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Direct and ^{18}O-Exchange Measurements Relevant to Possible Activated or Phosphorylated States of Myosin*

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ABSTRACT: A careful search has been made for a covalently linked phosphoryl group in myosin which has cleaved one $AT^{32}P$ per mole in a "burst" reaction. No evidence for a phosphoryl myosin was obtained upon extraction of protein with phenol near neutral pH, or by measurement of ^{18}O incorporation into inorganic phosphate (P_i) formed when trichloroacetic acid–$H^{18}OH$ was used to stop adenosine triphosphate (ATP) hydrolysis. Further, with EDTA or phenol as stopping agents in the presence of $H^{18}OH$, lack of appreciable ^{18}O incorporation into P_i does not support possible presence of a labile acyl phosphate. When the "burst "reaction is carried out in $H^{18}OH$, the initial P_i formed shows extensive exchange with water oxygen. In continued hydrolysis, water oxygen appears exclusively in the P_i and not in the adenosine diphosphate (ADP) formed or in the unhydrolyzed ATP. Presence of 2,4-dinitrophenol slightly accelerates the oxygen-exchange reaction, but the *p*-mercuribenzoate inhibits the exchange. This *p*-mercuribenzoate inhibition appears distinct from the activation of adenosine triphosphatase (ATPase) by the mercurial.

Among the myriad studies on the ATPase[1] action of myosin, various experimental findings have raised the possibility of the formation of a phosphorylated or an activated form of myosin during ATP cleavage. These include the occurrence of an initial rapid phase or "burst" of ATP hydrolysis (Weber and Hasselbach, 1954). This has recently been studied in considerable detail by Tonomura and associates under conditions where the "burst" of P_i liberated is approximately 1 mole/mole of myosin (Tonomura and Kanazawa, 1965; Imamura *et al.*, 1965). The suggestion has been made that this reflects a transient phosphorylation of myosin (Tonomura and Kanazawa, 1965). Other pertinent findings are the extra oxygen incorporation from HOH into inorganic phosphate (P_i) that accompanies ATP hydrolysis (Levy and Koshland, 1959; Levy *et al.*, 1960), and the induced exchange between medium P_i and HOH (Dempsey *et al.*, 1963). Some plausible explanations for these exchanges involve reversible phosphorylation of an active site by P_i.

Although a considerable search, likely mostly unpublished, has been made for a phosphorylated myosin, no convincing evidence for a covalent phosphoryl intermediate has been reported. One purpose of the present experiments was to apply more sensitive methodology to the possible direct demonstration of a phosphorylated myosin, with particular attention to preparations just after occurrence of an approximately stoichiometric "burst" of P_i release. Correlated with these studies, the incorporation of water oxygen into P_i during or at the time of stopping the ATPase action was measured as a possible means of revealing formation of any very labile phosphorylated intermediate. Other experiments with ^{18}O are reported which indicate lack of formation of activated ADP or ATP during ATP hydrolysis, and suggest that myosin SH groups may be of particular importance in the extra oxygen exchange accompanying ATP cleavage.

Experimental Section

Myosin Preparation. Myosin was prepared from

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[1] ATPase, adenosine triphosphatase; ATP and ADP, adenosine tri- and diphosphates.

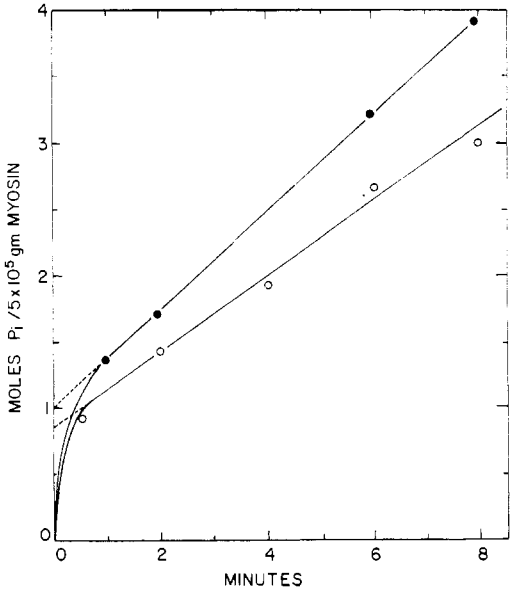


FIGURE 1: Extent of "burst" reaction observed with perchloric acid or $CuSO_4$ precipitation of myosin. Incubation mixtures contained at 0° 1 M KCl, 0.1 mM ATP, 20 mM Tris–maleate buffer at pH 7, 1 mM $MgCl_2$, and 5 mg of myosin/ml. Reactions were started by ATP addition. At the indicated times, myosin was precipitated by adding to a 2-ml aliquot an equal volume of 0.1 M $CuSO_4$ or of 0.9 M perchloric acid. P_i was isolated as described in the Experimental Section. Closed circles give results with $HClO_4$ and open circles with $CuSO_4$ as the stopping agent.

