The Adenosine Triphosphatase and Calcium Ion-Transporting Activities of the Sarcoplasmic Reticulum of Developing Muscle

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1. The ATPase (adenosine triphosphatase) specific activity and the total nitrogen content of the myofibrillar fraction per g. wet weight of rabbit longissimus dorsi muscle increased steadily during the late foetal stages and the first few weeks after birth. 2. The ATPase specific activity of the sarcoplasmic-reticular fraction isolated by a sucrose-density-gradient procedure rose to a sharp peak 8–10 days after birth and then declined to the adult value, which was about 25% of the maximum. 3. The peak in ATPase activity was a feature of the sarcoplasmic reticulum isolated from muscle, and the time at which it occurred in relation to birth was related to the degree of development and the activity pattern of the muscle. 4. The peak in ATPase activity of the sarcoplasmic reticulum occurred at an earlier age if newborn animals were made to exercise earlier than was normal. 5. The 'extra' ATPase associated with the sarcoplasmic reticulum and the ability to concentrate Ca^{2+} increased in a similar manner over the period of development studied. 6. It is postulated that the Ca^{2+} -transport system of the sarcoplasmic reticulum consists of two components, namely the ATPase and the system coupling this enzyme to Ca^{2+} transport. During development the ATPase develops first and has almost reached maximum activity in the longissimus dorsi muscle of the rabbit after 8–10 days. Subsequently the activity of the coupling system rises rapidly, leading to an increase in the capacity and efficiency of Ca^{2+} transport.

During development marked changes can be observed in the composition and enzymic activity of the sarcoplasm (Hartshorne & Perry, 1962; Perry & Hartshorne 1963; Kendrick-Jones & Perry, 1967), in the myofibrillar and sarcoplasmic-reticular fractions of the skeletal-muscle cell. The last two fractions are the principal structural components arising from specialization in this tissue, and with each are associated specific ATPases* that can be used to follow the changes in these intracellular components during development.

In the present study attention has been directed to the granular material sedimented from myofibril-free muscle supernatants by prolonged high-speed centrifugation. In adult whole muscle this fraction is derived principally from the sarcoplasmic reticulum, but in muscle from foetal and from newborn rabbits it contains appreciable amounts of mitochondria, which are relatively more abundant in developing tissue. This heterogeneous fraction was investigated initially, for it was hoped

that by study of the types of ATPase activity present it might be possible to detect in this fraction the presence of precursors of the myofibril or myosin itself. It became apparent at an early stage of the investigation that changes in the sarcoplasmic reticulum were largely responsible for the enzymic changes observed in the crude granular fraction and attention has therefore been focused on the reticulum. The results obtained are best interpreted by the hypothesis that there is a rapid development of the ATPase associated with the Ca^{2+} -transport system in the reticulum followed by a later development of the system coupling the ATPase to Ca^{2+} transport. Evidence is provided that the pattern of development of both ATPase and the Ca^{2+} -transport system is related to the contractile activity pattern of the muscle. Some details of this investigation have been reported in a preliminary form (Holland & Perry, 1968) and during the progress of this work reports by others have appeared on the changes occurring in the Ca^{2+} pump during development (Szabolcs, Kover & Lovacs, 1967).

* Abbreviation: ATPase, adenosine triphosphatase.

METHODS

Animals. New Zealand White rabbits were killed by a blow on the neck followed by exsanguination. The back muscle and, when required, other muscles and the liver were dissected out and placed in ice. Young and foetal animals were killed by decapitation. The mean gestation period was 31 days.

Eggs of the White Leghorn hen were incubated in an automatic incubator at 38° and hatched at 21 days. The chicks were removed from the eggs, blotted, killed by decapitation and the leg muscles dissected out and placed in ice. Newly hatched chicks were treated in the same way.

Method of enforced exercise. Five female rabbits were mated on the same day, enabling the simultaneous birth of at least three litters. As soon after birth as possible, usually within 1 day, the nests made by the female rabbits were removed and the young rabbits were forcibly made to move about the cage for 15 min. periods at least every 2 hr. of the day until day 14 (Kendrick-Jones & Perry, 1967). At various postnatal ages muscle from young rabbits from each litter was used for the preparation of subcellular fractions. Similar control preparations from undisturbed litters were made for comparison.

