TABLE III
LENGTHS AND ANGLES FORMED BETWEEN 5-5′ AND 7-7′ AXES OF BIS(5- AND 7-SUBSTITUTED 8-QUINOLINOLATO)COPPER(II)

Compd	5-5′ axis[a] Substituent	5-5′ axis[a] Length, Å	7-7′ axis Substituent	7-7′ axis Length, Å	Angle formed between axes, deg
	H	14.2	H	11.5	59
IVa	H	14.2	NO_2	14.9	70
	F	15.0	H	11.5	60
IVb	F	15.0	NO_2	14.9	71
	Cl	16.6	H	11.5	62
IVc	Cl	16.6	NO_2	14.9	73
	Br	17.4	H	11.5	63
IVd	Br	17.4	NO_2	14.9	74
	I	18.0	H	11.5	64
IVe	I	18.0	NO_2	14.9	75
	NO_2	18.1	H	11.5	64
	NO_2	18.1	NO_2	14.9	75

[a] Revision of data reported in ref 2.

filtration, washed free of acid with deionized H_2O, and dried at 70° overnight. The yield of compound was 6.9 g (66%), mp 215–218° dec.

Bis(5-fluoro-7-nitro-8-quinolinolato)copper(II) (IVb).—A solution of 12.7 g (0.061 mole) of 5-fluoro-7-nitro-8-quinolinol in 300 ml of DMF was mixed with a solution of 7.8 g (0.77 mole) of copper(II) acetate monohydrates in 600 ml of H_2O and the mixture was stirred for 1 hr. The product was removed by filtration, washed (H_2O, Me_2CO), and dried at 70° overnight. The complex was obtained in 87% yield, mp >500°.

7-Amino-5-fluoro-8-quinolinol Hydrochloride (IIIb).—A suspension of 20.8 g (0.1 mole) of 5-fluoro-7-nitro-8-quinolinol and 150 mg of PtO_2 in 100 ml of DMF was shaken under 5 atm of H_2. After 0.3 mole of H_2 had been taken up the catalyst was removed by filtration and the filtrate was acidified with 20 ml of concentrated HCl and diluted with 5 vol of acetone. After cooling in a freezing compartment overnight, the product was filtered off and washed (Me_2CO) until the wash liquid was nearly colorless. The compound which was dried at 70° overnight was obtained in 66% yield, mp >500°.

7-Nitro-8-quinolinol (IIa).—A suspension of 5.0 g (0.016 mole) of 5-iodo-7-nitro-8-quinolinol in 125 ml of AcOH was heated under reflux with agitation for 36 hr. Insoluble material was removed by filtration, and I_2 was reduced with aqueous $NaHSO_3$. The solvent was concentrated to a small volume by flash evaporation, and the residue was diluted with H_2O. After adjusting with 10% NaOH to pH 5, the product was obtained by filtration, washed (H_2O), and dried at 70° overnight. The yield of compound was 1.7 g (60%), mp 150°.

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Methyl Triphosphate, a Substrate for Myosin Adenosine Triphosphatase[1]

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The investigation of the molecular mechanism of the myosin–actin–adenosine triphosphate (ATP) interaction, basic to muscle contraction,[2] may require as a first step the elucidation of the mechanism of the adenosine triphosphatase (ATPase) activity of myosin. An approach to this problem has been to study the interaction with and the hydrolysis by myosin of a number of compounds structurally related to ATP in an attempt to establish some relationship between the structure of the substrate and the strength of its binding as well as the velocity of its hydrolysis by myosin.[3] Blum in particular,[3a] after studying various nucleoside triphosphates, has proposed that the purine ring and, more precisely, the 6-NH_2 group play an important role in giving to ATP specific properties as a substrate for myosin. But myosin can also hydrolyze simple triphosphates, such as ribose triphosphate[4a] or even inorganic tripolyphosphate,[4] and there has been some interest in these simple substrates as a means of evaluating theories such as that of Blum.

Some time ago we synthesized monomethyl tripolyphosphate (MTP) by the condensation of methyl phosphate and inorganic phosphate in the presence of dicyclohexylcarbodiimide according to the method of Smith and Khorana.[5] At the same time Brintzinger and coworkers[6] briefly reported that this compound could also be obtained by methylation of inorganic tripolyphosphate. This simple ATP analog has the same number of acidic groups as ATP. This is not the case for inorganic tripolyphosphate whose study, furthermore, is complicated by its property of giving insoluble Ca^{2+} salts.[4] Since then MTP has attracted the interest of workers in the myosin field.[7]