rabbit skeletal muscle essentially as described by Perry (1955), with additional centrifugation of the final myosin solution at 45,000*g* for 1 hr in 0.3 M KCl. The concentration of myosin protein was determined by phenol extraction and acetone precipitation as described in Table I, followed by evaporation under infrared lamps to determine dry weight, or by a biuret procedure (Layne, 1956).

Determination of P_i Release. P_i determinations were made by an adaptation of the procedure of Berenblum and Chain (1938) and Martin and Doty (1949). The extracted molybdate complex was read directly at 310 or 400 mμ. The $FeSO_4$ reduction procedure (Sumner, 1944) was also occasionally used. Reactions were stopped and myosin was removed by conventional procedures with perchloric or trichloroacetic acid preparations unless otherwise stated.

$CuSO_4$ precipitation of myosin (Bowen *et al.*, 1963) was performed by mixing with final 50 mM $CuSO_4$ using a vibrating "buzzer" mixer. Upon centrifugation, the protein pellet floated, and was removed for extraction with 2 ml of 20 mM $CuSO_4$. After breaking the pellet with a glass rod, the protein precipitate was removed and the combined extracts were used for P_i isolation or determination.

P_i Isolation and ^{18}O Analyses. To avoid use of excessively large amounts of reactants, nonlabeled carrier P_i was usually added with the stopping agents. For experiments concerned with the "burst" phenomenon, isolation of the small amounts of P_i from the relatively large reaction volume was accomplished by extraction as the phosphomolybdate complex (Berenblum and Chain, 1938; Martin and Doty, 1949). For example, for the experiments reported in Table II, 25 ml of cold 0.9 M perchloric acid was added to a 25-ml incubation mixture, and 200 mg of charcoal (acid-washed Norit A) was added to absorb nucleotides. A 4-ml aliquot was used, after centrifugation for protein removal, for P_i determination. Carrier P_i was added to the remaining suspension, which was then filtered through a Hirsch funnel into a separatory funnel. Ammonium molybdate solution (10 ml, 7.5 mg of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$/100 ml of 2.5 N H_2SO_4) was added, and the solution was extracted successively with 10, 15, and 10 ml of 1:1 isobutyl alcohol–benzene. The combined extracts were washed with 10 ml of molybdate solution (1.5 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$/100 ml of 0.5 N H_2SO_4). Concentrated ammonia (1 drop) was added to the washed extract, and the P_i was transferred to the aqueous phase by successive extraction with 2, 3, and 1 ml of 0.2 M NH_4Cl–0.5 M NH_4OH. The P_i was precipitated as $MgNH_4PO_4$ and the ^{18}O content was determined essentially as described previously (Dempsey *et al.*, 1963; Boyer *et al.*, 1961).

As a check of the adequacy of the above procedure for isolation and ^{18}O analyses, control experiments were performed using typical incubation mixtures with perchloric acid added before the myosin, and with P_i–^{18}O of amount and ^{18}O content as expected in a typical experiment. Observed ^{18}O contents in separate experiments, corrected for carrier addition, were 73 and 95% of the theoretical. This represents adequate recovery under rather restrictive conditions. Contamination and errors tend to lower observed ^{18}O contents of samples, particularly when small P_i samples and relatively large volumes and amounts of reagents must be used as in the present experiments. Hence observed values probably represent minimal values.

In experiments where many moles of ATP were hydrolyzed per mole of myosin, the myosin was precipitated by perchloric or trichloroacetic acid, nucleotides were removed by charcoal absorption and purified by reprecipitation, and ^{18}O was determined essentially as described previously (Dempsey *et al.*, 1963; Boyer *et al.*, 1961). For the analyses reported in Table III, ATP and ADP were separated chromatographically (Cohn and Carter, 1950) and hydrolyzed in 1 N HCl at 100° for 12 min, and the P_i formed was isolated.

