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γ-Fluoroadenosine Triphosphate. Synthesis, Properties, and Interaction with Myosin and Heavy Meromyosin†

Boyd Haley and Ralph G. Yount*

ABSTRACT: The adenosine triphosphate analog, γ-fluoroadenosine 5′-triphosphate (FATP), has been synthesized by the reaction of P^1-diphenyl P^2-fluoropyrophosphate with adenosine diphosphate and isolated as the trisodium salt in 30% yields. FATP is stable (<4% loss) in solutions, pH 7.4, 25°, for at least 2 days and as the sodium salt at −20° for several months. FATP binds Ca^{2+}, Mg^{2+}, and Mn^{2+} with affinity constants of 242, 200, and 505, respectively, at pH 8.2, 0.1 M NaCl while comparable constants measured for ATP were 5150, 8600, and 46,000. FATP was not a substrate for myosin, heavy meromyosin, hexokinase, or *Escherchia coli* alkaline phosphatase. It was about 0.4% as effective at ATP in the pyrophosphate-exchange assay of the mixed aminoacyl-tRNA synthetases from *Bacillus brevis*. Snake venom phosphodiesterase cleaved FATP to AMP and fluoropyrophosphate; this latter product established that the fluorophosphate was on the β-phosphate of adenosine diphosphate. FATP would not support the contraction of glycerol-extracted muscle fibers or prevent ATP-induced contraction. It did not irreversibly inhibit heavy meromyosin or myosin under a variety of conditions. FATP does competitively inhibit heavy meromyosin catalyzed ATP cleavage with $K_{iMg^{2+}} = 4.3 \times 10^{-4}$ M and $K_{iCa^{2+}} = 3.4 \times 10^{-3}$ M. FATP protects myosin against heat inactivation almost as well (50%) as ATP in the presence of Mg^{2+} but has no effect when Ca^{2+} is used. The stability and properties of FATP indicate it should be useful as an analog to minic $HATP^{3-}$. The fluorine nuclear magnetic resonance of FATP should be a useful probe of the binding sites of ATP-requiring enzymes.

Recently several new analogs of nucleotides with modified phosphate side chains have been synthesized (Hampton and Chu, 1970; Cook, 1970). Analogs of ATP are especially interesting in view of the central role ATP plays both as an energy source and as a metabolic control agent (Atkinson, 1966). Examples of phosphate-modified ATP analogs include those with bridge oxygens replaced with CH_2 groups

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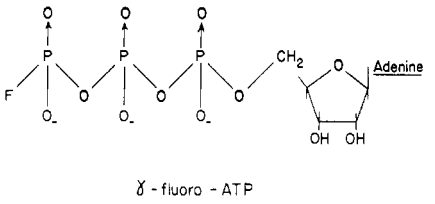


FIGURE 1: γ-Fluoroadenosine triphosphate.

(Myers *et al.*, 1963; Trowbridge and Kenyon, 1970) or with an NH group (Yount *et al.*, 1971a). Others include the γ-methyl ester of ATP (Wehrli *et al.*, 1964), an analog in which sulfate replaces the terminal phosphate of ATP (Yount *et al.*, 1966a), an analog in which the terminal bridge oxygen is absent (Remy *et al.*, 1967, 1970), and analogs in which a sulfur replaces a nonbridge oxygen on the α-phosphate (Eckstein and Gindl, 1967, 1970) and on the γ-phosphate of ATP (Goody and Eckstein, 1971).

Our primary interest is in the active-site properties of myosin and other contractile proteins. Since myosin appears to form no stable covalent intermediates during its catalytic cycle, ATP analogs with reactive groups which could covalently label the active site of contractile proteins would be of considerable interest and utility. Murphy and Morales (1970) have recently covalently labeled myosin using a purine-modified analog of ATP in which a thiol replaces the 6-amino group. These important studies indicate that a cysteine residue is near the binding site for the purine portion of ATP. Our aim has been to modify the phosphate chain of ATP since information about the catalytic amino acid residues would likely come from reactions with ATP analogs of this type.

As a first step we have synthesized γ-fluoroadenosine 5-triphosphate[1] (Figure 1) and tested it as potential substrate and/or inhibitor of myosin, heavy meromyosin, and muscle fiber ATPase. We have also tested it as a substrate with hexokinase, alkaline phosphatase (*Escherichia coli*), aminoacyl-tRNA synthetases, and snake venom phosphodiesterase. This paper describes the synthesis, pH stability, and metal-binding properties of FATP and details the nature of its interaction with the above enzymes. The potential usefulness of the fluorine atom of FATP as a nuclear magnetic resonance (nmr) probe of ATP-binding sites on enzymes is discussed.[2]

Experimental Section

Materials. Diphenyl chlorophosphate, triethylamine, and tributylamine (Aldrich Chemical Co.) were redistilled before using. Pyridine was refluxed over NaOH and distilled through a short column. Both pyridine and dimethylformamide were stored over Linde (Tonawanda, N. Y.) Type 4A 1/16-in. pelleted molecular sieves for 10 days before using. Sodium monofluorophosphate of high purity was a generous gift from Dr. Wayne E. White of the Ozark-Mahoning Co. ADP·Li_2·$5H_2O$ was from Schwarz BioResearch and [^{32}P]sodium pyrophosphate and phosphoric acid were from New England Nuclear Corp. Triethylammonium bicarbonate solutions were prepared as previously described (Yount *et al.*, 1971a). Alkaline phosphatase (*E. coli*) and hexokinase (yeast, Type C-300, 300 units/mg) were crystalline suspensions in ammonium sulfate from the Sigma Chemical Co. Snake venom (*Crotalus atrox*) containing phosphodiesterase and 5′-nucleotidase activity were from Calbiochem. Purified phosphodiesterase I (venom) was from Worthington Biochemical Corp.

Enzyme Preparations and Assays. Myosin was prepared from rabbit skeletal muscle by the method of Kessler and Spicer (1952) except actomyosin contamination was removed by two separate centrifugations of 0.3 M KCl solutions at 14,000*g* instead of by an ATP-induced superprecipitation. Heavy meromyosin was prepared as described by Yount and Koshland (1963) except the myosin hydrolysis by trypsin was at pH 8.2 in 0.04 M $KHCO_3$. Glycerinated muscle fibers were from rabbit psoas muscle (Szent-Györgyi, 1951).

