The Bound Nucleotide of the Isolated Myofibril

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Since Straub & Feuer (1950) reported the presence of adenosinetriphosphate (ATP) in globular actin (G-actin) the nucleotide content of this protein has been the subject of considerable investigation. The original claim that ATP was split during the transformation of G-actin to fibrous actin (F-actin) has been substantiated by the work of Laki & Clark (1951) and Szent-Gyorgyi (1951), but the work of these authors does not support the view that depolymerization is accompanied by resynthesis of ATP. On the other hand, Dubuisson & Mathieu (1950), whilst confirming that ATP is present in G-actin preparations, could not detect any change in phosphate distribution or any change in free phosphate during polymerization. Although the above investigators do not agree as to the role of ATP in the polymerization of actin, all confirm that ATP is a component of normal G-actin preparations, whereas Snellman & Gelotte (1951), using chromatographic techniques, were unable to detect any ATP or adenosinediphosphate (ADP) in either G-actin or F-actin. These authors consider that the prosthetic group of actin is a dinucleotide containing adenine and a so-far unidentified base which can occur in phosphate rich and phosphate poor forms, corresponding to G-actin and F-actin respectively.

Actin is not alone in its property of binding adenine nucleotides, for Buchtal, Deutsch, Knappeis & Munch-Petersen (1949) have shown that when myosin and actomyosin are treated with ATP, some nucleotide is bound to the protein in such a way that it cannot be removed by repeated washing.

The investigations so far mentioned have been carried out on extracted purified myofibrillar proteins and the nucleotide content of the intact myofibril has received little attention. Using ultraviolet absorption methods Caspersson & Thorell (1942) claimed to have demonstrated the localization of ATP in the I band of resting muscle. It is not possible, however, to decide whether their results were due to adenine nucleotide or to the small amount of nucleic acid which occurs in the myofibril (Perry, 1952). In an earlier communication (Perry, 1951) a method employing collagenase was described for the isolation of myofibrils in comparatively small quantities. Preliminary analyses of these preparations indicated that the iso-

lated myofibril contained small amounts of nucleic acid, phospholipid and acid-labile phosphorus. In the present paper a study of this latter phosphorus fraction is described, together with a method for isolating myofibrils in larger quantities which has considerably facilitated the investigation. The acid-labile phosphorus of myofibrils obtained from skeletal and cardiac muscle of the rabbit, and rat skeletal muscle, is rather constant in amount. In rabbit skeletal muscle it arises from ATP and ADP bound to the myofibril in a manner which renders these nucleotides inaccessible to the enzymes which normally attack them. This nucleotide fraction consists predominantly of ADP, and the quantities present are compatible with the view that it is associated with F-actin in the isolated myofibril.

METHODS

Preparation of myofibrils. Muscle from the hind legs and back of a rabbit was quickly dissected out, chilled in ice and minced. The mince was homogenized in a Waring Blendor for 2 min. with 8 vol. of 0.08 M-borate buffer, pH 7.1. This and all subsequent operations were carried out at 0°. Centrifugation of the homogenate for 15 min. at 600 g was usually sufficient at this stage to give a compact sediment which was resuspended in the same volume of borate buffer as was used to make the original homogenate. The suspension was homogenized again for 2 min. This homogenate was centrifuged for 20 min. at 600 g, the turbid supernatant discarded and the lighter-coloured upper layer of the sediment carefully removed with the aid of a little borate buffer. This upper layer, which consisted mainly of myofibrils, was freed from coarser material by centrifugation for 5 min. at 300 g.