$AT^{32}P$ Preparation. The $AT^{32}P$ was prepared using glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase, with purification on Dowex 1 anion-exchange resin, according to Glynn and Chappell (1964).

Results

"Burst" Reaction with $CuSO_4$ or $HClO_4$ Stop. The data in Figure 1 show the extent of P_i release at 0°

TABLE I: Lack of Formation of Phosphoryl Myosin Extractable by Phenol.[a]

Sample	Observed Cpm in Protein Fraction: Acid Labile	Observed Cpm in Protein Fraction: Acid Stable	Total Possible Moles of Bound Phosphoryl/ 5×10^5 g of Myosin
Control	18	39	0.008
Incubated	38	49	0.013

[a] A 2.5-ml incubation mixture contained 0.5 M KCl, 10 mM $MgCl_2$, 2 mM $AT^{32}P$ (1.7×10^6 cpm), 20 mM Tris–maleate buffer at pH 6.5, and 4 mg of myosin/ml. After 1 min of incubation at 25°, the solution was mixed with 6 ml of ice-cold liquid phenol (brought to pH 7 with NH_4OH). For the control sample, phenol was added before $AT^{32}P$. The phenol extract was washed carefully in a cold room seven times with 0.01 M P_i–0.01 M EDTA, pH 7, buffer saturated with phenol using centrifugation and careful suction to separate layers. Interfacial material encountered during washings was dispersed by small amounts of 7.5 M urea–0.3 M NH_3. Protein was precipitated from the washed phenol by addition of about 6 volumes of −25° acetone, with standing for 1 hr at −25°. The protein was washed with 75% ethanol, the ^{32}P was liberated by 6 min in boiling 0.3 M trichloroacetic acid, and the ^{32}P remaining with the protein was determined.

under conditions as used by Tonomura and Kanazawa (1965), with use of either 0.45 M perchloric acid or of 50 mM $CuSO_4$ (final pH 4.5) to stop the ATPase and remove the myosin. The results with perchloric acid are in good accord with the findings of Tonomura and Kanazawa (1965), and confirm that one may obtain an initial "burst" reaction about stoichiometric with the myosin present. This "burst" is also observed with the $CuSO_4$ stop, indicative that if any phosphoryl derivative is formed it is labile at this pH as well as in strong acid. Bowen *et al.* (1963) have previously shown that the more extensive "burst" which occurs under their conditions was also apparent with $CuSO_4$ as a stopping agent.

Apparent Lack of Phosphoryl Myosin Formation. In previous experiments, myosin-hydrolyzing $AT^{32}P$ was not observed to yield any detectable phosphoryl-protein after addition of concentrated urea–ammonia solution and removal of $AT^{32}P$ and $^{32}P_i$ by anion-exchange resin (Dempsey *et al.*, 1963). With mitochondria and particles therefrom, a phenol extraction procedure has given a more sensitive test for phosphoryl protein formation (Bieber *et al.*, 1964). Results given in Table I were obtained by application of the phenol extraction procedure to myosin hydrolyzing

TABLE II: Absence of ^{18}O Incorporation into P_i Released in the "Burst" Reaction with $HClO_4$–$H^{18}OH$ Stopping Reagent.[a]

Expt	Moles of P_i Formed/ 5×10^5 g of Myosin	Atom % Excess ^{18}O in P_i: Expected[b]	Atom % Excess ^{18}O in P_i: Obsd
1	1.12	0.033	<0.002
	1.04	0.031	<0.002
2	0.75	0.013	<0.002
	0.85	0.014	<0.002

[a] A 25-ml reaction mixture was used under conditions reported with Figure 1. After 30 sec, 25 ml of 0.9 M $HClO_4$ in $H^{18}OH$ was added to give a final concentration of 0.65 atom % excess ^{18}O. P_i isolation and analyses were made as described in the Experimental Section, with addition of 1 or 2 μmoles of non-isotopic carrier P_i in expt 1 and 2, respectively. [b] Calculated value for incorporation of one oxygen from HOH into each P_i formed.

