diaphragms from laboratory mice, rats and rabbits. Assay methods and units of activity are described in the text. ■, Mouse; ●, rat; ○, rabbit. (a) Total hexokinase; (b) phosphorylase; (c) phosphorylase/hexokinase ratio.

Fig. 3. Age-dependent variation in hexokinase and succinate oxidase activities and in myoglobin content of rat diaphragm and heart. Assay methods and units of activity are described in the text. ●, Myoglobin; ○, total hexokinase; ■, succinate oxidase. (a) Diaphragm; (b) heart.

Table 2. *Effect of age on glycogen phosphorylase, fructose diphosphate aldolase and glucose phosphate isomerase activities in diaphragms of mouse, rat and rabbit*

Assay methods and units of activity are described in the text.

Animal	Age	Glycogen phosphorylase (units)	Fructose diphosphate aldolase (units)	Glucose phosphate isomerase (units)
Mouse	1 day	905	1690	8250
	3 weeks	1240	2860	14000
	6 weeks	1560	2840	15800
	12 weeks	1800	2980	17700
	24 weeks	1910	2980	18500
Rat	1 day	602	1010	7000
	3 weeks	850	1940	8530
	6 weeks	1760	3050	14450
	12 weeks	2940	5010	23500
	32 weeks	3060	4950	25300
Rabbit	1 day	1010	2160	8740
	3 weeks	2940	5940	—
	6 weeks	3210	5700	20200
	14 weeks	3610	5750	25400
	28 weeks	3540	5620	24500
	52 weeks	3680	5420	29100

Fig. 2 shows hexokinase and glycogen phosphorylase activities in mice, rats and rabbits of differing ages and it also includes ratios of phosphorylase to hexokinase activity. Though age-dependent variations in enzyme activity exist, it is clear that by the third month, and in most cases after 6 weeks, they have sufficiently stabilized to show the existence of species-dependent differences between the adult animals. Phosphorylase/hexokinase activity ratios in rats 3 months old and older tend to approach the lower end of the range of values observed in rabbit.

Hexokinase and succinate oxidase on the one hand and phosphorylase and the glycolytic enzymes on the other clearly develop on different time-sequences.

In each species hexokinase is invariably at or near its maximum activity even 1 day after birth. Relatively minor changes then occur and there is a tendency for activity to decline from the third week onward (Fig. 2a). Succinate oxidase behaves similarly (Fig. 3). In contrast, phosphorylase activity (Table 2) and those of fructose diphosphate aldolase and glucose phosphate isomerase are low immediately after birth; in rat and rabbit they increase sharply by about three- to five-fold in the next 10 weeks and thereafter increase more slowly or not at all. In mouse increases also occur from birth onward, but only over an approximately twofold range.

Table 2 also shows that in each animal activities of glycogen phosphorylase, fructose diphosphate aldolase and glucose phosphate isomerase tend to increase simultaneously. Amounts of glycogen

phosphorylase and glycolytic enzymes vary in concert when different muscles are compared (see Pette, 1966; Burleigh & Schimke, 1968) and the present results are perhaps better evidence that synthesis of these enzymes is co-ordinated.

In Fig. 3, variations in hexokinase, succinate oxidase and myoglobin content are plotted as a function of age in rat diaphragm and heart. The increase in myoglobin is not accompanied by corresponding variations in hexokinase and succinate oxidase. The pattern of pigment development actually correlates best with increases in glycogen phosphorylase, fructose diphosphate aldolase and glucose phosphate isomerase activities. These enzymes are usually found to be most active in white muscle. In the heart and diaphragm of the horse, myoglobin and succinate oxidase do increase in concert for approximately 2 years after birth (Lawrie, 1953).

Myoglobin and haemoglobin content of various muscles. Table 3 gives means $\pm$ s.d. of myoglobin and haemoglobin concentrations in the various muscles we have examined. Comments on relationships with enzyme activities are reserved for the Discussion section. It suffices here to note that our values are generally in agreement with those of other authors where these are available. Thus in Fig. 3 the respective myoglobin contents of approx. 3.5 and 4.7 mg./g. in rat diaphragm and heart fall within the ranges 3.0–3.6 and 4.5–5.8 mg./g. quoted by Reynarfarje (1963) for these muscles. Dawson & Romanul (1964) obtained a value of 4.1 mg./g. for rat heart by a method that involved initial separation of haemoglobin. Somewhat lower values of

Table 3. *Myoglobin and haemoglobin content of various muscles*

Of the animals used in these experiments, mice and rats were between 6 weeks and 6 months of age, and age-dependent differences account for some of the variation. Rabbits and oxen were adult animals of unspecified age. Mean values $\pm$ S.D. are given with the numbers of determinations in parentheses.