Preparation of skeletal-muscle fractions. The chilled muscle was minced in a small hand-operated stainless-steel mincer of the Latapie type and homogenized for 3 min. in a Potter-type test-tube homogenizer of 70 ml. capacity with a Teflon pestle driven at 2000 rev./min. for 3 min. with 3 vol. of 0.25 M-sucrose. The clearance between pestle and tube was 0.001 in. The homogenate was centrifuged for 15 min. at 800g to sediment myofibrils and cell residues. The sediment was washed twice and the washings were combined with the first supernatant, and the combined supernatants were centrifuged twice for 15 min. at 800g to remove traces of myofibrils. This combined supernatant was known as the 'myofibrillar-free supernatant'. Liver was treated in the same way. The procedures described below were then used to sediment fractions from the myofibrillar-free supernatant.

(a) Fractionation with constant sucrose concentration.

(i) Centrifugation for 1 hr. at 100000g (crude granular fraction); (ii) centrifugation for 15 min. at 10000g (mitochondrial fraction) followed by 1 hr. at 100000g (microsomal fraction).

(b) Fractionation through zones of different sucrose concentrations. Myofibril-free supernatant (6 ml.) was carefully layered on top of two 12 ml. steps of 0.32 M- and 1.3 M-sucrose in a tube. Centrifugation was carried out for 110 min. at 45000g in an MSE 3 × 35 ml. swing-out rotor. The microsomal fraction, containing vesicular elements of the sarcoplasmic reticulum, formed a band at the 0.32 M-sucrose/1.3 M-sucrose junction, and the mitochondria formed a pellet at the bottom of the tube. The microsomal fraction was removed by inserting a hypodermic needle fitted to a syringe immediately below the band and carefully withdrawing the sample. Each fraction prepared by methods (a) and (b) was carefully washed by resuspension in 0.25 M-sucrose followed by centrifugation. The fractions were finally dispersed in 0.25 M-sucrose.

The supernatant fraction remaining after centrifugation for 1 hr. at 100000g was taken as the sarcoplasmic fraction.

Myofibrils were prepared from the sediment obtained

after centrifugation of the whole homogenate for 15 min. at 800g by the method of Perry & Corsi (1958).

Assay of ATPase. Assays were carried out in all cases at 25° in a total volume of 2 ml. Incubation conditions varied as follows: for the Mg²⁺-activated ATPase, 25 mM-tris-HCl buffer, pH 7.6, 2.5 mM-ATP and 2.5 mM-CaCl₂ or -MgCl₂ (these are referred to below as the standard conditions); for the 'basal' ATPase, 25 mM-histidine-HCl buffer, pH 7.2, 5 mM-ATP, 5 mM-MgCl₂, 0.1 M-KCl and 5 mM-dipotassium oxalate; for the 'extra' ATPase, as for the basal ATPase, with added CaCl₂, final concn. 0.12 mM unless otherwise stated.

In all cases tubes were preincubated and the reaction was started by the addition of 0.2 ml. of suspended fraction. Appropriate controls were also carried out. After 5 min. incubation, the reaction was stopped by the addition of 1 ml. of 15% (w/v) trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925), and the colour development, after 15 min., was read at 750 nm. on the Beckman DB spectrophotometer.

Inhibitory action of granular fractions. ATPase assays were carried out as described by Baird & Perry (1960) in a final volume of 2 ml. containing 25 mM-histidine-HCl buffer, pH 7.2, 5 mM-tris ATP, 5 mM-MgCl₂, 5 mM-dipotassium oxalate and 0.1 M-KCl.

Assay of succinate dehydrogenase. Succinate dehydrogenase was assayed by the method of Pennington (1961) with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride with the modification described by Lennie & Birt (1967). The substrate was 59 mM-succinate, and the formazan produced from 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride was extracted with ethyl acetate and the *E*₄₉₀ determined on the Beckman DB spectrophotometer.

Assay of cytochrome c oxidase. Cytochrome c oxidase activity was determined by the method of Smith (1955) with the modification that the cytochrome c was reduced with ascorbic acid and dialysed overnight against the phosphate buffer.