TABLE III: Lack of Oxygen Incorporation into ADP or ATP during ATP Hydrolysis by Myosin.[a]

Source of P_i for Anal.	Obsd Atom % Excess ^{18}O	Water Oxygen Incorpd/ P_i Formed
P_i	0.56	2.33
ADP	<0.004	<0.03
ATP	<0.004	<0.03

[a] Samples contained 4.6 mM ATP, 9.2 mM Mg^{2+}, 31 mM Tris–Cl at pH 7.4, 110 mM KCl, and 1.8 mg of myosin/ml in 6.5-ml total volume, with 0.96 atom % excess ^{18}O in the H_2O. Incubation for 3 hr at 25° gave 2.4 mM P_i. Separation and analysis of P_i, ADP, and ATP were made as described in the text.

$AT^{32}P$ under conditions in which an initial "burst" would occur (Tonomura and Kanazawa, 1965) and extensive water oxygen incorporation into the P_i released would be expected (Levy and Koshland, 1958; Dempsey *et al.*, 1963). The stopping of the reaction and subsequent extraction and washings were performed near neutral pH and at low temperature. Nonetheless, no detectable phosphoryl myosin was found within experimental error. The small number of counts observed with the protein may represent contamination, and one may conclude that less than one out of a hundred and possibly none of the myosin molecules were present as a phosphoryl derivative stable to the mild isolation procedure.

Oxygen Exchange Accompanying the "Burst" Reaction. Measurement of the amount of oxygen incorporation into the P_i released during the stoichiometric "burst" reaction was of particular interest. If prior myosin activation by cleavage of at least one ATP per mole of myosin were required for the oxygen-exchange reaction to commence, less exchange might be found with the initial than with subsequent P_i molecules released. Indeed, if a group on myosin furnished the oxygen for the initial cleavage, the possibility existed that the initial P_i released might even contain less than one solvent oxygen per mole. The data presented in Table IV show that the water oxygen incorporation in the initial P_i released is strikingly high.

TABLE IV: Rapid Oxygen Exchange Accompanying the Initial P_i Release.[a]

Expt	Moles of P_i Formed/ 5×10^5 g of Myosin	Obsd Atom % Excess ^{18}O	No. of Water Oxygens in P_i
1	1.2	0.059	2.86
2	1.0	0.055	3.16

[a] A 20-ml incubation contained additions as reported with Figure 1 and had 0.935 atom % excess ^{18}O in the HOH. The reaction was stopped after 30 sec by adding 200 ml of 0.4 M perchloric acid. Carrier P_i (2.5 μmoles) and 300 mg of charcoal were added, and extractions were performed as described in the text but with larger volumes, followed by precipitation and ^{18}O determination in the P_i.

Lack of Water Oxygen Incorporation into P_i in an Acid Stop. If a labile phosphorylated intermediate were formed in the "burst" reaction, and this intermediate were hydrolyzed upon perchloric acid addition, water oxygen might be incorporated into the P_i at the time of acid addition. This can be assessed with use of $H^{18}OH$ when total P_i liberation is small compared to the molarity of myosin present. Presence of a labile phosphoryl attached to N or S would require water oxygen incorporation. A labile phosphoryl attached to a carboxyl oxygen, however, might undergo C–O cleavage and give P_i without water oxygen incorporation. The results presented in Table II show that no detectable water oxygen appeared in P_i when perchloric acid was added at 0° after about 1 mole of ATP had been cleaved/mole of myosin.

Water Oxygen Incorporation with Other Stopping Agents and Near Neutral pH. As mentioned previously, a phosphorylated myosin carboxyl group might be cleaved at acid pH without water oxygen incorporation. Acetyl phosphate undergoes predominantly P–O cleavage at pH 2.7–7.5, but C–O cleavage in strong acid or alkali (Park and Koshland, 1958; Di Sabato and Jencks, 1961). For this reason, measurement of possible ^{18}O incorporation without strong acid or alkali present was desirable. For this purpose acetone, EDTA, and phenol were evaluated as stopping agents.

The results in Table V show that with addition of acetone together with $H^{18}OH$, followed 5 sec later by perchloric acid, incorporation of more than one oxygen per P_i formed was observed. This clearly cannot result only from hydrolysis of an unknown phosphoryl derivative as this could give incorporation of only one oxygen from water into P_i. The exchange reaction thus must continue at a rapid rate even though acetone is added. With the inclusion of EDTA to complex Mg^{2+} (expt 2, Table V) the extra exchange is diminished.