The myofibrils were washed free from granules (Perry, 1952) and soluble sarcoplasmic components by repeated resuspension in borate buffer followed by centrifugation for 20–30 min. at 600 g. To obtain a suspension of myofibrils which contained less than 2% of protein in solution, 4–5 centrifugations were necessary. These centrifugations were carried out as rapidly as possible because, after standing some hours in borate buffer, the myofibrils became increasingly difficult to centrifuge down at 600 g. It is desirable to keep the centrifugal field at this figure to ensure a satisfactory separation of granules and myofibrils. After an additional centrifugation at 300 g for 5 min. to remove any remaining large particles and myofibrils which may have denatured and clumped, the preparation was stored at 0° in the presence of a trace of toluene. The stored suspension was very viscous and usually contained 10–15 mg. protein per ml. By this method 5–10 g. of myofibrils could readily be

obtained from 200 g. of minced muscle. When examined microscopically with the aid of methylene-blue stain the preparations could be seen to be free from other components.

Isolation of nucleotide preparation. Myofibrils were washed twice by centrifugation and resuspension in distilled water to remove most of the borate buffer and the free phosphate which leaches out of these structures on standing. One volume of cold 60% (w/v) trichloroacetic acid (TCA) was well stirred into 11 vol. of the myofibril suspension. After standing for 20 min. at 0° the fibrous myofibrillar

by following the fall in absorption at 265 m μ . when the nucleotide was treated with (1) adenylic 5-deaminase, (2) myokinase and adenylic 5-deaminase, (3) potato pyrophosphatase and adenylic 5-deaminase. The enzyme preparations and the experimental procedures were carried out as described by Kalckar (1947).

Acid-labile P. The inorganic phosphate liberated when the TCA extract of the myofibrils, or an aqueous solution of the nucleotide preparation, were heated with N-HCl for 10 min. at 100°, was estimated by the method of Fiske & Subbarow (1925).

Table 1. *Acid-labile phosphorus content of isolated myofibrils*

Preparation	Condition	Source	Acid-labile P (μ g./g. myofibril)
9/5	Myofibrils stored at 0°: 5 days	Rabbit skeletal	111
	26 days	Rabbit skeletal	82
5/6	Myofibrils stored at 0°: 1 day	Rabbit skeletal	121
	7 days	Rabbit skeletal	121
	9 days	Rabbit skeletal	100
	24 days	Rabbit skeletal	100
	Treated 2 days with 0.5M-KCl	Rabbit skeletal	83
	Dialysed 2 days against 0.5M-KCl	Rabbit skeletal	63
7/8	Freshly prepared	Rabbit psoas	108
3/8	Muscle kept 3 days at 0° (rigor)	Rabbit psoas	114
17/10	Freshly prepared	Rat hind leg	116
24/10	Freshly prepared	Rat hind leg	92
18/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	146
31/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	100
24/10	Freshly prepared	Rabbit heart	88
11/10	Freshly prepared	Rabbit heart	88
31/10	Muscle stored 4 days at 0° (rigor)	Rabbit heart	111

precipitate was centrifuged down and the supernatant filtered. When the clear filtrate had been extracted four times with ether to remove the bulk of the TCA, the aqueous layer was freed from ether by distillation *in vacuo* in the cold and finally freeze-dried.

The dry, white residue consisting mainly of borate, which acted as a convenient carrier for the nucleotide, was extracted three times with ether to remove the last traces of TCA, and then dried and stored in a vacuum desiccator at 0°. All stages of this preparation were carried out in the cold to avoid the breakdown of acid-labile phosphate. The efficacy of these precautions was shown by the fact that the ratios of free to acid-labile P in the original TCA extract and the final nucleotide preparation were identical.

Enzymic analyses. Myosin, freed from myokinase by repeated precipitation, was prepared by the method of Bailey (1942), and myokinase as described by Colowick & Kalckar (1943). Nucleotide preparation corresponding to 10–20 μ g. of acid-labile P was incubated with the enzymes individually, and then with both together, in the presence of 0.1M-glycine buffer, pH 9.0, and 0.005M-CaCl₂; final vol. 2 ml. Incubations were carried out for 30 min. at 33°. Extra enzyme added at the end of this period induced no further splitting of phosphate, indicating that the reaction had gone to completion.

