measurement it should be possible to increase the sensitivity by a considerable extent. The rate of creatine liberation by a particular nucleotide sample will vary with the enzyme preparations and it is necessary to determine the rate of creatine liberation with graded amounts of known standard nucleotide at the same time and under identical conditions.

The experiments indicate that even dialysed purified preparations of creatine phosphokinase are liable to be contaminated with very small amounts of ADP and ATP. It should be emphasized, however, that although these nucleotides contaminate the enzyme, the amounts must be extremely small as in the experiment shown in Fig. 6 none could be demonstrated. Here, in the absence of added ADP, using the same creatine phosphokinase as was used in the experiments illustrated in Fig. 7, there was no liberation of creatine. Nevertheless, such small amounts of ADP or ATP would be adequate to prime the autocatalytic generation of ATP from adenylic acid by the combined action of creatine phosphokinase and myokinase.

SUMMARY

1. A method for the assay of creatine phosphokinase is described. The method involves creatine estimation under conditions in which creatine phosphate is completely stable.

2. The coupled creatine phosphokinase-hexokinase system has been demonstrated to be an extremely sensitive means of determining 0.5–5.0 $\mu\text{g.}$ of ADP and ATP.

3. By the addition of myokinase the creatine phosphokinase-hexokinase system can be used to determine small amounts of adenylic acid.

4. Some aspects of the coupled creatine phosphokinase-myokinase system have been studied.

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Creatine Phosphokinase and the Enzymic and Contractile Properties of the Isolated Myofibril

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Myofibrils from rabbit skeletal muscle which have been carefully isolated and freed from other intracellular components contain a small but constant amount of nucleotide (Perry, 1952, 1953). This nucleotide consists mainly of adenosine diphosphate (ADP), and in view of the importance of the adenosine polyphosphates in cell metabolism, and in the contractile process in particular, the close associa-

tion of ADP with the isolated contractile unit itself appears to be of some significance. Wherever ADP is found in the cell, enzyme mechanisms exist for its phosphorylation to adenosine triphosphate (ATP), and it is therefore reasonable to suggest, as was done in an earlier communication (Perry, 1952), that some system must be present in the muscle cell for the conversion of the myofibrillar ADP into ATP.

Such a system would have the advantage of maintaining ATP precisely localized where it is needed for contraction. At the same time, if we accept the assumption that the bound nucleotide of the myofibril is associated with the actin moiety of this structure, such an enzyme system might be involved in the transformation between F-actin (fibrous) and G-actin (globular). From the work of several investigators (Straub & Feuer, 1950; Laki, Bowen & Clark, 1950; Szent-Györgyi, 1951; Mommaerts, 1952), evidence has accumulated indicating a change in the level of phosphorylation of the nucleotide bound to actin when this protein is transformed from the globular to the fibrous form.

The present paper describes investigations made in an attempt to elucidate the mechanism of the phosphorylation of the myofibrillar nucleotide with particular reference to the role of creatine phosphokinase. The results indicate that the myofibrillar ADP does not catalyse the breakdown of creatine phosphate in the presence of creatine phosphokinase as does free ADP in the presence of the latter enzyme and myofibrils. By coupling the creatine phosphokinase system to the myofibril in the presence of extremely low concentrations of ADP, shortening is produced under conditions which strongly suggest that ATP must be split by the myofibril during this process.

A preliminary account of some of the results of this investigation has already been presented Perry (1954).

METHODS

Myofibril preparations. Myofibrils were prepared from rabbit back and hind-leg muscle by the method already described (Perry, 1953a). They were stored at 0° as concentrated stock suspensions in the same medium as was used for their preparation, consisting of 0.025 M-KCl and 0.039 M borate, buffer pH 7.1. Even after storage for 3-4 weeks under such conditions myofibrils shortened readily with ATP or with the creatine phosphate system. Immediately before use the stock suspensions were diluted with 4-5 vol. of the KCl:borate medium, centrifuged in the cold and the supernatant discarded. This process was repeated twice and finally the suspensions were diluted with medium to give a fivefold dilution of the original stock suspension. This procedure removed most of the slight myokinase activity, but not the adenylic deaminase activity, associated with the stock suspensions of myofibrils.