The activity of myosin was determined on serial samples by measuring the release of inorganic phosphate from ATP (Yount and Koshland, 1963) using Fiske–Subbarow reagents (1925). The activity of alkaline phosphatase, hexokinase, phosphodiesterase, and heavy meromyosin was followed by paper chromatography and paper electrophoresis. Where appropriate, the ultraviolet-absorbing compounds were eluted from the paper with 0.1 N HCl and quantified spectrophotometrically at 260 nm.

The identity of the phosphate products of snake venom phosphodiesterase action on FATP was determined after chromatography in solvent C using an ammonium molybdate spray for detection (Kolloff, 1961). The conversion of glucose to glucose 6-phosphate in the hexokinase reaction was followed by chromatography in solvent A using a periodate–benzidine spray for detection of glucose-containing compounds (Gordon *et al.*, 1956).

The aminoacyl-tRNA synthetases were prepared from *Bacillus brevis* (ATCC 10068). The cells were grown at room temperature, harvested in log-phase growth, sonicated, centrifuged at 104,000*g*, and the supernatant was collected and fractionated with ammonium sulfate. The material that precipitated between 25 and 75% saturation was collected, dissolved in 0.02 M Tris-Cl (pH 7.4), and dialyzed at 0° against three changes of 2 l. of the buffer. The dialyzed solution was centrifuged at 104,000*g* and the supernatant was used as the aminoacyl-tRNA synthetase preparation. [^{32}P]Pyrophosphate–ATP exchange was measured by a modification of Berg's (1956) method

The K_i values for FATP with heavy meromyosin were determined using [γ-^{32}P]ATP and the enzyme assay described by Yount *et al.* (1971b). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (1964).

Analytical Methods. Ultraviolet spectra were recorded with a Cary Model 15. Fluorine nmr spectra were obtained using a Varian DP 60 spectrometer at 56.4 MHz. Elemental analyses were by A. Bernhardt, Mulheim, W. Germany. Acid-labile phosphate (1 N HCl, 100°, 15 min) and total phosphate (Ames and Dubin, 1960) were determined using Fiske–Subbarow (1925) reagents. When triethylamine was present, the sample solutions were first evaporated to dryness since phosphomolybdate precipitates with trialkylamines.

[1] Abbreviations used are: FATP, γ-fluoroadenosine 5′-triphosphate; FPP_i, inorganic fluoropyrophosphate; FP_i, inorganic fluorophosphate; ADP-sulfate, adenosine 5′-sulfatopyrophosphate.

[2] An additional use of FATP results from its similarity in size and net charge to $HATP^{3-}$. This ionic form of ATP has been implicated in the inhibition of fumarase (Penner and Cohen, 1969), brain phosphofructokinase (Lowry and Passonneau, 1966), and creatine kinase (Noda *et al.*, 1960). To test this hypothesis, studies of ATP and FATP inhibition of fumarase (*Neurospora crassa*) have been performed (B. Haley and R. Yount, 1971, manuscript in preparation). Experiments show that the effect of pH on the inhibition is the result of changes in the ionization state of the enzyme rather than conversion of ATP^{4-} to $HATP^{3-}$ since FATP inhibition parallels ATP inhibition over the critical pH range of 6.5–7.5, where ATP is changing its ionic form but FATP is not.

TABLE I: Relative Mobilities of Adenosine-Containing Compounds.[a]

Nucleotide	R_F Solvent A	R_F Solvent B	Electrophoresis, Nucleotide: ATP Ratio
ATP	0.25	0.38	1.00
ADP	0.36	0.42	0.85
AMP	0.48	0.48	0.58
FATP	0.26	0.58	1.08

[a] Conditions were as follows. Ascending chromatography: approximately 25-cm solvent movement on Whatman No. 3MM paper at room temperature; solvent A: isobutyric acid–concentrated NH_4OH–H_2O (66:1:33), pH 3.7; solvent B: 1-propanol–NH_4OH–H_2O (6:3:1), pH 11.0. Electrophoresis was performed as described in the text.

Chromatography and Electrophoresis. Paper chromatography was done on Whatman No. 3MM and 31ET paper by both ascending and descending methods. Representative R_F values for adenine nucleotides are given in Table I for solvents A and B. Solvent C was isopropyl alcohol, 90% formic acid, and concentrated NH_4OH (90:80:1). Paper electrophoresis (Table I) was done on a water-cooled, Mylar-covered copper plate (30 × 45 cm) using Whatman No. 3MM paper and 0.05 M sodium citrate (pH 5.8) at 50 V/cm for 90 min.

Adenine-containing compounds were detected by their absorption of ultraviolet light using a Chromato-Vue cabinet and a short-wavelength transilluminator from Ultra-Violet Products, Inc. This allowed detection of less than 2×10^{-3} μmole/cm² of adenine-containing compounds.

Results

Preparation of FATP. Na_2PO_3F (1.084 g, 7.5 mmoles) was dissolved in deionized H_2O and converted to the tributylammonium form by passing the solution through a column (2.5 × 20 cm) of Dowex (50-X8, H^+, 50–100 mesh) at room temperature, and collecting the effluent, with stirring, in a 500-ml round-bottom flask containing 2.4 ml (10 mmoles) of tributylamine. The solution was immediately evaporated to dryness at 30°, using a rotary evaporator with a mechanical vacuum pump and a Dry Ice–isopropyl alcohol cooled trap. The residue was further dried by evaporation with three 5-ml portions of dimethylformamide. Dimethylformamide (1 ml), diphenyl chlorophosphate (1.6 ml, 7.7 mmoles), and tributylamine (2.4 ml, 10 mmoles) were added with stirring and allowed to react at room temperature (25°) for 2–3 hr. The reaction mixture was evaporated to dryness as before and the residue containing diphenyl fluoropyrophosphate was saved for the coupling reaction.

$ADP \cdot Li_3 \cdot 5H_2O$ (1.009 g, 1.37 mmoles) was dissolved in deionized H_2O and converted to the tributylammonium form by passing the solution through a column (2.5 × 20 cm) of Dowex (50-X8, H^+, 50–100 mesh) at room temperature, and collecting the effluent in a 1-l. round-bottom flask containing 4 ml (16.7 mmoles) of tributylamine. The solution was evaporated to dryness, as described above, leaving a transparent gum. This residue was further dried by evaporation with three 10-ml portions of absolute ethanol. The residue containing the tributylammonium salt of ADP was transferred with three 2-ml portions of dimethylformamide to the diphenyl fluoropyrophosphate previously prepared. Pyridine, the coupling reaction catalyst (Michelson, 1964), was added dropwise to the mixture after each addition of dimethylformamide solution until a precipitate started to form. Approximately 3 ml of pyridine was added.[3] The reaction was allowed to proceed for 30 min at 25°, the solvents were removed as before, and the residue was washed three times with 20-ml portions of ice-cold diethyl ether. The residue was transferred to a separatory funnel with three 20-ml portions of an ether–H_2O mixture (1:1, v/v). The aqueous phase was removed and evaporated almost to dryness. The residue was transferred with three 5-ml volumes of H_2O to a Sephadex K-50–100 column (5.0 × 90 cm bed volume) containing DEAE-cellulose (HCO_3^- form) and eluted with a linear gradient of 3 l. of H_2O and 3 l. of 1.0 M triethylammonium–HCO_3 (pH 7.5).