Assay of cholinesterase. The method of Augustinsson (1957), based on the technique of Hestrin (1949), was used for the measurement of cholinesterase activity.

Preparation of tris ATP. Tris ATP was prepared from the disodium salt of ATP by a modification of the method of Schwartz, Bachelard & McIlwain (1962) and the concentration determined by the spectrophotometric method of Bock, Ling, Morell & Lipton (1956).

Determination of nitrogen. Nitrogen was determined by the Nessler technique by the method of Strauch (1965).

Determination of RNA. RNA was determined by the orcinol technique. Protein suspensions were prepared by the method of Schneider (1957) and the RNA was determined by the method of Zamenhof (1957).

⁴⁵Ca²⁺-uptake experiments. Samples of microsomal fraction (containing 0.05–1.0 mg. of protein N) prepared by centrifugation through zones of sucrose (fractionation procedure b) were incubated under the following conditions, which are similar to those used by Martonosi & Feretos (1964): 5 mM-potassium oxalate, 25 mM-histidine-HCl buffer, pH 7.2, 5.0 mM-MgCl₂, 5.0 mM-tris ATP, 0.1 M-KCl and 0.120 mM-⁴⁵CaCl₂ in a total volume of 10 ml. The reaction was started by the addition of the ⁴⁵CaCl₂, and 1 ml. samples were removed at zero time and subsequently at 1 min. intervals up to 8 min. The samples were pipetted

into 5 ml. syringes attached to Millipore Swinnex-13 filter holders holding Millipore filters of $0.45\mu\text{m}$. pore size previously dampened with 25 mM-histidine-HCl buffer, pH 7.2. The samples of incubation medium, containing less than 0.1 mg. of microsomal protein, were gently forced through the filter disc and the filtrate was collected. Filtrate (0.5 ml.) was mixed in a counting vial with 2.5 ml. of Triton X-100 (Sigma) and 7.0 ml. of scintillation fluid, consisting of toluene containing 0.4% of 2,5-diphenyl-oxazole and 0.01% of 1,4-bis-5-(phenyloxazol-2-yl)benzene.

The ^{45}Ca was counted on a Nuclear-Chicago series 720 automatic scintillation counter with the system adjusted for ^{14}C , at an efficiency of 60%. Initial rates for $^{45}\text{Ca}^{2+}$ uptake were determined by taking the zero-time concentration as 0.120 mM- CaCl_2 .

Materials. C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, supplied the specially purified tris and the disodium salt of ATP. Cytochrome c and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride were supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A. $^{45}\text{CaCl}_2$ was obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Preliminary studies on the Mg^{2+} and Ca^{2+} -activated ATPase of the crude granular fraction (see the Methods section) and the myofibrillar fraction of rabbit longissimus dorsi muscle from 11 days before birth up to 6 weeks after birth indicated that the myofibrillar fraction was responsible for the major part of the total ATPase activity of the homogenate throughout this period. As development of the muscle progressed, the contribution of the Mg^{2+} -activated ATPase of the myofibrillar fraction rose from 55% of the total at the earliest stage studied to almost 80% 3 weeks after birth (Fig. 1). These results essentially confirmed those reported briefly by Perry & Hartshorne (1963).

When the enzymic activities in the presence of Ca^{2+} or Mg^{2+} were related to the nitrogen content of the fractions (Figs. 2 and 3), it was noted that, whereas the specific activity of the myofibrillar fraction increased steadily to the adult value, that of the crude granular fraction rose sharply to a maximum value 8–10 days after birth and then fell to the adult value, which was about 25% of the maximum. Although at the age corresponding to the peak value the specific ATPase activity of the crude granular fraction was appreciably higher than that of the myofibrils, in adult muscle the converse was the case.