Muscle acetone powder. Minced rabbit muscle was treated for 30 sec. in a Waring Blendor with 3 vol. of KCl:borate medium, pH 7.1. After being stirred for 30 min. the muscle was filtered through muslin and the residue re-extracted with 2 vol. of medium. The combined filtrates were centrifuged for 15 min. at 600 g to sediment myofibrils and the turbid supernatant was then precipitated with 3 vol. of acetone. Finally the precipitate was washed and dried with ether as described by Needham & Pillai (1937). The KCl:borate medium was used for the initial extraction instead of water to minimize the extraction of myofibrillar proteins (Perry, 1953).

Some acetone-powder preparations were also made from the first supernatants obtained during the preparation of myofibrils.

The acetone powder was stored at 0°, and for use 2 g. were extracted for 15 min. with 25 ml. distilled water. The extract was centrifuged and dialysed at 0° against frequently changed, stirred distilled water, for 1-2 days. Any precipitate was filtered off and discarded.

Creatine phosphokinase. Preparations were made according to Banga (1943) and Askonas (1951) as described in the previous paper (Chappell & Perry, 1954). Usually the Banga preparation, taken to the fourth stage, was concentrated by precipitation with 70% saturated $(\text{NH}_4)_2\text{SO}_4$, dissolved in a small volume of water and dialysed until free from $(\text{NH}_4)_2\text{SO}_4$.

Enzyme experiments. Experiments involving the creatine phosphokinase system were carried out essentially as described by Chappell & Perry (1954). The reaction medium, of total volume 2 ml., contained 0.05 M phosphate buffer, pH 7.4, 0.005 M-MgCl₂, 0.005 M creatine phosphate, 0.1-0.2 ml. acetone-powder extract or creatine phosphokinase and 0.5 ml. of washed myofibril suspension (stock diluted 1 in 5). For those experiments in which ADP was added to the system, usually 0.2 ml. of $2.0-3.0 \times 10^{-5}$ M ADP was used. The reactions were started by addition of either the creatine phosphokinase or the creatine phosphate. The enzyme was inactivated using phenylmercuric acetate, and deproteinization carried out by the method of Somogyi (1945) as described by Chappell & Perry (1954).

Adenosinetriphosphatase (ATPase) experiments were carried out as described earlier (Perry, 1951) using amino-tris(hydroxymethyl)methane (Tris) buffer.

Observation of shortening of myofibrils. Samples of reaction mixture were withdrawn at frequent intervals and examined under the microscope with the aid of methylene-blue stain. Complete contraction was considered to have taken place when all the myofibrils in the field appeared granular and had clumped in irregular masses.

Creatine estimations. These were carried out as described in the preceding paper (Chappell & Perry, 1954).

RESULTS

In preliminary attempts to phosphorylate the bound nucleotide of the myofibril, creatine phosphate was incubated with a well-dialysed aqueous extract of muscle acetone powder and a suspension of repeatedly washed myofibrils. With such a system, which will be referred to as the glycolytic extract-myofibril (GEM) system, there was a rapid production of creatine. As Table 1 indicates, creatine was not produced in the absence of myofibrils or acetone-powder extract. When magnesium ion was omitted from the incubation mixture, creatine formation was usually very low and in some cases absent. Concomitant with the liberation of creatine, the myofibrils shortened and settled to the bottom of the incubation tube.

Simultaneous determinations of the creatine and phosphate liberated after incubation clearly showed that these substances were being produced in the amounts to be expected for the hydrolysis of creatine phosphate.