TABLE V: ^{18}O Incorporation from Water into P_i with Acetone, EDTA, or Phenol as Stopping Agents.[a]

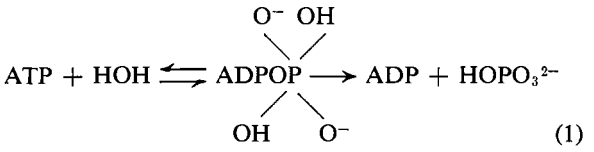
Expt	Condn for Stopping ATP Hydrolysis	Moles of P_i Released/ Mole of Myosin	No. of Water Oxygens in P_i
1	Acetone–$H^{18}OH$ (30 sec) Perchloric acid (35 sec)	0.97	1.7
2	EDTA and acetone–$H^{18}OH$ (30 sec)	1.07	1.3
3	EDTA (30 sec) acetone–$H^{18}OH$ (35 sec)	1.05	0.2
4	Phenol–$H^{18}OH$, pH 7 (30 sec)	1.30	0.16
5	Phenol–$H^{18}OH$, pH 1 (30 sec)	1.12	0.02

[a] Conditions were as given with Figure 1 except myosin was present at 20 mg/ml. The total incubation volume was 25 ml. ATP hydrolysis was stopped by additions as indicated in the following amounts; 50 ml of acetone and 25 ml of $H^{18}OH$ (1.5 atom % excess ^{18}O), 0.3 M final concentration of perchloric acid, 50 mM final concentration of EDTA, or 25 ml of liquified phenol and 25 ml of $H^{18}OH$ at the pH indicated.

Experiment 3 of Table V is of particular importance as it shows that exposure to EDTA for 5 sec before addition of acetone–$H^{18}OH$ nearly abolishes ^{18}O incorporation into the P_i. Thus if any acyl phosphate were present it must have been hydrolyzed in the 5

sec at neutral pH and 0° prior to the acetone–$H^{18}OH$ addition. This seems unlikely. Experiment 4 of Table V is also important as it demonstrates lack of any appreciable ^{18}O incorporation from $H^{18}OH$ when phenol is used to liberate the P_i near neutral pH. Such results clearly do not support the presence of any acyl phosphate

Possible Oxygen Incorporation into ADP or ATP during ATP Cleavage. One possible explanation for the extra oxygen incorporation into P_i released by myosin ATPase would be the reversible formation of a pentavalent intermediate from ATP, as indicated by eq 1. This would be analagous to the intermediate formed in hydrolysis of carboxyl esters, which gives



(1)

water oxygen incorporation into the unhydrolyzed ester (Bender, 1951). An experiment was thus performed to find if solvent oxygen might be incorporated into unhydrolyzed ATP. Results as shown in Table III demonstrate that no detectable water oxygen appears in the unhydrolyzed ATP. Earlier data had indicated that a small incorporation of water oxygen into the unhydrolyzed ATP might occur (Levy and Koshland, 1959).

Other modes of ATP cleavage can be formulated in which ADP would gain some solvent oxygen even though most of the oxygen would appear in the P_i. Such a result would be obtained, for example, if ATP were cleaved with P_i liberation accompanied by formation of a covalent myosin-ADP and with subsequent hydrolysis of this derivative by water to give myosin and ADP. If the ADP formed dissociated readily from the myosin, ^{18}O incorporation only into the ADP would be observed. If, however, the ADP remained bound to the myosin (as in the Michaelis complex), was rephosphorylated by a second ATP molecule, and the cycle of P_i formation repeated, oxygen from water would appear in the P_i formed. The route of water oxygen to the P_i would be indirect, that is, through the poorly dissociable ADP. Only to the extent that the ADP dissociated from the enzyme would oxygen incorporation into the ADP be observed. It thus seemed worthwhile to check carefully the ADP formed for possible oxygen incorporation. The results in Table III show no detectable incorporation of water oxygen into the ADP.