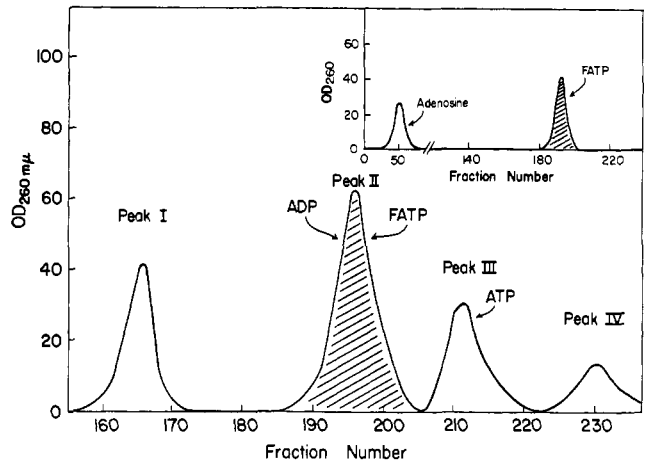


FIGURE 2: Chromatographic purification of FATP. Conditions: 18°; flow rate 4 ml/min; 20-ml fractions collected. The inset shows the rechromatography of FATP and ADP (peak II) after treatment with *E. coli* alkaline phosphatase. Peak I is a mixture of AMP and adenylylfluorophosphate. Peak III is ATP plus a second unknown adenine compound. Peak IV is a compound containing two AMP molecules linked *via* three acid-labile phosphates (adenine:P total = 1:2.5; adenine:acid-labile P_i = 1:1.5).

Three major and one minor peak(s) were detected (Figure 2). The fractions corresponding to FATP plus ADP peak (peak II) were pooled and evaporated to dryness. Residual triethylammonium bicarbonate was removed by three evaporations to dryness with 10-ml portions of absolute methanol. The residue was dissolved in 3 ml of 0.05 M sodium bicarbonate buffer (pH 8.2) containing 2 mg of alkaline phosphatase and allowed to react at room temperature for three hours. This converted the contaminating ADP and ATP to adenosine and inorganic phosphate, while FATP was unaffected. The enzyme was denatured by agitating the solution (Cyclomixer) for several minutes with 1 ml of chloroform and denatured protein was removed by filtering through glass wool. The aqueous layer was removed and chromatographed on DEAE-cellulose as before to yield one major peak, FATP,

[3] The order of addition is important. If pyridine is added first, a precipitate forms and subsequent addition of ADP gives an incomplete reaction. If too much pyridine is added, a precipitate forms and again low yields of FATP result. The above method gives the greatest reaction of ADP with a minimum of side products.

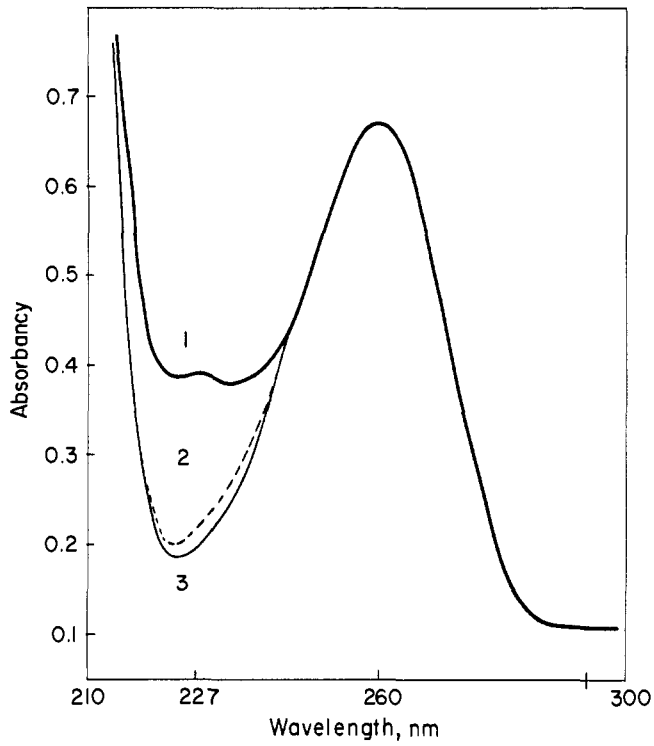


FIGURE 3: Ultraviolet absorption spectra of FATP (3) and ATP (2) at pH 6.5. The spectrum labeled 1 is impure FATP (see text).

and one minor peak, adenosine (see inset, Figure 2.). The FATP fractions were pooled and evaporated to dryness to remove triethylammonium bicarbonate as before. The residue was transferred with three 3-ml portions of methanol to a centrifuge tube, evaporated to 4–5 ml with an air stream, and 20 ml of acetone was added. NaI (1 M) in acetone (Moffatt, 1964) was added dropwise until no more precipitate formed. The precipitate was collected by centrifugation in the cold, washed three times with 25 ml of cold acetone and finally with 25 ml of cold ether. After partially air-drying the product was dried overnight *in vacuo* at room temperature in a desiccator over anhydrous $Mg(ClO_4)_2$. The yield was 460 mg (35%) of a free-flowing white powder. Yields from five preparations averaged 27% with a range of 15–45%.