At an early stage in the investigation the results were explained by postulating that the bound ADP of the myofibril was converted by the creatine phosphokinase system into ATP which itself was then dephosphorylated by the myofibrillar ATPase to re-form ADP (Perry, 1954). In this manner the bound ADP could catalyse the breakdown of creatine phosphate. A similar system with hexokinase and small amounts of free ADP has already been described in the preceding paper (Chappell & Perry, 1954).

Indications that this explanation was not satisfactory were given by several observations. Creatine phosphokinase preparations purified by the method of Banga (1943) neither liberated creatine nor induced shortening when incubated with myofibrils, creatine phosphate and magnesium ions. Also, after prolonged ageing, acetone-powder extracts lost their ability to liberate creatine in the GEM system but retained their creatine phosphokinase activity. The activity of these aged acetone-powder extracts could be restored by the addition of very low concentrations of ADP (2–4 μg . per enzyme tube), and also such trace amounts of ADP similarly produced a liberation of creatine and shortening of

the myofibrils when added to a reaction mixture containing purified creatine phosphokinase and myofibrils (CPM system).

This suggested that the dialysed acetone-powder extracts were active in the GEM system because they contained ADP or ATP in amounts sufficient to catalyse appreciably the breakdown of creatine phosphate. That this was the case was proved by the following experiments. When acetone-powder extracts were incubated with creatine phosphate, hexokinase, glucose and magnesium, creatine was produced only with those preparations which liberated creatine and shortened myofibrils in the GEM system. This means that these dialysed acetone-powder extracts contained enough available nucleotide to allow reactions (1) and (2) to proceed at a measurable rate (cf. Chappell & Perry, 1954).

The total amount of ADP and ATP present in various creatine phosphokinase preparations was determined using the creatine phosphokinase-hexokinase system described in the preceding paper (Chappell & Perry, 1954). Table 2 shows the results of such determinations, and it is apparent that only those preparations active in the GEM system contain significant amounts of ADP or ATP. Clearly the routine 48 hr. dialysis of acetone-powder extracts with stirring and frequent changes of distilled water, as used in these experiments, was not an effective method of removing trace amounts of ADP or ATP. In one experiment an acetone-powder extract, which was treated in this way and then dialysed for a further 12 days, still catalysed the liberation of creatine in the GEM system. The remarkable feature is that these nucleotides should survive for so long in an extract which contains some myokinase and very slight ATPase activity, and yet be readily available to the creatine phosphokinase and myofibrillar ATPase systems.

The myofibrillar ADP, however, appeared to be unavailable to the combined action of the latter two enzymes. Once removed from the myofibril by trichloroacetic acid treatment the extracted nucleotide readily catalysed the liberation of creatine by a system containing nucleotide-free creatine phosphokinase and myofibrils. For example if, as illustrated in Table 3, 1 ml. of a myofibril suspension was incubated with purified creatine phosphokinase (Banga) and creatine phosphate no creatine was liberated. When, however, half of the volume of myofibril suspension was supplemented by the nucleotide extracted from the other half, there was a considerable formation of creatine. In both cases the total nucleotide in the system, i.e. bound and free, is identical.

Table 1. *Liberation of creatine by the acetone-powder extract-myofibril system*

Myofibril preparations diluted 1 in 5. Incubations, carried out under conditions described in the Methods section, for 5 min. at 30°. Preparations *a* and *b* were 3 and 9 days old, respectively. Values quoted are μg . creatine liberated.

	Myofibril preparations	
	<i>a</i>	<i>b</i>
Complete system	106	109
Same without Mg^{2+}	15.2	4.3
Same without creatine phosphate	Nil	Nil
Same without myofibrils	4.5	2.0
Same without acetone-powder extract	3.0	Nil

Table 2. *Concentration of ADP + ATP in myofibril and creatine phosphokinase preparations*

Unless otherwise stated, creatine was liberated when 0.2 ml. creatine phosphokinase preparation either as the purified enzyme as an acetone-powder extract was incubated with 0.5 ml. of the 1-in-5 diluted myofibril suspension for 4 min. at 30°. ATP and ADP were extracted from the preparations by trichloroacetic acid treatment as described by Perry (1952).