Experimental Section

Synthesis of MTP.—Monomethyl phosphate[8] (disodium salt tetrahydrate, 752 mg, 3.3 mmoles) was dissolved in 70 ml of water and converted to the free acid by passage of the solution through a column of Dowex 50, H^+ (50W-X4, 20–50 mesh; 55 ml). The acidic eluate was taken to dryness at 40° in a flash evaporator and the residue was further dried overnight over P_2O_5 in a vacuum desiccator. To 40 ml of anhydrous pyridine in a 100-ml volumetric flask was added 4.82 g (42 mmoles) of 85% H_3PO_4 and 20.9 ml of n-Bu_3N and the volume was made up to 100 ml with pyridine. The methyl phosphate was dissolved in 80 ml of this solution and 34 g (165 mmoles) of dicyclohexylcarbodiimide was added. The stoppered reaction mixture was occasionally stirred during the first hour, then left at room temperature for 48 hr. The precipitate, which had begun to appear after 30 min, was filtered off and washed with 250 ml of H_2O. The combined filtrates were extracted four times with 80 ml of ether. The ether extracts were washed with H_2O (40 ml). The combined aqueous solutions were concentrated at 26° in a flash evaporator under high vacuum to a volume of about 25 ml, the condensing flask being cooled in i-PrOH–Dry Ice. To the residue was added H_2O (675 ml) and the solution was brought to pH 8.4 by the addition of 5 N NaOH. The volume was made up to 750 ml with H_2O.

After filtration, the solution was fractionated on a 4.4 × 15.5

(1) This work was supported by a Dernham Fellowship (D-105) of the American Cancer Society, California Division.

(2) H. E. Huxley in "Muscle," W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, Ed., Pergamon Press Ltd., Oxford, 1965, p 3.

(3) (a) J. J. Blum, *Arch. Biochem. Biophys.*, **55**, 486 (1955); (b) Y. Tonomura, K. Imamura, M. Ikehara, H. Uno, and F. Harada, *J. Biochem.* (Tokyo), **61**, 460 (1967).

(4) (a) N. Azuma, M. Ikehara, E. Ohtsuka, and Y. Tonomura, *Biochim. Biophys. Acta*, **60**, 104 (1962); (b) E. T. Friess and M. F. Morales, *Arch. Biochem. Biophys.*, **56**, 326 (1955).

(5) M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 1141 (1958).

(6) P. W. Schneider, H. Brintzinger, and H. Erlenmeyer, *Helv. Chim. Acta*, **47**, 992 (1964).

(7) (a) J. R. Swanson and R. G. Yount, *Biochem. Z.*, **345**, 395 (1966); (b) K. Imamura, J. Duke, and M. F. Morales, *Federation Proc.*, **27**, 519 (1968).

(8) Available from K and K Laboratories, Plainview, N. Y.

cm column of Dowex 2 resin (X8, 200–400 mesh, Cl^-) at 4°. The entire sample was passed, at 15 ml/min, through the column, which was then washed with H_2O (1 l.) followed by 1 l. of 0.003 *N* HCl. The fractionation of the products was achieved by elution, at the same rate, with successive 1-l. portions of 0.003 *N* HCl containing increasing concentrations of LiCl, the first portion being 0.0055 *M* in LiCl, the second 0.011 *M*, the third 0.0165 *M*, etc. One-liter fractions were collected and stored in the cold room. Total and acid-labile (10 min at 100° in 1 *N* H_2SO_4) P were determined on 0.5 ml of each fraction by the procedure of Bartlett.[9] The acid-stable P (total minus acid labile) was found in three well-separated peaks. Peak I (1.8 mg of acid-stable phosphorus) appeared in fraction 4, eluted with 0.022 *M* LiCl. It was probably a mixture of methyl phosphate and inorganic phosphate. Peak II (fractions 13–15) contained 23.7 mg of acid-stable P but very little acid-labile P. It is believed to consist primarily of P^1,P^2-dimethyl pyrophosphate. Peak III (fractions 27–31) contained approximately 60 mg of acid-stable P (59% of the starting methyl phosphate). All fractions in this peak had a ratio of total P/acid-labile P of close to 1.5. Inorganic polyphosphates were eluted shortly after this peak III.

Isolation of the Lithium Salt of MTP.—The fractions of peak III were pooled and brought to pH 7.3 with 1 *N* LiOH. The solution was concentrated at 40° in a flash evaporator to about 50 ml, then 64 ml of MeOH was added. The solution was filtered through sintered glass and the flask and the filter were rinsed with MeOH (30 ml). To the filtrate was added acetone (1880 ml). The precipitate, which formed immediately, was collected, after 48 hr in the cold, by decantation and centrifugation and was washed six times with MeOH–Me_2CO (1:20) and three times with dry ether. The product was dried overnight under vacuum at room temperature over P_2O_5 and weighed 644 mg. *Anal.* Calcd for $CH_3Li_4O_{10}P_3 \cdot H_2O$: P, 29.6. Found: P, 29.1. The ratio of total/acid-labile P was 1.48. Less than 1% of the P was present as inorganic phosphate. No Cl^- was detected. Titration between pH 8.5 and 4 gave one acid group per three P atoms.

Hydrolysis of MTP by Myosin.—A sample of myosin from rabbit skeletal muscle was kindly provided by Dr. G. Richards of the Department of Nutrition, University of California, Berkeley. It had been prepared by the method of Tonomura and coworkers[10] and preserved at −18° in 50% glycerol. The protein concentration was determined by Lowry procedure[11] with standardization by Kjeldahl. Possible changes in the color yield of the Lowry procedure were checked using albumin as a standard. A solution in 0.5 *M* KCl was obtained after precipitation of the myosin by diluting with water (10:1), centrifugation, and redissolution in and dialysis against 0.5 *M* KCl (pH 8.1 with 1 m*M* Tris-chloride buffer). Glass-distilled water was used in all procedures. The myosin solution so obtained (200 μg/ml) was kept in a plastic (cellulose nitrate) tube and in ice.