FATP was normally pure on chromatography in three solvents (less than 1% of other ultraviolet-quenching adenine compounds) and on electrophoresis at pH 5.8. On occasion, however, as much as 2.6% of an alkaline phosphatase resistant ultraviolet-absorbing compound was observed on paper chromatography (solvent A) with a R_F of 0.80. This impurity gave a negative periodate test indicating the absence of two adjacent hydroxyl groups on the ribose ring. Phosphate to adenine ratios were zero for acid-labile phosphate and 2:1 for total phosphate. This implies that the compound contains two phosphate groups, each bound to separate carbon atoms and that at least one is bound to the 2′ or 3′ position of the ribose ring. *Anal.* Calcd for $C_{10}H_{12}FN_5Na_3O_{12}P_3 \cdot 4H_2O$: C, 18.55; H, 3.11; F, 2.93; N, 10.82; P, 14.35. Found: C, 18.80; H, 3.21; F, 3.00; N, 10.85; P, 14.62. Adenine to acid-labile phosphate to total phosphate gave ratios of 1.00:2.01 ± 0.09:2.97 ± 0.09 where theory requires 1.00:2.00:3.00. The ultraviolet absorption of FATP was essentially identical with ATP at pH from 1.5 to 11.0. However, FATP preparations which contained the alkaline phosphatase resistant impurity had increased absorption in the region of 227 nm (Figure 3). This property provided a convenient assay for the purity of FATP preparations.

The fluorine nmr spectrum of FATP (0.1 M, pH 7.0, 27°) was a doublet arising from the spin–spin coupling of fluorine to the γ-phosphorus with no evidence of further splitting by the β-phosphorus atom. Coupling constants (J_{f-p}) of FATP and Na_2PO_3F were 934 and 867 Hz, respectively, with corresponding chemical shifts of 4085 and 4120 Hz relative to fluorotrichloromethane as an external standard.

FATP has been stored as a solid for 9 months at -20° with less than a 4% breakdown. Less than 2.5% of FATP was degraded after 24-hr incubation at pH 5.0, 7.0, and 9.0 at 28° as judged by paper chromatography using solvents A and B.

Determination of Apparent Formation Constants. Schubert's (1956) ion-exchange resin competition method as adapted by Walaas (1958) for nucleotides was used with little modification. Formation constants for ATP were also determined for reference. The Dowex 1-X4 (200–400 mesh; Bio-Rad) anion-exchange resin used was washed with two alternate cycles of 5% NaOH and 5% NaCl, equilibrated with 0.2 M NaCl, brought to pH 8.2 with 1 N HCl, filtered, washed repeatedly with deionized water, and air-dried. The percentage H_2O in the resin was determined to be 22.2% by drying to constant weight in a 110–115° oven. Resin (50 mg) was used in each determination. This weight corresponded to a region of nucleotide binding (~90%) where a slight error in resin weight would not affect the binding data.

The reaction system (20 ml) was agitated in 25-ml glass-stoppered flasks on a Burrell wrist-action shaker at 23° for 3 hr. Six different metal ion concentrations over at least a tenfold concentration range were run in duplicate for each determination. Other control flasks contained either no divalent metal ions or no resin. After equilibrium was attained, the resin was removed by a brief centrifugation and the absorbancies of the supernatant were read at 259 nm. These values were used to calculate the distribution coefficient, K_d (Walaas, 1958). Values of the formation constant, K_f, were obtained from plots of $1/K_d$ *vs.* (M^{2+}) as given in eq 1 where K_d° is the distribution coefficient in the absence of metal ions and

$$1/K_d = K_f/K_d(M^{2+})^n + 1/K_d^0 \quad (1)$$

n is the number of metal ions in the complex. With ATP only straight lines were obtained within the metal ion concentrations ranges given in Table II. Similarly, FATP formed 1:1 complexes with Mg^{2+}, Ca^{2+}, and Mn^{2+} up to 5 mM divalent metal. Above this concentration $1/K_d$ values had a linear dependence on the square of Ca^{2+} or Mg^{2+} concentrations indicating the formation of a 1:2 complex of nucleotide to metal ion. For comparison, the values of formation constants of $HATP^{3-}$ and ADP-sulfate with Mg^{2+} and Ca^{2+} are given in Table II. It can be seen FATP binds metal ions with approximately the same affinity as $HATP^{3-}$ but appreciably stronger than ADP-sulfate. ATP^{4-}, on the other hand, binds all three metal ions tested some one to two orders of magnitude stronger than FATP, a reflection of the greater charge density on γ-phosphate of ATP.

Snake Venom Phosphodiesterase Hydrolysis of FATP. Venom (*C. atrox*) converted FATP to adenosine, P_i, and FPP_i. Figure 4 shows the time course for release of adenine components as determined by electrophoretic analysis. These results indicate that the following reaction sequences were catalyzed by phosphodiesterase (step 1) and 5′-nucleotidase (step 2).

$$\text{FATP} \xrightarrow[1]{\text{slow}} \text{AMP} + \text{FPP}_i \xrightarrow[2]{\text{fast}} \text{adenosine} + \text{P}_i + \text{FPP}_i \quad (2)$$

TABLE II: Formation Constants of FATP and ATP with Mg^{2+}, Ca^{2+}, and Mn^{2+}.

Nucleotide	Range of [M^{2+}] (mM) [Ca^{2+}] and [Mg^{2+}]	Range of [M^{2+}] (mM) [Mn^{2+}]	K_f[a] Mg^{2+}	K_f[a] Ca^{2+}	K_f[a] Mn^{2+}
ATP[b]	0–2.5	0–0.2	8600	5150	46,000
FATP[b]	0–5.0	0–8.0	200	242	505
$HATP^{3-}$			100[c]	63[c]	790[d]
ADP-sulfate[e]	0.5–15		42	49	

[a] $K_f = (LM)/(L)(M^{2+})$, where (L) = nucleotide concentration and (M^{2+}) the divalent metal ion concentration. [b] Conditions: 0.1 N NaCl, 0.02 M Tris·HCl buffer (pH 8.2), and 0.6 × 10^{-4} M nucleotide, 23°. Standard error of mean is ±5% for these values. [c] Taken from Martell and Schwarzenbach (1956). Supporting electrolyte, 0.1 M KCl, 20°. [d] Taken from Handschin and Brintzinger (1962). Supporting electrolyte, 0.1 M KCl, 20°. [e] Taken from Yount *et al.* (1966b). Supporting electrolyte 0.2 M KCl–0.025 M Tris·Cl buffer (pH 7.4).

With purified phosphodiesterase (Worthington) only AMP and FPP_i were detected. In addition, the acid-labile phosphate products from venom treatment were identified (Table III). The detection of a compound with properties appropriate for FPP_i is considered additional proof of the proposed structure of FATP.