Spectrophotometric analysis for adenylic acid, ADP and ATP was carried out with the Beckman spectrophotometer

RESULTS

TCA extracts of myofibrils from fresh rat and rabbit skeletal muscle contained acid-labile P ranging in amount from 90 to 140 μ g./g. of myofibril (the dry weight of the myofibril was taken as six times the nitrogen content). Values within this range were also obtained from muscle (rabbit psoas and rat hind-leg) which had been allowed to go into rigor by storing at 0°.

Continued washing with 0.08M-borate, pH 7.1, did not remove the acid-labile P from the myofibrils; after storing the suspensions in this buffer for 3–4 weeks at 0° the amount had fallen by only 20–30%. During storage the acid-labile P remained bound to the myofibril and no significant amount could be detected in the clear supernatant obtained by centrifuging the suspension for 10 min. at 7000 g. Table 1 summarizes these results and also indicates that treatment with M-potassium chloride, which rendered 75–85% of the total protein of the myofibril soluble, only slightly reduced the acid-labile P level. A rather greater decrease was obtained in one experiment when a 0.5M-potassium

chloride extract of the myofibrils was dialysed against 0.5M-potassium chloride for 2 days at 0°.

Experiments with heart muscle indicated that the acid-labile P level is similar to that obtained with skeletal myofibrils. It should be noted, however, that preparations from heart were not usually as satisfactory as those from skeletal muscle, because cardiac tissue does not break down so readily into single myofibrils and the latter are not so easily separated from granules.

Fig. 1 is the ultraviolet absorption curve given by a preparation of nucleotide from well washed skeletal muscle myofibrils. The curve shows a maximum at 259 m μ . and is in general similar to that given by the adenosine polyphosphates. For a

acid-labile P from the nucleotide. On prolonged incubation in the presence of large amounts of enzyme, phosphorus was split from the nucleotide in amounts which approached the total organic P content. Under comparable conditions, strangely enough, the enzyme split only the acid-labile P of an ATP preparation, though it did slowly attack adenylic acid. Bailey (1949) and Krishnan (1949) have pointed out that there is some phosphatase (or

Fig. 1. Ultraviolet absorption of nucleotide from washed muscle myofibrils.

number of reasons it seemed likely that ADP or ATP could be responsible for the acid-labile P of the myofibril; to determine whether this was the case, and if so to estimate the amounts of the two polyphosphates, the nucleotide preparation was analysed by determining the amount of phosphate split from it by myosin, and then by myosin in the presence of myokinase. Table 2 shows that myosin alone split only a fraction of the acid-labile P whereas with myokinase 75–90% was liberated as inorganic phosphate. From these experiments it can be concluded that the acid-labile P arises from a mixture of ATP and ADP, with the latter making up about 80% of the whole. In no case was the total acid-labile P split by these enzymes acting in combination. This does not necessarily mean that other compounds containing acid-labile P are present, for the experience of other workers (cf. Bailey, 1949) with myosin and myokinase acting in combination indicates that it is extremely difficult to get complete hydrolysis of the acid-labile P even with the purest samples of ATP and ADP.

Potato pyrophosphatase prepared by the method of Kalckar (1947) liberated rather more than the

Table 2. *Analysis of nucleotide preparation using myosin and myokinase*

(Results expressed in μ g./g. of nucleotide preparation.)

Nucleotide preparation	N2	N3
Free P	503	1213
Acid-labile P	831	1395
Total organic P	—	2762
Total organic P	—	1.98
Acid-labile P	—	1.98
Acid-labile P calculated from amounts of ATP and ADP determined spectrophotometrically	770	1485
P liberated by myosin	107	179
P liberated by myosin and myokinase	604	1168
Acid-labile P due to ATP	214	358
Acid-labile P due to ADP	390	810
Moles ADP as % moles ADP plus ATP	79	82

nucleotidase) associated with the Kalckar potato pyrophosphatase preparation. If the pyrophosphatase is used in low concentration and particularly if the adenylic acid is removed as it is formed, as in the Kalckar spectrophotometric estimation of the adenine nucleotides, interference by the phosphatase activity is small.