The ATPase activities of the crude granular fraction and the myofibrils were in each case activated to a similar extent by Ca^{2+} and Mg^{2+} (Figs. 2 and 3), but the enzymes in the two fractions were clearly of different types, as has been reported by Perry (1952). Whereas the Mg^{2+} -activated ATPase of the myofibrils was sensitive to ethylene-

Fig. 1. ATPase activity of myofibrillar and crude granular fractions from rabbit longissimus dorsi muscle during development. The fractions were prepared as described in the Methods section. Conditions of assay: 25 mM-tris-HCl, pH 7.6, 2.5 mM-tris ATP and 2.5 mM- MgCl_2 or $-\text{CaCl}_2$. Adult values are indicated by arrows on the ordinate. Activities at various ages are expressed as percentages of the activity of the whole homogenate. ●, Myofibrillar Mg^{2+} -activated ATPase; ○, myofibrillar Ca^{2+} -activated ATPase; ▲, granular-fraction Mg^{2+} -activated ATPase; △, granular-fraction Ca^{2+} -activated ATPase.

dioxybis(ethyleneamine)tetra-acetic acid and much decreased in activity at higher ionic strength, the enzyme associated with the crude granular fraction was relatively unaffected by these conditions.

The marked peak of specific activity of the crude granular fraction was a feature of skeletal muscle, as it was not shown by a fraction isolated under similar conditions from rabbit liver (Fig. 4). In skeletal muscle, however, the precise age at which it occurred appeared to be correlated with the degree of development and activity pattern of the muscle. For example, in the diaphragm of the rabbit and in whole leg muscle of the chick the peak occurred before birth (Figs. 4 and 5). In rabbit heart the activity slowly declined from a high foetal value, although assays were not made early enough to determine whether the Mg^{2+} -activated ATPase of the crude granular fraction of foetal heart showed an optimum at an early stage of development.

In all the examples of skeletal muscle studied there was a striking correlation between the ages at which the peak in ATPase activity occurred and the rapid increase in the activities of a number of

Fig. 2. Comparison of the contributions of myofibrillar and crude granular fractions to the total N and the total Mg²⁺-activated ATPase activity of developing rabbit longissimus dorsi muscle. Conditions of ATPase assay were as given in Fig. 1. ●, Myofibrillar Mg²⁺-activated ATPase activity; ○, myofibrillar N; ▲, granular-fraction Mg²⁺-activated ATPase activity; △, granular-fraction N.

Fig. 3. Mg²⁺- and Ca²⁺-activated ATPase specific activities of the crude granular fraction from rabbit longissimus dorsi muscle during development. Adult values are indicated by arrows on the ordinate. Conditions of assay: 25mm-tris-HCl, pH 7.6, 2.5mm-tris ATP and 2.5mm-MgCl₂. ●, Mg²⁺-activated ATPase; ○, Ca²⁺-activated ATPase.

enzymes, particularly those concerned in the metabolism of adenine nucleotides, reported by Kendrick-Jones & Perry (1967). A rise in enzymic activity usually implies an increase in synthesis of the enzyme concerned. Certainly over this period a great deal of new protein is laid down in muscle, and direct determination showed that the total wet weight of rabbit back muscle increased about five-fold during the first 3 weeks after birth. This increase in protein was not paralleled by a rise in nucleic acid content, for determination of the RNA contents of the fractions separated from rabbit back muscle during this period indicated a steady fall in content relative to total nitrogen, from the high prenatal values (Fig. 6). In particular the RNA content of the crude granular fraction fell steadily throughout the period of investigation and no correlation could be observed with the pattern of change of the specific ATPase activity. It is noteworthy that with increasing development the fraction of the total RNA associated with the myofibrils increased (Fig. 7).

The possibility that the rise in Mg²⁺-activated ATPase activity might be associated with changes in the innervation of the muscle associated with development was also considered. Although there are difficulties in following such changes bio-

chemically it was considered that the cholinesterase activity of the muscle fractions might give an index of the development of the innervation. Certainly fragments of nerve and end-plates would be expected to accumulate in the crude granular fraction. The results of such a survey, which are summarized in Fig. 8, give no indication of a direct correlation of the cholinesterase activity with that of the ATPase of the microsomal fraction, although a slight fall in activity occurred at later stages of development.