Muscle Fiber Contraction. Bundles of fibers approximately 0.2–0.4 mm in diameter and 2 cm long were bathed in a solution of 0.05 M KCl, 0.05 M Tris·HCl (pH 7.4), and 2 mM Mg^{2+} at 31°. Their rest length was determined and they were transferred by jewelers tweezers to an identical solution containing (a) 1 mM FATP or (b) 1 mM ATP. Typical results are shown in Table IV. Adding Mn^{2+} or Ca^{2+} to give final concentrations from 0.1 to 10 mM did not change the results nor did FATP seem to inhibit contraction irreversibly.

Attempted Hydrolysis of FATP by Heavy Meromyosin. FATP was tested to see if it was a substrate for heavy meromyosin. The reaction conditions were 2 mg of heavy meromyosin/ml, 4 mM FATP or ATP, 50 mM Tris·HCl (pH 7.4), and 50 mM KCl at 31°. Aliquots of 20 μl (0.08 μmole of nucleotide) were taken at 0, 0.5, 1, 6, and 20 hr and electrophoresed as in Table I. In 1 hr under these conditions, more than 95% of the ATP was converted to ADP while in 10 hr less than 3% of FATP was hydrolyzed. Similar results were obtained when Ca^{2+} (5 mM) was added.

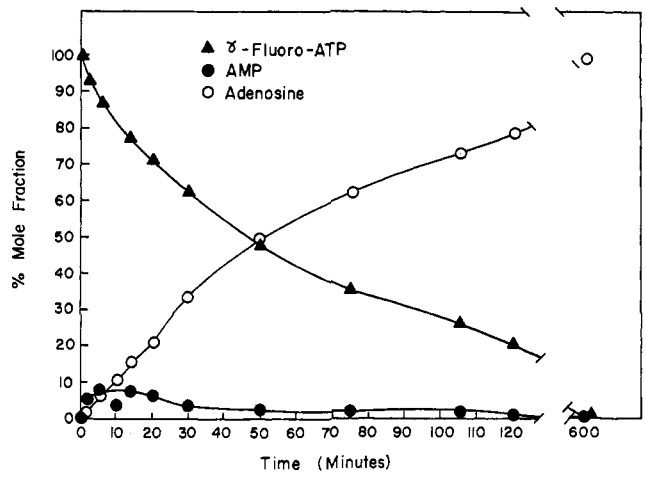


FIGURE 4: Time course of FATP cleavage by snake venom (*C. atrox*). Conditions and assay methods are as given in the text.

Attempted Irreversible Inhibition of Heavy Meromyosin and Myosin. Preliminary chromatographic studies indicated that a 330-fold excess of FATP (2 mM) to heavy meromyosin (6×10^{-6} M) did not irreversibly inhibit heavy meromyosin cleavage of ATP even after 16-hr incubation at pH 7.4 at 31°. A more rigorous check of the ability of FATP to inhibit irreversible was made with myosin. Incubating myosin (4×10^{-6} M) with a 500-fold molar excess of FATP (2 mM) for 20 hr under various conditions (Table V) gave no measurable inhibition of its CaATPase activity.

Competitive Inhibition of Heavy Meromyosin ATPase by FATP. FATP was tested to see if it interacted reversible with the active site of heavy meromyosin. Competitive inhibition of both the Ca^{2+}- (Figure 5) and Mg^{2+}- (Figure 6) ATPase

TABLE III: Relative Mobilities of Inorganic Phosphate Compounds.[a]

Compound	Solvent C R_F	Electrophoresis, P_i: Phosphate Compound Ratio
P_i	0.49	1.0
PP_i	0.17	
FPP_i[b]	0.27	0.80
FP_i	0.57	0.75

[a] Conditions were as follows. Descending chromatography: approximately a 40-cm solvent movement on Whatman No. 3MM paper at room temperature; electrophoresis: Whatman No. 3MM paper, 0.05 M citrate (pH 5.8) and 50 V/cm. [b] From snake venom treated FATP samples (see legend, Figure 4).

TABLE IV: Contraction of Glycerol-Extracted Muscle Fibers in the Presence of FATP and ATP.[a]

Nucleotide	Fiber Diameter (mm)	Original Length (cm)	Length after Initial Nucleotide Treatment (cm)	Length after Adding 25 μl of 0.04 M ATP[b] (cm)
FATP	0.2	1.7	1.7	0.6
ATP	0.2	1.7	0.8	0.6
FATP	0.4	2.0	2.0	0.7
ATP	0.4	2.0	0.9	0.7

[a] Conditions: 0.05 M Tris·HCl (pH 7.4), 0.05 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM nucleotide at 31°, 1-ml total volume. [b] Fibers were allowed to stand 20 min before adding 25 μl of 0.04 M ATP. Fiber lengths were measured after 4-min treatment.

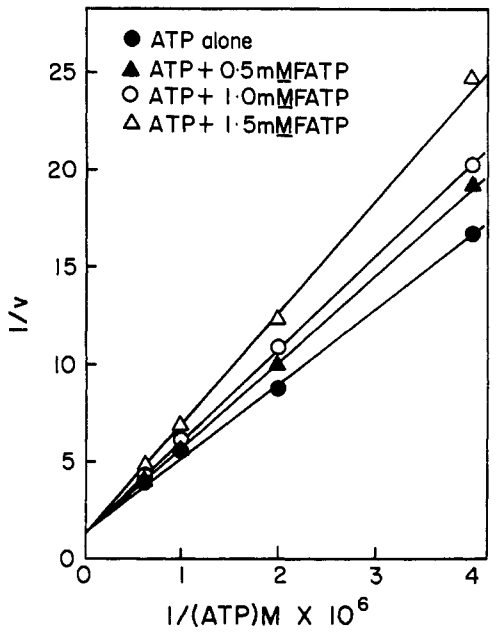


FIGURE 5: Inhibition of heavy meromyosin Ca^{2+}ATPase activity by FATP. Conditions were 0.08 M KCl, 0.013 M Tris·HCl (pH 7.4), 5 mM $CaCl_2$, 1.0 μg of heavy meromyosin/ml, and 16 μg of bovine serum albumin/ml, at 25°. Reactions were started by adding 1 ml of [γ-^{32}P]ATP of appropriate concentrations. Final volume, 6 ml. Five 1-ml aliquots taken at 1-min intervals were pipeted into 1 ml of 10% trichloroacetic acid. Five milliliters of acid molybdate solution (Fiske and Subbarow, 1925) containing 0.5 μmole of P_i plus 10 ml of isobutyl alcohol was added and the mixture was shaken 15 sec. Six milliliters of the isobutyl alcohol layer was added to 15 ml of scintillation fluid (*cf.* Table VII) and counted in a Packard Tri-Carb spectrometer. The best straight line drawn through each set of five points was used to calculate the specific activity.

activity of heavy meromyosin was observed with K_i values of 3.4×10^{-3} and 4.3×10^{-4} M, respectively. These are some 10^3 times larger than the comparable K_m values measured for ATP of 3.2×10^{-6} M (Ca^{2+}) and 1.1×10^{-7} M (Mg^{2+}).