Muscle	Myoglobin (mg./g.)	Haemoglobin (mg./g.)	Haemoglobin (% of total)
Mouse heart	3.9 $\pm$ 0.4 (3)	2.9 $\pm$ 0.4 (3)	43
Rat heart	3.9 $\pm$ 0.7 (4)	2.8 $\pm$ 0.4 (4)	42
Rabbit heart	3.2 $\pm$ 0.6 (3)	3.4 $\pm$ 0.7 (3)	51
Ox heart	6.6 $\pm$ 0.5 (3)	4.4 $\pm$ 0.4 (3)	40
Mouse diaphragm	3.0 $\pm$ 1.0 (3)	1.7 $\pm$ 0.4 (3)	36
Rat diaphragm	3.1 $\pm$ 0.7 (5)	0.8 $\pm$ 0.2 (5)	20
Rabbit diaphragm	1.7 $\pm$ 0.5 (3)	1.6 $\pm$ 0.3 (3)	48
Ox diaphragm	7.9 $\pm$ 0.3 (3)	1.7 $\pm$ 0.4 (4)	18
Rabbit semitendinosus	2.7 $\pm$ 0.5 (5)	0.6 $\pm$ 0.3 (5)	18
Rabbit adductor magnus	< 0.5	< 0.5	

Table 4. *Glycogen-UDP glucosyltransferase (I + D) activities in rabbit skeletal muscles*

Rabbit muscle extracts were assayed in the presence of glucose 6-phosphate as described in the Materials and Methods section. I and D refer respectively to the glucose 6-phosphate-independent and -dependent forms of the enzyme.

Muscle	Incubation time	Glycogen-UDP glucosyl- transferase activity (μ moles of UDP formed at 37°/hr./g.)	
		10 min.	20 min.
Semitendinosus		146	135
Diaphragm		177	139
Semimembranosus		211	179
Adductor magnus		203	163

2.5 mg./g. for diaphragm and 2.8 mg./g. for heart were reported by Anthony, Ackerman & Strother (1959).

The average myoglobin content for ox diaphragm of 7.9 mg./g. is consistent with a range of 7.6–8.7 mg./g. observed in the horse, an animal of roughly equivalent size (Lawrie, 1950). The same author estimated myoglobin at 2.9–4.3 mg./g. in horse heart and found myoglobin and haemoglobin concentrations to be approximately equal. Our results on ox heart are in fair agreement, though our quoted myoglobin concentration is about 33% greater than Lawrie's (1950) maximum value in horse. Of all muscles assayed, ox heart was the only one in which myoglobin estimates based on readings at 576 and 568 nm. significantly differed from those at 568 and 538 nm., being about 25% greater when derived from the pair of longer wavelengths. The results were averaged and may therefore be somewhat high if those at the lower wavelengths are in fact correct.

Glycogen-UDP glucosyltransferase activity in rabbit muscles. Table 4 illustrates one of three experiments in which glycogen-UDP glucosyltransferase activity was measured in various rabbit muscles with rabbit liver glycogen as acceptor. Assays were performed in the presence of glucose 6-phosphate and thus represent the total activity of the so-called I and D forms. Only minor differences in activity are apparent despite a progressive increase of up to 120-fold in the ratio of glycogen phosphorylase to hexokinase activity as muscles range from red to white (Burleigh & Schimke, 1968). Results obtained in the other experiments differed from those quoted by 20% or less. A single determination on rabbit soleus muscle gave a rate of 143 units based on a 20 min. reaction time. The values we obtained in rabbit muscles are similar to those obtained by the same assay method in rat skeletal muscle (220 μ moles of UDP formed/hr. at 37°/g. of tissue) and rat heart (166 μ moles of UDP/hr./g.) (Leloir *et al.* 1959).