	ATP + ADP ($\mu\text{moles} \times 10^{-3}/\text{ml.}$)	Creatine liberated ($\mu\text{g.}$)
Myofibrils (stock suspension)	68	—
Creatine phosphokinase	1	Nil
Acetone-powder extract	36	86
Acetone-powder extract*	69	101
Acetone-powder extract	Nil	Nil
Acetone-powder extract	2	3

* 0.6 ml. myofibrils used.

Table 3. *Bound nucleotide and the creatine phosphokinase-myofibril system*

Reaction mixture contained 0.05M phosphate buffer, pH 7.4, 0.005M-MgCl₂, 0.005M creatine phosphate and 0.2 ml. of purified creatine phosphokinase (Banga, 1943). Total volume 2 ml., incubated for 5 min. at 30°.

Myofibril suspension added (ml.)	Nucleotide added ($\mu\text{moles} \times 10^{-3}$)	Creatine liberated ($\mu\text{g./5 min.}$)
0.5	Nil	Nil
1.0	Nil	Nil
0.5	5.6 (ADP)	51
0.5	2.8 (ADP)	29
0.5	Extracted from 0.5 ml. myofibrils	37

It is also clear that the nucleotide did not leach out of the myofibrils when stored in the KCl:borate medium, for neither freshly prepared myofibrils nor the same preparations stored for several weeks and tested without further washing, liberated creatine on incubation with the purified creatine phosphokinase system (Banga). Any leaching out of nucleotide from the myofibril would be made apparent by a production of creatine in this system.

Effect of cations on the creatine phosphokinase-myofibril system

In view of the fact that the creatine phosphokinase-myofibrillar ATPase system is probably of considerable importance within the cell for the supply of energy-rich phosphate to the myofibril, this system was investigated in some detail. For the experiments described in this section, dialysed aqueous extracts of muscle acetone powder which produced creatine liberation in the GEM system, were used.

The liberation of creatine by the GEM system has already been demonstrated to be markedly dependent on the presence of Mg²⁺. Fig. 1 shows that in the absence of this ion, the activity of the system was slight at pH 7.4, but maximal activation was obtained at quite low concentrations of Mg²⁺, i.e. 0.002–0.003M. Increasing the Mg²⁺ concentration up to 0.04M produced little further effect on the amount of creatine liberated. On the other hand, with CaCl₂ concentrations up to 0.025M the activity remained the same as that shown in the absence of added cations, but slight activation was obtained at higher concentrations of CaCl₂ (Fig. 2).

In contrast to their effects on the GEM system, Ca²⁺ and Mg²⁺ both activate the myofibrillar ATPase (Perry, 1951). Hence in the former enzyme system when Ca²⁺ alone is present the limiting feature cannot be the splitting of ATP formed, but rather the enzymic phosphorylation of ADP, a process which requires Mg²⁺.

Fig. 1. Effect of Mg²⁺, and Mg²⁺ in the presence of Ca²⁺ on the liberation of creatine by the acetone-powder extract-myofibril system. Incubations carried out in 0.05M phosphate buffer, pH 7.4; 5 min. at 30°. O, Mg²⁺; ×, Mg²⁺ in presence of 0.006M-CaCl₂.

Fig. 2. Effect of Ca²⁺ on the liberation of creatine by the acetone-powder extract-myofibril system. Incubation carried out as in Fig. 1. O, Ca²⁺; ×, Mg²⁺ for comparison.