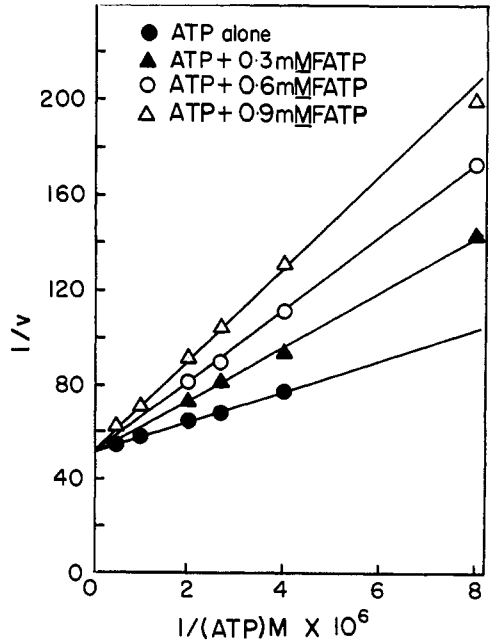


FIGURE 6: Inhibition of heavy meromyosin Mg^{2+}ATPase activity by FATP. Conditions were the same as in Figure 5 except 5 mM $MgCl_2$ replaced the 5 mM $CaCl_2$ and the heavy meromyosin concentration was 20 μg/ml.

TABLE V: Effect of Incubating Myosin with FATP on Myosin ATPase Activity.[a]

System	μM P_i/min per mg	% Retention of Act.
Control	0.221	100.0
K^+	0.200	90
K^+, FATP	0.207	94
Ca^{2+}	0.210	95
Ca^{2+}, FATP	0.208	94
Mg^{2+}	0.210	95
Mg^{2+}, FATP	0.210	95

[a] Incubation conditions were as follows. 2 mg of myosin/ml, 2 mM FATP, 0.025 M Tris HCl (pH 7.4), 0.025 M KCl, and 2 mM Mg^{2+} or Ca^{2+} at 20° for 20 hr. Control was stored at 0° during incubation of other reaction mixtures. Assay conditions were as follows. Myosin was freed of FATP by precipitation after dilution with cold deionized water and washed twice with 10 ml of 0.03 M KCl at 4°. Myosin activity was determined at 25°, 5 mM ATP, 0.1 M KCl, 0.075 M Tris-Cl (pH 7.4), and 5 mM Ca^{2+}. Samples were run in duplicate and conversion of ATP to ADP and P_i was determined using Fiske–Subbarow (1925) reagents.

Protection of Myosin against Heat Inactivation. ATP, other nucleoside triphosphates, and certain ATP analogs are known to protect myosin against heat inactivation (Blum, 1960; Yount *et al.*, 1971b), presumably by their interaction at the active site. FATP was also tested in this manner and was shown (Table VI) to protect myosin about one-half as well

TABLE VI: Effect of FATP on the Heat Denaturation of Myosin in the Presence of Calcium and Magnesium.[a]

System	Treatment Temp (°C)	μmole of P_i/min per mg	% Retention of ATPase Act.
Ca^{2+}	0	0.307	100
Ca^{2+}	40	0.147	48
Ca^{2+}, FATP	40	0.145	47
Ca^{2+}, ATP (ADP + P_i)[b]	40	0.237	77
Mg^{2+}	0	0.324	100
Mg^{2+}	40	0.108	33
Mg^{2+}, FATP	40	0.218	67
Mg^{2+}, ATP	40	0.318	98

[a] Conditions were as follows. Solutions of 0.05 M Tris·HCl (pH 7.4), 0.05 M KCl, 2 mg of myosin/ml, and 1.2 mM Mg^{2+} or Ca^{2+} with or without 2 mM nucleotide were heated at 40° for 20 min. The solutions were then cooled in an ice bath and myosin precipitated by adding 20 ml of cold deionized water. The myosin was pelleted by centrifugation (3000 rpm, RC-3 Servall), washed twice with 15 ml of ice-cold water, and dissolved in 0.6 N KCl. Protein recovery was greater than 95% in each case as determined by OD_{280} readings. Enzyme assays were at 25° (pH 7.4), 0.075 M Tris·Cl, 0.1 M KCl, and 5 mM Ca^{2+}. [b] Under the conditions of the heat treatment most of the ATP was converted to ADP and P_i.

TABLE VII: Effect of Aminoacyl-tRNA Synthetases on FATP-[^{32}P]PP$_i$ Exchange.[a]

Time (min)	Counts/10 min						
	Without Nucleotide	ATP − aa	ATP − E	ATP Complete	FATP − aa	FATP − E	FATP Complete
0	430	470	360	400	460	370	440
2	625	20,000	520	36,000	960	725	650
4	656	45,000	740	86,000	1225	900	1350
6	650	81,000	650	173,000	1260	675	1600
8	680	143,000	670	278,000	1300	750	1650

[a] Conditions were as follows. 0.1 M Tris·HCl (pH 8.2), 5 mM $MgCl_2$, 0.1 mM amino acids (AA-5 standard amino acid solution from Calbiochem), 2 mM [^{32}P]PP$_i$, 2 mM nucleotides, and 5 mM NaF at 23°. Assay conditions were as follows. The amount of ATP labeled with ^{32}P was determined by the method used by Berg (1956) with the exception that the solutions used to wash the Norit charcoal contained 2 mM PP$_i$ and 2 mM P$_i$ to facilitate removal of labeled PP$_i$ and P$_i$. Radioactivity was measured by use of a Packard Model 3003 liquid scintillation counter, using 1 ml of sample and 19 ml of scintillation fluid (700 ml of toluene, 300 ml of ethanol, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 1.0 g of 2,5-diphenyloxazole).