Myosin SH Groups and the Oxygen Exchange Reactions. To test the relationship of SH groups to the extra oxygen exchange, measurements were made of ^{18}O incorporation with increasing molarity of added *p*-mercuribenzoate. Increase in *p*-mercuribenzoate concentration is known to first accelerate then inhibit ATP cleavage by myosin (Kielley and Bradley, 1956; Kielley, 1961). Results presented in Table VI show that little

TABLE VI: *p*-Mercuribenzoate Effect on ATP Cleavage and Oxygen Exchange by Myosin at 25°.[a]

p-Mercuribenzoate Added (μM)	P_i Formed (mM)	Water Oxygen/ P_i
0	1.12	1.87
8	1.40	1.83
19	1.70	1.77
38	0.78	1.06
57	0.38	1.11

[a] Conditions were similar to those used for experiments reported in Table III.

change occurs in the amount of extra oxygen incorporated into each P_i liberated when the liberation of P_i is considerably enhanced by addition of the mercurial. With further increase in *p*-mercuribenzoate concentration, however, a pronounced inhibition of the extra oxygen incorporation occurs. Experiments not reported have shown that addition of ATP prior to the *p*-mercuribenzoate gave little or no protective effect.

In four other experiments, similar to those reported in Table VI but with extra oxygen incorporation ranging from 1.4 to 2.1/mole of P_i formed, a similar pronounced inhibition of the extra oxygen exchange was observed with 50–90 mM *p*-mercuribenzoate. Also, as shown in Table VII, the inhibitor effect of the

TABLE VII: *p*-Mercurbenzoate Inhibition of Oxygen Exchange by Myosin ATPase at 0°.[a]

p-Mercuribenzoate Added (μM)	P_i Formed (mM)	Water Oxygen/ P_i
0	0.52	1.91
20	0.53	1.48
39	0.43	1.20

[a] Experimental conditions were as given with Table III, except incubation was for 5 hr at 0°.

mercurial was evident at 0°. At this temperature, *p*-mercuribenzoate does not activate myosin ATPase (Gilmour and Griffiths, 1957).

Relation of 2,4-Dinitrophenol to the Exchange Reaction. Both *p*-mercuribenzoate (Kielley and Bradley, 1965) and 2,4-dinitrophenol (Greville and Needham, 1955; Chappell and Perry, 1955) accelerate myosin ATPase (Kielley, 1961). Similarly, both reagents are

known to uncouple oxidative phosphorylation by mitochondria and decrease the prominent $P_i \rightleftarrows HOH$ exchange catalyzed by mitochondria. Thus comparison of the effects of 2,4-dinitrophenol and *p*-mercuribenzoate on the myosin-exchange reaction was of interest. Koshland and Levy (1964) have previously reported that 2,4-dinitrophenol did not have any pronounced influence on the extra oxygen incorporation. Data in Table VIII confirm the lack of any pronounced effect.

TABLE VIII: Increased Oxygen Exchange during Hydrolysis of ATP by Myosin in the Presence of 2,4-Dinitrophenol.[a]

2,4-Dinitrophenol Added (mM)	P_i Formed (mM)	Water Oxygen/P_i
0	2.13	2.24
0.9	2.29	2.36
1.5	2.58	2.50
4.6	3.23	2.62
9.1	3.90	2.82

[a] Experimental conditions were as given with Table III.

They do show, however, the interesting point that 2,4-dinitrophenol addition actually slightly accelerates the exchange. In three other experiments, addition of 9 mM 2,4-dinitrophenol gave an average increase of 23% in the water oxygen incorporation into P_i. The oxygen exchange in the presence of 2,4-dinitrophenol is readily inhibited by *p*-mercuribenzoate, as shown by the data of Table IX.

Lack of Effect of Hydroxylamine and of p-Nitrothiophenol on the Oxygen Exchange. Carboxyl groups could conceivably participate in the exchange reactions and ATP cleavage of myosin by transitory acyl-S formation (Boyer, 1965). As one means of assessing such a possibility, effects of hydroxylamine on the exchange were measured. Little or no effect of 0.01 M hydroxylamine on either the exchange or on the extent of hydrolysis was observed.

The ability of *p*-nitrothiophenol to form an acyl-S derivative with myosin in the presence of ATP (Kitagawa *et al.*, 1964) also suggests possible participation of a carboxyl group in myosin action. In hydrolysis of ATP by the *p*-nitrothiophenol derivative of myosin, prepared as described by Kitagawa *et al.* (1964), only a slight decrease in the exchange reaction was noted. With the *p*-nitrothiophenol derivative an incorporation of 2.5 water oxygens into each P_i formed was observed as compared to 2.8 by the untreated myosin control. In addition, the exchange reaction continued unabated during hydrolysis of ATP in the presence of 0.01 M *p*-nitrothiophenol.