Table 3. *Relative proportions of adenosine phosphates in nucleotide fraction isolated from rabbit myofibrils*

(Figures for each preparation represent the average of two or more estimations.)

Nucleotide preparation	Age of myofibril preparation (days)	(Moles % total moles adenine nucleotides)		
		Adenylic acid	ADP	ATP
N2	10	17	75	8
N3	1	13	79	8
Average		15	77	8

In Table 3 are shown the results of enzymic analyses for the adenosine phosphates carried out by the method of Kalckar (1947). Although these estimations indicate a higher relative proportion of ADP than was obtained by assay with myosin and myokinase, the results given by the two methods are in satisfactory agreement, considering their accuracy (Table 2).

The work of Buchtal *et al.* (1949), on the adsorption of adenine nucleotides on myosin and actomyosin, suggests that during the preparation of

myofibrils, nucleotides could be picked up from solution and adsorbed on these structures in a similar way. The rather constant composition and amount of the nucleotide fraction associated with the myofibrils makes such an explanation unlikely, a view which is supported by the following experiment. ATP was added to myofibril suspensions in 0.08M-borate buffer, pH 7.1, so that the final concentrations of acid-labile P were 10 and 100 times that bound to the myofibrils. At the higher ATP concentration the myofibrils had very obviously synaerased, but in all cases after washing by centrifugation, and resuspension in the borate buffer, there was no significant difference between the acid-labile P content of the control myofibrils and those treated with ATP. For (a) control myofibrils, (b) myofibrils treated with ATP equivalent to 10 times, and (c) myofibrils treated with ATP equivalent to 100 times their acid-labile P content, the results were 127, 143 and 131 μg . of acid-labile P/g. respectively.

the myofibril. The ultraviolet absorption spectrum of the nucleotide preparation did not show any evidence of a maximum at 250 $\text{m}\mu$., the position of maximum absorption by inosinic acid.

The fact that the acid-labile P is not split off from the myofibrils when muscle passes into rigor probably accounts, in part at least, for the residue of easily hydrolysable phosphate reported by Bendall (1951) to be present in rigor muscle.

The amounts and relative proportions of adenylic acid, ADP and ATP are similar to those which would be expected if all the nucleotides were considered to be associated with actin in the fibrous form in the intact myofibril. If actin makes up 20% of the myofibrillar protein (cf. Hasselbach & Schneider, 1951), the actin present in the myofibril would have a nucleotide content which is similar to that of the extracted F-actin analysed by Szent-Gyorgyi (1951). It is a matter of some interest to decide whether actin exists in the globular or fibrous form in the living cell. To assess the true significance

Table 4. *Nucleotide content of actin and isolated myofibrils*

(Results expressed as $\mu\text{moles/g.}$)

Materia	Adenylic acid	ADP	ATP	Authority
Myofibrils*	0.88	2.7	0.47	—
Actin in myofibrils†	4.4	13.5	2.3	—
F-actin	—	—	8.5	Laki, Bowen & Clark (1950)
F-actin	1.6	11.7	2.4	Szent-Gyorgyi (1951)
G-actin	—	—	3.4	Laki <i>et al.</i> (1950)
G-actin	—	—	9.8	Laki <i>et al.</i> (1950)
G-actin	—	—	23	Straub & Feuer (1950)
G-actin	—	—	12-39	Dubuisson & Mathieu (1950)
G-actin	2.1	1.9	7.8	Szent-Gyorgyi (1951)

* These values are obtained by taking 5.9 as the molar ratio of ADP to ATP (average of the results obtained by the two methods described) in rabbit skeletal myofibrils containing 113 μg . of acid-labile P/g.

† Calculation made on the basis that all the nucleotide is associated with the actin which makes up 20% of the total myofibrillar proteins.