Effect of enforced exercise on the Mg²⁺-activated ATPase of crude granular fraction. In view of the fact that the peak in ATPase activity occurred just at the age when the activity of a number of enzymes of special importance for muscle increased rapidly (Kendrick-Jones & Perry, 1967), it was decided to investigate whether the pattern of development of the ATPase of the microsomes could be modified by increasing the contractile activity of the muscle. Such an effect has already been reported for creatine phosphokinase, one of the sarcoplasmic enzymes that starts to increase in amount rapidly at 8–10 days after birth (Kendrick-Jones & Perry, 1967). When newborn rabbits were encouraged to move about earlier than

Fig. 4. Mg^{2+} -activated ATPase specific activity of crude granular fractions obtained from muscle and liver of the rabbit during development. Adult values are indicated by arrows on the ordinate. Conditions of assay: 25 mM-tris-HCl, pH 7.6, 2.5 mM-tris ATP and 2.5 mM- $MgCl_2$. $\circ$, Diaphragm; $\square$, heart; $\triangle$, liver.

Fig. 5. Mg^{2+} - and Ca^{2+} -activated ATPase specific activities of the crude granular fraction from developing chick leg muscle. Adult values are indicated by arrows on the ordinate. Conditions of assay: 25 mM-tris-HCl, pH 7.6, 2.5 mM-ATP and 2.5 mM- $MgCl_2$ or - $CaCl_2$. $\bullet$, Mg^{2+} -activated ATPase; $\circ$, Ca^{2+} -activated ATPase.

is normal by removing the nest from the cage as soon as was practical, the specific activity of the Mg^{2+} -activated ATPase of the granular fraction rose to a peak somewhat earlier than in the control animals and declined to the adult value at a significantly earlier age (Fig. 9). The response of the granular-fraction ATPase system to these conditions was similar to that reported for creatine phosphokinase.

Separation of the microsomal and mitochondrial fractions. It is well known that the crude granular fraction isolated as described in the Methods section contains principally material derived from the sarcoplasmic reticulum. Although there are relatively few mitochondria in rabbit longissimus dorsi muscle, these organelles would be also present in this fraction. Preliminary attempts to remove the mitochondria selectively by separating the fractions sedimented from the myofibril-free supernatant after 15 min. at 10000g, 20000g and 60000g were only partially successful, in that the fraction obtained by subsequent centrifugation of the resulting supernatants for 60 min. at 100000g still contained succinate dehydrogenase and showed the peak in ATPase activity. Thus such procedures did not effectively concentrate the peak

of ATPase activity in either the mitochondria-rich or the sarcoplasmic-reticulum-rich fractions.

More satisfactory separation was achieved by using a sucrose-density-gradient procedure. In this way a microsomal fraction consisting essentially of fragments of the sarcoplasmic reticulum was obtained that was virtually free of mitochondria, as judged by the absence of succinate dehydrogenase and cytochrome *c* oxidase activities and by appearance in the electron microscope. The mitochondrial fraction that sedimented to the bottom of the tube was usually slightly contaminated with sarcoplasmic vesicles. Fig. 10 shows that the specific cytochrome *c* oxidase activity of the mitochondrial fraction increased steadily with increasing age, as did the Mg^{2+} - and Ca^{2+} -activated ATPase, whereas the microsomal fraction showed a peak at 8 days. Occasionally a small peak in the ATPase activity was observed in mitochondrial preparations from 8–10-day-old rabbits. Examination of these preparations in the electron microscope showed the presence of significant amounts of vesicles.

Fig. 6. RNA content of the intracellular components of rabbit longissimus dorsi muscle during development. Adult values are indicated by arrows on the ordinate. ●, Crude granular fraction; ○, myofibrils; □, whole homogenate; Δ, sarcoplasm.

Relation of relaxing-factor activity and the ATPase activity of the vesicles. It is well known that ATPase activity is associated with the active accumulation of Ca^{2+} within the sarcoplasmic vesicles brought about by the action of the so-called Ca^{2+} pump. The close association of the peak of ATPase activity with the microsomal preparation, the appearance of which was very similar to that of the vesicular preparations with which other workers (see Hasselbach, 1964) have shown the Ca^{2+} pump to be associated, suggested that it might reflect some aspect of the development of this system.