TABLE IX: Ability of a *p*-Mercuribenzoate to Abolish the Extra Oxygen Exchange in the Presence of 2,4-Dinitrophenol.[a]

Additions			
2,4-Dinitrophenol (mM)	*p*-Mercuribenzoate (μM)	P_i Formed (mM)	Water Oxygen/P_i
0	0	1.30	1.54
8.6	0	2.52	1.92
8.6	18	2.38	1.68
8.6	53	0.22	1.0

[a] Experimental conditions were as given with Table III.

Discussion

The formation of a phosphoryl myosin represents one logical hypothesis for explanation of the rapid initial cleavage of 1 mole of ATP/mole of myosin (Tonomura and Kanazawa, 1965; Imamura *et al.*, 1965). The two approaches used herein, namely search for incorporation of water oxygen into the P_i liberated upon addition of $H^{18}OH$ and a stopping agent and the phenol extraction of possible phosphoryl protein, have not revealed the presence of a covalently linked phosphoryl group.

The phenol extraction procedure allows separation of myosin from P_i and ATP near neutral pH and under mild conditions. In addition, transfer of the protein to the phenol would be expected to eliminate secondary and tertiary protein structural features that might confer extreme lability on a phosphoryl group. Similar considerations hold for an earlier study, based on urea dispersal and an anion-exchange column for separation of protein from P_i and ATP that likewise showed no detectable phosphoryl protein during ATP hydrolysis by myosin (Dempsey *et al.*, 1963). The possibility remains, however, that an exceptionally labile phosphoryl group is hydrolyzed prior to or during the separation.

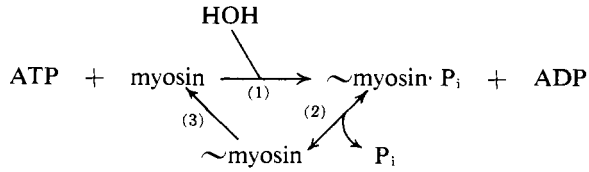
Hydrolysis of a phosphoryl derivative must be accompanied by an incorporation of water oxygen into a product of the cleavage. In the formation of P_i with hydrolysis of a P–N or a P–S linkage, water oxygen must appear in the P_i. Our results eliminate such a possibility for myosin. In cleavage of a C–O–P bond, as in a carboxyl phosphate, water oxygen will appear in the P_i or carboxyl group depending upon whether P–O or C–O cleavage occurs. As mentioned earlier, with acetyl phosphate, P–O cleavage predominates in the pH range of 3–8 but C–O cleavage occurs in more acidic or basic solutions (Park and Koshland, 1958; Di Sabato and Jencks, 1961). The lack of water

oxygen incorporation into P_i when myosin, just after a "burst" of ATP cleavage, is extracted with perchloric acid–$H^{18}OH$ thus does not eliminate the presence of a labile carboxyl phosphate. The absence of detectable incorporation with the EDTA or the phenol stop near neutral pH (Table V) makes the presence of a carboxyl phosphate considerably less likely. Again, however, the possibility remains that an unusual acyl phosphate might undergo C–O cleavage under these conditions. An answer could be obtained by measurement of any ^{18}O incorporation from water into carboxyl groups of myosin. Such studies may be undertaken in this laboratory in connection with development of more sensitive ^{18}O procedures.

Other possible forms of phosphoryl myosin, in which the phosphoryl is attached to an oxygen group, include a phosphorylated tyrosine, serine, threonine, or another phosphoryl group on the protein. Such structures seem unlikely both because they would be expected to be stable to the phenol extraction procedure and to show incorporation of water oxygen into the P_i formed if hydrolysis occurred upon extraction with perchloric acid–$H^{18}OH$. Alkyl OPO_3 compounds undergo P–O cleavage in acid solution (Cox and Ramsay, 1964).