DISCUSSION

This paper attempts to systematize biochemical parameters that distinguish muscles of varying pigmentation and physiological performance. The quantities most commonly measured are concentrations of soluble pigments and activities of phosphorylase and the enzymes of glycolysis and terminal respiration. Hexokinase has been of less concern though, recently, more extractable activity has been found in the so-called red (tonic) muscles than in the white (phasic) muscles (Burleigh & Schimke, 1968; Peter *et al.* 1968; Pette, 1966, 1968). Pette (1966) also found that the enzyme is several times more active in extracts of rat heart than in red (tonic) muscle of the rabbit.

In addition to our earlier observations on rabbit

muscles (Burleigh & Schimke, 1968), the present results show that the hexokinase and succinate oxidase activities of heart and diaphragm decline markedly in animal species of progressively greater size (Table 1). Table 2 and Fig. 2 indicate that this conclusion is not significantly affected by differences due to growth in the size of mice, rats and rabbits. We did not obtain comparable information on the ox.

In parenthesis, we assume that we have been measuring total capacity for glucose phosphorylation in the muscle extracts and that they contain no glucokinase, an enzyme species with a relatively high K_m for glucose. This seems justified because the enzyme is not detectable in rat heart (Katzen & Schimke, 1965) or in skeletal muscle of seven animal species, including all those examined in this paper (Grossbard, Weksler & Schimke, 1966).

Except in ox diaphragm, succinate oxidase/hexokinase activity ratios differ by less than four-fold (Table 1). However, there is no clear relationship between capacity for glucose phosphorylation and the visible redness of muscle or its content of soluble pigments. Thus differences in the hexokinase activities and myoglobin contents of ox diaphragm and rabbit adductor magnus are quite disproportionate (Tables 1 and 3), and in heart and diaphragm of the rat the same parameters do not vary in concert with increasing age (Fig. 3). Though hexokinase catalyses the phosphorylation of glucose taken up from the blood, its activity also seems to bear no consistent relationship with post-mortem concentrations of blood haemoglobin (Tables 1 and 3).

Other activities commonly linked with muscle pigmentation are those of phosphorylase and the glycolytic enzymes. In rabbit muscles these are inversely related to hexokinase and succinate

oxidase activities (see Table 1) as well as to myoglobin content (Burleigh & Schimke, 1968). Phosphorylase is barely detectable in the red semitendinosus muscle of the rabbit. But the latter's capacity for succinate oxidation is less than that of any heart or diaphragm examined, and therefore one finds no consistent inverse relationships within these groups between phosphorylase activity and those of either hexokinase or succinate oxidase (Table 1).

This might seem anomalous since the proportion of white fibres in diaphragm is said to increase with animal size (Gauthier & Padykula, 1966). However, these authors' criterion of 'redness' was qualitative, histochemical estimates of mitochondrial numbers. Though ox diaphragm consists solely of white fibres, thus defined, its absolute succinate oxidase activity is still twice that of the red semitendinosus muscle of the rabbit. Ox diaphragm is also intensely red to the naked eye and is rich in myoglobin. One is led to agree with James (1968) that neglect of soluble pigment by histologists has caused much of the confused literature on 'red' and 'white' muscle fibres.

Hexokinase and succinate oxidase activities in the muscle groups examined diminish in the order heart, diaphragm and rabbit skeletal muscles and, in the case of hexokinase, the ranges overlap (Table 1). Thus muscles are not readily classified into so-called tonic, phasic and repetitively contracting forms by these criteria.

A muscle's enzyme content also seems unrelated to the duration of its individual contractions. Thus mouse heart beats approximately 500 times/min. (Table 5). Assuming that the durations of individual beats and those of the time gaps between them are approximately equal, then one contraction cycle takes approximately 60 msec. The isometric

Table 5. *Heart rates and respiration rates in different mammalian species*

Heart rates and respiratory rates are taken from Altman & Dittmer (1964). Hexokinase activities are the mean values from Table 1 of the present paper.