Conditions for the MTPase and ATPase assays were as follows: 0.5 *M* KCl, 5 m*M* $CaCl_2$, 0.5 m*M* substrate, pH 8.2 (Tris-chloride buffer 0.02 *M*), temperature 25°. The substrates were used as K^+ salts, obtained by passing their solutions through a column of Dowex 50, K^+. The enzymic reaction was started by adding, with magnetic stirring, 0.5 ml of the 3 m*M* substrate to 2.5 ml of a solution containing the other components, including myosin (100 μg). The reaction was stopped by adding 1 ml of a 20% trichloroacetic acid solution. After filtration, Pi was determined in 3 ml of the filtrate by the phosphomolybdate extraction method of Dreisbach,[12] 4 ml of the xylene–*i*-BuOH solvent being used for the extraction.

The enzymic activity of the myosin, obtained from the amount of Pi released after a given time and expressed as micromoles of Pi per minute per gram, was found to be 390 with ATP and 48 with MTP. Azuma and coworkers[4a] have reported that ribose triphosphate, another simple ATP analog, is hydrolyzed, also in the presence of Ca^{2+}, at one-twentieth the rate of ATP. However, we noted that the loss of activity during storage in ice was not parallel for the two substrates, the ATPase being better preserved than the MTPase. After 35 days the ATPase

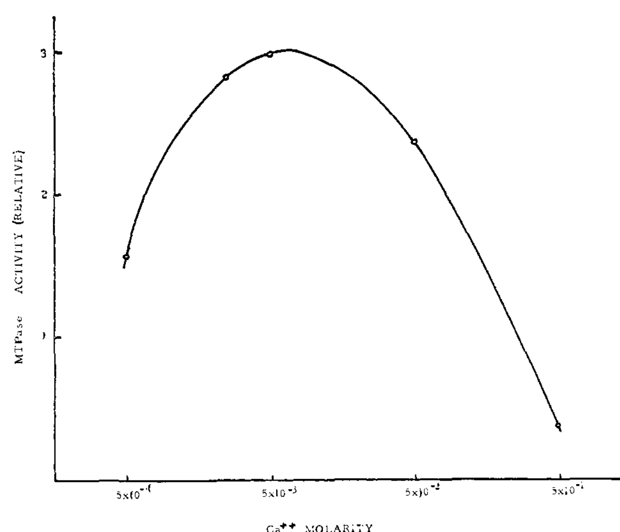

Figure 1.—Ca^{2+} dependence of myosin methy triphosphatase Experimental conditions: 0.5 *M* KCl, 0.5 m*M* MTP, pH 8.2, 25°

activity was still at 70% of the original value while the MTPase had declined to 27%. The loss of MTPase activity with time appears to be a first-order reaction with a half-life period of about 19 days in our conditions of storage of the myosin.

The dependence of MTPase activity on Ca^{2+} concentration is shown in Figure 1. Maximum activity was obtained when Ca^{2+} was about 5 m*M*. No activity was observed in the absence of Ca^{2+}. Green and Mommaerts,[13] working in somewhat different conditions (0.15 *M* KCl, pH 9.0), have reported an optimal Ca^{2+} concentration of approximately 1 m*M* in the case of ATP.

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(9) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).
(10) (a) Y. Tonomura, P. Appel, and M. Morales, *Biochemistry*, **5**, 515 (1966); (b) E. G. Richards, C.-S. Chung, D. B. Menzel, and H. S. Olcott, *ibid.*, **6**, 528 (1967).
(11) R. J. Henry, "Clinical Chemistry, Principles and Technics," Hoeber Medical Division of Harper and Row, New York, N. Y., 1964, p 190.
(12) R. H. Dreisbach, *Anal. Biochem.*, **10**, 169 (1965).
(13) I. Green and W. F. H. M. Mommaerts, *J. Biol. Chem.*, **210**, 695 (1954).

2-Hydroxy-3-naphthoic Acid Anilide Phosphate as a Fluorescent Histochemical Substrate for Phosphatase[1]

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The development of fluorogenic substrates for histochemistry of normal and cancer cells is of importance because of the inherent sensitivity of fluorescence measurement over that of the absorbance measurement. Several years ago, Rutenberg, *et al.*, reported[2] the use of 2-hydroxy-3-naphthoic acid anilide phosphate for histochemical demonstration of phosphatase. Because of the presence of a small amount of naphthol-AS, a simultaneous coupling procedure was recommended and fluorescent study was not possible. A more detailed study was therefore undertaken in order

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(2) A. M. Rutenberg, R. J. Barrnett, K. C. Tsou, B. Monis, and R. Teague, *J. Histochem. Cytochem.*, **6**, 90 (1958).