The dependence of the creatine liberation on the splitting of ATP by the myofibril was demonstrated with myofibrils treated so that Mg²⁺ produced little activation of the ATPase. Such preparations were obtained by treating myofibrils with M-KCl, a procedure which largely destroys the structure of the myofibril and profoundly modifies the Mg²⁺ activation of the ATPase (Perry, 1951). Table 4 presents the results of an experiment in which the rate of creatine liberation by the GEM system and the myofibrillar ATPase activity are compared before and after M-KCl treatment. The experiment was arranged so that the control and potassium chloride-treated myofibrils were compared under identical ionic conditions, and it clearly shows that simple disorganization of the myofibril as is induced by the salt treatment, diminishes in a comparable way the amount of creatine liberated and the ATP split by the respective systems.

This relation between creatine liberation and the ATPase activity of the myofibrils was further demonstrated by a comparison of the behaviour of the two systems when the ionic strength of the medium used for the enzymic tests was increased. Addition of 0.3M-KCl decreased both the Mg^{2+} -activated ATPase and the rate of creatine liberation, although in the latter case not quite to the same extent.

When the effect of Mg^{2+} concentration on the creatine liberated by the GEM system was studied in the presence of 0.006M- $CaCl_2$ (the value given for

muscle by Dubuisson, 1951) very little modification of the curve obtained for $MgCl_2$ alone (see Fig. 1) was obtained. This suggested that these ions did not have the same antagonistic effect on myofibrillar ATPase activity such as has been demonstrated for myosin (Mommaerts & Seraidarian, 1947). A study of the effect of these two ions on myofibrillar ATPase activity confirmed this point. 0.006M- Ca^{2+} increased the ATPase activity at low concentrations of Mg^{2+} , and when the concentration of the latter ion was 0.01M, activities with Mg^{2+} alone and with Mg^{2+} plus 0.006M- $CaCl_2$ were practically identical.

Under the usual test conditions the amount of creatine liberated was not affected very much by considerable variation in creatine phosphate concentration; thus, increasing the creatine phosphate concentration from 0.0005 to 0.02M raised the amount of creatine produced in a given time by only 30%. Increase in the amount of dialysed acetone-powder extract or in the myofibril concentration increased the rate of creatine liberation. In some cases the response to increases in the amount of dialysed acetone-powder extract was linear, but more usually it was of the type illustrated in Fig. 3.

Table 4. *Effect of treatment with M-KCl on creatine liberation and splitting of ATP*

Creatine was estimated after 0.5 ml. myofibril suspension and 0.1 ml. of acetone-powder extract had been incubated together with 0.05M phosphate buffer, pH 7.4, and the usual additions. Phosphate liberated from ATP determined by simultaneous independent experiments carried out in 0.05M Tris buffer, pH 7.3, using 0.3 ml. of myofibril suspension. In both cases 0.005M- $MgCl_2$ and 0.1M-KCl were present. Incubations for 4 min. at 30°.

	Creatine liberated (μ g.)	Phosphate liberated (μ g.)
Myofibrils treated with M-KCl	9.9	4.6
Untreated myofibrils	36.0	15.8

The creatine phosphate-induced shortening of isolated myofibrils

During the course of the experiments on the splitting of creatine phosphate by the GEM system it was noticed that the myofibril suspensions flocculated and settled rapidly during incubation. More detailed study of this shortening was carried out in a medium similar to that used for the enzymic studies. This consisted of 0.05M phosphate buffer, pH 7.4, 0.005M- $MgCl_2$ and 0.005M creatine phosphate (in addition to 0.006M-KCl and 0.01M borate buffer introduced with the myofibril suspension). When freshly prepared myofibrils and dialysed acetone-powder extract were added to this medium shortening was complete in 1–2 min. at 16°. Shortening was also readily observed with myofibril preparations which had been stored as a concentrated suspension for 3–4 weeks. Seen under the microscope the shortened myofibrils presented a very different appearance from the original structures, the characteristic myofibrillar form being absent and replaced entirely by granular bodies which had clumped together in irregular masses.