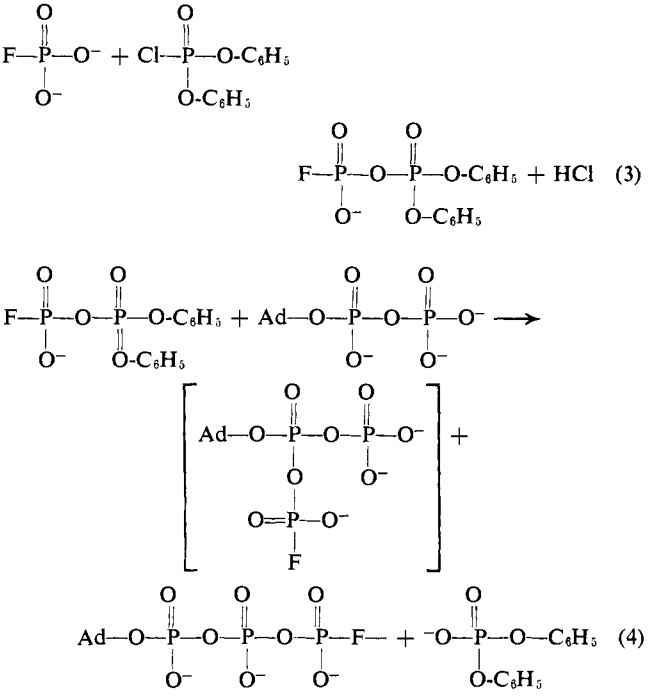
as ATP in the presence of Mg^{2+}. FATP with Ca^{2+} protected no better than Ca^{2+} alone, further indicating that the metal ion present markedly affected the degree of interaction of myosin and FATP. Note, however, at the FATP concentration used (2 mM) myosin would be less than 50% saturated with nucleotide, assuming the K_i from heavy meromyosin Ca^{2+}ATPase studies also holds for myosin.

Aminoacyl-tRNA Synthetases. FATP was tested to see if it would replace ATP with a mixture of aminoacyl-tRNA synthetases from *B. brevis*. The relative activity of ATP and FATP was measured by the amount of exchange that occurred between the nucleotides and [^{32}P]PP$_i$. Under the conditions used FATP was slightly effective as a substrate giving only 0.4% as much exchange as ATP (Table VII). The sizable amino acid independent ATP $\rightleftharpoons$ PP$_i$ exchange activity gave a background which made it difficult to quantify the results.

Hexokinase was incubated with FATP to see if FATP could replace ATP in the conversion of glucose to glucose 6-phosphate (glucose 6-fluorophosphate). Conditions were 0.8 mg/ml of hexokinase, 0.03 M nucleotide, 0.18 M Tris·HCl (pH 8.4), 0.05 M glucose, and 3 mM $MgCl_2$ at 24°. Aliquots of 10 μl were removed every 10 min for 1 hr and both were electrophoresed at pH 5.8 (Table I) and chromatographed using solvent A. The adenine products were monitored by ultraviolet absorption and the phosphate and glucose spots were found by using the sprays described in Materials and Methods. In a control reaction all of the ATP was converted to ADP in 20–30 min with a concomitant increase in glucose 6-phosphate. With FATP there was no formation of other adenine products and no detectable synthesis of glucose 6-fluorophosphate within 4.5-hr period. With the system used a 2% conversion of glucose to glucose 6-fluorophosphate could be detected. Addition of ATP after one hour to the system containing FATP resulted in the synthesis of glucose 6-phosphate. Therefore, FATP did not completely inactivate hexokinase.

Discussion

The structure proposed for FATP (Figure 1) would appear to be firmly established, particularly since a compound with properties appropriate for FPP$_i$ was found to be a product of snake venom cleavage (Table III). The synthetic reactions used (eq 3 and 4) could theoretically lead to a branched isomer



with FP$_i$ attached to the α-phosphate of ADP (product in brackets, eq 4). Little of it appears to have been formed although an analogous α isomer is known to exist for the sulfate analog of ATP (Yount *et al.*, 1966a).

This mode of synthesis represents an extension of the anion-exchange method of Michelson (1964). Normally the most expensive or least available intermediate phosphate is activated by reaction with diphenyl chlorophosphate and the product formed by displacing diphenyl phosphate with an excess of the commoner anion. When this was tried by activating ADP with diphenyl chlorophosphate and then reacting with an excess of FP$_i$ a large number of products resulted, of which FATP was a minor constituent. The alternate approach, to make the diphenyl phosphoryl derivative of FP$_i$ and react it with an excess of ADP was not economically sound. The best procedure was found to be the short-term reaction of ADP with an excess of diphenyl phosphorylfluorophosphate. This way the large majority of the ADP reacted to give prin-

cipally FATP with some ATP and higher phosphate containing compounds being formed. This approach depends on ADP being a better nucleophile than FATP. The ATP presumably arose because of P_i contamination in the FP_i either there initially or from hydrolysis of FP_i at some stage before the coupling step. The hydrolysis of FATP to ATP during the work-up of the reaction was never detected.

The formation constants of FATP for Mg^{2+}, Ca^{+}, and Mn^{2+} (Table II) are low compared to ATP^{4-} but are approximately the same as $HATP^{3-}$ (see Phillips, 1966, for a comprehensive review of the formation constants of ATP^{4-} and $HATP^{3-}$ with various metal ions). The similarity of metal binding by FATP and HATP is a further indication of the suitability of FATP as an analog to mimic $HATP^{3-}$. ADP-sulfate, on the other hand, binds metals with significantly less affinity than FATP and is therefore, less suitable as an $HATP^{3-}$ analog. It should be pointed out that the formation constants reported here have not been corrected for binding by Na^{+} or the Tris cation and would be much larger if such corrections were made (see, *e.g.*, O'Sullivan and Perrin, 1964). Also no corrections have been made for possible Tris–M^{2+} interactions. However, the relative affinities (our major concern) would be changed little by such corrections.

Of the enzymes tested with FATP, myosin, heavy meromyosin, alkaline phosphatase, and hexokinase cleave ATP between the β- and γ-phosphate groups while aminoacyl-tRNA synthetases and snake venom phosphodiesterase cleave ATP between the α- and β-phosphates. Only for the latter two does FATP appear to be a substrate. Based on these limited studies it would be predicted FATP would work only with enzymes which normally cleave the α–β-phosphate linkage of ATP, *e.g.*, the enzymes responsible for the synthesis of fatty acids and most biopolymers.

The fact that FATP is not a substrate for alkaline phosphatase proved essential in its purification since it was not possible to separate FATP and ADP by column chromatography using triethylammonium bicarbonate buffers. Since alkaline phosphatase readily hydrolyzes ADP and ATP to adenosine and P_i, subsequent chromatography yielded FATP free of these substances. Attempts to treat the reaction mixture directly with alkaline phosphatase to destroy ADP and ATP were unsuccessful and an initial chromatographic purification was found to be necessary. Eckstein and Gindl (1967) have previously used the resistance of phosphorothioate esters to alkaline phosphatase hydrolysis to determine the percentage of normal triphosphate in chemically synthesized thymidine 5′-triphosphorothioate, a TTP analog with sulfur replacing a nonbridge oxygen in the α-phosphate.