In the first place the effectiveness of the vesicular microsomal preparations at different ages of development was studied on the inhibition of the Mg^{2+} -activated ATPase of myofibrils isolated from adult muscle. There was no parallel between the activity of the Mg^{2+} -activated ATPase of the microsomal fraction and the amount, expressed as total nitrogen, required to bring about 50% inhibition of the myofibrillar ATPase (Table 1). The inhibitory activity of the mitochondrial fraction on the myofibrillar ATPase also increased regularly with age, although in adult muscle it was about ten times less effective than the microsomal preparation.

Thus it appears that with increasing age the inhibitory activity of the microsomes on the myofibrillar ATPase became steadily more effective

Fig. 7. Contribution of various intracellular components of rabbit longissimus dorsi muscle to the total RNA content of the muscle during development. Adult values are indicated by arrows on the ordinate. ●, Myofibrils; ○, crude granular fraction; Δ, sarcoplasm.

Fig. 8. Cholinesterase activity of the intracellular components of rabbit longissimus dorsi muscle during development. The fractions were prepared by the sucrose step technique [(b); see the Methods section]. Adult values are indicated by arrows on the ordinate. ●, Microsomal fraction; Δ, mitochondria; ○, sarcoplasm.

per unit weight of microsomal nitrogen, and there was no maximum of effectiveness associated with the vesicles from 8–10-day-old animals that could be correlated with their peak in ATPase activity. Although the inhibitory activity of microsomal preparations on the myofibrillar ATPase is known to be closely related to the effectiveness of the Ca^{2+} pump (Hasselbach, 1964), the Mg^{2+} -activated

Fig. 9. Mg^{2+} -activated ATPase specific activity of the crude granular fraction isolated from the longissimus dorsi muscle of control (●) and exercised (○) young rabbits. Conditions were as given in Fig. 2.

Fig. 10. Mg^{2+} -activated ATPase specific activity of the mitochondrial and microsomal fractions from developing rabbit longissimus dorsi muscle and the mitochondrial cytochrome c oxidase activity. The fractions were prepared by the sucrose step technique (b) described in the Methods section. Adult values are indicated by arrows on the ordinates. Standard conditions of assay were used. ●, Microsomal Mg^{2+} -activated ATPase; ○, mitochondrial Mg^{2+} -activated ATPase; △, mitochondrial cytochrome c oxidase activity.

ATPase as measured under the conditions of the experiments illustrated in Figs. 1–5 is not, however, a very sensitive index of this system. A more effective measure is the 'extra' ATPase

activity (Hasselbach, 1964), which is the increase above the basal level of ATPase activity of microsomes that occurs when they are actively pumping Ca^{2+} .

The conditions used for demonstrating the concentration of $^{45}Ca^{2+}$ by the microsomal preparation were somewhat similar to those used for assaying the Mg^{2+} -activated ATPase described in Figs. 1–5, with the difference that the Mg^{2+} and ATP concentrations were both increased from 2.5 mM to 5 mM and the addition that 5 mM-potassium oxalate and various concentrations of Ca^{2+} were present. In the absence of added Ca^{2+} the ATPase activity of the microsomal fraction was much lower than that reported in Figs. 1–5 owing to the presence of oxalate. Despite its lower value the 'basal' ATPase activity changed during the post-natal period in a parallel way to that of the Mg^{2+} -activated ATPase measured in the absence of oxalate, i.e. that measured in the experiments summarized in Figs. 1–5. The 'basal' ATPase activity of microsomes of adult animals was much lower than that of microsomal fraction from muscle of 8–10-day-old animals (Table 2). The relationship was reversed with the 'extra' ATPase, however, the activity of which rose from a low value in the newborn animal to the high value associated with microsomal fraction from adult muscle. Experiments comparing the 'basal' ATPase and 'extra' ATPase activities and the uptake of $^{45}Ca^{2+}$ by microsomal fraction as development proceeded are summarized in Fig. 11 and Table 2. Over the period studied the 'extra' ATPase activity and the rate of $^{42}Ca^{2+}$ uptake increased to the adult value.

These findings suggest that over the later stages of development there exists an inverse relationship between the Mg^{2+} -activated ATPase on the one hand and the 'extra' ATPase or the inhibitory activity on the myofibrillar ATPase on the other. After the Mg^{2+} -activated ATPase activity reaches its peak, its fall is accompanied by a rise in the 'extra' ATPase activity.