Control experiments showed that the acetone-powder extract and creatine phosphate were necessary additions for shortening to take place, but that in the absence of added Mg^{2+} slight changes could sometimes be observed after several hours. Although shortening of myofibrils was readily obtained when fresh dialysed aqueous extracts of acetone powder were used, if the latter was replaced

Fig. 3. Effect of variation in myofibril and acetone-powder extract concentrations on the liberation of creatine by the acetone-powder extract-myofibril system. Incubations carried out as in Fig. 1. O, 0.1 ml.; x, 0.2 ml.; Δ, 0.3 ml. acetone-powder extract.

by purified creatine phosphokinase (Banga, 1943) no changes could be detected under the microscope. In fact it was apparent from the results of a number of experiments that in the absence of creatine liberation in the CPM system, shortening did not take place. The addition of trace amounts of ADP (2–4 μg . in 2 ml.) to systems containing purified creatine phosphokinase produced shortening and creatine was liberated. ADP was ineffective in the absence of added creatine phosphokinase or creatine phosphate, but if the ADP concentration was varied in the presence of these two substances the time taken to complete the contraction of the myofibrils could be changed from less than a minute to several hours.

An interesting feature of the system was that contraction of myofibrils could be induced within a few minutes at room temperature with low ADP concentrations, whereas the same concentration of ATP produced little change in myofibrils under conditions identical except for the absence of creatine phosphokinase. In other words, ADP which was being actively phosphorylated by the creatine phosphokinase system was much more effective than the same molar concentration of ATP. It was usually not possible to observe any significant change in myofibrils which had been treated with ATP at the same concentration as the ADP which in the presence of creatine phosphokinase produced rapid contraction. To obtain the same degree of shortening with ATP alone it was necessary to increase its concentration 10–100 times that of the ADP, suggesting very strongly that for a given low ATP concentration shortening takes place much more readily when phosphate turnover can be maintained by the creatine phosphokinase system.

In the experiments illustrated in Table 5, 5.6×10^{-3} μmole ATP itself was not adequate to bring about the shortening but when this concentration was maintained by supplying 5.6×10^{-3} μmole ADP and the creatine phosphokinase system,

so that appreciable turnover of ATP resulted from the action of the myofibrillar ATPase, contraction rapidly proceeded to completion.

DISCUSSION

It is clear that for the isolated myofibril to shorten in the presence of purified creatine phosphokinase and creatine phosphate small amounts of available ADP or ATP had to be present in the system. The myofibril itself contained ADP but this did not catalyse the breakdown of creatine phosphate as did added ADP in the presence of creatine phosphokinase and myofibrillar ATPase. The possibility that the myofibrillar ADP is phosphorylated to ATP by the creatine phosphokinase system has not been excluded by the experiments carried out so far, but it is apparent that if this does take place the ATP formed is not subsequently dephosphorylated by the myofibrillar ATPase.

The observation that aqueous extracts of muscle powder contain small amounts of ADP or ATP which are not readily removed by conventional means, raises the question as to whether the small quantities of nucleotides associated with other components from muscle, e.g. myofibrils and actin, are simply present as contaminants with no functional significance. Such an association might be produced by the formation of polyvalent metal complexes with the nucleotide and the protein (cf. Buchthal, Deutsch, Knappeis & Munch-Petersen, 1951). In the case of actin, with which the bound nucleotide of the myofibril is presumably associated, the evidence of changes in nucleotide composition during the G-actin-F-actin transformation (Straub & Feuer, 1950; Laki *et al.* 1950; Szent-Györgyi, 1951; Mommaerts, 1952) certainly suggests a functional significance. The present investigation shows that the ADP is specifically bound to the myofibril and is not available to the creatine phosphokinase-myofibrillar ATPase system, in clear

Table 5. *Shortening of isolated myofibrils by ADP in the presence of the creatine phosphokinase system and by ATP*

All tubes contained 0.05 M phosphate buffer, pH 7.4, 0.005 M-MgCl₂, 0.005 M creatine phosphate and 0.5 ml. myofibril suspension. ADP and 0.2 ml. of nucleotide-free creatine phosphokinase was added to one series and ATP alone to the other. Total volume 2 ml.