DISCUSSION

From the evidence presented it would appear that the nucleotides associated with the myofibrils are bound to its structure in such a way that although the system contains myokinase and an active adenosinetriphosphatase (Perry, 1951) they are not attacked by these enzymes; that is, so far as can be judged by the maintenance of the acid-labile P level in stored myofibrils. In this respect the bound ADP of the myofibrils showed some differences from that associated with F-actin, for Laki & Clark (1951) have reported that a short time after the addition of salt to G-actin, practically no ADP could be found. This effect they attributed to the myokinase activity of their actin preparations.

In addition to ADP and ATP, adenylic acid is also present and this compound also must be unavailable to adenylic deaminase which is present in

of the state of the nucleotide in the isolated myofibril it must be known whether the system is in the relaxed or the resting condition because it is possible, although it has yet to be proved, that contraction is accompanied by a change in the state of polymerization of actin. Electron microscope examinations (kindly carried out by Mr R. W. Horne of the Cavendish Laboratory) of myofibrils isolated by the method described in this paper show the I band to be shorter than the A band, suggesting that at some stage of the preparation a certain degree of contraction had taken place. It must be concluded then that the actin in the isolated myofibril corresponds to the contracted or partly contracted state.

In Table 4 the nucleotide analyses of actin carried out by various authors are presented. The considerable variations in these values probably reduce their reliability, and they should be compared with the rather constant values for the acid-labile P of

isolated myofibrils as shown in Table 1. Many of the actin preparations have higher ATP values than might be expected in view of the low amount of this nucleotide in the well washed muscle residue from which actin is extracted, and which must consist mainly of myofibril residues. Straub & Feuer (1950) found insignificant amounts of ATP in the acetone-dried fibre from which they extracted G-actin and concluded that ATP was produced during the water extraction of the fibre.

Assuming that the adenine content of the isolated myofibril is compatible with that found *in vivo* it is possible to speculate on the ATP distribution within the muscle cell. Normal muscle contains powerful ADP phosphorylating systems and it is likely that in the resting state all the adenine exists as ATP. This would mean that about 10% of the ATP content of the cell is associated with the myofibrils. As the latter occupy about two-thirds of the cell volume, the concentration of ATP in the extra-myofibrillar space, which is occupied by the sarcoplasm, will be 18 times that in the myofibril, assuming that the myofibrils are not interpen-

trated by the sarcoplasm, or alternatively that any ATP which does penetrate into them is broken down. This concentration gradient must be an important factor affecting the accessibility of ATP to the enzyme associated with the contractile structure, the myosin adenosinetriphosphatase.

SUMMARY

1. A non-enzymic method for the isolation of myofibrils from rabbit skeletal muscle has been described.

2. Myofibrils from cardiac and skeletal muscle of the rabbit and skeletal muscle of the rat contained 90–140 μg . of acid-labile phosphorus per gram. Similar values were obtained with rigor muscle.

3. The acid-labile phosphorus of myofibrils isolated from rabbit muscle arises from adenosine-diphosphate, and to a lesser extent, adenosinetriphosphate. These nucleotides are bound to the myofibril in such a way that they are not acted upon by the enzymes occurring in myofibril which normally attack them.

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Histidine α -Deaminase and the Production of Urocanic Acid in the Mammal

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Urocanic acid (β -4(5)-glyoxalinylacrylic acid) (I), was discovered by Jaffe (1874) in the urine of one dog and was considered after its rediscovery by Siegfried (1898) to be a possible normal breakdown product of histidine. Evidence produced by Raistrick (1917, 1919) showing that it was formed from histidine by the action of certain micro-

organisms, and the determination of its structure by Hunter (1912) fostered the suggestion that direct deamination played a part in the normal degradation of histidine. This point of view was supported by the later work of Kotake & Konishi (1922), Kiyokawa (1933) and Harrow & Sherwin (1926). Cox & Rose (1926), however, and Darby & Lewis