FATP does not seem to inactivate irreversibly any of the enzymes tested, although only limited variations in reaction conditions for enzymes other than heavy meromyosin were tried. Conceivably FATP could react with myosin or related enzymes to form either (i) an adenosine triphosphoryl de-

$$\text{FATP} + \text{myosin} \longrightarrow \text{ATP–myosin} + F^{-} \qquad \text{(i)}$$

rivative of moysin or (ii) a fluorophosphoryl derivative (eq 6).

$$\text{FATP} + \text{myosin} \longrightarrow \text{FP–myosin} + \text{ADP} \qquad \text{(ii)}$$

Reaction i is analogous to the reaction of fluorophosphate nerve gases with various esterases (see Saunders (1957) and references therein). Reaction ii would result if FATP were hydrolyzed in a two-step reaction in which step 1 was fast (the formation of fluorophosphoryl-enzyme plus ADP) and step 2, the hydrolysis of the enzyme derivative, was slow. This reaction would be analogous to the acetylation of chymotrypsin by its pseudosubstrate, *p*-nitrophenyl acetate. Although neither reaction i nor ii appears to occur with myosin, FATP (and related fluorophosphate analogs) retains the potential to react with other enzymes. For example, Sporn *et al.* (1969) have recently shown that thymidine 3′-fluorophosphate reacts irreversibly to inhibit exoribonucleases from both Ehrlich ascites tumor and Hela cell nuclei.[4] The reaction appears to be specific in that thymidine 5′-fluorophosphate is not an inhibitor. Neither of these compounds inhibit RNA polymerase. Thus, although fluorophosphate analogs have the potential to inactivate enzymes, it is not possible at the present time to predict which enzymes these will be.

FATP does intereact with the active site of heavy meromyosin as evidenced by its competitive inhibition of both the Ca^{2+}- and Mg^{2+}ATPase activity. Earlier data obtained with myosin indicated ADP-sulfate was an uncompetitive inhibitor of myosin's Ca- and MgATPase activity (Yount *et al.*, 1966b). However, these data were obtained at ATP concentrations well above the K_m and at relatively high concentrations of inhibitor. It is now clear that inhibition studies of myosin or heavy meromyosin ATPase activity will normally require the use of [^{32}P]ATP in order to obtain valid K_m and V_m values (see *e.g.*, Schliselfeld and Bárány, 1968, and Yount *et al.*, 1971b). The usual methods, such as colorimetry or pH-Stat measurements, generally are not sensitive enough to measure the hydrolysis of the first few per cent of ATP and errors enter in, due to product inhibition by ADP. Thus, it may be that ADP-sulfate would give results comparable to those obtained with FATP if run under conditions of low ATP since both analogs are similar in structure and net charge and might be expected to interact with heavy meromyosin and myosin in a similar fashion.

In experiments to be reported elsewhere FATP has been shown to be an effective cofactor for the Mn^{2+}-stimulated nucleotide-dependent $P_i \rightleftharpoons H_2O$ exchange reaction (Swanson and Yount, 1966) catalyzed by heavy meromyosin and myosin (Herrman, 1970; J. Herrman and R. Yount, in preparation). The best available evidence indicates the nucleotide cofactor must bind at the active site to be effective (Herrman, 1970). This requirement can be taken as further evidence for direct binding of FATP to the active site, though it means FATP must bind in a manner similar to ADP in order to allow P_i access to the active site. Compounds similar to ATP, *e.g.*, adenylylimidodiphosphate (Yount *et al.*, 1971a), will *not* act as cofactors (Yount *et al.*, 1968; Herrman, 1970) presumably because the last phosphate blocks the entry of P_i to the exchange site. This means FATP binds in such a way that at least part of the time the fluorophosphate group does *not* occupy the normal binding site for the γ-phosphate of ATP. If this type of binding holds for Ca^{2+} and Mg^{2+} complexes as well, then the inability of FATP to inhibit irreversibly may be a reflection of the mode of binding rather than chemical inactivity.

Several reasons might be advanced to explain the general

[4] An alternate explanation exists for the effects observed by Sporn *et al.* (1969). The inhibition depended on incubating thymidine 3′-fluorophosphate with intact nuclei at 37° for 30 min prior to assay. It is possible that the analog was acting simply to make the enzyme *less* heat stable. Substrates or substrate analogs are known to have such an effect for a number of enzymes (Grisolia and Joyce, 1959; Grisolia, 1964).

metabolic inertness of FATP. One of these, given above, is the indication that FATP does not bind to the active site with the right configuration, possibly because it does not form the correct metal ion complex. The general weak binding of divalent metal ions to FATP may be an additional factor, especially if the role of the metal ion is to polarize a P–O bond to promote bond breaking at the P atom. The basis for these properties of FATP undoubtedly lies in the nature of the F–P bond. The $d\pi$–$p\pi$ overlap of this bond decreases the net positive charge on the phosphorus atom (Cox and Ramsay, 1964) and makes it less susceptible to attack by a nucleophilic amino acid. One possible way to minimize this would be to make the γ-methyl ester of FATP. Although structurally this analog would be further removed from ATP, chemically it would be more akin to the organophosphorus nerve gases and hence should be more reactive. The attempted synthesis of such analogs is planned.

Finally, the fluorine of FATP should prove to be a useful nmr probe of the binding sites for ATP. As pointed out by Spotswood *et al.* (1967) and Zeffren and Reavill (1968) the use of fluorine nmr has two main advantages over proton nmr: (1) ^{19}F chemical shifts are larger and considerably more sensitive to environmental effects than ^{1}H chemical shifts, and (2) the spectra are not cluttered by proton resonances from other sources. In addition, it should now be possible to measure the interatomic distance between the fluorine of FATP and Mn^{2+} bound to myosin by nmr relaxation techniques as has been done by Mildvan *et al.* (1967) for the fluorine of FP_i and Mn^{2+} bound to pyruvate kinase. The tight binding of Mn^{2+} to the active site of heavy meromyosin (R. Yount and M. Cohn, unpublished results) should facilitate such studies.

Acknowledgment

We thank Dr. James Magnuson for determining the fluorine nmr properties of FATP and Dr. R. J. Foster for many helpful discussions.

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