Serial no.	ADP + creatine phosphokinase		ATP alone		Temp. (°)
	ADP present ($\mu\text{moles} \times 10^{-3}$)	Contraction time (min.)	ATP present ($\mu\text{moles} \times 10^{-3}$)	Contraction time (min.)	
13/6/53	5.6	15 (completely granular)	5.6	50 (no obvious change)	—
			56	48 (contraction obvious but not complete)	18
18/6/53	5.6	20 (completely granular)	5.6	100 (little change)	—
			56	100 (obviously shortened but not completely granular)	18
26/6/53	11.2	8 (completely granular)	11.2	240 (very slight contraction)	—
			112	12 (completely granular)	23

distinction to the nucleotide found in the acetone-powder extracts.

Since this work was completed, Goodall & Szent-Györgyi (1953) have published observations on the role of creatine phosphate and a factor *X* isolated from aqueous muscle extract, in the relaxation of glycerated fibres. Factor *X* is precipitated between 50 and 70% saturation with ammonium sulphate and must contain creatine phosphokinase, for the latter enzyme precipitates in this range. The enzymic system employed by Goodall & Szent-Györgyi is very similar to that reported here and contains in addition to factor *X*, creatine phosphate, ATP and Mg^{2+} . Under these conditions the ATP level will be maintained in the same way as has been described for the myofibril systems, despite continuous breakdown by the glycerated fibre. Relaxation was measured by the fall in tension when the pH value of the medium containing the isometric fibre was lowered to 5.7. A possible explanation is that relaxation takes place under these conditions simply due to the inhibition of the fibre ATPase by the low pH. Under these conditions little splitting of ATP can take place, the fibre shows little tendency to shorten, and tension is not maintained, although the ATP level is kept constant by the creatine phosphokinase system. It is significant that a similar effect is reported by these authors with ATP alone, although as one might expect the relaxation is much less than that obtained when factor *X* and creatine phosphate are present, for under the conditions of assay the latter system is much more effective in maintaining the ATP level.

The system described involving myofibrils, creatine phosphokinase, ADP and creatine phosphate offers a means of studying both physical change and enzyme activity of the myofibril in the presence of a constant concentration of ATP. Furthermore, by decreasing the ADP concentration the rate of contraction can be made correspondingly slower, and at the same time the rate of splitting of creatine phosphate falls in a similar manner (cf. creatine phosphokinase and hexokinase system, Chappell & Perry, 1954). These facts, like the evidence already produced by Weber & Portzehl (1952), support the view that the splitting of ATP induces contraction. Other investigators, e.g. Szent-Györgyi (1953) and Hill & Morales (1952), maintain that for contraction to take place only the adsorption of ATP by the contractile system is necessary, the splitting of ATP taking place during the relaxation process. This latter suggestion is extremely difficult to disprove experimentally, but the above facts together with the observation that a given low concentration of ATP, maintained by the creatine phosphokinase system, is much more effective for inducing contraction than the same

concentration of ATP alone, indicates that the splitting of phosphate from ATP is very closely associated with the shortening of the isolated myofibril. If adsorption alone is sufficient to induce contraction it is difficult to see why a given ATP concentration should be less effective in bringing about contraction of the myofibril than a much lower concentration of ATP maintained by the creatine phosphokinase system.

SUMMARY

1. At pH 7.4 in the presence of well-dialysed aqueous extracts of muscle acetone-powder, creatine phosphate and magnesium ions, isolated myofibrils shorten and simultaneously creatine phosphate is split.

2. This shortening process and splitting of creatine phosphate has been demonstrated to be due to low concentrations of ADP or ATP which are associated with the acetone-powder extracts and not readily removed by dialysis.

3. The bound ADP of the myofibril does not catalyse creatine phosphate breakdown in the creatine phosphokinase-myofibril system.