Although the 'extra' ATPase activity and the initial rate of concentration of Ca^{2+} both increased during the first few weeks after birth the rate of concentration of Ca^{2+} rose more rapidly than the 'extra' ATPase activity. This is reflected in the increased efficiency of the Ca^{2+} pump, which increased from 0.3 mole of Ca^{2+} transported/mole of ATP split by the 'extra' ATPase activity in the 1-day-old rabbit to 1.6 moles/mole in the adult (Table 2).

It is presumed throughout that the ATPase activities of the microsomal fraction measured under different conditions and with different bivalent cations were all due to the same enzyme. This has not been unequivocally proved, but all the evidence obtained in this study and by

Table 1. *Inhibitory action of mitochondrial and microsomal fractions from developing rabbit longissimus dorsi muscle on the Mg^{2+} -activated ATPase of myofibrils isolated from adult rabbit muscle*

Results are expressed as the means $\pm$ S.E.M. of the numbers of experiments given in parentheses. The inhibitory activity represents the amount of protein required to produce 50% inhibition of myofibrillar ATPase under standard conditions (see the Methods section).

Age of rabbits (days after birth)	Inhibitory activity		Mg^{2+} -activated ATPase sp. activity of microsomal fraction (μ moles of P_i /min./mg. of N)
	Mitochondrial fraction (μ g. of protein)	Microsomal fraction (μ g. of protein)	
2 days	565 $\pm$ 76 (6)	105 $\pm$ 13 (6)	2.10 $\pm$ 0.3 (6)
9 days	500 $\pm$ 66 (5)	69 $\pm$ 8 (5)	2.80 $\pm$ 0.32 (7)
Adult	305 $\pm$ 55 (7)	35 $\pm$ 6 (7)	1.20 $\pm$ 0.16 (7)

Fig. 11. 'Basal' and 'extra' ATPase activities of microsomal fractions isolated from rabbit longissimus dorsi muscle. 'Basal' and 'extra' ATPase activities were assayed as described in the Methods section. $\circ$, Δ and $\square$, 'Basal' ATPase; $\bullet$, $\blacktriangle$ and $\blacksquare$, 'extra' ATPase. $\circ$ and $\bullet$, Adult; Δ and $\blacktriangle$, 1-day-old; $\square$ and $\blacksquare$, 8-day-old.

many other investigators (e.g. Hasselbach, 1964; Martonosi & Feretos, 1964) suggests that it is. Certainly Ca^{2+} -transport activity and Mg^{2+} -activated ATPase activity could not be separated by fractionation procedures employing centrifugation in sucrose density gradients as described by Dallner, Orrenius & Bergstrand (1963).

DISCUSSION

The peak of ATPase specific activity associated with the microsomal fraction implies some changes in composition of this fraction during development.

Although it is not unusual to find changes in enzymic activity of organelles occurring during development the usual pattern is a steady rise or decline to the adult value. The change in ATPase activity of the myofibrils is a typical example of this pattern. With a few enzymes, however, peaks in activity at certain ages have been reported (Sippel, 1954; Kretchmer, 1959; Solomon, 1959; Fedorov, 1966).

The pattern of the changes in the Mg^{2+} -activated ATPase activity of the microsomal fraction appears to be unique to muscle, and the exact age at which the peak occurs depends on the functional activity of the muscle and the stage of development at birth of the species from which the muscle is obtained. In this respect there is striking correlation between the age at which the activities of certain enzymes associated with the specialized requirements of muscle begin to increase rapidly (Kendrick-Jones & Perry, 1967) and the peak in specific activity of the Mg^{2+} -activated ATPase of the microsomes. There is evidence that the increases in enzymic activity reported by Kendrick-Jones & Perry (1967) are associated with the stage at which the contractile activity of the muscle begins to increase markedly, and in view of the results obtained with the disturbed litters it is tempting to suppose that the change in ATPase activity of the microsomes is likewise related. Some caution is necessary, however, in interpreting the results of such an experiment, in which a number of factors may be involved.