	Heart rate (beats/min.)	Heart hexokinase activity (units)	Respiratory rate (breaths/min.)	Diaphragm hexokinase activity (units)
House mouse (<i>Mus musculus</i>)	500 (450–550) (wt. 17.4 g.) 624 (480–738) (wt. 10–20 g.)	1070	163 (84–230) (wt. 12–26 g.)	632
Norway rat (<i>Rattus norvegicus</i>)	352 (260–450) (wt. 252 g.) 305 (270–350) (wt. 92–210 g.)	623	86 (66–114) (wt. 63–152)	241
European rabbit (<i>Oryctolagus cuniculus</i>)	251 (167–330) (wt. 1350 g.)	471	51 (38–60) (wt. unspecified)	118
Ox (<i>Bos bovis</i>)	46–53 (wt. 500 kg.)	248	27–30 (wt. 403–514 kg.)	48

twitch time of a typical fast (phasic) muscle is of the same order. In cat, for example, fast muscles take approximately 30 msec. to attain maximum tension, the slow muscles taking approximately 70 msec. (Buller, Eccles & Eccles, 1960). Mere differences in contraction speeds of mouse heart and of the white (phasic) rabbit adductor muscle thus seem unlikely to explain their widely divergent enzyme contents. One might postulate that rabbit white muscles have to overcome greater inertia in achieving speeds of contraction comparable with those of the smaller mouse heart, and that this is what is facilitated by phosphorylase-mediated glycogen breakdown. A counter-argument is the fact that in the small, fast, sartorius muscle of the frog, glycogen degradation predominates over hexose uptake during contraction (Helmreich, Danforth, Karparkin & Cori, 1965).

It seems, therefore, that muscle disuse is somehow the major cause of high extractable phosphorylase activities in rabbit white muscles. It would be worthwhile to see if the enzyme is less active in leg muscles of the wild rabbit. These are visibly redder than their counterparts in laboratory strains (Watzka, 1939).

Table 5 shows that hexokinase activities in heart and diaphragm correlate reasonably well with frequencies of contraction. Thus it seems that the only physiological attribute that unifies the present findings is the average rate of contractile energy expenditure per unit weight of muscle in an extended period of time. The results are best explained if this simply varies in rather a continuous way from the infrequently used white leg muscles of the laboratory rabbit to the rapidly contracting hearts of small mammals.

Final conclusions must await comparisons of capacities for fat and fatty acid metabolism and substrate utilization *in vivo*. The present meagre literature suggests that what will emerge is a straightforward, if approximate, relationship between average rates of contractile energy expenditure per unit weight of muscle and rates of fatty acid and blood glucose utilization.

In muscles other than so-called white (phasic) forms, fatty acids (or lipids) will be the predominant substrates and glycogen will probably prove to be the least significant. Thus, in the perfused contracting rat heart, little glycogen breakdown occurs unless adrenaline is added, and even then glycogen utilization is exceeded by glucose uptake over a 30 min. period (Williamson, 1964). However, in the contracting white (phasic) frog sartorius muscle, glycogenolysis predominates markedly over uptake of the glucose analogue 2-deoxyglucose (Helmreich *et al.* 1965). We are not aware of comparable information on the so-called red (tonic) muscles. Fatty acids are utilized *in vivo* more than carbo-

hydrates in muscles that are not of the visibly white variety, pertinent examples being rat heart (Williamson, 1962), human heart (Bing, 1955) and the skeletal muscle of the human forearm (Rabinowitz, Klassen & Zierler, 1965). Pette (1966) also found that capacities for fatty acid metabolism in rabbit adductor magnus and soleus muscles and in rat heart varied in the proportions approx. 1:4:70 respectively. Lipase and succinate oxidase activities in pigeon breast muscle are also directly related (George & Talesara, 1961) though to our knowledge the respective roles of blood fatty acids and stored lipid as substrates have not been systematically surveyed in muscles of diverse physiological properties.

Clearly a wider study of substrate utilization *in vivo* is needed and some observations in the literature require clarification. For example, skeletal muscles that differ in visual redness apparently do not convert phosphorylase *b* into the *a* form to the same extent during contraction (Stubbs & Blanchaer, 1965). Though present results indicate there is no correlation between total extractable glycogen synthetase activity and capacity for glucose phosphorylation, there do seem to be differences between so-called red and white fibres in the degree to which glycogen synthesis is promoted by accumulations of intracellular glucose (Bär & Blanchaer, 1965; Bocek, Petersen & Beatty, 1966). However, perhaps the major problem that should now be investigated is the mechanisms determining the very widely divergent enzyme contents that muscle fibres can exhibit.

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