4. The effects of calcium and magnesium ions on the liberation of creatine by the acetone-powder extract-myofibril system have been described.

5. In the presence of the creatine phosphokinase system, low concentrations of ADP will bring about rapid contraction of isolated myofibrils, whereas a similar concentration of ATP added in the absence of creatine phosphokinase induces little or no change. This observation is taken as evidence that removal of phosphate from ATP is essential for contraction of the isolated myofibril.

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Studies in Carotenogenesis

12. THE EFFECT OF TEMPERATURE AND THIAMINE CONCENTRATION ON CAROTENOGENESIS BY *PHYCOMYCES BLAKESLEEANUS*

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In *Rhodotorula glutinis*, variations in the temperature of cultivation have a profound qualitative effect on carotenogenesis (Skoda, 1951; Nakayama quoted by Mackinney, 1952). Low-temperature cultivation (5°) results in the production of yellow cultures containing predominantly α - and β -carotene, whilst at higher temperature (25°) the cultures become red owing to the production of the more unsaturated xanthophylls, torulin and torularhodin. In *R. sarnieii*, however, temperature changes between 14 and 28° have no qualitative effect on pigmentation (Fromageot & Tschang, 1938).

Turian (1953) has recently also observed a temperature effect on carotenogenesis by *Mycobacterium phlei*. At 30° leprotene, γ -carotene, a 'rhodopin-like' carotene, a hydroxycarotene and traces of phytofluene are produced together with very small amounts of the acidic chrysophlein. At 37° only small amounts of these neutral compounds were formed, but the synthesis of chrysophlein was considerably increased.

Starr & Saperstein (1953) observed that *Corynebacterium poinsettiae* cultured in a medium low in thiamine (0.1 μ g./100 ml.) is pink and contains mostly spirilloxanthin and lycioxanthin. On a high-thiamine medium (100 μ g./100 ml.) orange colonies are formed which contain much less spirilloxanthin, the same amount of lycioxanthin and some cryptoxanthin, which was not present in the organisms grown in the low-thiamine medium.

The present paper describes an investigation undertaken to find out the effects of variation in both the temperature of cultivation and in the thiamine content of the medium on carotenogenesis in *Phycomyces blakesleeanus*.

EXPERIMENTAL

Cultures. *Phycomyces blakesleeanus* (—strain) was grown in 8 oz. medicine bottles in 15 ml. of our standard medium. This medium is that described by Schopfer & Jung (1935), but contains 2.5% instead of 10% (w/v) glucose; this concentration was found to give maximal β -carotene production (Garton, Goodwin & Lijinsky, 1951). In the experiments on temperature variation, two glass incubators (Garton *et al.* 1951) were placed close together in the laboratory, one being kept at 25° and the other at either 20 or 30°. Thus both the control (25°) and the experimental runs were always carried out under comparable conditions of illumination. The experiments at 3–5° were carried out in the dark in a 'warm' corner of a large refrigerator. It was not possible to illuminate the cultures in these experiments.

In the experiments on thiamine-deficient media, inoculation was not carried out by the usual means of a spore suspension made by shaking up a stock agar culture of *Phycomyces* with sterile water (Garton *et al.* 1951), but by a very small direct transfer with a needle from an agar slope, care being taken not to transfer any agar. This precaution was necessary to eliminate the possibility of carrying over significant amounts of thiamine. That this was achieved, was shown by the fact that no growth (or occasionally very little) occurred on media containing no thiamine.

On repeating the experiments on thiamine-low media some time after the first series was carried out, it was found that no growth could be obtained. This failure was eventually traced to the age of the parent culture. Inoculations from cultures up to 6 months old will grow quite well on the standard medium, but in order to get growth in thiamine-low media, young parent cultures (less than 4 weeks old) must be used.

Determinations of dry weight and carotene. Dry weight and carotene determinations were carried out according to the methods described by Garton *et al.* (1951). The chromatographic separation of the carotenoids was carried out according to the method of Goodwin (1952).