The microsomal fraction of muscle is a complex fraction containing vesicles resulting from homogenization of the sarcoplasmic reticulum, and fragments of nerve, end-plates and possibly other structures. Material derived from the innervation of muscle is present in this fraction, judging from the presence of cholinesterase activity. The lack of direct correlation between the pattern of development of this enzyme and the Mg^{2+} -activated ATPase suggested, however, that the latter was not directly related to the development of innervation of the muscle. It should be emphasized that

Table 2. *ATPase activity and $^{45}\text{Ca}^{2+}$ uptake in the microsomal fractions from developing rabbit longissimus dorsi muscle*

The procedure for measuring the 'basal' ATPase and 'extra' ATPase activities and $^{45}\text{Ca}^{2+}$ uptake is described in the Methods section. Results are expressed as the means of the initial rates $\pm$ S.E.M. of the numbers of experiments given in parentheses.

Age of rabbits (days after birth)	ATPase activities ($\mu\text{moles of P}_1/\text{min.}/\text{mg. of N}$)			Rate of Ca^{2+} transport ($\mu\text{moles}/\text{min.}/\text{mg. of N}$)	Efficiency (moles of $\text{Ca}^{2+}/\text{mole of ATP}$)
	'Basal'	'Extra'	'Basal' + 'extra'		
1 day	1.5 ± 0.1 (6)	0.6 ± 0.05 (6)	2.1	0.18 ± 0.01 (6)	0.3 ± 0.02
8 day	2.4 ± 0.2 (6)	1.2 ± 0.09 (6)	3.6	0.54 ± 0.01 (5)	0.45 ± 0.03
Adult	0.9 ± 0.01 (9)	3.0 ± 0.2 (9)	3.9	4.8 ± 0.4 (7)	1.6 ± 0.2

some cholinesterase may, however, be associated with the sarcoplasmic reticulum (Ulbrecht & Kruckenberg, 1965).

On the other hand evidence from many laboratories strongly suggests that the Mg^{2+} - and Ca^{2+} -activated ATPase of the muscle microsomal preparations is a component of the Ca^{2+} pump present in the vesicular system. It is therefore reasonable to suppose that the peak in enzymic activity studied in this investigation was associated with this system. Certainly the mitochondrial Mg^{2+} -activated ATPase did not show the peak and was presumably of a different type. Nevertheless the development of the Ca^{2+} -pumping capacity followed a different pattern from that of the Mg^{2+} -activated ATPase. It was significant, however, that after the peak in activity at 8–10 days, when the Mg^{2+} -activated ATPase activity fell three- to four-fold, the Ca^{2+} -pumping ability and the 'extra' ATPase activity increased three- to four-fold. Over the same period the total ATPase activity of the microsomes measured under the conditions used to demonstrate Ca^{2+} transport, i.e. the 'basal' rate plus the 'extra' ATPase rate when Ca^{2+} was pumped at the maximal rate, increased by only 20–30%.

Although it is appreciated that there are problems in equating the ATPase activities under different assay conditions the facts could be explained by the following hypothesis. It is assumed that the Ca^{2+} pump consists of an ATPase system associated with some as yet ill-defined and probably complex component that couples the enzymic activity to Ca^{2+} transport. It is suggested that these two components, the ATPase and the Ca^{2+} -transport coupling system, are synthesized independently during development, the ATPase arising earlier and almost reaching the adult value after 8–10 days in rabbit longissimus dorsi muscle, by which time the young animal is just beginning to use its muscles and move around. This process occurs earlier in muscles that function earlier. Whereas after the peak in Mg^{2+} -activated ATPase activity there is

little increase in the total capacity of the microsomes to hydrolyse ATP, the coupling system now begins to increase rapidly in activity and functions in an organized way with the ATPase to act as the Ca^{2+} -transport system, so that when the enzyme is coupled in this way it is not stimulated by Mg^{2+} unless Ca^{2+} is being concentrated within the vesicles. Further, there are suggestions that the development of the coupling system is in some way associated with the increased contractile activity of the muscle. Such a scheme would explain satisfactorily the changes described in the present paper, but depends on the assumption that the Mg^{2+} -activated ATPase activity that rises to a peak at 8–10 days is a component of the Ca^{2+} -transport system. All the evidence suggests that this is the case, but further experiments are required to confirm this view and test the hypothesis proposed.

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