The chemical events associated with the "burst" phenomena are likely to be closely correlated with the extra oxygen exchange that accompanies cleavage of ATP by myosin. The data reported in Table IV show that such extra exchange is found even with the first mole of P_i released per mole of myosin. Several possibilities for explanation of the extra oxygen incorporation into the P_i released may be considered. The reversible formation of a pentavalent intermediate from ATP (eq 1) is made unlikely by the lack of detectable incorporation of water oxygen into the unhydrolyzed ATP (Table III). Similarly, the possibility of a reversible cleavage of ATP appears unlikely because of the lack of detectable incorporation of $^{32}P_i$ as well as water oxygen into ATP (Dempsey *et al.*, 1963). That P_i can reversibly combine with the exchange site on the enzyme is indicated by the catalysis of a $P_i \rightleftarrows HOH$ exchange (Dempsey *et al.*, 1963; Swanson and Yount, 1965). The reversible formation of a phosphoryl myosin from ATP obviously cannot give water oxygen incorporation into P_i. One must further postulate reversible formation of a pentavalent intermediate in the hydrolysis of the phosphoryl myosin, or a reversible hydrolysis of the phosphoryl myosin by P–O cleavage with delayed liberation of the P_i formed to the reaction medium.

The relation between ATP cleavage and the extra oxygen is not obvious. Either a transient covalent intermediate formed from the terminal phosphoryl group must possess unusual properties to allow exchange with HOH or the cleavage of the ATP has modified the myosin active site so it is able to induce exchange of HOH with the noncovalently bound P_i formed prior to dissociation of the P_i to the medium. Some features of the latter possibility are indicated in the following scheme, where ~myosin indicates a modified form capable of inducing oxygen exchange between bound P_i and HOH.



Step 1 would be relatively rapid and account for the "burst" phenomenon. Upon protein denaturation P_i would be liberated and measured as such. Either step 2 or 3 or both would be slow steps in the catalysis. Step 2 is depicted as reversible to allow for the relatively slow exchange that occurs with medium P_i and favored by ATP cleavage. Step 3 gives the original form of myosin that is again capable of combining with and activating hydrolysis of ATP.

If phosphoryl myosin were cleaved with concomitant formation of an acyl-S linkage, a reversible hydrolysis of the acyl-S linkage as well as a reversible cleavage of the phosphoryl enzyme would be necessary for the observed ^{18}O exchange. A myosin carboxyl group may be induced by ATP to bind *p*-nitrothiophenol in an acyl-S linkage (Kitagawa *et al.*, 1964). The unabated ^{18}O exchange catalyzed by the *p*-nitrothiophenol derivative eliminates this carboxyl group as a participant in the exchange reaction.

An alternative that could explain both the "burst" phenomenon and the exchange reaction is the formation of monomeric metaphosphate at the active site, with subsequent hydration to form P_i. Under conditions favoring exchange, the P_i formed would be reversibly dehydrated to metaphosphate more readily than liberation of P_i to the medium. The occurrence of the $P_i \rightleftarrows HOH$ exchange would mean that medium P_i gained access to the active site and underwent reversible hydration. Cleavage of ATP would not be required for an exchange based on metaphosphate formation, in harmony with the data of Swanson and Young (1965). Their data on requirement of ADP for the $P_i \rightleftarrows HOH$ exchange could reflect need of ADP binding for proper configuration at the active site. If continued search fails to reveal a covalently linked phosphoryl group, such a metaphosphate hypothesis must be given serious consideration.

The data showing that *p*-mercuribenzoate inhibits the extra oxygen-exchange reaction could reflect an essential role for an SH group in the reaction. As with other effects of SH reagents on catalysis, it is difficult to distinguish between a direct role and an effect accompanying protein conformation change. The activation of myosin by *p*-mercuribenzoate as well as by 2,4-dinitrophenol has been regarded as likely reflecting a conformation change (see Sekine and Kielley, 1964; Rainford *et al.*, 1964; Gergely, 1966). The inhibition of the oxygen exchange by the mercurial appears to reflect reaction of the mercurial with SH groups other than those associated with the initial activation reaction, as the pronounced inhibition of exchange

requires *p*-mercuribenzoate concentration in excess of that for maximal activation of ATPase. Also, our data show that *p*-mercuribenzoate can nearly abolish the oxygen-exchange reaction even though ATPase continues. Thus SH groups that might participate in the exchange reaction are distinct from any required for the ATPase reaction. Heterogeneity in reactivity and various effects of blocking of SH groups of myosin have been shown in a number of investigations (Gergely, 1966). More information on the relations between or identity of SH groups involved in modification of the oxygen-exchange reaction, the ATPase reaction, and the binding to actin is desirable.

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